



Plasma corticosterone of city and desert Curve-billed Thrashers, *Toxostoma curvirostre*, in response to stress-related peptide administration

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ABSTRACT

We compared the activity and responsiveness of the hypothalamo–pituitary–adrenal (HPA) axis of an urban (Phoenix, Arizona) and desert population of a male songbird species (Curve-billed Thrasher, *Toxostoma curvirostre*), by measuring plasma corticosterone in response to acute administration of corticotropin-releasing factor, arginine vasotocin, or adrenocorticotropin hormone. Urban adult male thrashers showed greater responsiveness than desert birds to an injection of arginine vasotocin or adrenocorticotropin hormone, suggesting a population difference in pituitary and adrenal gland sensitivity. Plasma corticosterone in response to corticotropin-releasing factor injection did, however, not differ between populations. The differential corticosterone response to arginine vasotocin and corticotropin-releasing factor may reflect effects of chronic stress or habituation, which are known to favor arginine vasotocin over corticotropin-releasing factor sensitivity. Efficacy of HPA negative feedback by glucocorticoids was determined by measuring plasma corticosterone in response to acute administration of the synthetic glucocorticoid dexamethasone. This administration decreased plasma corticosterone similarly in urban and desert thrashers, suggesting that the negative feedback of glucocorticoids on the HPA axis in the two populations was equally effective. The higher sensitivity of urban than desert thrashers to adrenocorticotropin hormone and arginine vasotocin may result from up-regulation of the HPA axis in urban birds. This up-regulation may in turn make it easier for city birds to cope with urban environment-associated stressors.

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1. Introduction

The hallmark of the vertebrate physiological response to stress is two-fold: activation of the sympathetic nervous system *via* catecholamines primarily released from the adrenal gland medulla and the release of glucocorticoids from the adrenal gland cortex. These responses enable an organism to mobilize energy stores and alter behaviors that maximize survival during a stressful event (Norris, 2006). Glucocorticoids are the end products of the hypothalamo–pituitary–adrenal (HPA) axis. At the onset of a perceived threat, parvocellular neurons of the avian hypothalamic paraventricular nucleus secrete corticotropin-releasing hormone (CRH) and arginine vasotocin (AVT), the avian homologue of mammalian vasopressin (AVP). In mammals, the actions of CRH during a stress response precede those of vasopressin, which has longer lasting effects (Herman et al., 1992; Ma and Aguilera, 1999). In mammals and birds, both neuropeptides stimulate the pituitary gland secretion of adrenocorticotropin hormone (ACTH; Zelena et al., 2004; Rich and Romero, 2005), which ultimately induces adrenal secretion of glucocorticoids, such as corticosterone (CORT) in birds.

Corticosterone regulates HPA activity through a classic negative feedback loop by acting at both the hypothalamus and pituitary gland (Young et al., 1995; Makino et al., 2002). This feedback may decrease CRH production and release (Aguilera et al., 2007) but research on rodents suggests that AVP is not directly affected by CORT (Dallman, 1993; Aguilera et al., 2007). A difference in feedback efficacy on CRH and AVT secretion may be important during “chronic” stress, here defined as persistently elevated plasma CORT combined with a reduced capacity to elevate CORT in response to acute stress. During chronic stress, the adrenal glands may continue to secrete CORT even after the stressor is no longer perceived. This can have deleterious impacts on energy balance, reproduction or immune function (Clinchy et al., 2004; Rich and Romero, 2005; Wingfield, 2005; Lightman, 2008). These processes have been extensively studied in mammals but are less studied in birds.

In nature, avian populations inhabiting different habitats often show varied seasonal responses to stress. For example, birds from climates where breeding opportunities are temporally limited (e.g., arctic, desert) can show lower CORT secretion during stress when breeding compared to other life-history stages (Astheimer et al., 1995; Wingfield et al., 1992; Fokidis et al., 2009). Limiting HPA activity during breeding may help avoid potentially deleterious consequences of elevated CORT levels, such as nest abandonment and suppression of

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reproductive physiology (Wingfield and Ramenofsky, 1997; Love et al., 2002), which could in turn decrease fitness.

Research investigating differences between populations have begun to focus on how anthropogenic environmental change can alter the HPA axis activity. Recent studies have identified differences in CORT secretion across urban–rural gradients (Partecke et al., 2006; Schoech et al., 2007; French et al., 2008; Fokidis et al., 2009), but the mechanisms involved are poorly understood. During the breeding season (March to July), urban Curve-billed Thrashers, *Toxostoma curvirostre*, increase plasma CORT in response to capture and handling stress more than conspecific birds living in desert areas (Fokidis et al., 2009). Desert thrashers may limit their acute stress response during the breeding season to avoid its interference with reproduction, since the reproductive window coincides with the food that becomes available shortly after the winter rains and before the very hot and dry summer (Wingfield et al., 1992; Fokidis et al., 2009). Outside the breeding season, initial plasma CORT (i.e., prior to application of handling stress or baseline) is higher in desert than urban birds, and during molt desert thrashers show stronger stress response than urban birds (Fokidis et al., 2009). Urban thrashers may be exposed to more frequent chronic (e.g., pollution, increased temperatures, and noise) and acute stressors (e.g., vehicles, humans, and feral predators). Exposure to such stressors, are far less common in desert-dwelling thrashers. Thus urban thrashers may require an active stress response during the breeding season, in contrast to desert birds that may need to modulate their acute stress response when breeding. An alternative situation can occur if consistent exposure to the above stressors results in habituation, here defined as attenuated stress responses as originally novel stressors over time are perceived as benign (Cyr and Romero, 2009).

We investigated the source of variation along the HPA axis that may account for these differences in CORT secretion between breeding urban and desert thrashers. Specifically, we compared (1) differences in sensitivity of the pituitary gland to CRH and AVT; (2) differences in the responsiveness of the adrenal glands to ACTH injection; and (3) differences in negative feedback induced by administration of the synthetic glucocorticoid dexamethasone (DEX). This information can be used to assess whether urban birds show symptoms of chronic stress, habituation, or differences in the regulation of their HPA axis activity compared to desert birds. If urban thrashers are chronically stressed, we predict they would produce greater amounts of CORT due to increased adrenal sensitivity to ACTH compared to desert birds. This increased CORT production would decrease CRH secretion, via a negative feedback loop. In contrast, AVT release which is thought to be impervious to the negative feedback action of CORT may increase which would translate to increased pituitary sensitivity to AVT in urban compared to desert birds. If urban birds down-regulate their HPA activity as a result of habituation, we predict decreased secretion of the entire HPA axis, which would ultimately decrease sensitivity to ACTH, AVT, and CRH injections, and DEX would have an increased ability to decrease CORT secretion in urban compared to desert birds. If urban thrashers up-regulate their HPA activity, we predict increased sensitivity in all aspects of the HPA axis so that ACTH, AVT, and CRH injections have a greater ability to increase CORT levels in urban birds compared to desert birds. In addition, we predict that DEX would also have a greater capacity to limit CORT secretion, in urban as compared to desert birds. This is the first study to investigate intraspecific differences at multiple levels of the HPA axis between rural and urban animal populations.

2. Methods

2.1. Model species and study populations

Curve-billed Thrashers are common Sonoran Desert songbirds in Phoenix, Arizona, although they are found at lower population

densities than in the surrounding desert (Green and Baker, 2003). This species is non-migratory and defends a permanent territory year-round against conspecific intrusion (Tweit, 1996). Thus physiological differences among populations likely result from local environmental factors and not from those associated with distant wintering grounds.

The study was conducted in a Sonoran desert site and in urban areas of the cities of Phoenix and Scottsdale. The desert site was the McDowell Mountain Sonoran Preserve and the adjoining Regional Park (940 acres and 21,099 acres, respectively), which is located at the northern periphery of Phoenix and 4 km from the fringe suburban developments of Troon and Fountain Hills. Urban sites included high- and low-income residential housing tracts, commercial areas, business districts, and manicured city parks of various sizes, and their sampling was governed primarily by authorized access. Previous research documented consistent differences in acute stress responses over several years between thrashers inhabiting various areas of Phoenix and those from several Sonoran Desert localities to the north, west, and south of the city, and there is little evidence for differences in stress physiology within urban localities (Fokidis, unpublished data). The current study is confined to two populations but the results likely apply to urban–desert comparisons across a number of localities.

2.2. Field capture and initial blood collection

The study was conducted between February and April 2008, which coincides with the early (incubation) to middle (early nestling) stages of the breeding season in this species (Tweit, 1996). Adult male thrashers (presumably defending active territories) were lured and captured in mist nets using conspecific playback recordings and all bird captures took place between 0500 and 1100 h.

Within 3 min of capture, approximately 300 μ L of blood was collected from the right jugular vein into a heparinized 0.3 mL syringe with a 29.5 gauge needle. These samples were used to determine pre-injection (*hereafter* initial) plasma CORT. Birds (mean body mass \sim 80 g) then received either an intrajugular control injection of 0.9% NaCl solution (*hereafter* “Saline”), or one of the following treatments (see below). The order of these treatments was originally randomized, but once this order was established, treatments were given systematically and independently between the populations. There were no apparent differences in the breeding phenology between urban and desert bird populations.

Some birds (6 urban and 6 desert) were bled using the standard 30 min capture and handling protocol outlined by Fokidis et al. (2008, 2009), but received no injection. Results from these birds (*hereafter* “Bleed”), were compared to those from saline-injected birds to determine whether the act of injection alters the stress response. All blood samples were kept on ice until plasma was separated by centrifugation and then stored at -80°C until assayed for CORT.

Sex of the birds was confirmed as male by the presence of a developed cloacal protuberance, and body mass (\pm 0.1 g) and wing chord (\pm 1 mm) was also measured for each bird. All birds received a uniquely numbered aluminum United States Geological Survey leg band and were released at the site of capture. All procedures followed the guidelines established by the Arizona State University Institutional Animal Care and Use Committee (protocol # 06-850R) and site access was authorized by the Arizona Game and Fish Department, the United States Fish and Wildlife Service, Cities of Phoenix and Scottsdale Parks and Recreation Departments, and various private landowners.

2.3. Pituitary sensitivity to CRH and AVT

To test the pituitary sensitivity to CRH and AVT, birds were injected *via* the jugular vein with either ovine CRH (*Sigma-Aldrich Co. Cat # C3167*) or AVT (*Sigma-Aldrich Co. Cat # V0130*). These injections

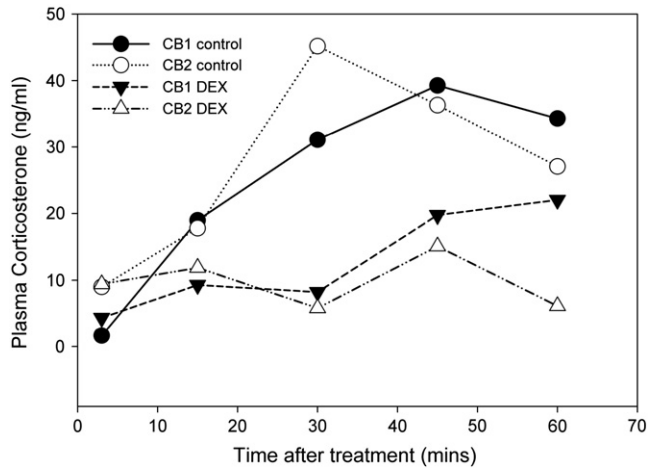


Fig. 1. Preliminary data showing efficacy and time course of dexamethasone (DEX) suppression (8 mg/kg dose) of stress-induced increases in plasma corticosterone in two captive Curve-billed Thrashers (CB: *Toxostoma curvirostre*) compared to control injections of 0.9% NaCl.

were administered as either a low (3 µg/kg) or high dose (6 µg/kg) in 100 µL of saline vehicle. These doses were chosen based on previously published research in free-living passerines which demonstrated responses in CORT levels to these concentrations (Romero and Wingfield, 1998; Romero et al., 1998a; 1998b; 1998c; Romero and Wingfield, 2001; Romero, 2006; Dickens et al., 2009). The focus of this study was to elucidate population differences in pituitary sensitivity to these two peptides, and not their relative effect on CORT secretion, thus doses were not equivalent in molar concentration. Instead, doses were designed to induce CORT levels that represented a maximal physiological secretion during acute stress.

After injection, birds were kept in a cloth bag for 30 min until a second 300 µL blood sample (*hereafter* treatment sample) was collected. Published studies of various bird species found that 30 min is sufficient time to induce a maximum increase in corticosterone levels in response to CRH and AVT injections (Romero and Wingfield, 1998; 2001; Romero et al., 1998a; 1998b; 1998c; Romero, 2006; Dickens et al., 2009). This time frame (30 min) also coincides with maximal CORT levels during acute stress in this species and enables comparisons of the present data with those of a previous study investigating differences in plasma CORT between thrasher populations (Fokidis et al., 2009).

2.4. Adrenal sensitivity to ACTH

Exogenous ACTH injection was used to assess adrenal gland sensitivity *via* its effect on CORT secretion. To isolate the effects of exogenous ACTH on the adrenal gland, birds first received an injection of 6 mg/kg injection of DEX (*Sigma-Aldrich Co. Cat # D1756*), which binds to pituitary glucocorticoid receptors thus inhibiting endogenous ACTH secretion (Dekloet et al., 1975). This dosage of DEX appears sufficient to minimize the increase in CORT that normally results from capture and handling stress based on preliminary data on captive thrashers (Fig. 1), and is well within the range used in previous studies in songbirds (Koch et al., 2002; Holberton et al., 2007; Dickens et al., 2009; Singh et al. 2010). Based on a preliminary investigation the decrease in the CORT response by DEX administration occurs within 15 min and can seemingly last for at least one hour (Fig. 1). After 15 min, birds received an intrajugular injection of porcine ACTH (*Sigma-Aldrich Co. Cat # A6303*) at either a 50 or 100 IU dose in 100 µL saline vehicle. These doses of ACTH have successfully increased CORT in other passerine species (Romero and Wingfield, 1998; Romero et al., 1998a; 1998b; 1998c; Romero and Wingfield, 2001; Romero, 2006; Dickens et al., 2009).

2.5. Testing negative feedback with DEX

To assess the efficacy of the negative feedback of the HPA axis, DEX was used at one of two concentrations (2 mg/kg or 8 mg/kg). After an initial blood sample was collected, birds received an injection of DEX, and a second blood sample was collected 30 min post-injection. Based on previous studies in songbirds, these doses are effective at simulating the negative feedback of glucocorticoids on the HPA axis (Koch et al., 2002; Holberton et al., 2007; Dickens et al., 2009; Singh et al. 2010).

2.6. Plasma corticosterone assay

Plasma total CORT concentrations were measured using a validated commercial competitive enzyme-linked immunoassay (ELISA; Assay Designs Inc. Ann Arbor, MI, USA; Fokidis et al., 2009). Samples were assayed in duplicate except for 17 samples that were assayed singly. Samples were distributed randomly across assay plates, but initial and treatment samples from the same individual were assayed on the same plate. Plasma CORT concentrations were calculated by interpolation from standard curves present on each assay plate and using GraphPad Prism v4 (La Jolla, CA, USA). The sensitivity of the CORT assay ranged from 6.4 to 15.7 pg/mL and the mean intra-assay and inter-assay coefficients of variation were 14.9% and 19.1%, respectively (n = 4 plates, 156 samples total).

2.7. Data analyses

Initial and stress-induced CORT is thought to interact with different receptors (mineralocorticoid and glucocorticoid receptors, respectively) and thus may exert different physiological functions (Norris, 2006). Individual variation in initial CORT levels prompted data analysis that incorporated both the initial and treatment levels of CORT. Thus data were analyzed using repeated measure analysis of variance (ANOVA), with initial and treatment CORT as the within-subject factor (dependent variable), and locality (urban or desert), treatment (high dose, low dose, control), and their interaction as between subject factors (independent variables). Date and time of capture, wing chord, and body mass were entered into the model as random factors, but were subsequently removed if not significant ($P > 0.05$). We also tested differences in body mass and wing chord

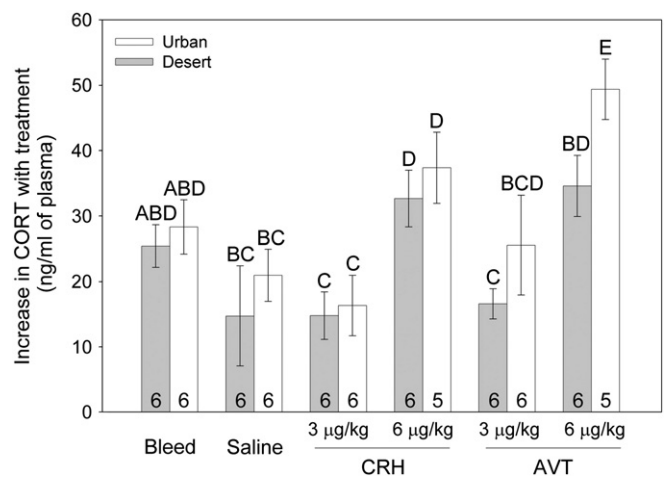


Fig. 2. Comparison of the effects of injection with 0.9% NaCl (Saline) vs. no injection received (Bleed), and also injection of either 3 or 6 µg/kg of corticotropin-releasing factor (CRH) or arginine vasotocin (AVT) on plasma corticosterone concentration of urban and desert Curve-billed Thrashers (*Toxostoma curvirostre*). Numbers indicate treatment sample sizes and different letters indicate significant differences between groups at $P < 0.05$ in the response to injection (i.e., difference between initial and post-treatment levels of corticosterone).

between urban and desert birds among the various treatments. Differences between specific treatment groups were tested using Fisher's least-significant difference (LSD) *post-hoc* tests. Statistical comparisons were only made between sites and within hormone or treatment types. All statistical analyses were performed using SPSS Version 13.0 (2004; Chicago, Illinois, USA) with alpha levels set at 0.05. Data sets conformed to assumptions of normality and are presented as means \pm 1 standard error (SE).

3. Results

3.1. Effect of injection on plasma CORT

Initial and treatment plasma CORT did not differ between birds receiving no injection or an injection of saline solution (urban: $F_{1,2} = 0.916, P = 0.442$, desert: $F_{1,2} = 0.071, P = 0.502$; Fig. 2). Urban and desert birds did not differ with respect to body mass (urban: 75.2 ± 1.83 g, desert: 73.8 ± 0.98 g; $P = 0.32$), wing chord (urban: 109 ± 2.3 mm, desert: 112 ± 3.4 mm; $P = 0.45$), or the time of day sampled (urban: 138 ± 34 min after sunrise, desert: 157 ± 16 min after sunrise; $P = 0.18$). Initial plasma CORT did not differ between the various treatment and control groups (all $P \geq 0.341$).

3.2. Pituitary gland sensitivity to CRH and AVT

An injection of a higher CRH dose increased plasma CORT ($F_{1,3} = 13.882, P \leq 0.001$; Fig. 2) but this increase was similar in urban and desert birds ($F_{1,2} = 2.594, P = 0.115$; Fig. 2). Both urban and desert thrashers injected with $3 \mu\text{g}/\text{kg}$ of CRH did not increase their plasma CORT above levels attained by saline-injected birds (both $P \geq 0.282$), but had lower CORT levels than birds that were only bled (both $P \leq 0.017$). Urban and desert birds injected with $6 \mu\text{g}/\text{kg}$ of CRH had higher plasma CORT than birds receiving either the lower CRH dose (both $P \leq 0.034$), or saline (both $P \leq 0.034$). However, CORT responses in high dose birds did not differ from birds that were only bled (both $P \geq 0.279$), and also did not differ between populations ($P = 0.454$).

The effect of AVT administration on plasma CORT varied as a function of the dose administered ($F_{1,3} = 16.495, P \leq 0.001$) and was habitat-related ($F_{1,2} = 10.128, P = 0.003$; Fig. 2). In urban and desert thrashers, an injection of $3 \mu\text{g}/\text{kg}$ of AVT did not elevate plasma CORT above that in saline-injected birds (urban: $P = 0.173$; desert: $P = 0.354$; Fig. 2). In desert thrashers, injection of $6 \mu\text{g}/\text{kg}$ of AVT

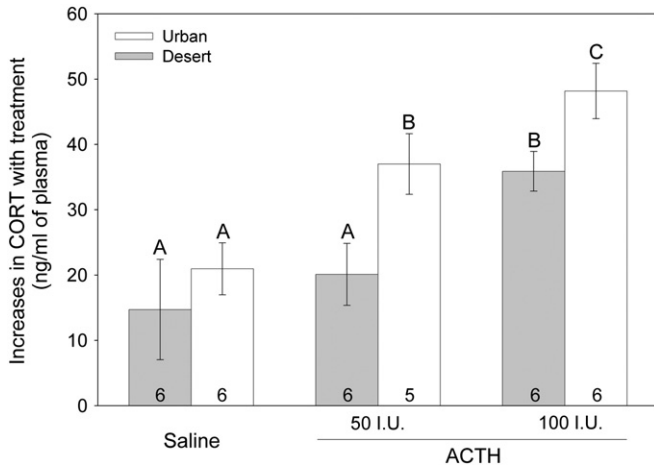


Fig. 3. Changes in plasma corticosterone in response to treatment with 100 (High) and 50 IU (Low) doses of adrenocorticotropic hormone (ACTH), after pre-treatment with dexamethasone (see methods) in urban and desert Curve-billed Thrashers (*Toxostoma curvirostre*) compared to control birds injected with 0.9% NaCl (Saline). Numbers indicate treatment sample sizes and different letters indicate significant differences between groups at $P < 0.05$ in the response to injection (i.e., difference between initial and post-treatment levels of corticosterone).

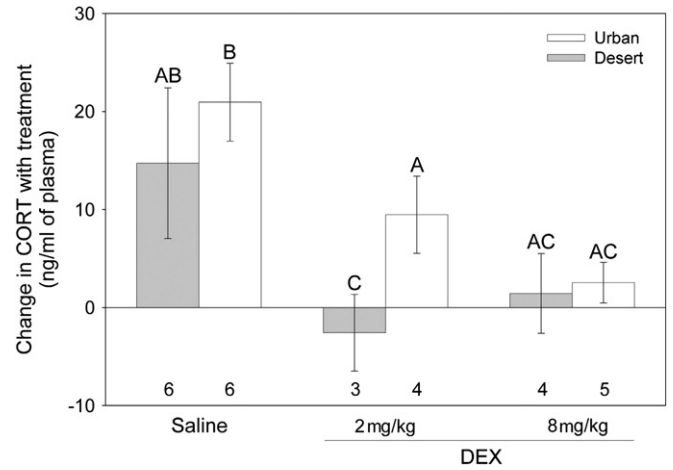


Fig. 4. Efficacy of negative feedback of the hypothalamic-pituitary-adrenal axis as induced by injection with 8 (High) and 2 mg/kg (Low) doses of the synthetic glucocorticoid dexamethasone in urban and desert Curve-billed Thrashers (*Toxostoma curvirostre*) compared to birds injected with 0.9% NaCl (Saline). Numbers indicate treatment sample sizes and different letters indicate significant differences between groups at $P < 0.05$ in the response to injection (i.e., increase between pre- and post-treatment levels).

elevated plasma CORT above levels in control ($P = 0.021$) or lower dose ($P = 0.009$) groups, but not above levels in birds that were only bled ($P = 0.155$). Similarly in urban thrashers, the high AVT dose group had higher plasma CORT post-treatment than control ($P = 0.014$), low dose ($P = 0.017$) and bled groups ($P = 0.030$). Levels of CORT were higher in urban birds injected with the high dose than those in corresponding desert birds ($P = 0.040$).

3.3. Adrenal sensitivity to ACTH

Injection of ACTH elevated plasma CORT and this was dose-dependent in both urban and desert birds ($F_{1,3} = 9.275, P = 0.007$; Fig. 3). Injection of 50 IU ACTH elevated CORT levels above that in birds injected with saline in urban ($P = 0.022$), but not desert thrashers ($P = 0.171$). Administration of 100 IU/kg of ACTH significantly increased CORT levels above saline injected birds in both urban ($P \leq 0.001$), and desert thrashers ($P = 0.019$). The levels of CORT in response to ACTH administration differed between populations, with urban birds being more responsive than desert conspecifics ($F_{1,2} = 2.374, P = 0.048$; Fig. 3).

3.4. Efficacy of negative feedback

Injections of DEX at both doses eliminated the increase in plasma CORT associated with capture and handling, as shown by lack of a difference with saline-injected controls ($F_{1,3} = 0.477, P = 0.216$; Fig. 4). The DEX injection-induced suppression of the CORT response did not differ between low and high doses ($F_{1,3} = 1.005, P = 0.091$) and was similar in urban and desert thrashers ($F_{1,3} = 0.0121, P = 0.780$). However, urban thrashers receiving 8 mg/kg of DEX had lower plasma CORT than control urban and desert birds ($P = 0.030$).

4. Discussion

The present study is the first to investigate sources of variation in the HPA axis activity between rural (i.e., desert) and urban populations of a free-living bird. Plasma CORT increased in response to CRH treatment in a dose-dependent fashion, but this increase did not differ between urban and desert thrashers. However urban thrashers demonstrated a higher pituitary and adrenal gland response to exogenous AVT and ACTH injections respectively, than desert conspecifics. The efficacy of negative feedback of the HPA axis as

induced by treatment with DEX did not differ between populations, with all birds showing a robust negative feedback to both doses of DEX tested.

4.1. Hypothalamic regulation of the HPA axis by CRH and AVT

The relative contribution of CRH and AVT in regulating the activity of the HPA axis in birds is unclear, because studies have produced contradicting results. In the chicken, *Gallus domesticus*, centrally administered CRH elevates plasma CORT more effectively than AVT treatment (Madison et al., 2008), but peripheral CRH and AVT injections suggest the opposite in Japanese quail, *Coturnix coturnix japonica* (Hazard et al., 2007). The latter study is consistent with findings in passerines (Romero and Wingfield, 2001; Romero et al., 1998a; Rich and Romero, 2005, 2007; Romero, 2006). Note however that in two of the above passerine studies, CRH administration did not elevate CORT above control levels (Romero and Wingfield, 2001; Romero et al., 1998a). Similarly in this study, AVT, but not CRH injection, increased CORT more than a saline injection in desert thrashers. One possible explanation for these observations is that this relates to the dosages of CRH and AVT given. In this study, although doses were equivalent by weight, four times as much CRH was given than AVT on a molar concentration basis. Despite the greater amount of CRH, a lesser response in terms of CORT levels was observed, compared to AVT injection. This suggests that AVT is a more potent secretagogue of CORT than CRH. However CRH and AVT dose-response curves for passerine species are to our knowledge unavailable and these would be necessary to draw specific conclusions about the relative potency of these two neuropeptides in regulating the HPA axis. Passerine CRH has to our knowledge not been isolated or sequenced and the affinity of mammalian CRHs for avian CRH receptors has not been determined. However, chicken CRH has an 83% sequence homology with ovine CRH, but is identical to rat and human CRH (Vandenborne et al., 2005). Future avian studies involving CRH administration may, therefore, benefit from the use of rat or human, rather than ovine CRH (Vandenborne et al., 2005).

4.2. Are urban thrashers chronically stressed?

An objective of this study was to evaluate whether urban thrashers, which are exposed to multiple anthropogenic factors (e.g. pollution, vehicular disturbance, noise), have greater HPA axis activity, a possible indicator of “chronic stress,” than desert thrashers. If this is the case, we predicted urban thrashers to have higher initial CORT, a lower acute stress response, and a decreased and increased sensitivity to CRH and AVT injection, respectively than desert birds. Both populations had similar initial CORT, but urban birds secreted more CORT than desert ones in response to an AVT injection during the breeding season. Previously, we found higher initial CORT in desert compared to urban thrashers outside the breeding season, but breeding levels were equivalent (Fokidis et al., 2009). Here saline-injected desert birds had similar initial CORT levels to urban birds, and thus, initial CORT data alone do not support the hypothesis that urban thrashers are chronically-stressed compared to desert birds.

Another characteristic of “chronic stress” is a compromised negative feedback effect of CORT resulting in a reduced ability to regulate the HPA axis (Young et al., 1995; Dallman et al., 2004). In mammals, DEX inhibits CORT secretion by blocking the pituitary gland release of ACTH (Dekloet et al., 1975). Administering DEX decreased CORT secretion in response to capture stress equally in urban and desert thrashers. It is possible that the doses of DEX used here inhibited ACTH secretion completely, thus masking subtle differences in feedback between study populations. The lack of an obvious dose effect of DEX treatment on CORT levels partly supports this hypothesis, but future investigations would benefit from comparing a wider range of DEX doses to determine their potency to decrease

ACTH secretion. Population differences in stress responses may also stem from variation in neural or “upstream” processes instead of pituitary gland resistance to CORT feedback. In this case differences due to DEX treatment would not be expected. Similar initial plasma CORT levels, increased responsiveness to AVT in urban birds, and the presence of a robust negative feedback system, collectively suggest that urban thrashers are not “chronically stressed” compared to desert birds.

4.3. Evidence for habituation to city life?

Urban birds are likely exposed to two stressor types: 1) Short-acting stressors that birds may be able to avoid by modifying their behavior (e.g., human disturbance, vehicular traffic, and noise) and 2) environmental stressors, such as pollutants and warmer ambient temperatures than surrounding undeveloped areas, that are presumably unavoidable (French et al., 2008; Fokidis et al., 2009). Habituation to a stressor is thought to involve a down-regulated CORT response. For example, studies have found decreased HPA axis responses to capture stress in free-living Magellanic penguins (*Spheniscus magellanicus*) exposed to frequent tourist-related disturbances, compared to undisturbed birds (Fowler, 1999; Walker et al., 2006). Similarly in laboratory rodents, repeated or sustained exposure to the same stressor desensitizes the CRH, but not the AVP response (Ma et al., 1999). In field studies, habituation to a stressor may be difficult to detect, partly due to the difficulty in documenting responses to the same stressor over time (Cyr and Romero, 2009). The thrashers in this study were presumably naïve to capture stress and the act of injection alone did not alter their stress response. However, even when subjected to capture stress, which is likely perceived as a severe stressor, thrashers remained capable of further secreting CORT within 30 min in response to AVT or ACTH treatment. According to recommendations for detecting habituation in field-sampled animals (Cyr and Romero, 2009) this observation may represent a physiological limit on CORT secretion, which may suggest habituation of the HPA axis. However, an alternate interpretation is that under natural conditions, endogenous AVT and ACTH may take time to increase CORT levels where as exogenous peptides induce a change in CORT more rapidly. Another explanation may be that repeated activation of the HPA axis may result in a self-preserving “down-regulation” of CORT production (Rich and Romero, 2005). For both injections with ACTH and AVT, the CORT responses of urban birds were greater than that of desert thrashers and this mirrors results from previous results (Fokidis et al., 2009), however further studies are needed to determine whether such observations constitute habituation, active down-regulation, and if various urban stressors can induce different HPA responses.

4.4. Population differences in adrenal sensitivity to exogenous ACTH challenge

We used changes in plasma CORT after ACTH injection to assess the adrenal gland's capacity to secrete glucocorticoids. This capacity depends on several factors including: ACTH receptor density, the concentration of steroidogenic enzymes, the amount of steroidogenic adrenal tissue, and the clearance rate of plasma CORT (Carsia and Weber, 2000). A direct assay for measuring plasma ACTH levels in birds is currently unavailable. This necessitates the use of ACTH challenge studies to address questions about pituitary regulation of CORT secretion in free-living birds. Previous research using such ACTH challenge protocols has reported that many birds modulate their CORT secretion seasonally, but nonetheless can always respond to exogenous ACTH injection (Astheimer et al., 1994; Romero et al., 1998c; Romero and Wingfield, 1999; Sims and Holberton, 2000; Rich and Romero, 2001; Wilson and Holberton, 2001; Meddle et al., 2003). An injection of ACTH into urban and desert thrashers elevated their

plasma CORT above saline-treated and non-injected birds, indicating that capture stress does not induce maximum CORT secretion by the adrenal glands over a 30 min timeframe. It is unclear why birds do not secrete CORT at a maximum rate in response to capture stress and this raises the question of whether maximum CORT secretion during acute stress is even advantageous. Retaining the capacity to further increase CORT secretion may prepare an individual to respond to additional challenges or to inflammation, where prolonged HPA activation can be beneficial (Maxwell and Robertson, 1998; Brydon et al., 2009).

In a previous study, desert and urban thrashers had similar initial and stress-induced plasma CORT only during breeding (Fokidis et al., 2009). Outside the breeding season desert thrashers had higher initial CORT levels than urban birds, but also had a greater stress response than urban birds during molt (Fokidis et al., 2009). Here, ACTH administration increased plasma CORT more in urban than desert thrashers, suggesting differences in adrenal ACTH sensitivity. One potential explanation is that the pituitary glands of urban birds secrete less ACTH than desert birds under resting conditions, but the adrenal glands of urban birds are more sensitive to ACTH than those of desert birds. This situation would result in baseline CORT levels not differing between populations and in urban birds secreting more CORT than desert birds in response to the same ACTH dose. Thus differences between urban and desert thrashers may depend on an upstream process regulating ACTH production and/or release. This hypothesis is consistent with studies in seasonal birds that show that stress-induced CORT levels vary seasonally, but without concurrent change in adrenal sensitivity to ACTH (Romero et al., 1998c; Romero and Wingfield, 1998; Romero, 2006). These studies also reported seasonal changes in baseline CORT levels (Romero et al., 1998c; Romero and Wingfield, 1998), as has been previously noted in our species (Fokidis et al., 2009). Future studies aimed at elucidating how environmental disturbance induces specific changes along the HPA axis are warranted to elucidate the physiological processes that underlie natural variation in CORT secretion.

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