Corticosterone and corticosteroid binding globulin in birds: Relation to urbanization in a desert city

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ABSTRACT

As cities expand worldwide, understanding how species adapt to novel urban habitats will become increasingly important to conservation. The adrenocortical stress response enables vertebrates to cope with novel environmental challenges to homeostasis. We examined total and estimates of free baseline and stress-induced corticosterone (CORT) concentrations and CORT binding globulin (CBG) levels in five passerine species within and around Phoenix, Arizona. We tested whether baseline and stress-induced CORT patterns differed among species living at varying densities in Phoenix and tested the hypothesis that, for species capable of successfully colonizing cities, individuals living in urban areas have a decreased acute stress response compared to individuals living in native desert. Baseline total CORT levels were generally similar in urban and rural birds. Capture and handling stress typically produced greater total CORT responses in urban birds than in rural birds, although these responses differed as a function of the life history stage (non-breeding, breeding or molt). CBG binding capacity did not change with life history stage or locality. Estimated free CORT concentrations differed less between groups than total CORT concentrations. Urban birds showed less variability in stress responses across life history stages than rural birds. We propose that more predictable resources in the city than in rural areas may decrease the need to vary stress responsiveness across life history stages. The results highlight the species-specific effects of urbanization on stress physiology and the difficulty to predict how urbanization impacts organisms.

Published by Elsevier Inc.

1. Introduction

As urban landscapes expand and encroach onto natural environments, the ability of organisms to respond to anthropogenic disturbance becomes an increasingly important factor governing their future persistence. Whether an organism can thrive in cities may depend on its capacity to deal with the novel and potentially adverse challenges of urban living. Cities present numerous stressors including human disturbance (Heil et al., 2007; Fernandez-Juricic, 2001), traffic (Bautista et al., 2004), noise (Slabbekoorn and Peet, 2003), feral animal predation (Woods et al., 2003; Baker et al., 2005), introduced competitors (White et al., 2005), exposure to toxins (Eens et al., 1999), artificial lighting (Longcore and Rich, 2004), and warmer nighttime temperatures (McLean et al., 2005). The hypothalamic–pituitary–adrenal (HPA) axis enables vertebrates to cope with environmental challenges (Sapolsky et al., 2000). Particularly, the acute release of glucocorticoids is considered beneficial for surviving such challenges (McEwen, 1998; Sapolsky et al., 2000; Landys et al., 2006; Wingfield, 2006). However, prolonged or repeated exposure to stressors and elevated glu-

cocorticoids may have deleterious effects, including inhibition of growth (Belden et al., 2005; Hull et al., 2007), impaired immune function (McEwen et al., 1997; Owen-Ashley et al., 2004; Martin et al., 2005; French et al., 2006, 2007), cessation of reproduction (Moore and Jessop, 2003; Wingfield and Sapolsky, 2003; Lendvai et al., 2007), and hypertension (Amstislavsky et al., 2006; Djordjevic et al., 2007; Markel et al., 2007). Elevated baseline glucocorticoid levels may indicate chronic stress (McEwen, 2001; Dallman et al., 2006; Cyr et al., 2007). However, exposure to chronic stressors may also be associated with decreased secretion and/or increased clearance of glucocorticoids, resulting in reduced baseline concentrations (Rich and Romero, 2005; Cyr and Romero, 2007; see review: Herbert et al., 2006). Baseline glucocorticoids may also increase during some life history transitions, such as pregnancy, even in the absence of true "stressors" (Sapolsky et al., 2000; Landys et al., 2006).

Urban animals may down-regulate their HPA activity to avoid having chronically elevated glucocorticoid levels. Partecke et al. (2006) measured plasma levels of corticosterone (CORT), the predominant avian glucocorticoid, in European Blackbird (*Turdus merula*) nestlings from rural and urban areas. A common garden experiment demonstrated that urban-raised birds show suppressed stress responses to capture and handling compared with

rural-raised birds (Partecke et al., 2006). In White-crowned Sparrows (*Zonotricha leucophrys*), baseline plasma CORT levels decreased between urban and rural populations in males, but not females (Bonier et al., 2007). This sex-specific variation may reflect differences in the cost of elevated baseline CORT levels, since females with higher baseline CORT levels fledged fewer offspring whereas male reproductive success was independent of baseline CORT levels (Bonier et al., 2007). Bonier et al. (2007) did not report stress-induced CORT levels and thus whether intersexual differences in stress responsiveness exist is unclear. In another study, Florida Scrub-Jays (*Aphelocoma coerulescens*) inhabiting suburban areas had lower baseline CORT levels (Schoech et al., 2004) but mounted a greater acute stress response, than rural birds (Schoech et al., 2007). This opposite pattern of stress responsiveness may reflect differences in access to human food sources between suburban and rural Scrub-Jays, as birds fed high protein diets showed higher stress responsiveness than birds receiving a low protein diet (Schoech et al., 2004, 2007).

The arid southwestern United States experiences rapid urbanization but despite drastic change to the desert landscape, some birds have adjusted to urbanization and indeed, are often found at higher densities in cities than in native habitats (Green and Baker, 2003). In contrast to the Sonoran Desert, cities have year-round water available which may increase food resource predictability. Thus cities embedded in arid regions may buffer the seasonal patterns of native habitat (Shochat, 2004) and this may alter seasonal patterns of HPA activity. Birds breeding in habitats with seasonal fluctuation in resource availability and/or unpredictable weather patterns often show dampened stress responsiveness during the breeding and nesting periods compared to other phases of their annual cycle (Wingfield et al., 1998; Boonstra and McColl, 2000; Wingfield, 2005). This dampened stress responsiveness has been demonstrated in arctic-breeding birds (Wingfield et al., 1994a,b; Romero et al., 1997) and to a lesser extent in birds breeding in desert habitats (Wingfield et al., 1992). In these habitats reproduction is limited to narrow temporal windows of resource abundance and in deserts this is largely influenced by the timing and magnitude of precipitation (Vleck, 1984; Deviche et al., 2006; Small et al., 2007). The decreased seasonality of urban areas may be favorable for some desert bird species, and this may alter CORT patterns by diminishing the need to depress stress responsiveness during breeding.

Seasonal variation in plasma CORT has been documented for many species but less is known about seasonal variation in factors that regulate CORT (but see Breuner and Orchinik, 2001). CORT is a hydrophobic steroid that circulates in plasma largely bound to proteins (corticosteroid binding globulins, CBG; Rosner et al., 1991; Rosner, 2006). The "free hormone" hypothesis postulates that only unbound ("free") steroids interact with their receptors and are biologically active (Breuner and Orchinik, 2002). CBG binding capacity in birds may vary with life history stage (Silverin, 1986; Love et al., 2004), season (Breuner and Orchinik, 2001; Romero et al., 2006a,b), hormonal environment (Deviche et al., 2001), and in response to food restriction (Lynn et al., 2003), but currently the significance of these changes is largely speculative.

To date studies of the stress physiology of urban animals have focused on single species living in cities embedded in forested regions (Schoech et al., 2004, 2007; Bonier et al., 2007; Partecke et al., 2006). Collectively, these studies suggest that variation in stress physiology resulting from urbanization is species- and development type-specific. Another important consideration is how species differ with respect to their ecology (i.e. migratory versus resident species) or the length of time populations have been exposed to urbanization (i.e. recent versus old city). In this study, we measured baseline and stress-induced levels of total (CBG-bound and unbound) and free (unbound to CBG only) CORT in urban and rural populations of a suite of desert songbirds with varying population densities across the urban landscape of Phoenix, Arizona. Phoenix is undergoing a relatively recent city expansion compared to those studied previously (Partecke et al., 2006; Bonier et al., 2007) and may enable us to elucidate early adaptations in the urbanization of birds.

We hypothesized that CORT profiles of urban-adaptable species (Kark et al., 2007) would differ from those of species less adept at using city habitats. Specifically we tested three predictions: (1) urban-adaptable species have lower plasma total or free CORT in cities than in rural areas; (2) urban-adaptable species show lower total or free CORT stress responsiveness to capture and handling stress in urban than rural areas and (3) urban-adaptable species from urban areas exhibit less seasonal variation in stress responsiveness than rural birds. The study constitutes the first comparative investigation of stress physiology with reference to the effects of urbanization.

2. Materials and methods

2.1. Study species and sites

We measured CORT levels in adult male songbirds of five species: the House Sparrow (HOSP; *Passer domesticus*), a successful introduced human commensal; two native species from the family Mimidae: the Northern Mockingbird (NOMO; *Mimus polyglottos*) and the Curve-billed Thrasher (CBTH; *Toxostoma curvirostre*); and two native desert towhees: the Abert's Towhee (ABTO; *Pipilo aberti*) and the Canyon Towhee (CANT; *P. fuscus*). These species are common sedentary residents of central Arizona and the four native species are territorial throughout the year (Derrickson and Breitwisch, 1992; Tweit and Finch, 1994; Johnson and Haight, 1996; Tweit, 1996). In contrast, the socially gregarious HOSP has a distribution within Arizona that is entirely restricted to human-populated areas (Lowther and Cink, 1992). The adaptability of each species to urban Phoenix was assessed based on differences in mean bird density between rural and urban sites (Green and Baker, 2003). Using these densities, species were ranked ordinally (hereafter urbanization rank) from the most to the least urban-adaptable, as HOSP > NOMO > ABTO > CBTH > CANT. One species, CANT was only sampled in rural desert areas since it is only occasionally observed in suburban areas at the periphery of Phoenix. However we included data on CANT as it inhabits more xeric habitats than does ABTO and provides a useful comparison against rural populations of its more urban-adaptable congener.

Birds were sampled at various locations within metropolitan Phoenix. As high income neighborhoods support greater plant diversity than low income neighborhoods (Hope et al., 2003), our samples were randomly collected from high (19% of urban sites) and low income (33%) residential housing tracts as well as commercial areas (7%), business districts (10%), and city parks of various sizes (31%). These localities ("urban sites") were classified as representing Central, South, and East Phoenix, for subsequent statistical analysis. Birds were also sampled from three unpopulated Sonoran Desert localities southwest of Phoenix ("rural sites"). These were the Hell's Canyon, Sierra Estrella Wilderness Areas, and the Robbins-Powers Butte Wildlife Management Areas. The two former sites consist of vegetation characteristic of upland Sonoran Desert including: columnar cacti, cholla (*Opuntia* spp.), ocotillo (*Fouquieria splendens*), mesquite (*Prosopis* spp.), and palo verde (*Prosopis* spp.). The latter site borders the Gila River and vegetation cover is a diverse mixture of mesquite, saltbush (*Atriplex* spp.), creosote (*Larrea tridentata*), bursage (*Ambrosia* spp.), and willows (*Salix* spp.). This area also contains dense thickets of the invasive salt cedar (*Tamarix* spp.) and retired agricultural lands. Rural HOSP were sampled at two dairy farms belonging

to the Cone Ranch and located near Buckeye, Arizona. All localities are located no more than 25 km apart (between 33°18′N and 33°57′N, and 111°59′W and 112°41′W) and are described in Fokidis et al. (2008).

2.2. Bird sampling

Birds were caught passively with mist nets (HOSP), or lured into nets using conspecific song playback recordings (native species). Each sampling locality was sampled weekly from January to October 2006 and all captures took place between 0500 and 1130 h. A blood sample ($\sim$200 µl) was taken within 3 min of capture from the right jugular vein using a heparinized 0.3 ml syringe with a 29.5 gauge needle for a baseline CORT sample. The bird was then held in a cloth bag for 30 min, a standard paradigm for inducing a stress response through handling and restraint (Wingfield et al., 1994a,b), after which another blood sample was collected to determine stress-induced CORT levels. The total blood volume collected varied among species, but ranged from 300 µl in the HOSP to 500 µl for CBTH. Samples were kept on ice until plasma was separated by centrifugation and then stored at −80 °C until assayed.

Since our capture method relied on stimulating territorial behavior, only adult male birds were targeted. Males were identified either by plumage (HOSP), by the presence of a developed cloacal protuberance (CP) during the breeding season, or by unilateral laparotomy during the non-breeding season. During laparotomies testis length was measured to the nearest 1 mm (for procedure see Small et al., 2007; Fokidis et al., 2008). Male birds were considered in breeding condition if testis length exceeded half the maximum length known for each species (Fokidis, unpublished data). This threshold is based on the assumption that testes can produce sperm when at half-maximal volume (Partecke et al., 2004). Laparotomies were performed only after blood samples were taken so as not to influence CORT results. Age (hatch-year; after-hatch year) was determined using flight feather and rectrix characteristics (Pyle, 1997). Molting body feathers were also recorded. Hatch-year HOSP undergo a complete molt during their first year and were distinguished from molting after-hatch year males by their incompletely pneumaticized skull, partial juvenile plumage, and bill color. A black bill in this species is indicative of breeding condition (Lowther and Cink, 1992; Pyle, 1997). For all species, birds were classified as being in pre-breeding condition, breeding condition, or post-breeding condition (i.e., molting). Body mass (+0.1 g), tarsus length (+1 mm), and wing chord length (+1 mm) were also measured. Each bird received a uniquely numbered aluminum US Geological Survey leg band and was released at the capture site. All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee with necessary permits from the Bureau of Land Management, the Arizona Game and Fish Department, and the City of Phoenix Parks and Recreation Department.

2.3. Total corticosterone assay

Total CORT concentrations were quantified using commercial competitive enzyme-linked immunoassay kits (ELISA; Assay Designs Inc., Ann Arbor, Michigan, USA). This ELISA uses a polyclonal antibody with low cross-reactivity (<0.2%) with other steroids (manufacturer's specifications). There was no difference for any species between the slopes of a curve produced by serial plasma dilution (2- to 32-fold) and a standard curve (all $p > 0.3$). Samples were assayed following a ten times dilution with assay buffer. All samples were assayed in duplicate and distributed randomly across assay plates. However, paired samples from a given individual (baseline and stress-induced) were assayed on the same plate. The optical density of assay wells was measured at 405 nm with a microplate absorbance plate reader. Plasma CORT concentrations were calculated via interpolation from the standard curve on the respective plate using GraphPad Prism vers. 4 (GraphPad Software Inc.: San Diego, California, USA). The sensitivity of the assay calculated from two standard deviations from a zero standard, ranged from 5.8–16.3 pg/ml and the mean intrassay coefficient of variation was 8.46% ($n = 8$ plates; 312 samples).

2.4. Corticosteroid binding globulin assay

Radioligand binding assays for CBG were based on Orchinik et al. (2000) and Deviche et al. (2001) with minor modifications. CORT binding capacity was determined using plasma stripped of endogenous steroids by incubation with 1% Norit A charcoal coated with 0.1% dextran in assay buffer (50 mM Tris–acetic acid; pH 7.4) at 4 °C or room temperature for 10 or 15 min, depending upon species. The incubate was then centrifuged for 10 min, and the supernatant was collected and then diluted with assay buffer (final dilutions vary between species). Radiolabelled CORT (1,2,6,7–³H-CORT, specific activity 70 Ci/mmol; Perkin–Elmer Inc. Boston, Massachusetts, USA) was diluted in assay buffer and 50 µl of solution was dispensed into polypropylene tubes containing 50 µl of assay buffer (total binding) or of unlabeled competitor solution, and 50 µl of diluted steroid-free plasma. Nonspecific binding was determined in alternate samples using 3 µM of progesterone (P4), the hormone with the highest affinity for CBG (see results). Free and bound ³H-CORT were separated by rapid vacuum filtration using a Brandel (M-24) harvester using glass fiber filters (GF-B) soaked for one hr in 0.3% polyethylenimine. Filters were immediately rinsed three times with 3 ml of ice-cold 25 nM Tris–HCl buffer. The radioactivity in sample-soaked filters was measured by liquid scintillation, using a Beckman LS 6500 liquid scintillation β-counter (Beckman Coulter Inc., Fullerton, California, USA).

For each species, the equilibrium dissociation constant (K_d) and binding capacity (B_{max}) of CBG were determined by incubating plasma pooled from five individuals with increasing concentrations of ³H-CORT (1 to 43 nM). Competition studies were performed using 1 nM ³H-CORT and the following unlabeled steroids: CORT, P4, dexamethasone (DEX), testosterone (T), 5α-dihydrotestosterone (DHT), and 17β-estradiol (E2) at concentrations ranging from 10^{-6} to 10^{-10} M. For these studies, samples were incubated at 4 °C for 1 or 2 h, depending upon species. Estimates of potency (EC50) derived from competition experiments were converted to inhibition constants (K_i) using the equation of Cheng and Prusoff (1973). Binding parameter estimates were calculated using nonlinear regression models based on the law of mass action (GraphPad Prism vers. 4). Comparisons of best-fit nonlinear regression models were done using two-tailed Student's t-tests with GraphPad Prism.

The CBG binding capacity of individual plasma samples was estimated using single point ³H-CORT binding assays, run in duplicate. Samples (10 µl plasma) were incubated with a total concentration of CORT equivalent to ten times the K_d to ensure that binding sites were nearly 90% saturated. To avoid using excessive amounts of radioactivity, this concentration was achieved using one part ³H-CORT to five parts unlabelled CORT. The raw data were converted to B_{max} by adjusting for percent saturation and the diluted specific activity of the radioligand. The B_{max} values from single point assays and the species-specific K_d values derived from pooled samples were then used to estimate free CORT concentrations in plasma using the equation of Barsano and Baumann (1989).

2.5. Statistical methods

Differences between bird populations were compared using four-way analysis of variance (ANOVA) with species, urbanization

rank, locality, and life history stage as independent factors. Both baseline and stress-induced total CORT levels were included as dependent variables in the same model. Other four-way ANOVA models were used to analyze free CORT (baseline and stress-induced) levels and CBG binding capacity with the same dependent variables included. Each model also included the following interactions: Species rank × locality × life history stage; species × locality × life history stage. Unequal sample sizes resulted in an unbalanced design and the general linear model, therefore, used a type III sum of squares. To satisfy assumptions of normality and homogeneity of variance, CORT and CBG concentrations were log-transformed prior to analysis. Tukey's honest significant difference (HSD) post-hoc tests were used to identify significant interactions between species, life history stage, and localities.

Comparative studies present the issue of phylogenetic autocorrelation, i.e., variation in traits that is constrained within phylogenetic groupings (Felsenstein, 1985). Although this needed to be tested for, the use of an unbalanced ANOVA design prevented the addition of nested terms (family or genus nested within family) in the model (Sokal and Rohlf, 1995). A separate analysis was done using a three-way ANOVA with the same independent variables as above but with family and genus (nested within family) as factors, and including three-way interactions with locality and life history stage. Nested ANOVA terms do not provide sufficient resolution to determine evolutionary patterns in the context of a cladistics-based phylogeny (Felsenstein, 1985). However, this type of ANOVA determines whether significant variation in the dependent variable is explained solely by taxonomic information (Felsenstein, 1985).

To examine whether seasonal variability in the stress response differed between urban and rural populations, we calculated the proportional increase in CORT above baseline levels for all individuals. These data were then tested using Levene's test of homogeneity of variances, to test for differences in variability between urban and rural birds (see Orlando and Guillette, 2001). These differences were expressed as a percent coefficient of variation (% cv) for each urban and rural population across all seasons for each species and for both total and free CORT. As we only had data for rural populations CANT was excluded from analysis. To compensate for multiple comparisons, we used a Bonferroni corrected alpha-level of 0.01. All statistical analyzes were done using SPSS version 13.0 (2004) and all tabular and graphical data are presented as mean + standard error (SE).

3. Results

Within species, birds captured at various rural or urban localities had similar baseline and stress-induced CORT and individuals were, therefore, classified for subsequent analyzes as "urban" or "rural". Phylogeny (family or genus nested within family) did not significantly influence locality or seasonal patterns of total or free levels of either baseline or stress-induced CORT (all $p \geqslant 0.16$) or CBG binding capacity (all $p \geqslant 0.08$).

3.1. CBG binding properties

In all species, specific and nonspecific binding of ^{3}H-CORT decreased linearly as plasma dilution increased (data not shown), and heating plasma samples for 15 min at 92 °C to denature proteins eliminated specific binding. Specific binding decreased significantly after three but not one or two freeze-thaw cycles (data not shown). Specific binding remained relatively constant with increasing incubation temperatures (4, 21, and 46 °C) and was always less than 7% of total binding. For kinetic studies we incubated pooled plasma samples with 1 nM ^{3}H-CORT at 4 °C in the presence or absence of 3 μM P_4 for 1–240 min. Specific binding of ^{3}H-CORT in-

creased rapidly with incubation time until a plateau was reached at around 20 min. This time course was consistent across species, although specific binding was decreased by 16% in NOMO after 240 min of incubation. The observed rate constants (k_{obs}) were similar between species, ranging from 0.36 ± 0.07 to 0.58 ± 0.03 min^{-1}. Based on these data, we terminated the binding reactions after 120 min in subsequent equilibrium studies.

In each species, equilibrium saturation binding data were consistent with a single population of high affinity binding sites. There were differences in the affinity of CBG for CORT (K_d) and binding capacity (B_{max}) between species (Table 1). Titration experiments using pooled plasma samples and unlabeled steroids indicated that P_4 competed with ^{3}H-CORT binding sites with the highest affinity, and E_2 competed with ^{3}H-CORT binding sites with the lowest affinity in all species (Table 2). The potencies of other steroids to compete for ^{3}H-CORT binding sites are presented in Table 2. For a random subset of samples ($n = 9$) for each species we tested whether CBG binding capacity changed within 30 min of capture and restraint. CBG binding capacity did not change following capture and restraint for any species ($t \leqslant 0.96$, $n = 9$, $p \geqslant 0.12$), and baseline and stress-induced CBG levels were correlated within species (Spearman's $r \geqslant 0.68$, $p \leqslant 0.03$).

3.2. Patterns of total CORT secretion in urban and rural birds

Baseline total CORT levels did not differ with respect to any factors under consideration (Table 3). Thirty minutes of handling and restraint significantly elevated total CORT concentrations in all species (Fig. 1). Stress-induced levels of total plasma CORT were influenced by life history stages and differed between urban and rural populations (Table 3). Furthermore there was a significant interaction between species, locality, and life history stage on stress-induced total CORT levels (Table 3).

Post-hoc tests revealed that stress-induced total CORT levels were significantly lower in rural than urban ABTO, but only in non-breeding and molting birds (Fig. 1). Baseline total CORT was significantly lower in breeding than non-breeding and molting CANT (Fig. 1). Baseline and stress-induced total CORT levels were significantly lower in CANT than in rural or urban ABTO.

Urban CBTH had significantly lower baseline total CORT than rural counterparts, except when in breeding condition (Fig. 1). During breeding rural CBTH birds had lower stress-induced total CORT levels than urban birds (Fig. 1). Baseline total CORT was higher in rural non-breeding than in urban non-breeding or breeding NOMO (Fig. 1). Rural NOMO had significantly lower baseline total CORT levels than rural CBTH, but baseline total CORT did not differ between urban NOMO and urban CBTH (Fig. 1).

Rural breeding HOSP had significantly lower baseline total CORT levels than urban birds (Fig. 1) but rural breeding HOSP had higher stress-induced total CORT than urban HOSP (Fig. 1).

Table 1
Equilibrium dissociation constants (K_d) for ^{3}H-CORT binding to CBG and CBG binding capacity (B_{max}). Equilibrium saturation binding assays were performed using pooled plasma for five species of birds inhabiting the Sonoran Desert. Different superscript letters indicate statistical differences at $p < 0.05$.

Species	K_d (nM)	B_{max} (nM)
ABTO	5.97 ± 0.89[a]	25.47 ± 0.95[a]
CANT	8.32 ± 1.71[b]	38.11 ± 2.28[a]
NOMO	6.58 ± 0.76[a]	80.53 ± 2.43[b]
CBTH	6.13 ± 0.78[a]	42.05 ± 1.36[a]
HOSP	5.10 ± 0.62[a]	60.15 ± 1.71[c]

ABTO—Abert's Towhee; CANT—Canyon Towhee; NOMO—Northern Mockingbird; CBTH—Curve-billed Thrasher; HOSP—House Sparrow.

Table 2
Species comparisons of CBG specificity profiles. Inhibition constants (K_i) (means + SE derived from goodness of fit curves to data) and% inhibition at 1 μM for steroid inhibition of ^{3}H-CORT specific binding. Assays were performed using pooled plasma for each of five species of birds inhabiting the Sonora Desert. See table 1 for species abbreviations. Within species, different superscript letters indicate statistical differences at $p < 0.05$.

	ABTO		CANT		NOMO		CBTH		HOSP	
	K_i (nM)	%	K_i (nM)	%	K_i (nM)	%	K_i (nM)	%	K_i (nM)	%
CORT	8.15 + 1.23[a]	85	6.49 + 1.27[a]	82	7.83 + 1.20[a]	89	5.82 + 1.10[a]	92	5.51 + 0.96[a]	91
DEX	6.97 + 1.25[a]	85	7.18 + 1.13[a]	82	5.14 + 1.24[a]	92	7.40 + 1.01[a]	93	6.43 + 1.00[b]	85
T	22.88 + 1.07[b]	86	19.02 + 1.10[b]	81	17.37 + 1.21[b]	93	15.97 + 1.05[b]	95	14.61 + 1.01[c]	90
DHT	41.37 + 1.10[b]	87	43.46 + 1.04[c]	80	23.43 + 1.05[b]	93	25.97 + 0.97[c]	92	24.62 + 0.87[d]	89
P_4	4.23 + 1.07[c]	90	5.56 + 1.16[d]	93	3.07 + 1.22[c]	95	4.13 + 1.04[d]	97	2.78 + 0.92[e]	91
E_2	1382.06 + 37.78[d]	43	1882.16 + 73.57[e]	47	2108.49 + 23.43[d]	27	387.89 + 1.41[e]	26	114.03 + 1.03[f]	47

Corticosterone (CORT); Dexamethasone (DEX); Testosterone (T); 5α-Dihydrotestosterone (DHT); Progesterone (P_4); Estradiol (E_2).

Table 3
Summary of results for an analysis of variance (ANOVA) examining the effects of species, urbanization rank, locality (urban vs. rural), life history stage (non-breeding, breeding, or molt), and relevant interaction terms on patterns of total and estimated free plasma corticosterone (CORT) concentrations and CORT binding globulin (CBG) in five passerine species in the Sonoran Desert. See text for variable definitions.

Factor	Baseline				Stress				Binding Capacity			
	Type III SS	df	F	P	Type III SS	df	F	P	Type III SS	df	F	P
	Total CORT								CBG			
Species	245.94	3	1.33	0.266	1756.59	2	1.41	0.248	130889.6	3	7.82	0.001[b]
Rank (Urbanization)	86.51	3	0.47	0.626	76.53	2	0.06	0.941	6906.55	3	0.41	0.663
Locality (urban vs. rural)	84.45	1	0.92	0.340	1983.33	1	3.18	0.008[b]	62733.58	1	7.49	0.069
Life history stage	115.26	2	0.63	0.537	6196.54	2	4.97	0.008[b]	8729.24	2	0.52	0.595
Species × Locality × Life history stage	245.94	1	1.33	0.266	1756.59	1	3.41	0.025[a]	130889.6	1	1.81	0.057
Rank × Locality × Life history stage	86.51	1	0.47	0.626	76.53	1	0.06	0.941	6906.55	1	0.41	0.663
Error	15305.8	166			103577.88	166			1381625	165		
	Free CORT											
Species	1.22	3	1.3	0.276	6.51	3	0.01	0.987				
Rank (Urbanization)	1.71	3	1.81	0.168	28.28	3	0.06	0.944				
Locality (urban vs. rural)	0.01	1	0.02	0.896	1489.87	1	6.04	0.015[a]				
Life history stage	0.98	2	1.04	0.356	2623.35	2	5.32	0.006[b]				
Species × Locality × Life history stage	1.22	1	1.3	0.276	6.51	1	5.01	0.010[b]				
Rank × Locality × Life history stage	1.71	1	1.81	0.168	28.28	1	0.06	0.944				
Error	77.9	165			40676.94	165						

[a] Significant at $p < 0.05$.
[b] Significant with Bonferroni correction ($p < 0.01$).

No other significant differences in total baseline or stress-induced CORT levels were observed for any species.

3.3. Variation in CBG binding capacity and free CORT concentrations

CBG binding capacity varied between species but there was no significant interaction between CBG binding capacity and other independent variables (Table 3). Molting CBTH from urban areas had lower CBG levels than molting CBTH from rural areas or breeding birds from either habitat (Table 4). In addition, urban non-breeding ABTO and rural molting ABTO had lower CBG levels than rural breeding conspecifics (Table 4). Molting HOSP from urban areas had significantly lower CBG levels than HOSP from rural areas or those at other life history stages (Table 4). No other significant differences in CBG binding capacity were observed.

CBG binding capacity estimates were used to calculate free ("unbound") CORT concentrations at each life history stage (Fig. 2). Baseline free CORT levels were not influenced by any independent variable (Table 3). However, stress-induced free CORT levels were influenced by life history stage, locality, and the interaction of species, locality, and life history stage (Table 3). CBG binding capacity was correlated with baseline total CORT in three species (Spearman correlations; NOMO: $r = 0.53$, $p = 0.048$; ABTO: $r = 0.64$, $p = 0.038$; CANT: $r = 0.51$, $p = 0.041$), but not in others (CBTH: $r = 0.29$, $p = 0.183$; HOSP: $r = 0.19$, $p = 0.80$). CBG binding capacity was not correlated with baseline free CORT concentration (all $p > 0.08$).

Urban CBTH had higher stress-induced free CORT than rural CBTH during molt, but the opposite was observed during the breeding season (Fig. 2). Rural NOMO had lower stress-induced free CORT than urban NOMO (Fig. 2). Urban ABTO also had significantly elevated stress-induced free CORT levels during the non-breeding season, compared to other life history stages or to levels in rural conspecifics (Fig. 2). Rural ABTO had lowest stress-induced free CORT levels during molt (Fig. 2). Baseline and stress-induced free CORT levels were higher in rural than urban HOSP, but only during breeding (Fig. 2). No other significant differences in baseline or stress-induced free CORT levels were observed for any species.

3.4. Seasonal variability in CORT concentrations

The stress response, based on total CORT levels, was significantly less variable in urban birds than rural for three of the four species compared (ABTO: urban 16% cv, rural 21%, $p = 0.023$; NOMO: urban 22%, rural 31%, $p = 0.039$; HOSP: urban 19%, rural 34%, $p = 0.001$; CBTH: urban 12%, rural 15%, $p = 0.067$). However, the stress response, determined by changes in free CORT levels was more variable in urban ABTO (urban 33%, rural 11%, $p = 0.008$), CBTH (urban 33%, rural 26%, $p = 0.005$), and NOMO (urban 40%, rural 18%, $p = 0.048$) than rural conspecifics. By contrast, the free CORT stress responses of urban HOSP were less variable than rural birds (urban 16%, rural 21%, $p = 0.001$).
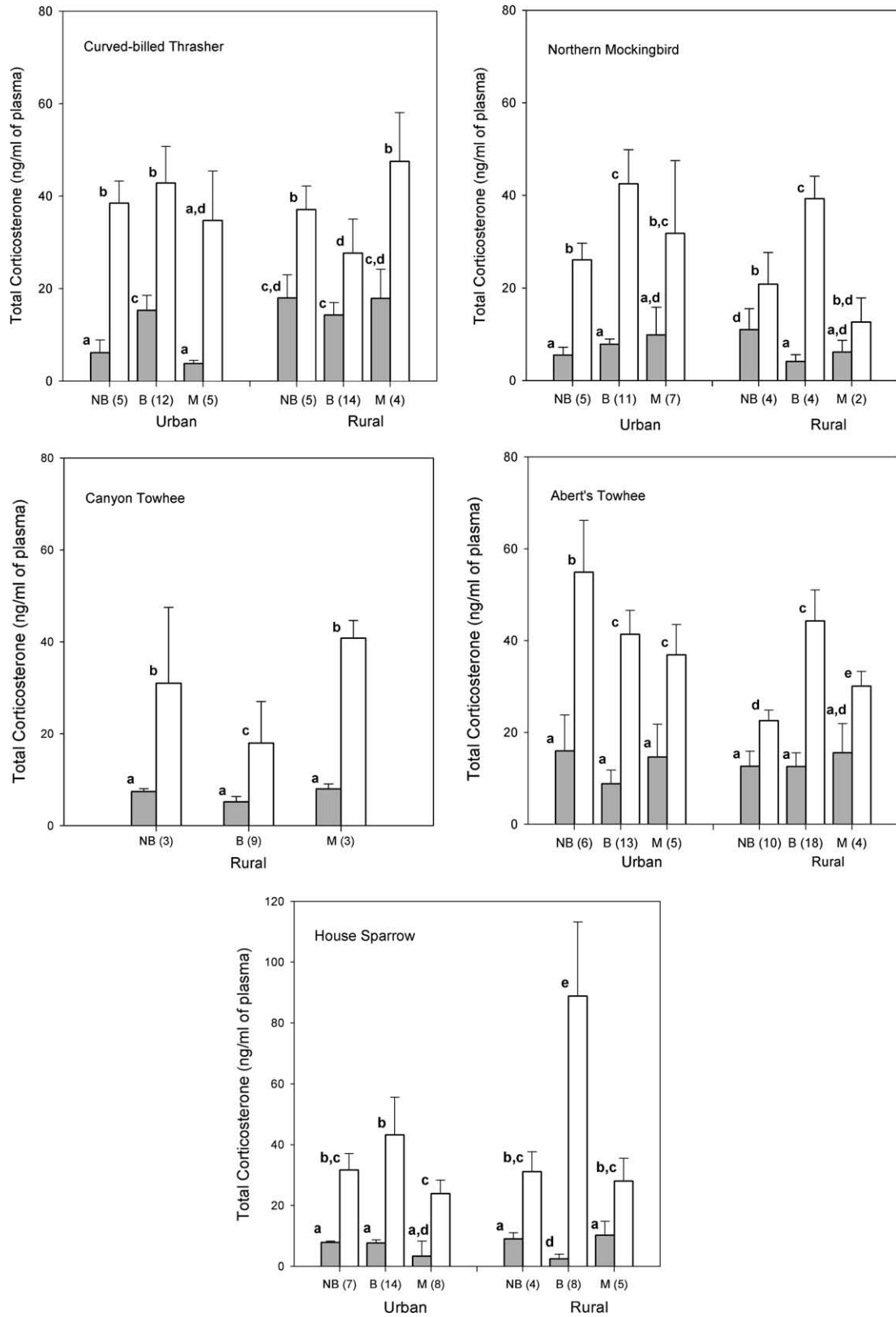
Fig. 1. Baseline (*gray bars*) and stress-induced (*white bars*) total plasma corticosterone (ng/ml of plasma) in urban and rural populations of five species across three life history stages; NB = non-breeding, B = breeding, M = molting. Numbers in brackets indicate sample sizes and bars sharing the same letter are not significantly different at the $p < 0.05$ level.

4. Discussion

Urban encroachment on native habitats poses unique and potentially "stressful" challenges to the animals that attempt to ex-

ploit these novel habitats. We investigated whether CORT levels differed between urban and rural populations of five bird species that vary in their abundance in the city of Phoenix. We predicted that urban-adaptable species would display lower plasma baseline

Table 4
Binding capacity of corticosteroid binding globulin (CBG) in urban and rural populations of five Sonoran Desert bird species across three life history stages. *n* indicates sample size and data is presented as mean ± standard error. Within species, rows sharing the same letter do not differ at the *p* < 0.05 level. See Table 1 for species abbreviations.

Species	Life history stage	Rural		Urban	
		n	Binding capacity	*n*	Binding capacity
ABTO	Non-breeding	10	491.34 ± 14.61[a,b]	6	441.75 ± 23.42[a]
	Breeding	18	510.40 ± 17.21[b]	13	486.95 ± 28.19[a,b]
	Molt	4	437.38 ± 17.09[a]	5	495.91 ± 13.64[a,b]
CANT	Non-breeding	3	327.58 ± 19.13[a]		
	Breeding	9	317.08 ± 15.65[a]		
	Molt	3	296.55 ± 21.02[a]		
NOMO	Non-breeding	4	197.94 ± 5.14[a]	5	213.38 ± 6.04[a]
	Breeding	4	215.43 ± 9.44[a]	11	199.31 ± 7.64[a]
	Molt	2	220.52 ± 12.39[a]	7	220.30 ± 8.81[a]
CBTH	Non-breeding	5	339.64 ± 13.94[a,b]	5	358.85 ± 36.01[a,b]
	Breeding	14	373.75 ± 22.51[a]	12	382.02 ± 23.08[a]
	Molt	4	401.18 ± 17.40[a]	5	330.43 ± 25.33[b]
HOSP	Non-breeding	4	419.50 ± 34.75[a]	7	368.53 ± 32.02[a]
	Breeding	8	375.98 ± 32.75[a]	14	414.22 ± 40.73[a]
	Molt	5	350.76 ± 32.42[a]	8	226.01 ± 31.23[b]

CORT levels and a suppressed CORT response to an acute stressor than less urban-adaptable species. We found that neither adaptability to urbanization nor phylogenetic relationships predicted total and free CORT levels or CBG concentrations. However, we detected species-specific differences in both total and free CORT levels between urban and rural birds.

### 4.1. Urban–rural differences in baseline CORT concentration are species-specific

Baseline total CORT levels did not differ between urban and rural populations of three of the four species compared. In other species (Florida Scrub-Jays: Schoech et al., 2004; Tree Lizards, *Urosaurus ornatus*: French et al., 2008), urban populations had lower baseline total CORT than rural populations, but the opposite pattern was observed in male, but not female, White-Crowned Sparrows (Bonier et al., 2007). Data on baseline CORT levels can be difficult to interpret, but generally reflects circulating "unstressed" levels prior to exposure to an acute stressor (McEwen, 2001; Romero and Reed, 2005; Dallman et al., 2006; Cyr et al., 2007). Variation in baseline CORT levels may be associated with permissive actions of CORT (Sapolsky et al., 2000), particularly the mobilization of energy during specific predictable physiological changes associated with particular life history stages (Landys et al., 2006). Thus differences in baseline CORT levels between urban and rural birds during similar life history stages may represent differences in HPA activity, such as repeated stimulation associated with chronic stress.

Urban non-breeding and molting CBTH had lower plasma CORT than rural birds during the same sampling periods, but this difference did not persist when we examined baseline free CORT levels. Interestingly a closely related species (NOMO) did not show a similar difference between urban and rural populations, and phylogeny did not explain patterns of CORT concentration across urban–rural comparisons. The observations in CBTH suggest that these birds in urban areas are less "chronically stressed" than rural birds, but data must be interpreted with caution due to low repeatability in sampling (Romero and Reed, 2008), diurnal variation (Breuner et al., 1999), and natural fluctuations associated with different life history stages (Landys et al., 2006). Another factor that may contribute to decreased baseline CORT levels is attenuation of the HPA axis activity resulting from repeated exposure to acute stressors (Rich and Romero, 2005).

4.2. The hormonal stress response is not suppressed in urban birds

We predicted that urban birds in Phoenix are repeatedly exposed to acute stressors and this may ultimately lower their glucocorticoid responses to stress. Urban animals are usually less wary and more approachable than their rural counterparts (Blumstein, 2006; Campbell, 2006; Rose and Nagel, 2006; Tarlow and Blumstein, 2007). The urban birds in this study are also superficially "tamer" (i.e., more closely approached by humans) than their rural counterparts (Fokidis, *personal observation*). In addition, European Blackbird nestlings from urban areas show decreased responses to the stress of capture and handling than rural conspecifics (Partecke et al., 2006). Novelty elicits a robust HPA response in laboratory rodents, but repeated exposure to a same stimulus can decrease HPA axis responsiveness (Bhatnagar and Dallman, 1998; Hazard et al., 2008). The desensitization that occurs in response to repeated exposure to a same stimulus can result in low wariness or tameness and may be mediated by a dampening of the CORT stress response (Berger et al., 2007; Rodl et al., 2007) even though robust stress responses can be elicited in response to a different stressor (Bhatnagar and Dallman, 1998; Armario et al., 2004a,b; Hazard et al., 2008). We did not find evidence for suppressed CORT responses or HPA desensitization in urban birds with the exception of breeding HOSP: In this species, rural birds had more robust stress responses than urban birds. Although urban birds may be accustomed to human approach, capture and handling likely represent a novel experience to which they have not been previously exposed. Alternately, "tameness" may be mediated not directly by glucocorticoids but by other factors, such as changes in the activity of the sympathetic nervous system.

In three study species (CBTH, NOMO, and ABTO) urban dwellers mounted more robust HPA axis responses than rural birds, which opposes our initial prediction. One hypothesis for explaining the increased HPA responsiveness of urban birds involves the ability to locate and use urban food sources (Chace and Walsh, 2006; Mennechez and Clergeau, 2006; Anderies et al., 2007; Schoech et al., 2007; Fuller et al., 2008). Elevated CORT may serve to mobilize energy needed for dealing with a perceived stressor, but the stress response itself can be influenced by the energetic state of an individual. Birds with greater energy stores (i.e., better body condition) may mount greater stress responses than those with fewer energy stores (Lynn et al., 2003; Landys et al., 2006; Schoech et al., 2007). Free-living birds supplemented with food show stronger CORT responses to acute stress than non-supplemented birds
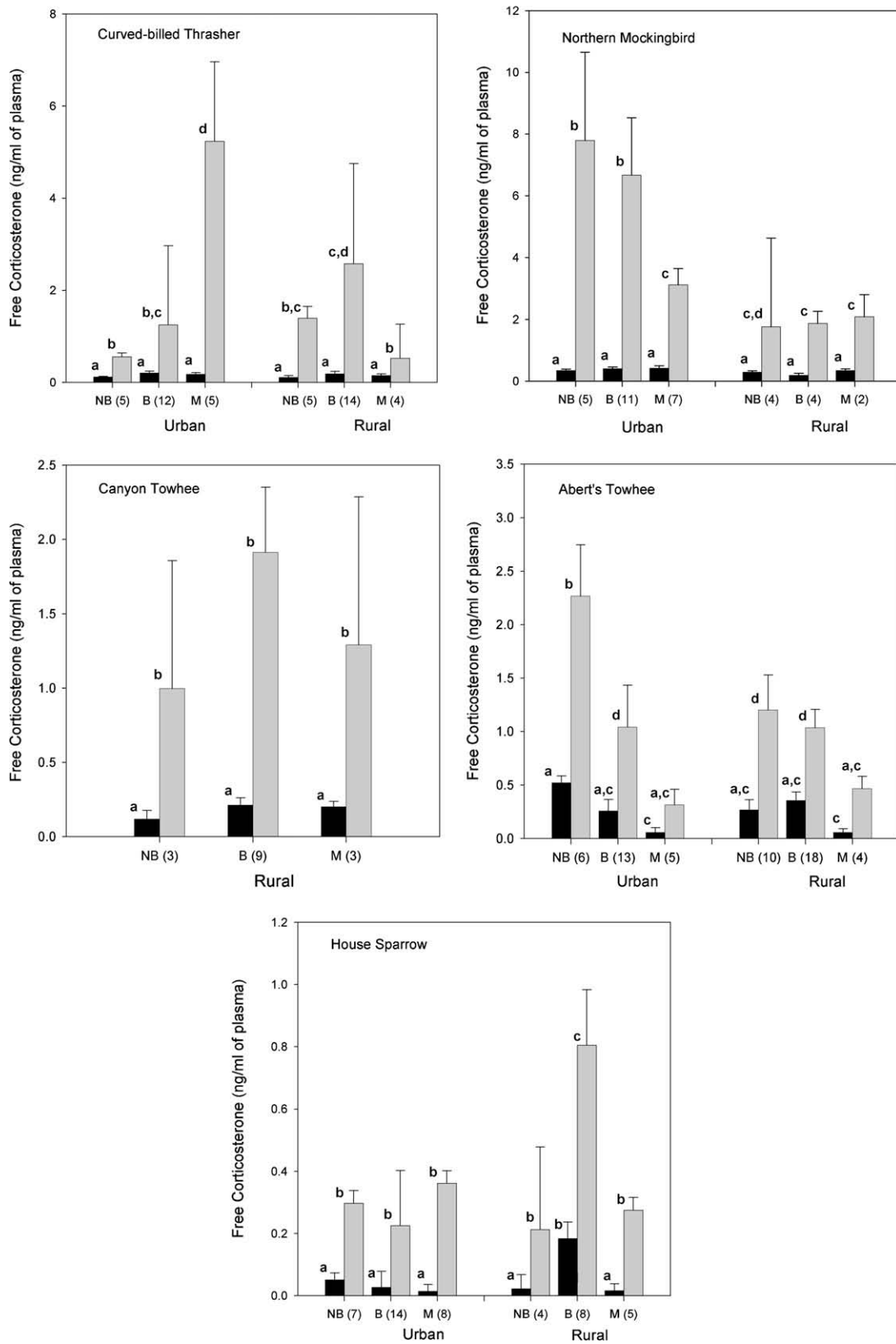
Fig. 2. Baseline (*black bars*) and stress-induced (*gray bars*) free (i.e., unbound to corticosteroid binding globulin) plasma corticosterone (ng/ml of plasma) in urban and rural populations of five species across three life history stages; NB = non-breeding, B = breeding, M = molting. Numbers in brackets indicate sample sizes and bars sharing the same letter are not significantly different at the $p < 0.05$ level.

(Schoech et al., 2007). In our study, birds from urban areas may have access to predictable food resources, enabling them to maintain a robust stress response that may be advantageous in the urban environment.

Several results in this study support the hypothesis that food resource differences between urban and rural habitats may explain differences in CORT levels. Urban CBTH, and to a lesser extent NOMO, mounted substantial stress responses during molt, whereas rural conspecifics showed decreased stress responses during this period. In many birds, molt is associated with decreased stress responsiveness (Romero et al., 2005, 2006a,b; Desrochers et al., 2006; Strochlic and Romero, 2008), which may relate to the fact that elevated CORT may interfere with protein deposition into feathers (Romero, 2002). However, whether short-term increases in CORT in response to stressors impact molt is unclear, as manipulating CORT levels did not have a clear effect on feather growth rate or quality (Strochlic and Romero, 2008). However, robust stress responses during molt may be indicative of a decreased need to restrict CORT secretion, possibly due to increased protein resources for urban birds.

Further evidence for a role of food resources stems from stronger stress responses in rural breeding HOSP compared to urban conspecifics. Rural HOSP were sampled at dairy ranches and these birds have access to abundant food that may enable them to maintain a high body condition (Fokidis et al., 2008). High body condition may in turn enable lipolytic and proteolytic actions of CORT to continue without compromising current energy stores.

4.3. Cities as buffers against seasonal resource availability

The Sonoran Desert is a seasonal environment where timing of precipitation is the primary factor driving life histories (Webb et al., 1978; Vleck, 1993). This desert is characterized by biannual rainfall, but the timing, extent, and amount of precipitation vary annually, resulting in temporally unpredictable food resources. In habitats where breeding opportunities are few and reproduction must continue despite environmental perturbations, some species down-regulate their stress responses, presumably to avoid deleterious energy expenditure and behaviors inhibiting reproduction (Wingfield et al., 1998). This down-regulation is observed in high latitude breeding species (Wingfield et al., 1995; Boonstra et al., 2001; Meddle et al., 2003; Boonstra, 2004; Holberton and Wingfield, 2003) as well as in Sonoran Desert passerines (Wingfield et al., 1992). In this study, we sampled birds at three life history stages: non-breeding, during breeding, and during molt, to determine whether urban birds show decreased seasonal variation in the stress response, compared to those from seasonal rural habitats. In considering rural samples, we observed lower stress-induced total CORT during breeding, compared to non-breeding in those species from open desert areas (CANT and CBTH). In contrast, species from more mesic "riparian" habitats (ABTO and NOMO) had higher stress-induced total CORT levels during breeding compared to other stages. Taken together, these data partially support the hypothesis that HPA axis responsiveness decreases during breeding in species that inhabit xeric "open desert" environments, as suggested by Wingfield et al. (1992).

In contrast to undeveloped desert environments, urban areas embedded within arid regions may provide year-round sources of food, shade, and water. Thus, cities effectively constitute mesic areas that buffer the stochasticity of native environments. Individuals of species that inhabit urban areas display earlier breeding dates (Antonov and Atanasova, 2003; Schoech and Bowman, 2003; Partecke et al., 2004, 2005; Beck and Heinsohn, 2006) and decreased propensity for migration (Partecke and Gwinner, 2007) relative to conspecifics in rural locations. Together these studies support the hypothesis that cities may act

as temporally stable environments with decreased fluctuations in resource abundance.

During the non-breeding period only urban ABTO had an increased total CORT stress response compared to rural birds. This sampling period coincides with winter conditions, characterized by mild daytime (18–24 °C) and cool nighttime temperatures (5–10 °C). Territorial behavior at this time is at its lowest seasonal intensity in all species (Fokidis, unpublished data), and ABTO often exhibit limited flocking behavior (Tweit and Finch, 1994). No study has examined food availability to ABTO in the Sonoran Desert, but more abundant winter resources available to urban than rural ABTOs may enable a stronger stress response to be maintained.

In each species, the breeding season coincides with the onset of winter precipitation, the timing of which varies from one year to another. During the course of this study the winter rains began in late March and birds were in breeding condition in early April (Fokidis et al., 2008). For one species (CBTH), urban birds did not suppress their total CORT response to acute stress during breeding, unlike their rural conspecifics. This may be interpreted as evidence for the suppression of the stress response during breeding in unpredictable habitats being buffered by the urban environment, may be as a result of resource predictability. This hypothesis however remains untested.

During molt, feather replacement is thought to necessitate a reallocation of energy and nutrient resources, particularly amino acids (Murphy and King, 1992). Studies in several avian species have demonstrated decreased stress responses during molt, and modulation of CORT may lessen protein degradation and inhibition of protein synthesis (reviewed by Romero, 2002). In our species, molt occurs in late summer and early fall. The timing of molt coincides with that of the summer monsoon, when food resources become more abundant. Rural CANT and CBTH showed substantial total CORT stress responses during this period, and in the former species the magnitude of the response was greater than at other times. Increased resource availability during the monsoon may partly offset the energetic costs associated with molt, thus permitting maintenance of a stress response in xeric species. The total CORT stress response was more robust in urban ABTO, CBTH, and to a lesser extent NOMO than in rural conspecifics. Resource availability during molt may provide a similar benefit to urban birds as it does in desert birds, but this question requires further study.

Data during non-breeding and molt periods were difficult to obtain for some species and this limitation in our sample sizes for some comparisons should be considered when interpreting results. To minimize the possibility of type I error and to better enable us to assess differences in the variability of the stress responses between habitats we also used tests for equality of variances between urban and rural birds. Although seasonal variation in the stress response differed between species, variation in the total CORT stress response was less in urban birds than in rural conspecifics for three of four species (ABTO, NOMO, and HOSP). This may be interpreted as reflecting the fact that the city constitutes a more stable environment than the native desert, resulting in a decreased need to modulate the activity of the HPA axis. Food availability may be a factor determining differences between the urban and rural environments, but decreased predation pressure in urban areas and year-round water availability may also play a role. However, it is important to n d on free CORT levels. For three species (ABTO, CBTH, and NOMO), greater variability in the stress response was observed in urban birds compared to rural ones, where as for HOSP the data were consistent with observations based on total CORT.

4.4. Free versus total plasma CORT levels

The free hormone hypothesis postulates that CBG modulates cellular responses to CORT because only free steroids have access

to intracellular receptors in target cells and can initiate action (Rosner et al., 1991; Rosner, 2006). We estimated that between 0.2 and 18% of total CORT was unbound to CBG and the binding kinetics, specificity, and affinities of CBG were similar across species, lending support to a conserved binding globulin among passerines. There were species differences in the binding capacity of CBG, but B_{max} estimates did not vary with life history stage or locality. In some species, CBG binding capacity varies with season, subspecies, nutritional state, and endocrine status (Breuner and Orchinik, 2001; Deviche et al., 2001; Lynn et al., 2003; Hattori et al., 2004; Seabury and Breuner, 2005; Wada et al., 2006). Binding capacity decreased during molt in urban HOSP, but not during the non-breeding season, coincident with winter, as found by Breuner and Orchinik (2001). There were few statistically significant changes in CBG binding capacity, but small changes in B_{max} resulted in free CORT estimates that either negated (CANT and NOMO) or amplified (urban CBTH) differences in total CORT at different life history stages. Thus, changes in binding capacity were sufficient to alter CORT profiles, supporting the hypothesis that CBG alters CORT bioactivity. Free CORT patterns across life history stages often differed from total CORT patterns. In NOMO, CANT, and ABTO, baseline total CORT levels were correlated with CBG binding capacity, suggesting that baseline CORT up-regulates CBG expression, as reported in some species (Romero et al., 2008; Shultz and Kitaysky, 2008) but not others (Cyr et al., 2007; Ward et al., 2007).

CBG may buffer the actions of environmentally-induced increases in CORT, thereby attenuating potentially deleterious actions of high CORT levels. Alternately, a decrease in CBG levels may increase the amount of CORT available to tissues, thus enhancing tissue responsiveness to CORT with chronic or repeated stressors. Petersen et al. (2006) demonstrated decreased total CORT production, increased adrenocorticotropin hormone secretion from the pituitary, and decreased CORT target gene expression in CBG-deficient mice compared to wildtype controls. These results were consistent with a HPA axis that was hyporesponsive to CORT, suggesting an active role for CBG in mediating HPA activity. Within species, we did not observe differences in CBG between urban and rural birds, or across life history stages and thus limit our ability to infer the status of HPA responsiveness from CBG data. However, comparisons of populations that differ in patterns of CORT secretion may provide opportunities to elucidate roles for CBG in mediating CORT responses to environmental changes such as urbanization.

As cities continue to expand, understanding how organisms cope with urban stressors and adapt to these novel habitats will become increasingly important in the context of conservation and management strategies. Variation in the activity of the HPA axis may represent a form of adaptive plasticity that facilitates persistence of species in modified environments. Further research into the role of the HPA axis in successful colonization may provide a valuable model for studying the mechanisms that mediate adaptations of wildlife to urbanization.

Acknowledgments

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