

From The Brain To The Barrio: Energy And Stress Interact To Facilitate The
Urbanization Of Sonoran Desert Birds.

by

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ABSTRACT

The impact of urbanization on wildlife is becoming an important topic in conservation. However little is known concerning the proximate mechanisms involved which enable some species to persist in cities, while others perish. Adapting to novel city environments requires individuals to maintain a functional physiological response to stressful stimuli, while concurrently using the necessary resources (food) needed to persist. A primary function of the stress response is the mobilization of intrinsic energy resources, and thus both requirements (energy and stress) are explicably linked.

This dissertation investigates the interaction of energetic reserves and the physiological stress response in a native bird species, the Curve-billed Thrasher, within the context of this species' colonization of Phoenix, Arizona. This research uses a combination of comparative studies, statistical modeling, and experimental approaches conducted in field and captive settings to demonstrate how urban and desert populations of these species differ in energetic state and stress physiology.

These studies reveal that the current energetic status of an individual bird influences the secretion of glucocorticoids (primary stress hormones) and can alter how energy reserves are used for gluconeogenesis to produce energy during acute stress. In addition, this research also identifies how differing levels of a hypothalamic neuropeptide (vasotocin) may play a role in mediating differences in stress physiology between populations. The quantity of food available and even temporal variability in its abundance may alter how native birds respond to stress. Increased body condition offsets the costs of maintaining the stress response in urban areas.

DEDICATION

My research has been supported along every step of the way by friends, family and my wonderful and tolerant girlfriend, Valerie. I dedicate this work to them all, but most of all I dedicate this research to the many birds whose lives I disrupted in an effort to learn more about them.

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PREFACE

A recent milestone in human history is the recognition that the majority of people now live in cities and that urban habitat is the most rapidly expanding environment on earth (United Nations Report). This revelation has prompted a great deal of research examining the impacts of cities on wildlife and establishing this subject as a predominant discipline within conservation biology. This is evidenced by the ever-increasing number of publications on this topic (Figure 1). As urban landscapes continue to encroach onto natural habitats, how wild animals will respond to anthropogenic disturbances will become an increasingly important factor governing their future existence (Marzluff *et al.* 2001). As we progress from simply documenting changes in natural communities with urbanization to establishing broad-scale responses of organisms to cities, our need for empirical studies exploring mechanisms of change becomes vital.

One important trend in urban biology is that urbanization tends to produce a homogenous community structure, which is characterized by the presence of a few highly successful and often introduced “urban-exploiting” species (e.g. pigeons, cockroaches, house mice) and a greater number of less successful but usually native “urban-adapting” species (Vitousek *et al.* 1997; Marzluff *et al.* 2001; Turner *et al.* 2004). However the exact reasons why some species thrive under heavy anthropogenic modification of existing habitats where as others are unable to cope are currently unclear.

Previous research have demonstrated the above shifts in community structure (Emlen 1974; Mills *et al.* 1989; DeGraaf *et al.* 1991; Green & Baker 2003;

Clergeau *et al.* 1998, 2006; Grimm *et al.* 2008). Studies have also documented other ecological changes including: earlier reproduction (Schoech & Bowman 2001; Partecke *et al.* 2004; Beck & Heinsohn 2006), changes in morphology (Yeh 2004), differences in behavior (Slabbekoorn & Peet 2003; Shochat *et al.* 2004; Beck & Heinsohn 2006; Partecke & Gwinner 2007), genetic structure (Rasner *et al.* 2004), altered trophic dynamics (Faeth *et al.* 2005), variation in host-parasite dynamics (Fokidis *et al.* 2008; French *et al.* 2008), and changes in species interactions (Petren & Case 1996; Parris & Hazell 2005). Although, informative, these previous studies have been primarily correlative and did not involve experimentation.

Challenges In Urban Ecology

One major limitation in predicting long-term consequences of urbanization to wildlife is assessing the degree to which a species is impacted by anthropogenic disturbance. Looking strictly at numbers, many species are found at higher population densities in cities than in appropriate natural habitats at the city periphery (Marzluff *et al.* 2001; Beckmann and Berger 2003). One often cited ecological factor accounting for such variation between habitats is increased quantity or quality of food in urban areas. This difference may result in a proportional increase in species density with food availability according to the “resource matching rule” (Parker 1978). Another important factor may be lower variability in urban resource abundance on a seasonal basis which can lead to population densities exceeding their carrying capacity and providing opportunities for “less competitive” individuals in a population to persist (“credit card hypothesis”; Shochat 2004).

An added difficulty in generalizing wildlife responses to urbanization stems from the spatial variation associated with regional and development-specific aspects of the urban landscape (Schoech & Bowman 2001; Grimm *et al.* 2008). For example, industrialized areas and vegetated suburban neighborhoods may represent different challenges to wildlife, inducing different responses within the same species. Full understanding of the effects of urbanization on wildlife will require examining responses at various levels and across numerous species. Thus responses to anthropogenic change represent the sum of both ecological factors in an individual's habitat and underlying characteristics of a species' biology (Shochat *et al.* 2006; Fokidis *et al.* 2008). However the mechanisms inspiring these responses remain largely speculative and poorly studied (Shochat *et al.* 2006).

Mechanistic Approaches To Questions Of Urban Adaptation

To overcome the above difficulties, it is essential to use mechanistic studies examining the influence of urbanization on specific physiological parameters. This approach allows us to test specific predictions concerning the advantages and disadvantages that certain traits confer in novel urban environments and to detect adaptive change occurring on a relatively short-term basis. For instance, a recent study on leaf-cutter ants (*Atta sexdens*) suggests that increased temperatures in the mega-city of Sao Paulo, Brazil, resulted in a shift in the ants' tolerance to warmer temperatures (Angilletta *et al.* 2007). Such differences may become genetically fixed over the course of only a few generations.

In another example, studies on European Blackbirds, *Turdus merula*,

demonstrated that urban birds breed earlier than rural counterparts (Partecke *et al.* 2004). A “common garden” experiment suggested this difference results from phenotypic flexibility and not genetic divergence between populations (Partecke *et al.* 2006). Thus environmental factors in this species may be primary determinants of this urban-related shift in reproductive physiology. However a genetic component could not be entirely ruled out (Partecke *et al.* 2005) as urban blackbirds have lost the propensity to migrate which is a genetically-based trait (Partecke & Gwinner 2007).

Similar research in Florida Scrub-Jays, *Aphelocoma coerulescens*, shows suburban birds also breed earlier than rural jays because of access to anthropogenic food sources (Bowman *et al.* 1998; Schoech 1996). Suburban jays also have lower baseline levels of glucocorticoid than rural birds, including those supplemented with a high protein diet (Schoech *et al.* 2004). Thus, in this species food predictability and quality may interact with glucocorticoid secretion to regulate the timing of reproduction (but see Schoech *et al.* 2007).

Understanding Stress In Urban Animals

Glucocorticoids such as corticosterone (CORT) in birds, are steroids secreted by adrenal glands that induce gluconeogenesis and promote feeding and avoidance behaviors that are ultimately geared towards persisting a stressful challenge to ensure survival (Sapolsky *et al.* 2000). In plasma, glucocorticoids dramatically increase in concentration in response to acute stress within minutes and can remain higher than background (i.e. baseline) levels if the stressor persists (Sapolsky *et al.* 2000). Considered an advantageous energy-mobilizing physiological system in the

short-term, the effects of glucocorticoids may be detrimental with long term stimulation, since consistently high levels of glucocorticoids may interfere with immune or reproductive physiology (Sapolsky *et al.* 2000). This stress response, here defined as an increase in plasma CORT in direct response to unpredictable aspects of an individual's environment or a disturbance of homeostasis, is ubiquitous among vertebrates and substantial research in wild animals has focused on its use as an indicator of physiological stress.

Studies of ecological factors that influence the stress response are many and include investigations of: social interactions (Goymann *et al.* 2001; Greenberg 2003; see review: Creel 2001; DeVries *et al.* 2003), resource availability (Clinchy *et al.* 2004), habitat structure (Brown & Fuller 2006), predation risk (Hik *et al.* 2001), parasitism (Opplinger *et al.* 1998), and direct anthropogenic disturbance (Wasser *et al.* 1997; Fowler 1999; Romero & Wikelski 2001). The conversion of natural areas to urban ones can potentially influence each of the above factors, and these studies have demonstrated variation in CORT secretion between urban and rural populations.

In some cases, urban populations exhibit a lesser CORT secretion than rural counterparts to an identical stressor (Partecke *et al.* 2006). This decreased stress responsiveness may represent “desensitization” to ever present and perhaps non-threatening urban stimuli (e.g. constant traffic, human presence, noise). Other studies report lower baseline levels of CORT in urban compared to rural populations of the same species (Florida Scrub-Jays, Schoech *et al.* 2004; Tree Lizards, *Urosaurus ornatus*, French *et al.* 2008), which may also indicate a similar desensitization, but these differences may depend upon the sex (Bonier *et al.* 2007) or age-group (Heiss *et al.*

2009) examined in the study. Although, these studies provide evidence for variation in CORT secretion being important in urban animals, it is important to note that the secretion of CORT involves a complex neuroendocrine cascade which has rarely been considered in these studies.

Is There More Food In The City?

A frequently cited benefit for animals that use urban habitats is gaining access to food resources (Figure 2) through: direct anthropogenic provisioning (e.g. refuse, food handouts); introduced plant species brought in for landscape purposes (e.g. fruits, nuts, and leaves), and/or the presence of introduced species of prey. However, in truth, very little empirical data is available on how animals use urban food resources (Figure 2), the few studies available comparing diets have largely focused on predatory species (e.g. Morey et al. 2007; Kristan et al. 2004), with much less known from insectivorous and herbivorous species.

In some species urban individuals are heavier or in higher body condition (i.e. more energy reserves) than their rural conspecifics (Cypher & Frost 1999). Other studies have reported opposite patterns where urban individuals have lower condition than rural ones (e.g. Liker et al. 2008). However in some species no differences have been observed between habitats (e.g. Rodewald & Shustack 2008). Together these observations suggest that for some species variation in body condition between urban and rural populations may result from using novel urban food resources, decreased access to traditional resources in cities, or on the contrary using resources that aren't negatively impacted by urbanization. Thus food

availability may be an important driver of ecological patterns in urban environments.

Few studies have examined dynamics of food use by wildlife within urban areas. Shochat et al. (2004) compared foraging decisions at artificial food patches between an urban (i.e. Phoenix) and desert habitat. Based on measuring giving up density (GUD), urban birds showed decreased susceptibility to predation during foraging compared to desert birds. In the same study, supplementation of food patches with water or excessive food availability influenced foraging in desert, but not urban birds. This suggests urban birds may have lowered predation risk and increased access to food, thus enabling them to maintain a higher body condition than desert conspecifics.

Thus cities may provide greater foraging opportunities because of higher food abundance, higher quality of food (e.g. high fat diet), or increased chances to forage due to lower predation risk. The latter is especially interesting since predation risk may be considered an environmental stressor, which can stimulate the secretion of CORT.

The Thrashers Of The Southwest USA

Since 2006 I have used the Curve-billed Thrasher (*Toxostoma curvirostre*), songbird model species to examine how food access in Phoenix, Arizona influences body condition, and its implications for the hormonal stress response. This is the most common member of the thrasher (*Toxostoma*) genus (family: Mimidae) in the Southwestern US deserts and is a common bird within Phoenix, and in other US cities (Emlen 1974; Tweit 1996; Green & Baker 2003). However, previous research

demonstrated this species is found at lower densities in Phoenix than in the surrounding Sonoran Desert (Green & Baker 2003). Based on this information, the Curve-billed Thrasher would thus be classified as an “urban-adaptor” based on the criteria of Blair (1996).

These thrashers are large (65 - 85g) insectivorous songbirds that hold permanent year-round territories (2 - 4.5 ha; Smith 1971; Fischer 1980) which they defend vigorously against conspecifics (Tweit 1996). Most studies of urbanization in birds have focused on social species (Ruiz *et al.* 2002; Partecke *et al.* 2006; Chamberlain *et al.* 2007), and migratory birds (Rodewald & Matthews 2005; Bonier *et al.* 2007). The sedentary nature of these thrashers suggests that once established, adult birds are unlikely to leave their home range. Thus physiological differences between populations likely result from local environmental factors and not those associated with distant wintering or migratory stopover grounds.

In the deserts the distribution of the Curve-billed thrasher is largely shaped by that of various cholla cacti (*Cylindropuntia* sp.) and other desert thorn scrubs on which they nest (Tweit 1996). However in Phoenix they also use exotic vegetation for nesting and roosting (Fokidis *pers. obs.*). Active nests in desert areas have been found from January to August (Bent 1948; Ricklefs 1965; Tweit 1996) but in Phoenix active nests have been observed in late September (Fokidis *pers. obs.*). Data on testicular size in desert-sampled thrashers suggests breeding may occur into October (Vleck 1993). Interestingly testicular development begins approximately two weeks earlier in Phoenix than in the surrounding desert habitats (Deviche *et al. in press*). Since other studies have proposed links between early breeding and fitness (reviewed

in Verhulst and Nilsson 2008) this suggests some benefit to city-living for thrashers.

The varied diet of thrashers includes arthropods, gastropods, berries, flowers, oats, and cactus fruits. This broad diet presumably confers a competitive advantage in seasonal desert environments, as proposed for other desert-dwelling bird species (Tweit 1996). It also suggests these birds have the capacity to exploit novel food sources within Phoenix such as fruit and seeds from the cities' extensive landscaping with exotic plants.

The Curve-billed Thrasher's behavior, diet, and ecology are very similar to other birds inhabiting the drier areas of the Sonoran Desert, including the Canyon Towhees, *Pipilo fuscus*, Cactus Wrens, *Campylorhynchus brunneicapillus*, and Black-throated Sparrows, *Amphispiza bilineata* (Wingfield *et al.* 1992; Vleck 1993). In addition, urban-rural comparisons of some desert species show similar physiological responses as in thrashers. With the rapid conversion of these upland Sonoran Desert habitats to housing developments at the periphery of Phoenix and other major southwest US cities, the thrashers and these sympatric species may share a similar fate. Additionally parallels can be drawn between my research and that on Florida Scrub-Jays where food is a likely driver of physiological patterns of wild and suburban populations (Bowman *et al.* 1998; Schoech 1996; Schoech *et al.* 2004; 2007).

Together these data suggest that thrashers are an appropriate model species to research effects of urbanization, with impacts on this species likely representative of other desert species, as well as species in other ecoregions. Studies on these birds are likely to provide important insight into how birds in general respond to urbanization.

Hypothesis

Species that colonize novel urban environments must overcome unfamiliar aspects of their new habitat by altering their responses to stressful stimuli. At the same time they must access the necessary resources to persist within a city. These concepts are inexplicably linked since responding to stress requires energy and fluctuating energy stores may influence the ability to respond to stress. Here I test the hypothesis that *urban-dwelling birds access urban food resources which alters their ability to respond to acute stress* (Figure 3).

Dissertation Overview

To investigate the above hypothesis I addressed how stress physiology and usage of energy reserves differs between urban and rural birds, and how variability to food abundance can influence these two concurrent physiological systems.

Specifically my dissertation research aims to answer the following questions:

- 1) How does the acute response to a stressful stimulus vary between urban and desert birds across different life history stages? (Chapter 1)
- 2) What is the source of variation in the acute stress response that differs between urban and desert populations? (Chapters 2 & 3)
- 3) How are intrinsic energy resources used during an acute stress response and does this vary with current body condition? (Chapter 4)
- 4) Can differences in stress physiology between urban and desert birds be negated in birds kept under similar environmental conditions? (Chapter 5)

- 5) How do periods of mass loss and gain alter the acute stress response?
(Chapter 5)
- 6) How does variation in daily availability of food alter the stress and metabolic physiology of birds and does this differ between urban and desert populations? (Chapter 6)
- 7) Does variation in acute stress physiology between urban and desert birds ultimately impact behavior? (Chapter 7)

In chapter 1, I investigated plasma CORT levels using a standardized acute stress protocol in both urban and outlying desert populations of five bird species during three life-history stages (non-breeding, breeding and molting). These species differ in their usage of urban habitat and establishes precedence for assessing how stress physiology in desert birds varies with urbanization in general.

Chapter 2 identifies the component of the hypothalamic-pituitary-adrenal (HPA) axis, the hormonal cascade that constitutes the stress response that differs between urban and desert Curve-billed Thrashers. This was done by comparing CORT secretion in response to injections with HPA peptides.

I further explore how this variation in stress physiology may arise in chapter 3 using immunocytochemistry to compare the relative amounts and distribution of arginine vasotocin (AVT), a predominant stress neuropeptide, in the brain of urban and desert wild-caught thrashers.

Chapter 4 models in wild-caught birds of five species, how acute stress alters plasma concentrations of metabolites associated with the intrinsic carbohydrate, lipid

and protein reserves. Using a path analytical approach I also determine whether body condition influences how plasma metabolites change during acute stress in wild birds.

In chapter 5, the acute stress responses of urban and desert thrashers are assessed in the field and after a period of acclimation in captivity to determine if differences in CORT secretion between populations depend on current (i.e. identical) environmental conditions. Also in this chapter, I manipulated food availability to induce periods of mass loss and mass gains in captive thrashers and investigated how changes in body mass (i.e. body condition) alters the acute stress response and plasma metabolite concentrations.

As well as greater food quantity or quality, cities may also provide less seasonal food sources than in desert areas. Chapter 6 tests how a variable food regime may impact how captive thrashers mount stress responses and whether they use intrinsic energy reserves during stress. To further characterize responses to variable food availability I also examined changes in locomotor activity, and masses of energy storage organs.

Finally, chapter 7 assesses how differences in stress physiology between urban and desert birds of two species may impact an important behavior for desert birds: territoriality. I measured behavioral responses to simulated territorial intrusions, and how CORT interacts with testosterone, the predominant hormone mediating aggression.

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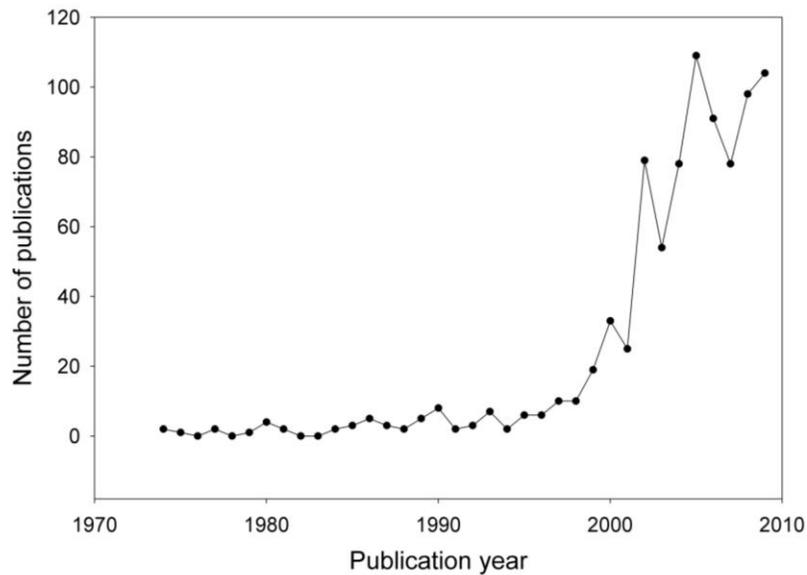


Figure 1. Trend in the number of publications investigating urban wildlife from 1974-2009, based on online database searches in Web of Science[®], Zoological Record[®], and Google Scholar[®].

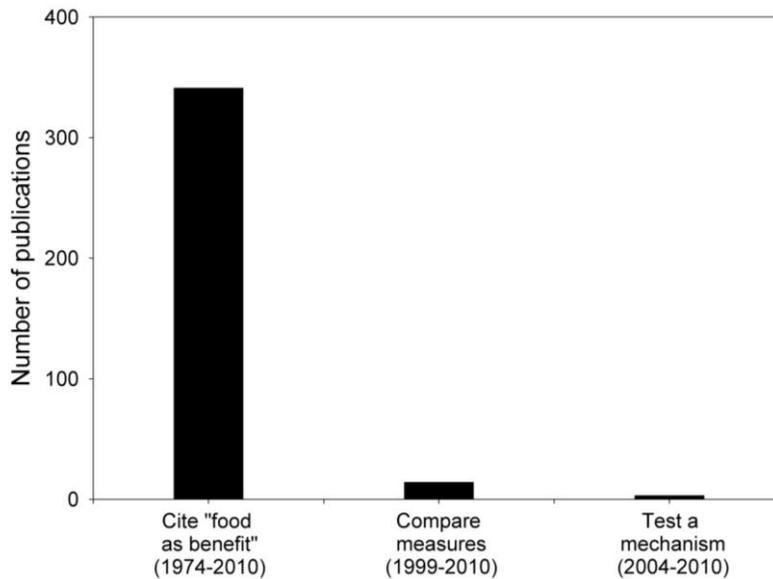


Figure 2. Number and year range of publications: 1) citing food as a benefit for the urbanization of wildlife; 2) comparing measures of either diet or body condition between urban and non-urban habitats; and 3) experimentally testing a mechanism involving food or body condition. Based on online database searches in Web of Science[®], Zoological Record[®], and Google Scholar[®].

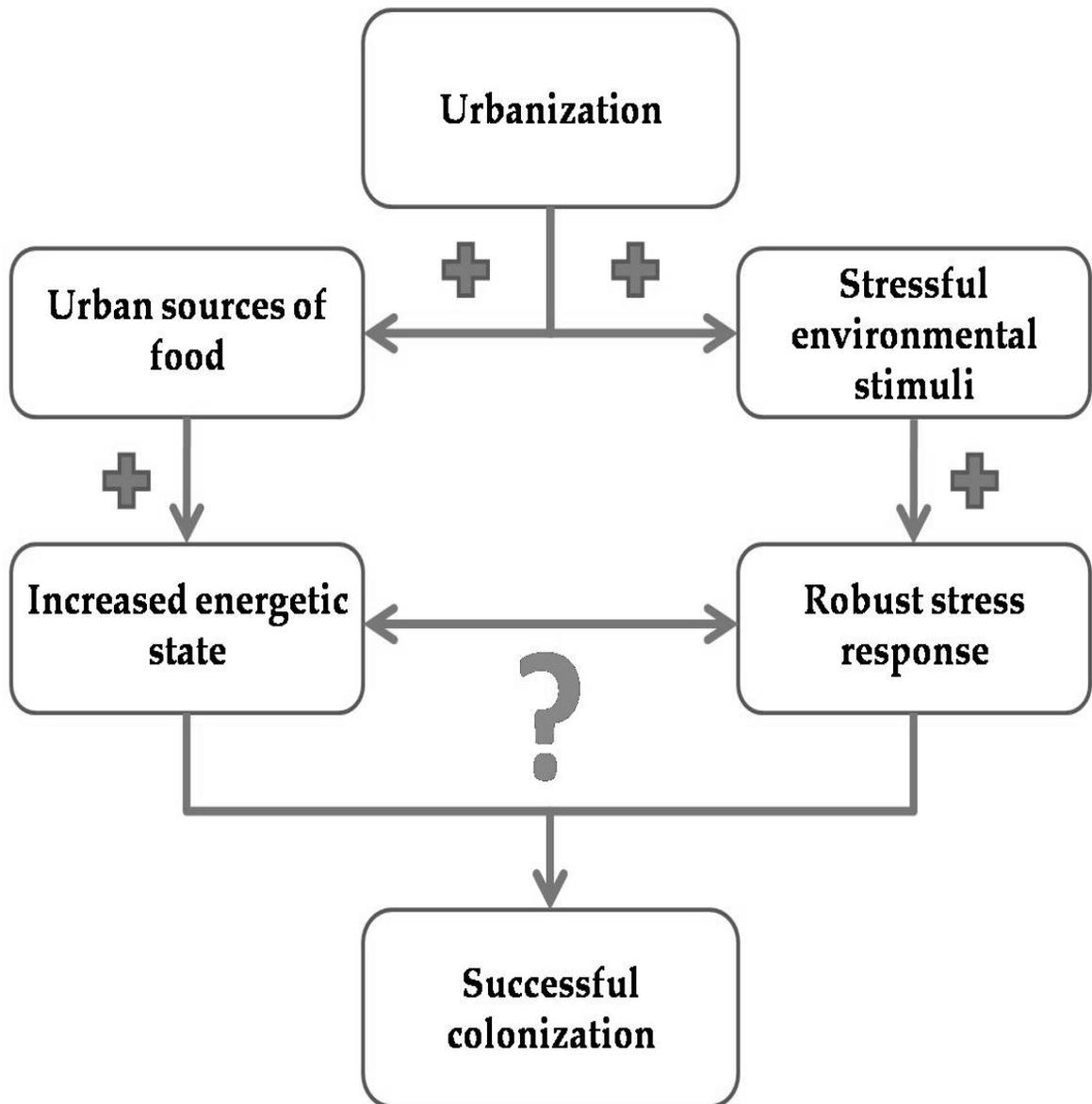


Figure 3. Proposed hypothesis for the colonization of urban environments by native wildlife. Here a species' ability to acquire urban food sources promotes a higher energetic state or body condition. This enables the maintenance of an effective short-term response to novel “urban” stressors.

Chapter 1

CORTICOSTERONE AND CORTICOSTEROID BINDING GLOBULIN IN BIRDS: RELATION TO URBANIZATION IN A DESERT CITY

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. I 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. I 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. I 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.

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 & 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 & 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 glucocorticoids 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 & 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 & Romero 2005; Cyr & 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 (*Zonotrichia 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 & 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 & 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 & 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 & Orchinik 2002). CBG binding capacity in birds may vary with life history stage (Silverin 1986; Love *et al.* 2004), season (Breuner & 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, I 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.

I 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 I 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

I 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 & Breitwisch 1992; Tweit & Finch 1994; Johnson & Haight 1996; Tweit 1996). In contrast, the socially gregarious HOSP has a distribution within Arizona that is entirely restricted to human-populated areas (Lowther & 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 & 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 I 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), my 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°180N and 33°570N, and 111°590W and 112°410W) 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 (~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 μ l in the HOSP to 500 μ l for CBTH. Samples were kept on ice until plasma was separated by centrifugation and then stored at -80 °C until assayed.

Since my 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 & 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 (\pm 0.1 g), tarsus length (\pm 1 mm), and wing chord length (\pm 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 (P_4), 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 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, P_4 , dexamethasone (DEX), testosterone (T), 5 α -dihydrotestosterone (DHT), and 17 β -estradiol (E_2) 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 (EC_{50}) 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 x locality x life history stage; species x locality x 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 & 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, I 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 & 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 I only had data for rural populations CANT was excluded from analysis. To compensate for multiple comparisons, I 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 \pm 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 analyses 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 $p > 0.16$) or CBG binding capacity (all $p > 0.08$).

3.1. CBG Binding Properties

In all species, specific and nonspecific binding of ^3H -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 I incubated pooled plasma samples with 1 nM ^3H -CORT at 4 °C in the presence or absence of 3 μM P_4 for 1–240 min. Specific binding of ^3H -CORT increased 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 $0.58 \pm 0.03 \text{ min}^{-1}$. Based on these data, I 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 3H -CORT binding sites with the highest affinity, and E2 competed with 3H -CORT binding sites with the lowest affinity in all species (Table 2). The potencies of other steroids to compete for 3H -CORT binding sites are presented in Table 2. Using a random subset of samples ($n = 9$) for each species I 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 ($t = 0.96$, $n = 9$, $p = 0.12$), and baseline and stress-induced CBG levels were correlated within species (Spearman's $r = 0.68$, $p = 0.03$).

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 (Figure 4). 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 (Figure 4). Baseline total CORT was significantly lower in breeding than non-breeding and molting CANT (Figure 4). 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 (Figure 4). During breeding rural CBTH birds had lower stress-induced total CORT levels than urban birds (Figure 4). Baseline total CORT was higher in rural non-breeding than in urban non-breeding or breeding NOMO (Figure 4). 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 (Figure 4).

Rural breeding HOSP had significantly lower baseline total CORT levels than urban birds (Figure 4) but rural breeding HOSP had higher stress-induced total CORT than urban HOSP (Figure 4). 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 (Figure 5). 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 (Figure 5). Rural NOMO had lower stress-induced free CORT than urban NOMO (Figure 5). 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 (Figure 5). Rural ABTO had lowest stress-induced free CORT levels during molt (Figure 5). Baseline and stress-induced free CORT levels were higher in rural than urban HOSP, but only during breeding (Figure 5). 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%, $p = 0.001$).

4. Discussion

Urban encroachment on native habitats poses unique and potentially “stressful” challenges to the animals that attempt to exploit these novel habitats. I investigated whether CORT levels differed between urban and rural populations of five bird species that vary in their abundance in the city of Phoenix. I predicted that urban-adaptable species would display lower plasma baseline CORT levels and a suppressed CORT response to an acute stressor than less urban-adaptable species. I found that neither adaptability to urbanization nor phylogenetic relationships predicted total and free CORT levels or CBG concentrations. However, I 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 I 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 & 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 & Romero 2005).

4.2. The Hormonal Stress Response Is Not Suppressed In Urban Birds

I 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 & Nagel 2006; Tarlow & Blumstein 2007). The urban birds in this study are also

superficially “tamer” (i.e., more closely approached by humans) than their rural counterparts (Fokidis *pers. obs.*). 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 & 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 & Dallman 1998; Armario *et al.* 2004a,b; Hazard *et al.* 2008). I 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 my initial prediction. One hypothesis for explaining the increased HPA responsiveness of urban birds involves the ability to locate and use urban food sources (Chace & Walsh 2006; Mennechez & 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 (Schoech *et al.* 2007). In my 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; Storchlic & 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 (Storchlic & 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 & Wingfield 2003) as well as in Sonoran Desert passerines (Wingfield *et al.* 1992). In this study, I 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, I 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 & Atanasova 2003; Schoech & Bowman 2003; Partecke *et al.* 2004, 2005; Beck & Heinsohn 2006) and decreased propensity for migration (Partecke & 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 & 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, maybe 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 & 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 the species chosen for the current study, 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 my 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 I 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 note data 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, whereas 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). I 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 & Orchinik 2001; Deviche *et al.* 2001; Lynn *et al.* 2003; Hattori *et al.* 2004; Seabury & 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 & 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 & 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, I did not observe differences in CBG between urban and rural birds, or across life history stages and thus limit my 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.

5. References

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Table 1. Equilibrium dissociation constants (K_d) for ^3H -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 $p < 0.05$. ABTO—Abert’s Towhee; CANT—Canyon Towhee; NOMO—Northern Mockingbird; CBTH—Curve-billed Thrasher; HOSP—House Sparrow.

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

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 μ M 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 $p < 0.05$. Corticosterone (CORT); Dexamethasone (DEX); Testosterone (T); 5 α -Dihydrotestosterone (DHT); Progesterone (P₄); Estradiol (E₂).

	<i>ABTO</i>		<i>CANT</i>		<i>NOMO</i>		<i>CBTH</i>		<i>HOSP</i>	
	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

Table 3. Results for an analysis of variance examining the effects of species, urbanization rank, locality, and life history stage 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. ^a Significant at $p < 0.05$. ^b Significant with Bonferroni correction ($p < 0.01$).

<i>Factor</i>	<i>Baseline Total CORT</i>				<i>Stress Total CORT</i>				<i>CBG Binding Capacity</i>			
	<i>Type III SS</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>Type III SS</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>Type III SS</i>	<i>df</i>	<i>F</i>	<i>P</i>
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		
	<i>Free CORT</i>											
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						

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 \pm standard error. Within species, rows sharing the same letter do not differ at the $p < 0.05$ level. See Table 1 for species abbreviations.

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

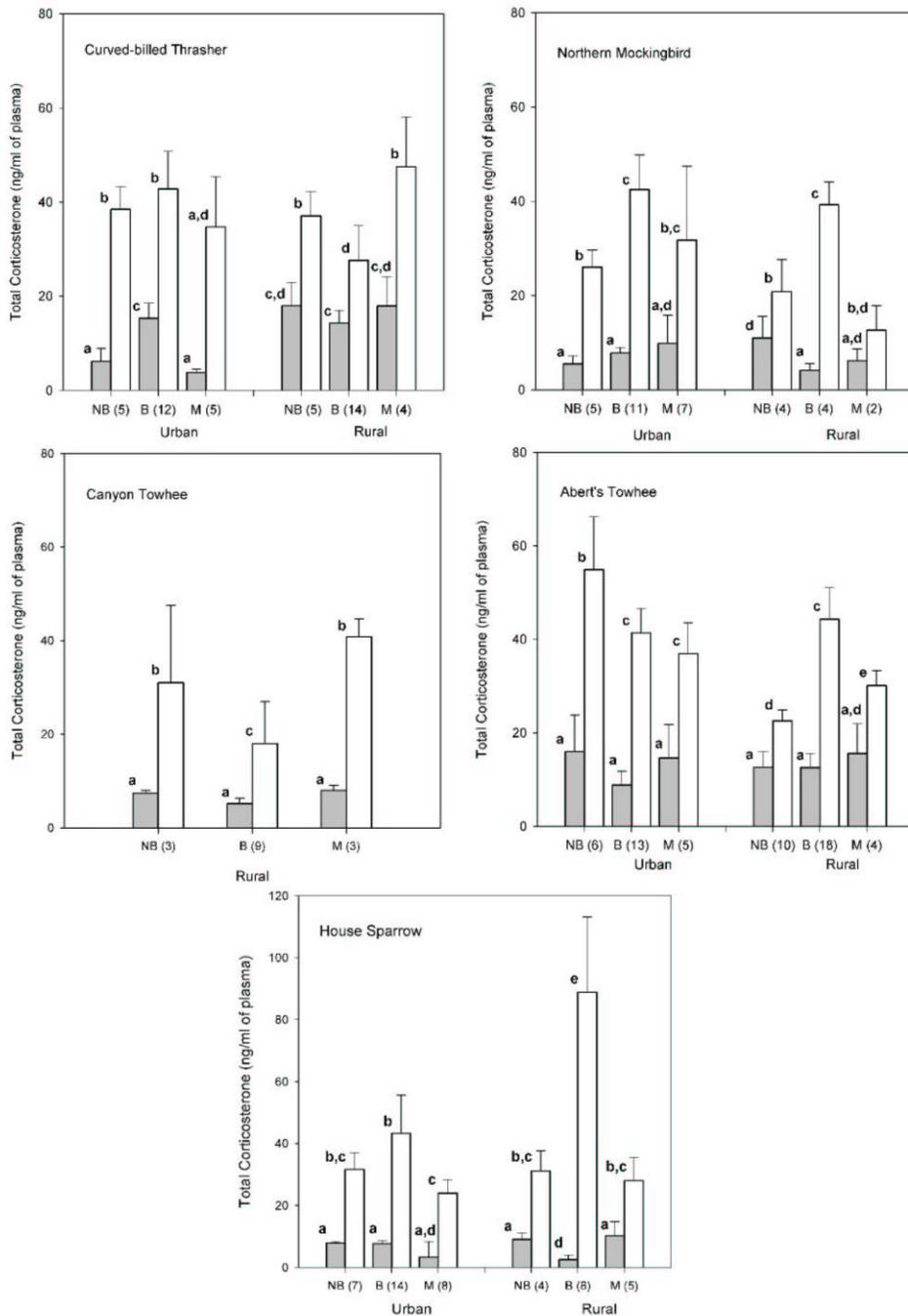


Figure 4. Baseline (*gray bars*) and stress-induced (*white bars*) total plasma corticosterone (ng/ml of plasma) in urban and rural populations of five species across three life history stages; NB = non-breeding, B = breeding, M = molting. Numbers in brackets indicate sample sizes and bars sharing the same letter are not significantly different at the $p < 0.05$ level.

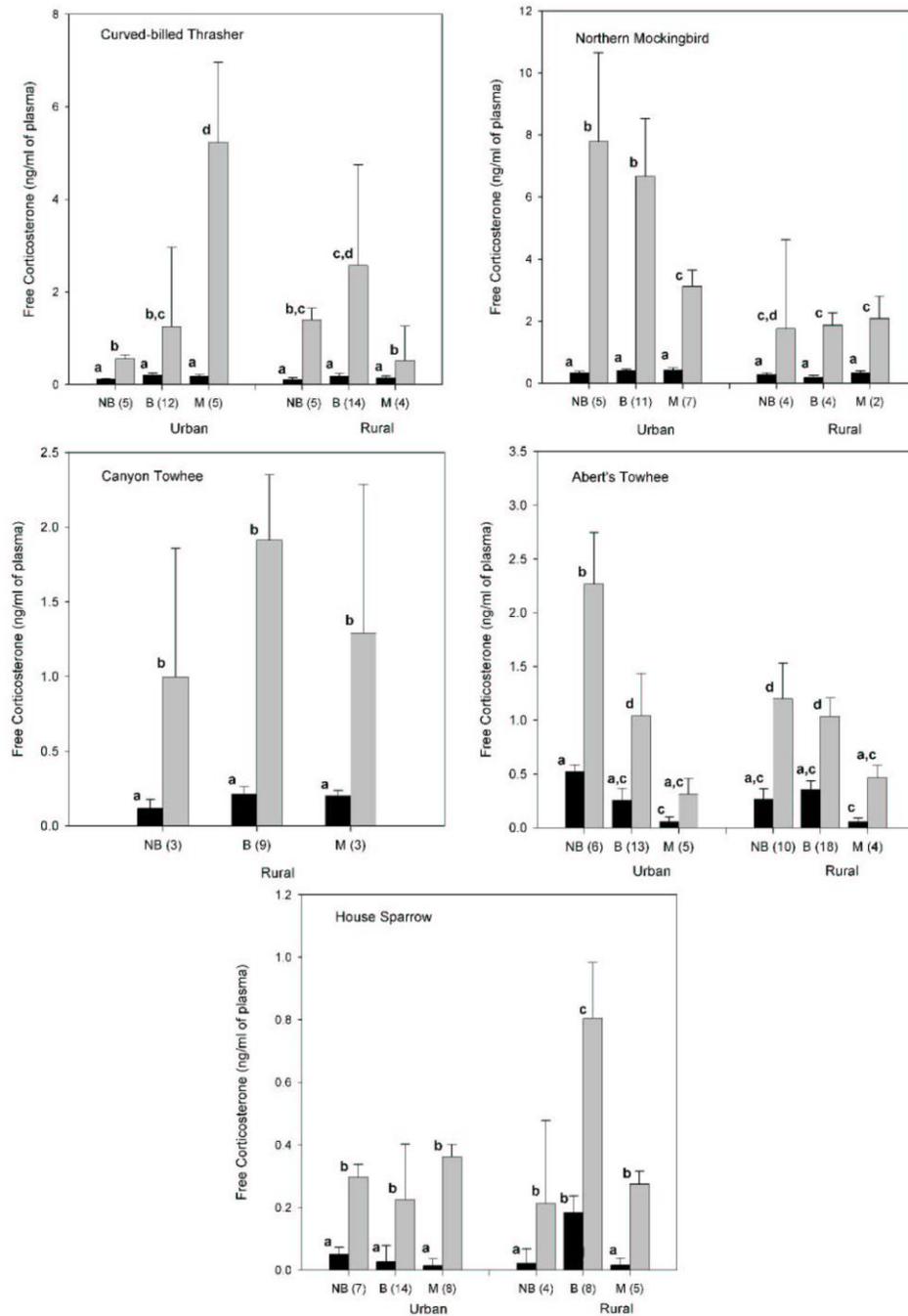


Figure 5. Baseline (*black bars*) and stress-induced (*gray bars*) free (i.e., unbound to corticosteroid binding globulin) plasma corticosterone (ng/ml of plasma) in urban and rural populations of five species across three life history stages; NB = non-breeding, B = breeding, M = molting. Numbers in brackets indicate sample sizes and bars sharing the same letter are not significantly different at the $p < 0.05$ level.

Chapter 2

PLASMA CORTICOSTERONE OF CITY AND DESERT CURVE-BILLED THRASHERS, *TOXOSTOMA CURVIROSTRE*, IN RESPONSE TO STRESS-RELATED PEPTIDE ADMINISTRATION

I compared the activity of the hypothalamo-pituitary-adrenal axis of urban (Phoenix, Arizona) and desert populations of a male songbird species (Curve-billed Thrasher, *Toxostoma curvirostre*) by measuring corticosterone in response to acute administration of corticotropin-releasing factor, arginine vasotocin, or adrenocorticotropin hormone. Urban thrashers showed greater responsiveness than desert birds to an injection of arginine vasotocin or adrenocorticotropin hormone, suggesting population differences in pituitary and adrenal gland sensitivity. Plasma corticosterone in response to corticotropin-releasing factor injection did 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. Resistance of the hypothalamo-pituitary-adrenal axis to the negative feedback effect of 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 the negative feedback of glucocorticoids on the hypothalamo-pituitary-adrenal 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 hypothalamo-pituitary-adrenal axis in urban birds. This up-regulation may in turn make it easier for city birds to cope with urban environment-associated stressors.

1. Introduction

The hallmark of the vertebrate physiological response to stress is two-fold: Activation of the sympathetic nervous system *via* catecholamines released from the adrenal gland medulla and release of glucocorticoids from the adrenal gland cortex. Together, 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 factor (CRF) and arginine vasotocin (AVT), the avian homologue of mammalian vasopressin (AVP). In mammals, the actions of CRF during a stress response precede those of vasopressin, which has longer lasting effects (Herman *et al.* 1992; Ma & Aguilera 1999). In mammals and birds, both neuropeptides stimulate the pituitary gland secretion of adrenocorticotropin hormone (ACTH: Zelena *et al.* 2004; Rich & Romero 2005), which ultimately induces adrenal secretion of glucocorticoids, such as corticosterone (CORT) in birds.

Corticosterone regulates the HPA axis activity through a negative feedback effect at the hypothalamic and pituitary gland levels (Young *et al.* 1995; Makino *et al.* 2002). This feedback may decrease CRF 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 CRF and AVT secretion may be important during “chronic” stress, loosely defined as persistently elevated plasma CORT combined with a reduced capacity to elevate CORT in response to acute stress. During chronic stress adrenal glands may continue to secrete CORT even after the stressor is no longer perceived,

which may have deleterious impacts on energy balance, reproduction or immune function (Clinchy *et al.* 2004; Rich & Romero 2005; Wingfield 2005; Lightman 2008). These processes have been extensively studied in mammals but less in birds.

In nature, avian populations inhabiting different habitats often show varied stress responses, particularly at certain times of year. For example, birds from climates where breeding opportunities are temporally limited (e.g. arctic, desert) can show lower HPA activity, as measured by CORT secretion in response to capture and handling, during breeding than at other times (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 reproductive physiology (Wingfield & Ramenofsky 1997; Love *et al.* 2002), which could in turn decrease fitness.

Research investigating differences between populations has 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 more than conspecific birds living in rural areas (Fokidis *et al.* 2009). Breeding desert thrashers may limit their CORT response to acute stress to minimize interference with reproduction, since nesting is timed to coincide with the winter rains that increase food availability (Wingfield *et al.* 1992; Fokidis *et al.* 2009). Outside the breeding season, initial plasma CORT (i.e. prior to application of handling stress) is higher in desert than urban

birds and during molt, desert thrashers show stronger stress responses than urban birds (Fokidis *et al.* 2009). Food availability may undergo less seasonal fluctuations in urban settings than in the desert (Fokidis *et al.* 2008; 2009), which may translate into a decreased need for seasonal modulation of the HPA axis activity in urban than desert birds. However, urban thrashers may be exposed to more persistent and largely unavoidable chronic (e.g. pollution, increased temperatures, and noise) and acute stressors (e.g. vehicles, humans, and feral predators) than desert birds.

I investigated mechanisms that may account for previously observed differences in HPA axis activity between breeding urban and desert thrashers. Specifically, I compared (1) differences in sensitivity of the pituitary and/or adrenal glands to an injection of CRH, AVT, or ACTH and (2) differences in the resistance to negative feedback induced by administration of the synthetic glucocorticoid dexamethasone (DEX). I attempted to determine whether urban birds show symptoms of chronic stress or differences in the regulation of their HPA axis activity compared to desert conspecifics. I predicted that if chronically stressed, urban birds would have higher sensitivity to ACTH and AVT injection, lower sensitivity to CRF injection, and higher resistance to negative feedback than desert birds. If urban birds down-regulate their HPA axis activity I predicted decreased sensitivity to ACTH, AVT, and CRF injections, and less resistance to DEX in these birds than in desert birds. If urban thrashers up-regulate their HPA activity, I predicted increased sensitivity to all hormone treatments and a robust negative feedback response. 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 desert specialist passerines in Phoenix, Arizona, but are found at lower densities in this city than in the surrounding desert (Green & Baker 2003). The species holds a permanent year-round territory that it defends vigorously against conspecifics (Tweit 1996). The sedentary nature of these birds suggest that once established, they are unlikely to leave their home range and thus physiological differences between populations likely result from local factors.

The study was conducted in a Sonoran desert locality and in urban areas of the cities of Phoenix and Scottsdale. The desert site was the McDowell Mountain Sonoran Preserve and 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. Urban sites included high- and low-income residential housing tracts, commercial areas, business districts, and manicured city parks of various sizes, and they were largely governed by authorized access. Previous research documented consistent differences in stress physiology between thrashers inhabiting various areas of Phoenix and those inhabiting several Sonoran Desert localities to the north, west, and south of the city (Chapter 1). In this study, urban thrashers showed a stronger response to capture and handling (i.e. greater plasma CORT increase) than birds sampled in the Sonoran Desert and this difference occurred regardless of capture locations. The current study is confined to two populations but the results, therefore, likely apply to urban-desert comparisons across a number of localities.

2.2. Field Capture And Injection Protocol

The study was conducted between February and April 2008 and all captures took place between 0500 and 1100 h. Male thrashers were captured in mist nets using conspecific playback recordings. 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 = 80 ± 6 g) then received either an intrajugular injection of 0.9% NaCl solution (*hereafter* “saline”) or one of the following treatments: 1) 8 mg/kg (2.51 pmoles/injection) DEX (*Sigma-Aldrich Co. Cat # D1756*) followed by 50 IU/kg porcine ACTH (*Sigma-Aldrich Co. Cat # A6303*); 2) 8 mg/kg DEX followed by 100 IU/kg porcine ACTH; 3) 3 μ g/kg (1.12 pmoles/injection) ovine CRF (*Sigma-Aldrich Co. Cat # C3167*); 4) 6 μ g/kg (2.24 pmoles/injection) CRF; 5) 3 μ g/kg (0.25 pmoles/injection) AVT (*Sigma-Aldrich Co. Cat # V0130*); 6) 6 μ g/kg (0.5 pmoles/injection) AVT; 7) 6 μ g/kg CRF and 3 μ g/kg AVT; 8) 2 mg/kg (0.63 pmoles/injection) DEX only; or 9) 8 mg/kg (2.51 pmoles/injection) DEX only. Peptides and DEX were dissolved in saline (100 μ l/injection) with the latter being first dissolved in 100% ethanol. The order of treatments was originally randomized. Once this order was established, treatments were given systematically and independently among the populations. Figure 6 illustrates the times of injection and sampling for each treatment.

The above treatments were designed to induce maximum physiological CORT secretion. However, peptide dose-response data for my species are unavailable and collecting these data would be unfeasible due to the large sample sizes that would be required. As with other peptide injection studies on free-living passerines, I addressed this limitation by using a low and high dose of each peptide and based the choice of dose on published avian data

(Astheimer *et al.* 1994; Romero & Wingfield 1998; Romero *et al.* 1998a; 1998b; 1998c; Romero & Wingfield 2001; Romero 2006). Treatment 7 involved injecting CRF and AVT at a 2:1 ratio to test for possible additive/synergistic effects of the two peptides. This ratio was chosen based on studies suggesting that AVT in birds is a more potent secretagogue of ACTH than CRF (Castro *et al.* 1986). Birds were kept in a cloth bag after receiving an injection and until a second 300 μ l blood sample (*hereafter* treatment sample) was collected 30 min later. The 30 minute delay between treatment and the collection of a second blood sample enabled us to compare the present data with those of a previous study investigating differences in plasma CORT between populations in response to 30 min of acute stress (Fokidis *et al.* 2009). Previous studies found that 30 min is sufficient time to induce a maximum increase in CORT levels in response to each of these peptide injections (Astheimer *et al.* 1994; Romero & Wingfield 1998; Romero *et al.* 1998a; 1998b; 1998c; Romero & Wingfield 2001; Romero 2006).

To isolate the effects of exogenous ACTH administration (treatments 1 and 2) birds received an injection of DEX 15 min before treatment with this hormone. Pilot data on two captive thrashers revealed that DEX injection abolishes the increase in plasma CORT that normally results from capture, and that this suppression occurs within 15 min of DEX administration and lasts for at least one hour (Figure 7). In addition, some urban and desert birds ($n = 6$ birds per group) were bled using the standard 30 min capture and handling protocol outlined in Fokidis *et al.* (2009) but received no injection. Results from these birds were compared to those from saline-injected birds to determine if the act of injecting alters the stress response. Blood samples were placed on ice until plasma was separated by centrifugation and then stored at -80° C until assayed for CORT.

Sex was determined by the presence of a developed cloacal protuberance, and body mass (± 0.1 g) and wing chord (± 1 mm) were measured. All birds received a uniquely numbered aluminum United States Geological Survey leg band and were released at the site of capture. All procedures followed 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, and the Cities of Phoenix and Scottsdale Parks and Recreation Departments.

2.3. Plasma Corticosterone Assay

Plasma total CORT concentrations were measured using a validated commercial competitive enzyme-linked immunoassay (ELISA; Assay Designs Inc. Ann Arbor, Michigan, USA; Fokidis *et al.* (2009)). Samples were assayed in duplicate except for 17 samples that were assayed singly due to limited availability of assay plates. 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, California, USA). The sensitivity of the CORT assay ranged from 6.4 to 15.7 pg/ml depending on the plate. The mean intra-assay coefficient of variation was 14.9 % ($n = 4$ plates, 156 samples total).

2.4. Data Analyses

Initial and stress-induced CORT is thought to interact with different receptors (mineralcorticoid 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 measures 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 ($p > 0.05$). I also tested differences in body mass and wing chord 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 (S.E.).

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: $F_{1,2} = 0.071$, $p = 0.502$; Figure 8). 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 minutes after sunrise, desert: 157 ± 16 minutes after sunrise; $p = 0.18$). Initial plasma CORT did not differ

between groups ($p = 0.341$) except that desert thrashers receiving a saline injection had higher plasma CORT than corresponding urban thrashers ($p = 0.027$) and than desert birds injected with a low AVT dose ($p = 0.019$).

3.2 Pituitary Gland Sensitivity To CRF And AVT

An injection of CRF increased plasma CORT ($F_{1,3} = 13.882, p < 0.001$) and this increase was similar in urban and desert birds ($F_{1,2} = 2.594, p = 0.115$; Figure 9). Urban thrashers injected with 3 $\mu\text{g}/\text{kg}$ of CRF did not increase their plasma CORT above levels attained by saline-injected control birds ($p = 0.282$). However, desert thrashers injected with this CRF dose had lower plasma CORT than control birds ($p = 0.046$). In contrast, desert birds injected with 6 $\mu\text{g}/\text{kg}$ of CRF had higher plasma CORT than birds receiving a lower CRF dose ($p = 0.034$), but did not differ from control birds ($p = 0.279$). Injection of the high CRF dose into urban thrashers elevated plasma CORT above levels measured in control ($p = 0.017$) and low dose birds ($p = 0.035$). Plasma CORT in response to administration of the high CRF dose did not differ between urban and desert birds ($p = 0.454$).

The effect of AVT administration on plasma CORT varied as a function of the dose administered ($F_{1,3} = 16.495, p < 0.001$) and was habitat-related ($F_{1,2} = 10.128, p = 0.003$; Figure 10). In urban and desert thrashers, an injection of 3 $\mu\text{g}/\text{kg}$ of AVT did not elevate plasma CORT above that in control birds (urban: $p = 0.173$; desert: $p = 0.354$). In desert thrashers, injection of 6 $\mu\text{g}/\text{kg}$ of AVT elevated plasma CORT above levels in control ($p = 0.021$) or lower dose ($p = 0.009$) groups. Similarly in urban thrashers, the high AVT dose group had higher plasma CORT post-treatment than control ($p = 0.014$) and low dose groups ($p = 0.017$), and indeed levels of CORT were higher than those in corresponding desert birds ($p = 0.040$).

Injected in combination, CRF (6 µg/kg) and AVT (3 µg/kg) did not elevate plasma CORT levels beyond those in a saline-injected control group ($F_{1,2} = 0.424, p = 0.317$) and the response to this treatment did not differ between urban and desert thrashers ($F_{1,2} = 0.205, p = 0.164$; Figure 8).

3.3. Adrenal Sensitivity To ACTH

An injection of ACTH elevated plasma CORT. The effect of this injection differed as a function of the ACTH dose administered in urban and desert birds ($F_{1,3} = 9.275, p = 0.007$), but especially in the former birds ($F_{1,2} = 6.815, p = 0.016$; Figure 11). Injection of 50 IU/kg of ACTH into urban and desert thrashers elevated CORT levels above those in DEX-injected control birds (urban: $p = 0.034$; desert: $p = 0.041$), and urban thrashers elevated their CORT levels higher than desert birds receiving the same treatment ($F_{1,2} = 2.374, p = 0.048$; Figure 11). Urban thrashers also showed a greater response than desert birds to administration of 100 IU/kg of ACTH ($p < 0.001$).

3.4. Resistance To 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 ($F_{1,3} = 0.477, p = 0.216$; Figure 12). 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 vertebrate. Plasma CORT increased above control levels in response to CRF treatment and this increase did not differ between urban and desert thrashers. In contrast, urban thrashers appear to have a higher pituitary and adrenal gland response to exogenous AVT and ACTH injections respectively, than desert conspecifics. The resistance of the HPA axis to the negative feedback induced by treatment with dexamethasone did not differ between populations, with all birds showing a robust negative feedback.

4.1. Relative Importance Of AVT And CRF To The Stress Response

The relative contributions of CRF and AVT to regulating the HPA axis activity of birds are poorly understood. In the chicken, *Gallus domesticus*, centrally administered CRF elevates plasma CORT more effectively than AVT treatment (Madison et al. 2008). However, peripheral CRF and AVT injections suggest the opposite in Japanese quail, *Coturnix coturnix japonica* (Hazard et al. 2007), which is consistent with findings in passerines (Romero & Wingfield 2001; Romero et al. 1998a; Rich & Romero 2005; 2007; Romero 2006). In two of the above studies, CRF did not elevate CORT above controls (Romero & Wingfield 2001; Romero et al. 1998a). Similarly in this study, AVT, but not CRF injection (equivalent by weight) increased CORT more than a saline injection in desert thrashers. The interpretation of these observations is complicated by the fact that on a molar concentration basis, birds received approximately four times more AVT than CRF. Additionally, passerine CRF has to my knowledge not been isolated or sequenced and the affinity of mammalian

CRFs for avian CRF receptors has not been determined. Chicken CRF has an 83% sequence homology with ovine CRF, but is identical to rat and human CRF (Vandenborne et al. 2005). Future avian studies involving CRF administration may, therefore, benefit from the use of rat or human, rather than ovine CRF (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, I predicted urban thrashers to have higher initial CORT, a lower acute stress response, and a decreased and increased sensitivity to CRF 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, I found higher initial CORT in desert than urban thrashers outside but not during the breeding season (Fokidis *et al.* 2009). In this study, saline-injected desert birds had higher initial CORT than urban birds, but initial CORT was similar across the other treatments. Thus, initial CORT data do not by themselves support the hypothesis that environmental factors impact urban and desert birds differently.

Another characteristic of “chronic stress” is a compromised negative feedback effect of CORT resulting in the inability to regulate the HPA axis (Young *et al.* 1995; Dallman *et al.* 2004). In mammals, DEX inhibits CORT secretion by blocking the pituitary gland secretion of ACTH (Dekloet *et al.* 1975). Administrating 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 resistance 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 desert birds, and the presence of a robust negative feedback system, together suggest that urban thrashers are not “chronically stressed” and, indeed, have a stronger acute stress response than desert birds.

4.3. Evidence For Habituation To City Life?

Urban birds are potentially exposed to two stressor types: 1) Short-acting stressors that birds can avoid by modifying their behavior and to which they may habituate (e.g. human disturbance, vehicular traffic, 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 stress results in a down-regulated CORT response. For example, studies have found decreased HPA 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 CRF, but not the AVP response (Ma *et al.* 1999). 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 in response to AVT or ACTH treatment. Further studies are needed to determine whether this constitutes habituation and if various stressor types induce different HPA responses.

4.4. Population Differences In Adrenal Sensitivity To Exogenous ACTH Challenge

I 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 & Weber 2000). Previous research using ACTH challenge protocols similar to that used here found that many birds modulate their CORT secretion seasonally, but nonetheless can always respond to exogenous ACTH injection (Romero *et al.* 1998c; Romero & Wingfield 1999; Sims & Holberton 2000; Rich & Romero 2001; Wilson & Holberton 2001; Meddle *et al.* 2003). An injection of ACTH into urban and desert thrashers elevated their plasma CORT above saline-treated, DEX-treated, and also non-injected birds, indicating that capture stress does not induce maximum CORT secretion by the adrenal glands. 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 & 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 (non-stressed) 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 & Wingfield 1998; Romero 2006). However, these studies also reported seasonal changes in baseline CORT levels (Romero *et al.* 1998c; Romero & Wingfield 1998), which were not observed in my study. 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.

5. References

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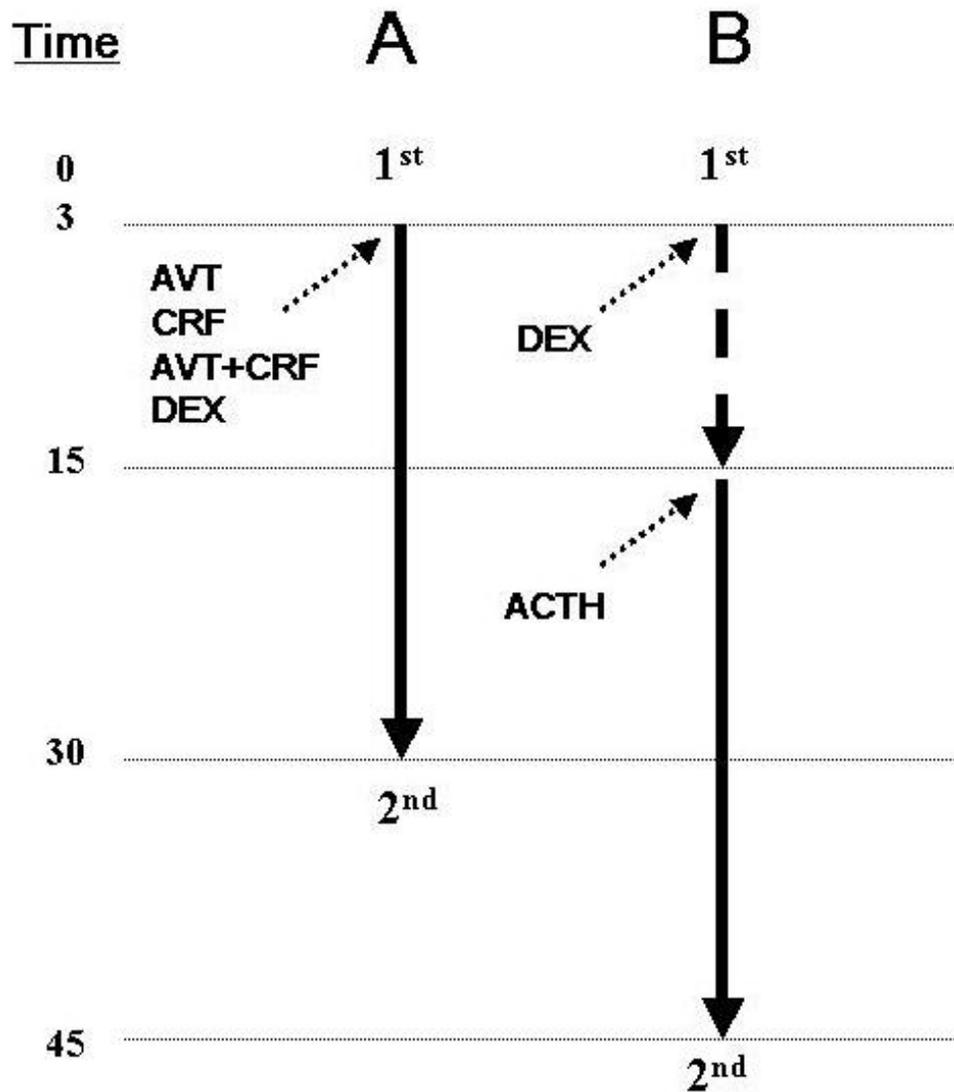


Figure 6. Injection (provided as minutes after capture) and blood sampling (1st – initial; 2nd – post-treatment) times for both neuropeptide (arginine vasotocin: AVT, corticotropin-releasing factor: CRF) and dexamethasone (DEX) treatments (A) or adrenocorticotropin hormone (ACTH) treatment, which involves pretreatment with DEX (B) in free-living Curve-billed Thrashers (*Toxostoma curvirostre*).

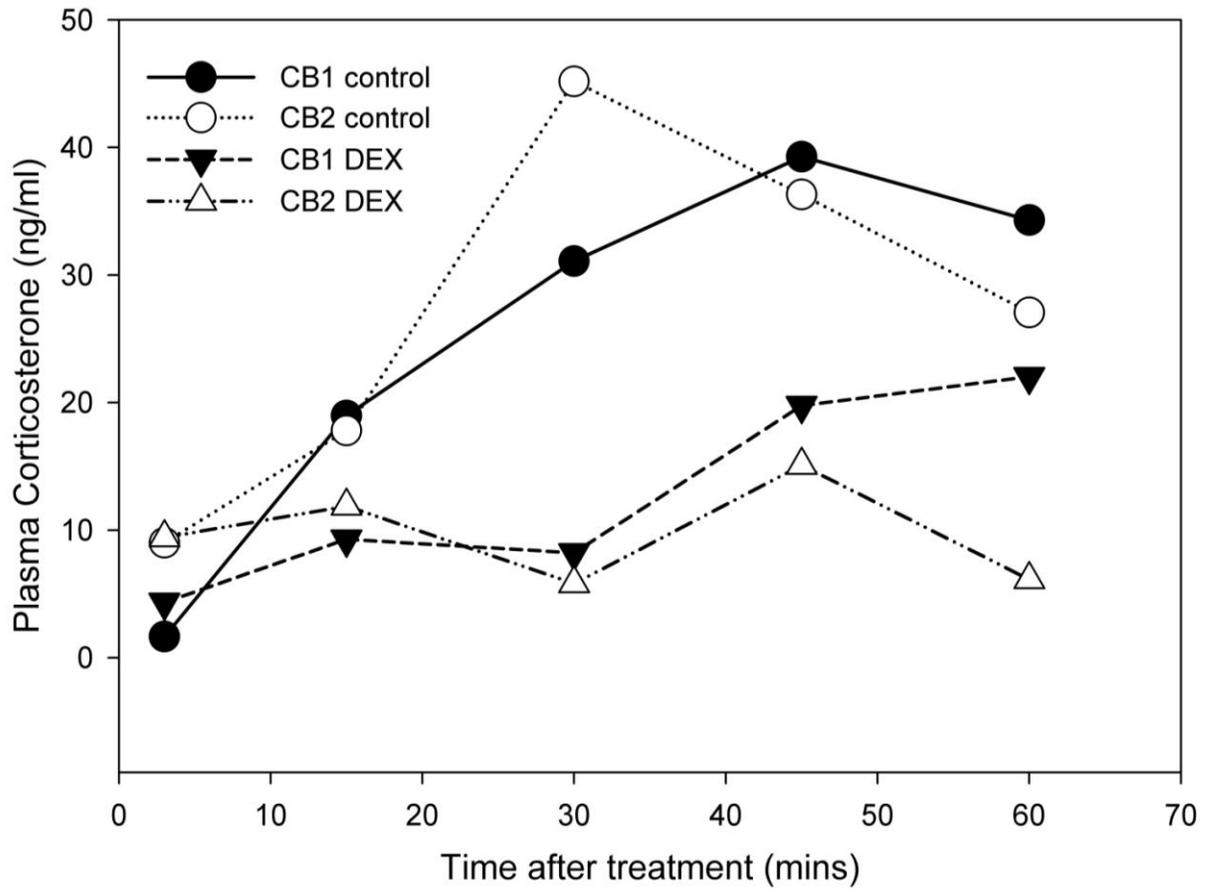


Figure 7. Efficacy and time course of dexamethasone (DEX) suppression (8 mg/kg dose) of stress-induced increases in plasma corticosterone in two captive Curve-billed Thrashers (CB: *Toxostoma curvirostre*) compared to control injections of 0.9% NaCl.

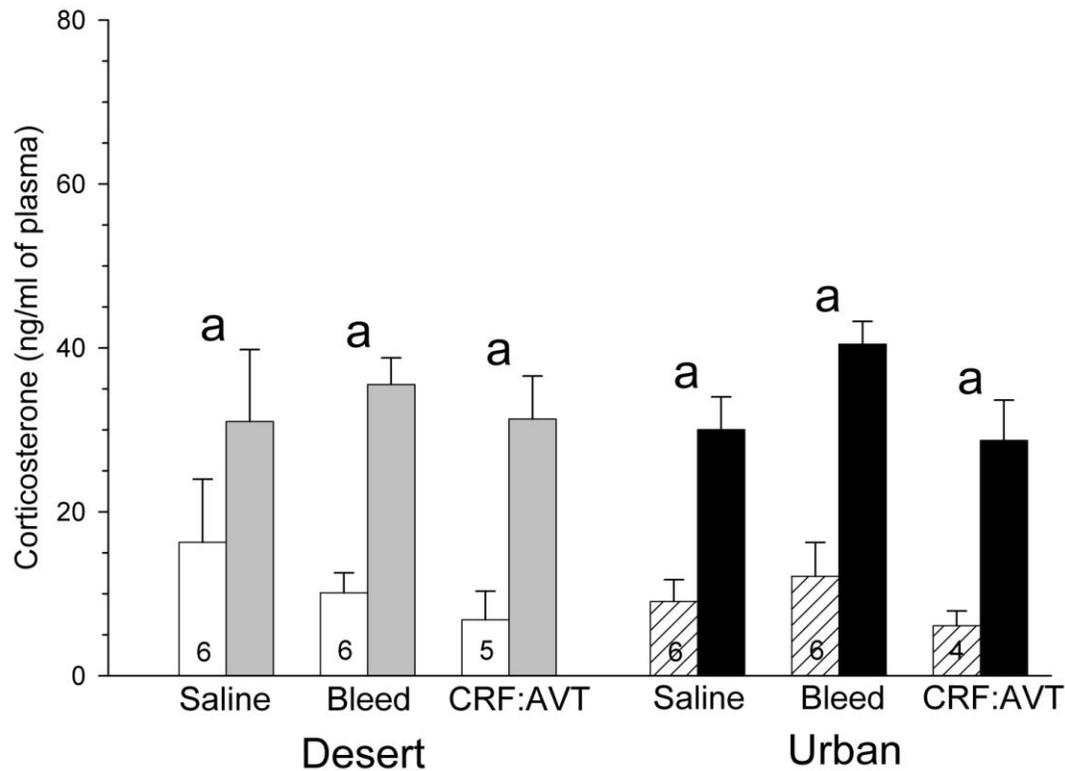


Figure 8. Comparison of the effects of injection with 0.9% NaCl (Saline), no injection (Bleed), and injection of a 2 to 1 ratio (6 to 3 $\mu\text{g}/\text{kg}$) of corticotropin-releasing factor to arginine vasotocin (CRF:AVT) on plasma corticosterone concentration of urban and desert Curve-billed Thrashers (*Toxostoma curvirostre*). Baseline corticosterone levels taken within 3 min of capture are indicated as white bars (*unfilled* = Desert; *patterned* = Urban), and 30 min post-treatment corticosterone samples are indicated as filled bars (*gray* = Desert; *black* = Urban). Numbers indicate treatment sample sizes and different letters indicate significant differences at $p < 0.05$ in the response to injection (i.e. increase between pre- and post-treatment levels).

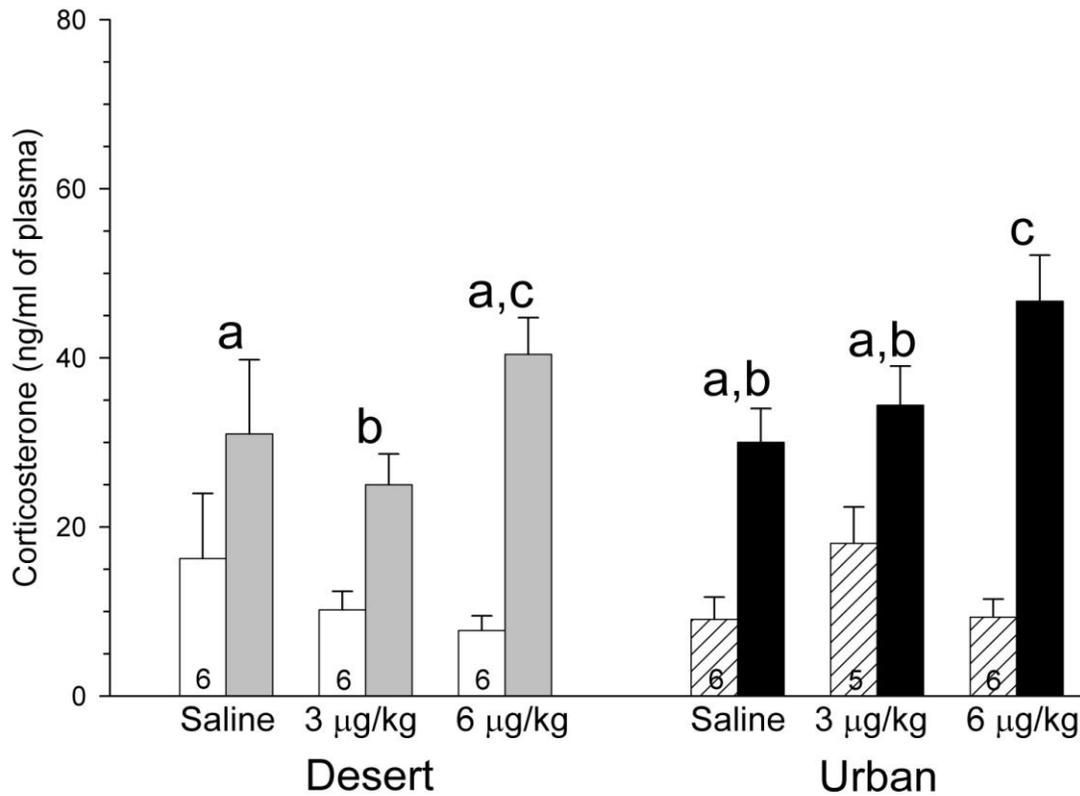


Figure 9. Changes in plasma corticosterone in response to treatment with 3 (Low) or 6 µg/kg (High) of corticotropin-releasing factor (CRF) in urban and desert Curve-billed Thrashers (*Toxostoma curvirostre*) compared to 0.9% NaCl (Saline) injected controls. Baseline corticosterone levels taken within 3 min of capture are indicated as white bars (*unfilled* = Desert; *patterned* = Urban). Plasma corticosterone 30 min after injection are shown as filled bars (*gray* = Desert; *black* = Urban). Numbers indicate treatment sample sizes and different letters indicate significant differences at $p < 0.05$ in the response to injection (i.e. increase between pre- and post-treatment levels).

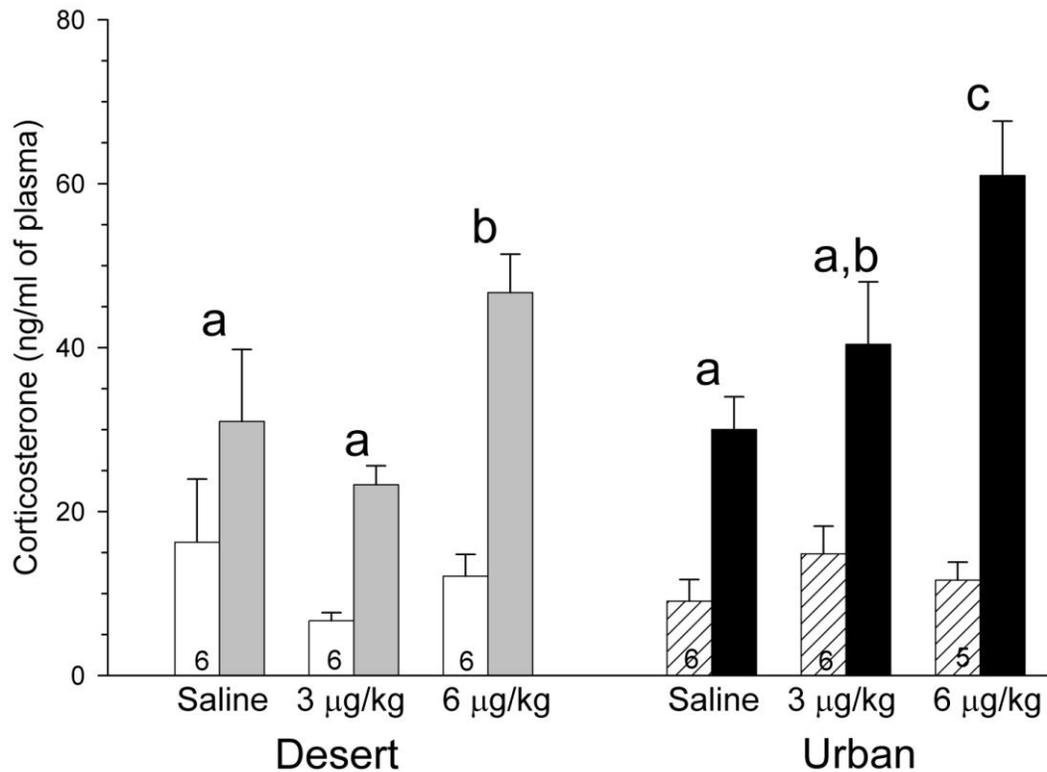


Figure 10. Changes in plasma corticosterone in response to treatment with 6 (High) and 3 µg/kg (Low) doses of arginine vasotocin (AVT) in urban and desert Curve-billed Thrashers (*Toxostoma curvirostre*) compared to 0.9% NaCl (Saline) injected controls. Baseline corticosterone levels taken within 3 min of capture are indicated as white bars (*unfilled* = Desert; *patterned* = Urban), and 30 min post-treatment corticosterone samples are indicated as filled bars (*gray* = Desert; *black* = Urban). Numbers indicate treatment sample sizes and different letters indicate significant differences ($p < 0.05$) in the response to injection (i.e. increase between pre- and post-treatment levels).

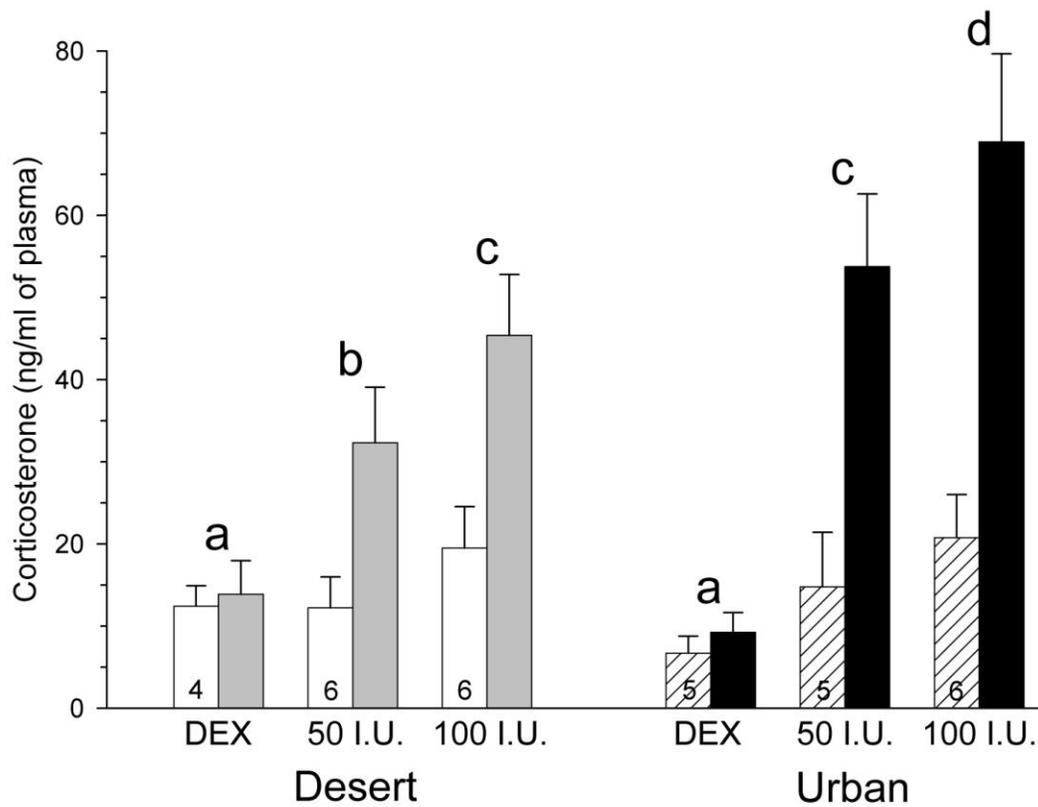


Figure 11. Changes in plasma corticosterone in response to treatment with 100 (High) and 50 I.U. (Low) doses of adrenocorticotrophic hormone (ACTH) in urban and desert Curve-billed Thrashers (*Toxostoma curvirostre*) compared to control birds injected with 8 mg/kg dexamethasone (DEX). Baseline corticosterone levels taken within 3 min of capture are indicated as white bars (*unfilled* = Desert; *patterned* = Urban), and 30 min post-treatment corticosterone samples are indicated as filled bars (*gray* = Desert; *black* = Urban). Numbers indicate treatment sample sizes and different letters indicate significant differences at $p < 0.05$ in the response to injection (i.e. increase between pre- and post-treatment levels).

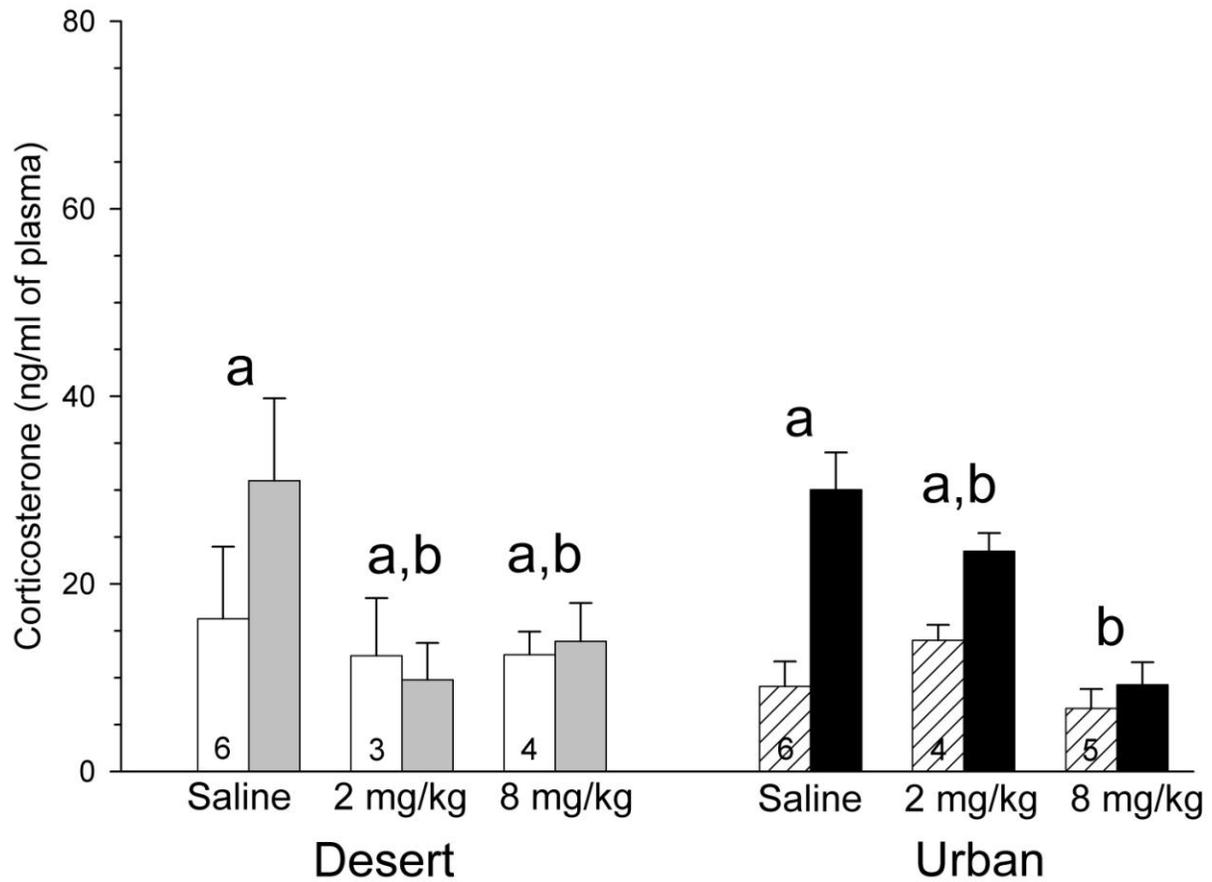


Figure 12. Resistance of the hypothalamic-pituitary-adrenal axis to negative feedback induced by injection with 8 (High) and 2 mg/kg (Low) doses of the synthetic glucocorticoid dexamethasone in urban and desert Curve-billed Thrashers (*Toxostoma curvirostre*) compared to birds injected with 0.9% NaCl (Saline). Baseline corticosterone levels taken within 3 min of capture are indicated as white bars (*unfilled* = Desert; *patterned* = Urban), and 30 min post-treatment corticosterone samples are indicated as filled bars (*gray* = Desert; *black* = Urban). Numbers indicate treatment sample sizes and different letters indicate significant differences at $p < 0.05$ in the response to injection (i.e. increase between pre- and post-treatment levels).

Chapter 3

ARGININE VASOTOCIN (AVT)-LIKE IMMUNOREACTIVITY DIFFERS BETWEEN URBAN AND DESERT CURVE-BILLED THRASHERS, *TOXOSTOMA CURVIROSTRE*: RELATIONSHIPS WITH TERRITORIALITY AND STRESS PHYSIOLOGY

The neuropeptide arginine vasotocin (AVT: an avian homologue of vasopressin) is a potent regulator of the adrenocortical stress response and water balance, and is involved in numerous social behaviors. These functions of AVT make it susceptible to environmental influence, yet little is understood concerning the variation in the AVT system across habitats. In this study, I compared AVT-like immunoreactivity between male Curve-billed Thrashers, *Toxostoma curvirostre*, from native Sonoran Desert locations and those within Phoenix, Arizona. These populations differ in that urban thrashers are more responsive to territorial intrusion, secrete more corticosterone (CORT) during stress, and have greater access to water than desert counterparts. I related AVT-like immunoreactivity with levels of plasma CORT and osmolality, and with behavioral responses to a simulated territorial intrusion. I detected population variation in AVT-like immunoreactivity in two brain regions: the paraventricular nucleus of the hypothalamus and the medial bed nucleus of the stria terminalis, a part of the limbic system. Immunoreactive AVT within the paraventricular nucleus was associated with plasma CORT levels in urban, but not desert birds, but was not associated with osmolality in birds from either site. Cell density within the medial bed nucleus of the stria terminalis corresponded to a decreased responsiveness to territorial intrusion. I found little evidence supporting differences in the AVT system was associated with variation in water availability between habitats. Collectively these data demonstrate divergence in the AVT system between urban and desert thrashers which may help explain observed variation in both the stress response and territorial behavior between populations.

1. Introduction

The nonapeptides arginine vasotocin (AVT) and its mammalian homolog vasopressin (VP) have a conserved distribution in the vertebrate brain (Moore & Lowry 1998). The wide distribution of these peptides across multiple brain regions (see below) reflects their multiple roles in peripheral and central physiological processes. Physiological actions include maintenance of hydromineral balance by increasing the permeability of the renal collecting ducts to water (Goldstein 2006; Lau *et al.* 2009), acting as a vasoconstrictor (Conklin *et al.* 1996; Donald & Trajanovska 2006), stimulating the secretion of adenohipophyseal hormones during stress (Madison *et al.* 2005; Romero 2006), and changing neuronal activity (Saito & Urano 2001). In addition, vasotocin acts centrally to modulate aggressive and reproductive behaviors (Viglietti-Panzica *et al.* 2005; Goodson *et al.* 2009b, Kabelik *et al.* 2009, Miranda *et al.* 2009, Kime *et al.* 2010). The distribution of AVT immunoreactivity in the avian brain has been described in several species (Panzica *et al.* 1999; Kimura *et al.* 1999; Viglietti-Panzica *et al.* 2001; Fabris *et al.* 2004; Klein *et al.* 2006; Singh & Chaturvedi 2008) and studies have linked behavior and physiological processes to specific AVT-like immunoreactive (hereafter AVT-ir) cell populations in the diencephalon and forebrain (see review Goodson & Kabelik 2009).

In birds as in mammals, the paraventricular nucleus (PVN) contains AVT-producing magnocellular and parvocellular neurons (Panzica *et al.* 1999; Kimura *et al.* 1999; Viglietti-Panzica *et al.* 2001; Fabris *et al.* 2004; Singh & Chaturvedi 2008). Magnocellular neurons of the PVN, as well as neurons secreting this peptide in the supraoptic nucleus (SON), release AVT into the blood via the neurohypophysis. Once in circulation, AVT plays an important role in osmoregulation (Chaturvedi *et al.* 2000; Seth *et al.* 2004). By contrast, PVN AVT-producing parvocellular neurons secrete the peptide into the median eminence (ME) to induce the secretion of adrenocorticotrophic hormone (ACTH) by pituitary gland corticotropes during acute stress (Jurkevich *et al.* 2008;

Kulczykowska *et al.* 2009; de Kloet 2010). As a result, AVT in birds is a potent secretagogue of corticosterone (CORT), the main glucocorticoid produced by the avian adrenal glands (Rich & Romero 2005; Romero 2006).

Parvocellular AVT-secreting neurons are found also in various “limbic” structures including the medial bed nucleus of the stria terminalis (BSTM). Studies in birds have focused on the role of these neurons in the control of social and aggressive behaviors (Goodson 2008; Goodson *et al.* 2009a). Cells in the BSTM form either a single group (Japanese quail, *Coturnix japonica*: Aste *et al.* 1998) or two distinct subnuclei (BSTM1 and BSTM2; chicken *Gallus gallus*: Jurkevich *et al.* 1999). AVT from the BSTM and the PVN is thought to be released into several brain areas including the lateral septum, where it presumably modulates social/aggressive behavior in a species-specific manner (Goodson & Kabelik 2009). Infusion of AVT into the lateral septum inhibits aggression in territorial species such as the Field Sparrow *Spizella pusilla* (Goodson 1998a; Goodson *et al.* 1999) and the Violet-eared Waxbill, *Uraginthus granatina* (Goodson 1998b), but has the opposite effect in gregarious species such as the Zebra Finch, *Taeniopygia guttata* (Goodson *et al.* 1999; Goodson & Adkins-Regan 1999). Simulated territorial intrusions did not alter the co-localization of AVT and the immediate early gene expression cell marker fos in the BSTM of Song Sparrows, *Melospiza melodia*, but AVT-fos co-localization in the PVN was reduced in the more relative to the less aggressive birds (Goodson & Kabelik 2009). Similar results have been obtained in laboratory mice (Ho *et al.* 2010). These data suggest a role for AVT in the regulation of aggressive behavior that involves actions of the neuropeptide in the BSTM and the PVN. However, the effects of the peptide in these regions appear to be species- and context-specific, and recent research suggests that differences between species in AVT’s effects on aggression reflect differences in procedures used to measure behavior (Goodson *et al.* 2009a). This research demonstrates that AVT exerts a similar influence on

aggressive behavior across species with different levels of sociality and, collectively, these studies highlight the multi-functional nature of the avian central vasotocin system. This system may be influenced by extrinsic factors (i.e., water availability or environmental stress), but little information is known about intraspecific variation in AVT particularly across habitat types.

Animal populations inhabiting urban areas often differ from their non-urban counterparts physiologically and behaviorally, such as in terms of their adrenocortical stress response (Partecke *et al.* 2006; Schoech *et al.* 2007; French *et al.* 2008; Fokidis *et al.* 2009) and territoriality (Newman *et al.* 2006; Chapter 7). The Curve-billed Thrasher, *Toxostoma curvirostre*, is a native passerine (family: Mimidae) of the Southwest USA that is at home in the Sonoran Desert whether within the city of Phoenix, Arizona, (*hereafter* urban birds) or in undisturbed natural habitat (*hereafter* desert birds). Compared to urban birds, desert thrashers have higher baseline (“pre-stress”) plasma CORT, particularly during molt, and a seasonally more variable plasma CORT response to a 30 min acute stress induced by capture and restraint (Fokidis *et al.* 2009). Desert thrashers also have a lower ratio of blood heterophils to lymphocytes (*hereafter* H:L ratio), which is often used as an alternate indicator of stress in birds (Vleck *et al.* 2000; French *et al.* 2008; Fokidis *et al.* 2008), than urban birds (Fokidis *et al.* 2008). In addition, desert thrashers secrete less CORT than urban birds in response to AVT administration and this variation may reflect a population difference in pituitary gland sensitivity to this peptide (Chapter 2). It is not known whether this putatively lower pituitary gland sensitivity to AVT of desert versus urban birds reflects a population difference in AVT secretion. Furthermore, urban and desert thrashers differ behaviorally. Urban thrashers are behaviorally more responsive than desert conspecifics to simulated territorial intrusion during and outside the breeding season (Chapter 7). This difference is not related to population differences in plasma testosterone or CORT (Chapter 7), suggesting that it is centrally mediated.

The “mesic” landscaping that commonly takes place in the Phoenix metropolitan area involves extensive water supplementation through irrigation. This supplementation results in alterations of the local ecosystem (Martin & Stabler 2002; Keys *et al.* 2007; Shen *et al.* 2008) and for practical purposes, in a habitat with unlimited water availability (Cook & Faeth 2006; Shen *et al.* 2008). By contrast, in the surrounding Sonoran Desert water availability is intermittent, resulting primarily from inconsistent precipitation events and playing a major role in the control of reproductive cycles in thrashers and other species (Dawson *et al.* 1989). Previous research found differences in plasma AVT within and among species of Australian honeyeaters (family Meliphagidae) occupying habitats that differ with respect to water availability (Goldstein & Bradshaw 1998). Death Valley pupfish, *Cyprinodon nevadensis*, showed variation in neural AVT-ir between isolated populations that differ with respect to their habitat temperatures and social organization (Lema & Nevitt 2004). Thus, populations subjected to different environmental conditions may demonstrate variation in the neural AVT system.

I compared AVT-ir neurons in the brains of thrashers inhabiting either an urban or native desert habitat to test two hypotheses. The first hypothesis was that urban thrashers have a higher level of AVT-ir in the PVN than desert birds, reflecting the above-described difference in activity of the adrenocortical stress response and increased access to water. According to this hypothesis, I predicted that AVT-ir expression in the PVN is positively associated with plasma CORT levels and osmolality. The second hypothesis was that AVT-ir expression in the BSTM is lower in “behaviorally more responsive” urban than in “behaviorally less responsive” desert thrashers. Accordingly, I predicted that AVT-ir expression in the BSTM is positively correlated with territorial behavior during a simulated territorial intrusion. To my knowledge, this is the first investigation to describe neuroendocrine differences associated with urbanization in a free-living vertebrate species.

2. Materials and Methods

All procedures described herein were approved by the Arizona State University Institutional Animal Care and Use Committee and were conducted under appropriate scientific collecting permits from the US Fish and Wildlife Service, the Bureau of Land Management, and the Arizona Game and Fish Department.

2.1. Study Locations

This study was conducted at a desert and an urban location. The desert site was the largely unpopulated Hummingbird Springs (12,626 ha) and the adjoining Bighorn Mountains Wilderness Area (8498 ha) located about 80 km west of the city of Phoenix, Arizona. The vegetation at this site is typical of upland Sonoran Desert and includes saguaro (*Carnegiea gigantea*), barrel cactus (*Ferocactus wislizeni*), prickly pear (*Opuntia* spp.), cholla (*Cylindropuntia* spp.), ocotillo (*Fouquieria splendens*), mesquite (*Prosopis* spp.), and palo verde (*Prosopis* spp.). Urban birds were captured within the city of Phoenix, in an area that primarily consists of middle and low income residential housing tracts with their associated commercial areas. Birds were captured between 11 Sept and 22 Oct 2008, after the species' breeding season and during the prebasic molt period. Captures took place between 0604 and 1035 h.

2.2. Behavior Recording

Thrashers (8 from the urban location and 8 from the desert location) were captured by luring them to mist nets using conspecific playback recordings, which simulates a territorial intrusion. I used only males, which are more readily captured than females using this technique. Playback recordings consisted of songs and calls of thrashers compiled from independent recordings of

several individual birds from sites in and around Phoenix, Arizona. Recordings were played on a Sony MD Walkman minidisk player through a handheld speaker at constant amplitude (74 dB at 2 m distance from the speaker).

The urban site was sampled during times of minimal human disturbance (early mornings and weekends). When a male bird was located, a mist net was erected and the bird was exposed to the recording with the observer (HBF) located 40-80 m away and having an unobstructed view of the mist net and surrounding area. The observer, remaining quiet and still, recorded the occurrence of several stereotyped behaviors previously reported as being associated with responses to playback (Chapter 7). These included: 1) the latency to first approach the playback speaker; 2) the number of approaches from the air and ground; and 3) the number of “whit-whit” calls, the predominant vocalization made by this species (Tweitt 1996; Chapter 7). All birds were observed during the entire time before capture, but behavioral observations were corrected for the total time each bird was observed. Sex was confirmed by examination of gonads after euthanasia (see below).

2.3. Bird Collection

Within 3 min of capture, thrashers were removed from the net and approximately 300 μ l of blood, for measuring plasma CORT and osmolality, was collected from the right jugular vein using a heparinized 0.3 cc syringe with a 29.5 gauge needle. Blood was collected rapidly to avoid increases in plasma CORT associated with the acute stress response (Fokidis *et al.* 2009). In addition, a 5 μ l blood sample was used to prepare a thin blood smear on a glass microscope slide for H:L ratio determination (Fokidis *et al.* 2008; French *et al.* 2008). Blood samples were kept on ice until the plasma was separated by centrifugation and then stored at -80° C until assayed. I measured the intensity of body molt on a scale ranging from 0 (no molt) to 2 (heavy generalized molt), and also

the body mass (± 0.1 g), tarsus and culmen length (± 1 mm), and wing chord length (± 1 mm) of each bird

prior to euthanasia. Replacement of primary feathers in this species is typically completed by August to early September in Arizona (Pyle 1997), and was completed by the onset of this study. Thus our wing chord measurements were unaffected by error associated with abraded feathers.

2.4. Tissue Processing

After the above measurements were taken, birds were deeply anesthetized using Metofane (methoxyflurane: Mallinckrodt, Mundelein, IL, USA) inhalation and euthanized by decapitation. Brains were removed from the skull (6-11 minutes post-capture) and placed into to 5% acrolein solution in 0.1 M phosphate buffer (PB) overnight at 4 °C (King *et al.* 1983; Luquin *et al.* 2010). Brains were then post-fixed and gelatin-embedded prior to cryostat sectioning following a modified protocol outlined in Saldanha *et al.* (1994). Briefly, they were rinsed three times with 0.1 M PB (30 min each) and then immersed in 4% gelatin solution for 30 minutes. Brains were then embedded in an 8% gelatin solution-filled mold and gelatin was allowed to solidify overnight at 4 °C. Embedded brains were post-fixed in 4% paraformaldehyde for 48 hrs and then immersed in 10%, 20%, and then 30% sucrose solutions in PB for 48 hours each. Brains were frozen on dry ice and stored at -80 °C until sectioned.

2.5. Arginine Vasotocin Immunohistochemistry

Brains were coronally sectioned at 30 μm and every 3rd section was collected into cryoprotectant solution (Watson *et al.* 1986). Free-floating sections were stained for AVT-ir using an indirect immunohistochemical procedure modified from (Small *et al.* 2008). Sections were washed

three times with 0.1 M PB for 30 min, incubated with 0.36% H₂O₂ in PB for 15 min, washed three times with 0.1M PB (5 min each), incubated with normal horse serum (1:30 in PBT: PB containing 0.3% Sigma Triton X-100; Sigma-Aldrich Co., St. Louis, MO, USA) for one hour, and then incubated overnight in 0.3% PBT containing anti-AVT polyclonal antibody (1:15,000; raised in rabbit and generously provided by Dr. M. S. Grober (Georgia State University, Atlanta, GA, USA). Sections were washed five times (10 min each) in 0.1 M PB, incubated for one hour in 1:100 biotinylated horse anti-rabbit IgG in 0.3% PBT (Vector Laboratories Inc. Burlingame, CA, USA), washed three times for 10 min each in 0.1 M PB, incubated in Vectastain ABC solution (Vector Laboratories Inc.) for one hour, washed three times (15 min each) in 0.1 PB, incubated for 3 min in Vector SG peroxidase chromagen (Vector Laboratories Inc.), and washed twice for 5 min in 0.1 PB. Immunostained sections were mounted onto gelatin-coated glass microscope slides, allowed to dry at room temperature for 24 hours, dehydrated with ethanol, cleared in xylene, and coverslipped using Cytoseal 60 (Stephens Scientific, Kalamazoo, MI, USA).

The antibody specificity was tested by incubating sections as above, but omitting the primary or secondary antiserum, or replacing the ABC solution with buffer. Sections incubated in these conditions showed no staining. Preabsorption with 200 µg/ml of AVT (# V0130, Sigma-Aldrich Co., St. Louis, MO, USA) also eliminated the staining which was, however, not affected by preabsorption with 200 µg/ml of mesotocin (# H-2505, Bachem Inc., Torrance, CA, USA).

2.6. Image Analyses

Images of brain sections were digitized using a camera (Olympus DEI-750D, Olympus Optical Co. Ltd., Tokyo, Japan) attached to a light microscope (Olympus BX60) at 40× magnification and using constant microscope, camera, and computer settings. All images were

analyzed blind with respect to all variables examined. In addition, for each brain section photographed, an “out of focus” image was taken of an area devoid of immunolabelling to correct for background staining as described in (Small *et al.* 2008). Images were analyzed using Image-Pro Plus version 4.0 (Media Cybernetics, Silver Springs, MD, USA). Briefly, all images were converted to black and white (Gray Scale 16 function) and flattened (Filter enhancement function), and the background staining image was “subtracted” from the image of interest (background correction function).

Brain regions were selected for analysis after visual inspection of immunolabelled brain sections (Figure 13). Regions were defined on the basis of easily recognizable neuroanatomical landmarks defined in Stokes *et al.* (1974) and Balthazart *et al.* (1996). The bed nucleus of the stria terminalis was further defined based on Aste *et al.* (1998). The nomenclature used throughout is based on the above studies and on revisions published by Reiner *et al.* (2004). Below are descriptions of areas where most AVT-ir was observed.

Discrete cell clusters were observed throughout the preoptic area (POA) with fibers primarily in the most rostral portion of this region. In a couple of brain sections from each bird, perikarya were confined to a narrow strip of overlapping cells bordering the third ventricle. Diffuse immunoreactive perikarya and fibers were also detected in the supraoptic nucleus (SON) medial to the nucleus geniculatus lateralis ventriculatus (GLV). Extensive immunostained fibers and few lightly stained perikarya were present in the lateral hypothalamus (LHy) and extending laterally throughout the lateral forebrain bundle (fasciculus prosencephali lateralis, FPL) and along the edge of the tractus thalamo-medialis (TFM) dorsal to the nucleus rotundus (Rt). Discrete populations of cells with few fibers were seen in the paraventricular nucleus (PVN), from which fibers apparently extended to the ME. The rostral portion of the bed nucleus of the stria terminalis (BSTM) contains

two discrete cell populations. One population of perikarya was located dorsal to the medial tractus occipitomesencephalicus (OM) and is referred to as the dorsolateral BST (BSTM1) population. A second population of perikarya was located lateral to the PVN and borders the OM, and I refer to this population as the ventromedial BST population (BSTM2). By contrast to previous studies (Aste *et al.* 1998; Jurkevich *et al.* 1999; Kabelik *et al.* 2008; Hattori & Wilczynski 2009; Barka-Dahane *et al.* 2010), no immunoreactive fibers or perikarya were observed dorsal to the anterior commissure or along the lateral septa. Extensive immunoreactive fibers were present dorsal of and within the ME.

Our analyses focused on AVT-ir perikarya and not fibers. I used five measurements to quantify differences in AVT-ir between urban and desert thrashers. First, I measured the overall optical density of AVT-ir within a 100 μm diameter circular area of interest (AOI) centered over the brain region of interest. Intensity of staining within AOIs (hereafter staining intensity) was defined as the optical density (0 = all black, 256 = all white) and not the staining hue or saturation (Kabelik *et al.* 2008). Second, for each brain region I measured the density (number of perikarya within the 100 μm AOI) of AVT-ir perikarya (hereafter AVT-ir cell density) using an automatic count function optimized to recognize cells based on optical density and shape. When necessary cells missed by the automatic count function (i.e. were not highlighted) were counted manually. Third, the cross-sectional area of a subset ($n = 7$ to 20 cells per section) of clearly delineated and non-overlapping AVT-ir perikarya within each brain region was determined using a manual tracing function. Fourth, I measured the optical density of the AVT-ir perikarya for which cross-sectional area was available. For both size and optical density of immunostained perikarya, means from multiple sections including both left and right hemispheres from each bird from the subset of cells examined for each brain region were used for statistical analyses. Perikaryon area and optical density data were not collected for the POA, LHy, and SON due to the presence of numerous AVT-ir fibers that made

individual perikarya difficult to distinguish. Fifth, to quantify AVT-ir in the ME, the optical density of ten circular AOIs (each 25 μm diameter) placed along the entire length of a representative image of the ME from each bird was determined. Mean optical densities of staining obtained for the ME of each bird were used for statistical analyses.

2.7. Corticosterone Assay And Plasma Osmolality

Total plasma CORT concentration was measured using validated commercial competitive enzyme-linked immunoassays (ELISA; Assay Designs Inc. Ann Arbor, Michigan, USA) as described by Fokidis *et al.* (2009). The sensitivity of the CORT assay was 11.9 pg/ml and the mean intra-assay coefficient of variation was 6.23 % with all samples assayed in duplicate.

Plasma osmolality (mOsm/kg of H₂O) was measured using a vapor pressure osmometer (Model 5500XR, Wescor Inc. Logan, Utah, USA) with 10 μl samples assayed in duplicate. The osmometer was calibrated to known concentration standards before use, and its use to determine the osmolality of thrasher plasma has been previously validated (Fokidis *et al.* 2010).

2.8. Heterophil To Lymphocyte Ratio

Blood smears were fixed for 10 min in absolute methanol within 5 days of collection and then stained using the Giemsa method (Bennett 1970). Stained slides were cleared using xylene, cover-slipped, and sealed using Cytoseal 60 (VWR, San Francisco, CA). Using an Olympus BX60 light microscope (Olympus Optical Co., Tokyo, Japan) heterophils and lymphocytes were counted at 400 \times magnification until a total of 100 cells of both cells types combined were counted (French *et al.* 2008; Fokidis *et al.* 2008). Cell types were identified using the criteria of Campbell (1996). Slides were examined without knowledge of the bird identity, collection site, or collection date.

2.9. Statistical Analyses

Comparisons of sampling date, sampling time, and morphological, physiological, and the latency to first response to playback between urban and desert populations were done using Student's t-tests. The number of approaches towards the speaker and the number of calls were analyzed using Mann-Whitney U-tests. Body condition was defined as the standardized residuals of a linear regression of body mass on tarsus length as described in a previous study (Fokidis *et al.* 2008). Body molt differences between urban and desert birds were assessed using a chi-squared (χ^2) test. Data for AVT-ir staining intensity were normally distributed, thus allowing for comparisons across brain regions (PVN, LHy, POA, SON, BSTM2, and BSTM1) using analysis of variance (ANOVA), with site as a fixed factor and body condition, molt intensity, sampling date, and time of sampling as covariates. Differences between brain regions were assessed using Fisher's least significant difference post-hoc tests. Cell cross-sectional area and cell optical density data followed a Poisson distribution and they as well as corresponding AVT-ir cell densities were compared using non-parametric Kruskal-Wallis tests with brain region as the main effect. Post-hoc comparisons were done using Dunnett's T3 test for non-equal variance.

Pearson product moment correlations were used to determine the degree to which AVT-ir was related between the different brain regions. For the sake of brevity, only significant correlations will be discussed in the results. Pearson correlations were also used to relate variation in AVT-ir within different regions with optical densities in the ME, and to examine the relationship between AVT-ir and plasma osmolality, CORT, H:L ratio, and territorial behaviors. All data were tested for the presence of outliers using Chauvenet's criterion outlier test (at $p < 0.05$), which identifies data points beyond two standard deviations from the corresponding group mean. A single outlying point (latency to first response (desert sample) = 1043 secs) was identified and was excluded from

subsequent analysis. Whether site (urban vs. desert) influenced the magnitude or direction of correlations between variables was determined using Fisher Z-transformed correlation coefficients. When the slopes of relationships differed significantly between sites, separate correlations are depicted for each site. All data are presented as either means \pm standard errors (SE) or medians \pm 95% confidence intervals. The critical alpha level for all tests was set at $p \leq 0.05$, except in the case of multiple univariate comparisons where a Bonferroni-corrected alpha value of $p \leq 0.014$ is also presented.

3. Results

3.1. Morphology, Physiology, And Behavior Of Urban And Desert Thrashers

Neither sampling date nor time of sampling differed between urban and desert birds (Table 5). Urban thrashers were heavier, but not structurally larger (i.e., no difference in wing chord or tarsus length) than desert conspecifics (Table 5). Brain mass of birds from the two study populations was similar and all birds were in non-reproductive condition as evident by regressed testes and undeveloped cloacal protuberances (Table 5). Desert birds had higher plasma baseline CORT than urban conspecifics (Table 5). Plasma osmolality did not differ between urban and desert thrashers (Table 5). All birds were undergoing body molt and urban and desert thrashers did not differ with respect to their molt intensity ($\chi^2 = 4.462, p = 0.106$). Consistent with previous research, during simulated territorial intrusion urban thrashers responded more quickly (Table 5) and with a greater number of approaches toward the speaker than desert birds (Mann-Whitney U test: $Z = -2.515, p = 0.011$). The number of calls given during playback did not differ between sites (Mann-Whitney U test: $Z = -0.095, p = 0.933$).

3.2. Brain AVT-Ir In Urban And Desert Thrashers

The AVT-ir staining intensity differed across brain regions (ANOVA: $F = 4.913$, $df = 2,14$, $p = 0.047$). Perikarya in various brain regions also differed with respect to their AVT-ir optical density (Kruskal-Wallis test = 19.983, $df = 2$, $p=0.014$) and cross-sectional area (Kruskal-Wallis test = 7.112, $df = 2$, $p=0.035$). In contrast, the density of AVT-ir stained perikarya did not differ between brain regions (Kruskal-Wallis test = 3.102, $df = 2$, $p=0.367$). No relationships were observed between any of these measures of AVT-ir and sampling date or time, body condition or molt intensity (all $p \geq 0.092$).

The density of AVT-ir staining in the BSTM2 was 19% higher in desert than urban birds (Figure 14). No site differences in overall optical density were observed for the other brain regions (Figure 14), and no site differences were observed in the AVT-ir cell density (Figure 14). However, AVT-ir cells within the PVN were 11% darker and 21% smaller in urban than desert birds (Figure 14). Urban thrashers also had a 73% darker ME than their desert conspecifics (Figure 15).

3.3. Covariation Of AVT-Ir Brain Regions

Staining intensity within the BSTM2 was positively correlated with staining intensity in the LH_y ($r = 0.499$, $p = 0.049$) and negatively correlated with staining intensity in the POA ($r = -0.505$, $p = 0.046$). There was also a positive correlation between AVT-ir perikaryon density and the cross-sectional area of these perikarya in the LH_y ($r = 0.869$, $p \leq 0.001$). The AVT-ir cell density in the PVN was correlated with cell cross-sectional area in the BSTM2 ($r = 0.507$, $p = 0.045$). Optical densities of cells within the PVN were also correlated with their cross-sectional area ($r = 0.832$, $p \leq 0.001$) and negatively with staining intensity within the BSTM1 ($r = -0.480$, $p = 0.038$).

Optical density within the ME was negatively correlated primarily with staining in the

BSTM2 in terms of the AVT-ir cell density ($r = -0.590, p = 0.014$), staining intensity ($r = -0.568, p = 0.022$), and cell optical density ($r = -0.490, p = 0.043$). Thus a darker ME was associated with lighter and fewer AVT-ir cells in the BSTM2. Optical density within the ME was also positively correlated with cell cross-sectional area in the LHy ($r = 0.537, p = 0.032$).

3.4. Stress, AVT-Ir, And The PVN

Plasma CORT was negatively correlated with PVN staining intensity (Figures 17-18) and positively correlated with AVT-ir cell cross-sectional area (Figure 18) in urban thrashers, but not desert birds. Thus urban birds with higher plasma CORT levels had a lighter PVN composed of smaller AVT-ir cells. The AVT-ir cell density within the PVN was correlated with H:L ratio ($r = 0.497, p = 0.050$), but other measures of the PVN were not associated with plasma CORT (all $p \geq 0.125$). There was a negative correlation between overall staining intensity of the LHy and the H:L ratio ($r = -0.525, p = 0.037$). Plasma CORT was also negatively correlated with H:L ratio (Figure 18). Neither plasma CORT nor H:L ratio was associated with optical density within the ME (both $p \geq 0.316$).

3.5. Plasma Osmolality And AVT-Immunoreactivity

There were no associations between plasma osmolality and any variable for any brain region (all $p \geq 0.069$), with the exception of the optical density within the ME in urban thrashers (Figure 16).

3.6. Territorial Behavior And AVT

Latency to the first response to playback was negatively correlated with the number of calls

($r = -0.699, p = 0.002$) and of approaches toward the speaker ($r = -0.501, p = 0.025$). The AVT-ir cell density within the BSTM2 was correlated with the latency to first response to playback (Figure 19), but not with either the number of approaches toward the speaker or the number of calls (both $p \geq 0.264$).

Staining intensity and AVT-ir cell numbers in the PVN was negatively correlated with latency to first responses to playback (overall: $r = -0.528, p = 0.018$; cell: $r = -0.799, p \leq 0.001$). However the former relationship was only significant for urban thrashers (Figure 18). The PVN staining intensity was correlated with the number of approaches birds made toward the speaker ($r = 0.521, p = 0.038$). Thus darker (i.e., more immunoreactive) cells within the PVN were associated with more responsive behavior.

Latency to first response to playback was also correlated with the cross-sectional area of AVT-ir cells with the LH_y ($r = 0.405, p = 0.044$). Optical density of the ME was negatively correlated with the latency to first response to playback (Figure 16). The number of calls was not associated with measures taken from any brain region (all $p \geq 0.092$).

4. Discussion

The central AVT system is involved in numerous functions, such as those associated with anthropogenic change, that may be susceptible to environmental influences. I demonstrated differences in AVT-ir within brains of free-living songbirds sampled from a native Sonoran Desert location and a nearby urban habitat. Consistent with previous work on birds, (Panzica *et al.* 1999; Kimura *et al.* 1999; Viglietti-Panzica *et al.* 2001; Fabris *et al.* 2004; Klein *et al.* 2006; Singh & Chaturvedi 2008), AVT-ir perikarya in thrashers were observed in multiple brain regions (PVN, POA, SON, BSTM1, BSTM2) and staining was also present within the ME. I also found habitat-

related differences in brain expression of AVT-ir and these differences were primarily confined to the PVN and the BSTM2. Neuropeptides from the PVN regulate the adrenocortical stress response (Singh & Chaturvedi 2006; Shibata *et al.* 2007), and water balance (Seth *et al.* 2004; Singh & Chaturvedi 2006). I observed that AVT-ir perikarya are 21% larger in the PVN of desert than urban thrashers. However, larger AVT-ir PVN perikarya and a lighter density of AVT-ir material in this region were associated with increased plasma CORT only in urban birds.

The BSTM2 is thought to regulate antagonistic behavior, including responses to territorial intrusion (Goodson *et al.* 2009a; Kabelik *et al.* 2010). Staining for AVT-ir in the BSTM2 was darker in desert than urban thrashers, but only birds with fewer AVT-ir cells within the BSTM2 responded quicker to a simulated territorial intrusion. Urban but not desert birds with a lightly stained PVN also responded quicker to a simulated intrusion on territory than birds with a darkly stained PVN. The intensity of AVT-ir staining in the ME was higher in urban than desert thrashers, suggesting that urban thrashers release more AVT into their hypophyseal portal system. The intensity of AVT-ir staining in the ME was also positively associated with the latency of thrashers to respond to simulated territorial intrusion, but not with plasma CORT. However, further research on this topic is warranted because few data are available regarding whether the intensity of peptide immunolabelling in the ME reflects their release into the hypophyseal circulation. These data may suggest that decreased AVT-ir in the BSTM2 and increased AVT-ir in the ME is related to increased territorial behavior. This conclusion supports the hypothesis that AVT inhibits aggressive behavior in territorial situations.

4.1. Hypothalamic AVT, Stress, And Water Balance

Corticotropin-releasing hormone (CRH) and AVT are predominant secretagogues of pituitary

gland ACTH during a stress response (Madison *et al.* 2008; de Kloet 2010). In birds, peripheral AVT administration increases plasma CORT (Romero & Rich 2007; Madison *et al.* 2008) and I recently demonstrated that this increase is larger in urban than desert thrashers (Chapter 2). This finding suggests elevated pituitary gland sensitivity to AVT of urban relative to desert birds. Here I demonstrate that urban thrashers have darker AVT-ir PVN perikarya than desert birds, a difference that may reflect a population difference in hypothalamic AVT production and/or storage. In addition, the intensity of AVT-ir staining in the ME is higher in urban than desert birds, suggesting higher secretion of the peptide into the hypophyseal portal system. As well, larger and lighter AVT-ir cells in the PVN were associated with elevated plasma CORT, but only in urban birds, and an increased AVT-ir cell density was associated with a higher H:L ratio, an alternate stress marker that in this species was negatively correlated with plasma CORT.

Increased AVT-ir staining in PVN perikarya may reflect cellular accumulation of the peptide resulting, e.g., from reduced processing rate or transport (Panzica *et al.* 2001; Goodson & Kabelik 2009). Most authors, however, consider that increased immunoreactivity in perikarya reflects elevated peptide production in response to higher levels of neuropeptide secretion, (Kabelik *et al.* 2008; Singh & Chaturvedi 2008, Hattori & Wilczynski 2009, Iwata *et al.* 2010; Sewall *et al.* 2010). This interpretation would be consistent with our observation that birds with more AVT-ir staining in the PVN also showed more immunostaining in the ME. Accordingly, urban birds apparently produce and secrete more hypothalamic AVT than desert birds. The larger AVT-ir PVN perikarya of urban than desert birds may reflect this difference. Urban thrashers had lighter AVT-ir PVN perikarya than desert thrashers. With the observation that AVT-ir staining in the ME was more intense in urban than desert birds, these observations suggest that in response to acute stress, urban thrashers secrete AVT into the hypophyseal portal system either in larger amount or faster than desert thrashers.

Consistent with this contention, acute stress often stimulates CORT secretion more in urban than desert birds (Fokidis *et al.* 2009).

Hypothalamic AVT also acts as an anti-diuretic hormone and previous research found that magnocellular cells in the PVN and SON play an important role in the control of water balance in birds (Chaturvedi *et al.* 2000; Seth *et al.* 2004). However, in this species I did not identify AVT-ir cell populations that could correspond to magnocellular and parvocellular perikarya. Plasma osmolality in the present study was similar to that measured in other bird species (Gray & Erasmus 1989; Gray & Brown 1995; Goecke & Goldstein 1997; Goldstein & Bradshaw 1998; Saito & Grossmann 1998; Sharma *et al.* 2009) and in Curve-billed Thrashers (Vleck 1993) and I found neither an association between brain AVT-ir expression and plasma osmolality nor a population difference in plasma osmolality, suggesting no difference in water stress between habitats. However, plasma osmolality was positively correlated with AVT-ir staining intensity within the ME in urban but not desert birds. As peptides secreted into the ME presumably act on the adenohypophysis and not the neurohypophysis, the significance of this correlation is currently unclear.

4.2. Effects Of AVT On Territorial Behavior

Secretion of AVT from the BSTM inhibits aggressive behavior in rats challenged with an intruder (Brown & King 1984), and has a similar effect in territorial birds (Goodson & Kabelik 2009). These observations led to hypothesize that AVT inhibits aggressive behavior in species that actively establish and defend a territory, an idea that is also supported by research on fish and reptiles (Larson *et al.* 2005; Lema 2006; Santangelo & Bass 2006; Dewan *et al.* 2008; Greenwood *et al.* 2008; Hattori & Wilczynski 2009). Thrashers in the city of Phoenix are more responsive to conspecific playback than desert conspecifics throughout the year (this study; Chapter 7). In this

study, urban thrashers expressed less AVT-ir in one BSTM subregion than desert thrashers, and birds that responded quicker to playback had fewer AVT-ir cells in the BSTM2. These data support the hypothesis that low brain expression of AVT in the BSTM is associated with high aggression in this territorial species. Establishing the generality of this hypothesis does, however, warrant further research because there was no relationship between BSTM levels of AVT-ir and two other behaviors (number of approaches towards the speaker and number of calls) that were stimulated by song playback exposure.

The behavioral response of thrashers to playback song involved exposure to an auditory stimulus but not a live decoy, and recent research has suggested that the decoy type used in this type of study can influence the behavioral response of challenged birds (Scriba & Goymann 2008). In Lincoln's Sparrows *Melospiza lincolni*, song quality (high vs. low based on song length, complexity, and trill performance) altered AVT-ir expression in the forebrain, particularly the BSTM and lateral septum (Sewall *et al.* 2010). Given these findings, future studies will benefit from investigating the activity of the central AVT system under different experimental conditions. The context of aggression may also be important for the regulation of the central AVT system. In territorial violet-billed waxbills, Goodson *et al.* (2009a) found that inhibiting the effect of AVT by administration of a V_{1a} receptor antagonist decreased aggression in males that were tested during a mate competition but not a resident-intruder paradigm, except for birds that were typically subordinate.

The BSTM is considered to be part of a neural network that also includes the POA and lateral septum and that regulates behavioral responses to social stimuli (Goodson 2005). I observed no AVT-ir staining in the lateral septum, and the expression of AVT in the POA did not differ between urban and desert thrashers. The close negative association between AVT-ir in the ME and the latency to first response to playback suggests that AVT release into portal circulation plays a role

in the control of this behavior. The observation that the intensity of AVT-ir staining in the ME was positively associated with the BSTM2 AVT-ir cell density further supports this idea. However, recent research has proposed that in territorial species, the secretion of AVT from the PVN, and not the BSTM, into responsive extrahypothalamic sites is the primary inhibitor of aggression (Goodson & Kabelik 2009). Thus the above-described association may reflect the “stress” of responding to territorial intrusion. However, in previous research in breeding thrashers (Chapters 5 and 7) and in this study of non-breeding thrashers, no association between CORT and behavior was observed. Additionally, previous research also demonstrated that breeding territorial behavior in this species was not directly linked to acute changes in testosterone (Chapter 7). These findings suggest a divergence in behavioral responses at the neural level that may be mediated by variation in the AVT within the BSTM between urban and desert thrasher populations.

4.3. Urban Influences On The AVT System

The urban environment is arguably the most rapidly expanding habitat on earth. Identifying characteristics that enable species to live in this environment requires an understanding of their physiological basis. I found that neuropeptide distribution and behavior can differ between urban inhabitants and their wild land counterparts. The study adds to a growing body of literature that shows differences in physiology associated with urbanization. Stress physiology and particularly CORT secretion can be altered by urbanization (Partecke *et al.* 2006; Schoech *et al.* 2007; French *et al.* 2008; Fokidis *et al.* 2009) and this study provides evidence that this alteration may involve a role for brain AVT. Other studies have uncovered behavioral shifts, especially in gregarious species, including increased tameness and approachability (Moller 2008; Valcarcel & Fernandez-Juricic 2009); increased vigilance (Partan *et al.* 2010); changes in singing behavior (Fuller *et al.* 2007; Slabbekoorn &

Ripmeester 2008); and altered migratory behavior (Partecke & Gwinner 2007) as a function of urbanization. Each of these differences may ultimately have a basis in divergence or plasticity in neural processes. Thus the urban environment provides an exciting “natural” system from which to investigate shifts in behavioral phenotypes and the underlying processes that mediate them.

5. References

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Table 5. Mean sampling date and time, as well as morphometrics, reproductive status, and variables associated with stress, water balance, and territorial behavior in adult male Curve-billed Thrashers sampled from urban and desert sites ($n = 8$ per site). * indicates significant differences between sites at $p \leq 0.05$ according to Student-t tests.

	Desert	Urban	t_{1,15}	p-value
Sampling date	7 Oct 08	07 Oct 08	0.372	0.291
Time of sampling	07:40 am	06:28 am	1.008	0.104
Body mass (g)	72.8 \pm 1.77	78.6 \pm 1.58	2.161	0.007 *
Wing chord (mm)	106.8 \pm 1.07	103.7 \pm 1.96	1.372	0.173
Tarsus length (mm)	34.5 \pm 0.38	33.8 \pm 0.39	1.308	0.191
Brain weight (g)	6.06 \pm 0.61	6.01 \pm 0.47	1.139	0.247
Cloacal protuberance (mm)	4.2 \pm 0.32	4.3 \pm 0.27	1.067	0.274
Testes width (mm)	5.7 \pm 0.72	5.5 \pm 0.61	1.092	0.623
Plasma corticosterone (ng/ml)	15.3 \pm 1.24	10.1 \pm 2.11	1.649	0.048 *
Heterophil to lymphocyte ratio	1.1 \pm 0.22	0.9 \pm 0.31	0.205	0.759
Plasma osmolality (mOsm)	302.7 \pm 7.96	293.5 \pm 6.35	0.907	0.343
Latency to first response (s)	196.8 \pm 56.0	91.8 \pm 16.2	2.438	0.026 *

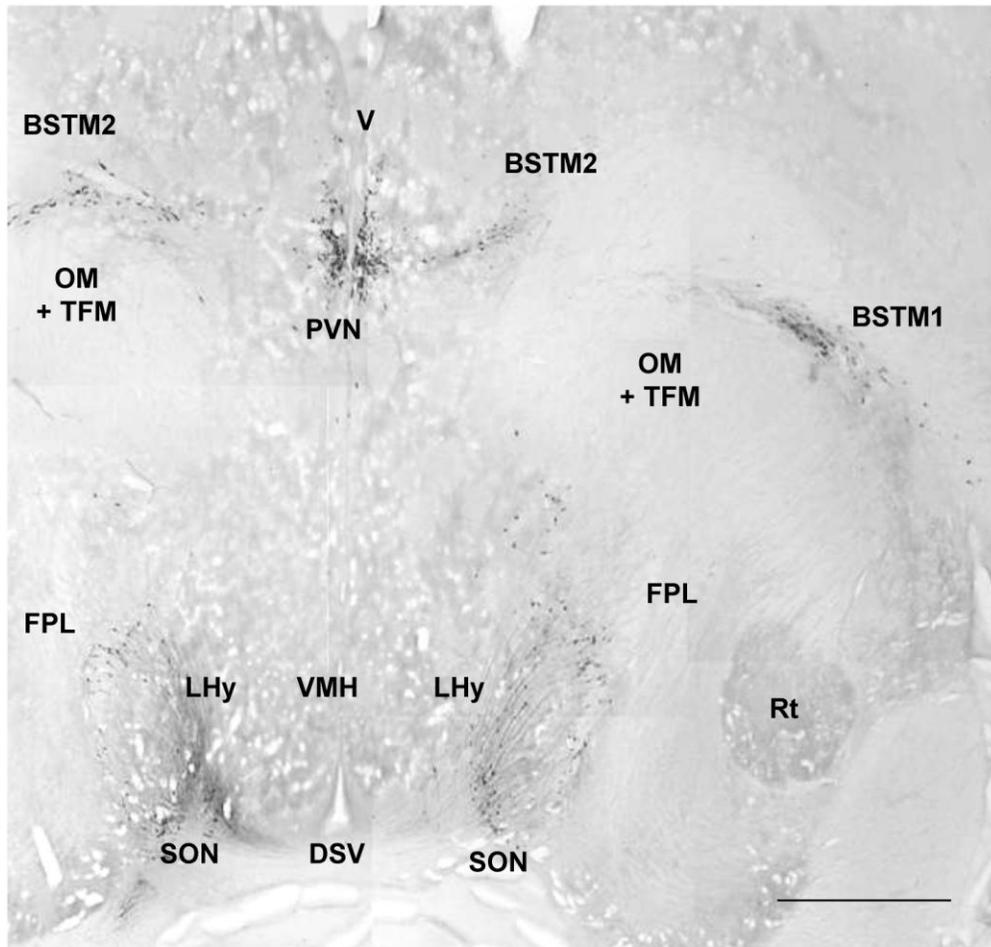


Figure 13. A map of AVT-like immunoreactivity (AVT-ir) within the Curve-billed Thrasher midbrain. Cells immunoreactive for AVT are present within the paraventricular nucleus (PVN), the lateral hypothalamus (LHy), the supraoptic nucleus (SON), and two cells clusters in the medial bed nucleus of the stria terminalis (BSTM1 and BSTM2). Immunoreactive cells are shown in relation to several neuroanatomical landmarks: occipitomesencephalic tract (OM); tractus-thalamo-frontalis et frontalis-thalamicus medialis (TFM); ventricle (V); fasciculus prosencephalicus lateralis (FPL); nucleus rotundus (Rt); ventro-medial hypothalamus (VMH); and the decussatio supraoptica ventralis (DSV). Scale bar represents 500 μ m.

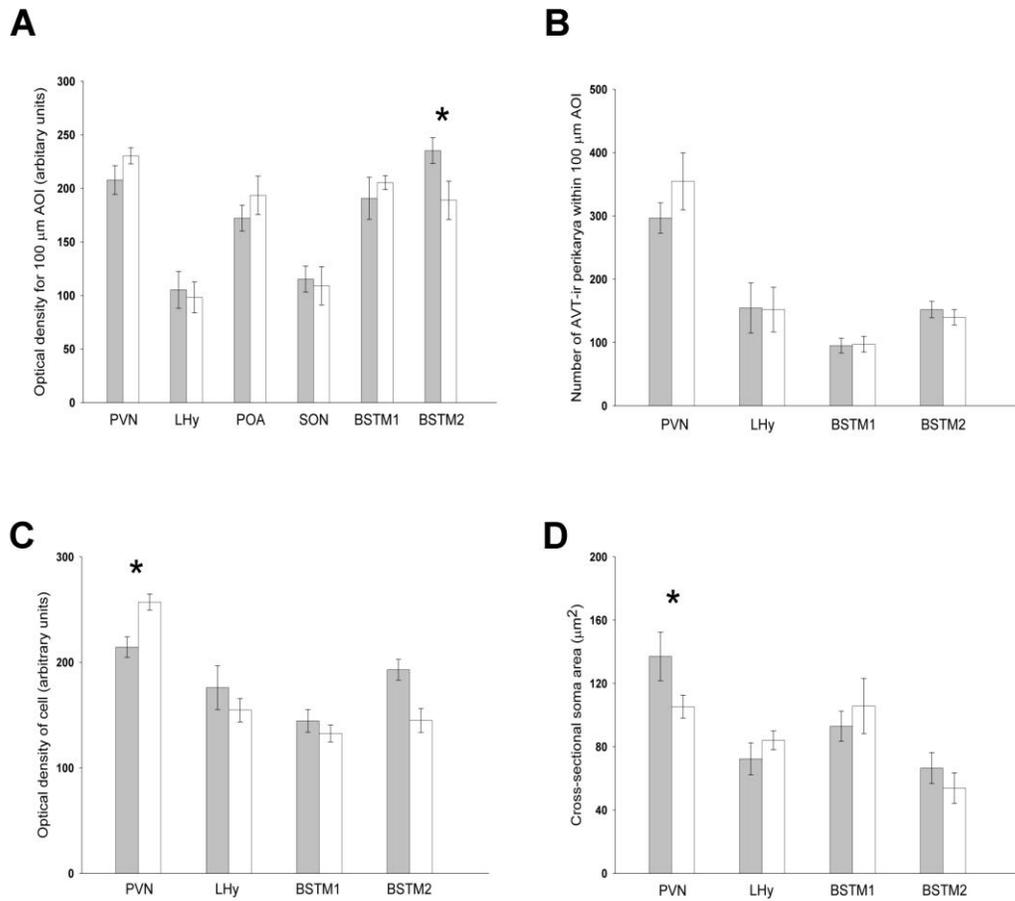


Figure 14. Variation AVT-ir between urban (white bars: $n = 8$) and desert (gray bars: $n = 8$) populations of thrashers with respect to: **(A)** the optical densities of AVT-ir brain regions; **(B)** the AVT-ir cell density within each brain region; **(C)** the optical density of individual AVT-ir cells; and **(D)** the sizes of individual AVT-ir cells. * indicates significant differences ($p \leq 0.05$) between urban and desert birds for specific brain regions.

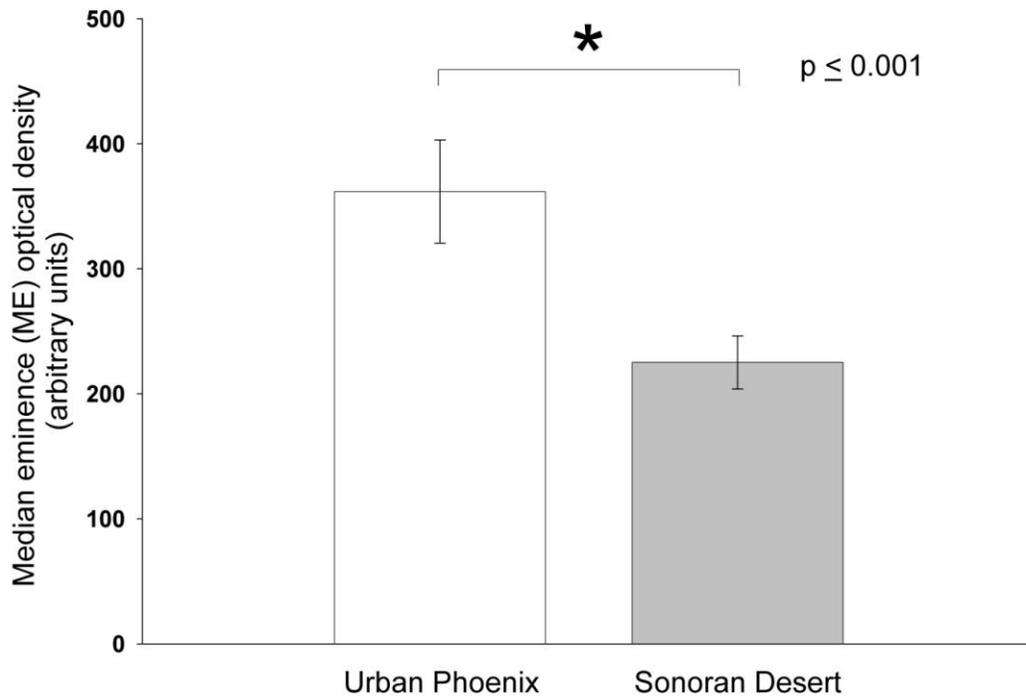


Figure 15. Images (above) and graph (below) illustrating average AVT-like immunoreactivity within the median eminence of urban and desert Curve-billed Thrashers. Scale bars indicate 200 μm .

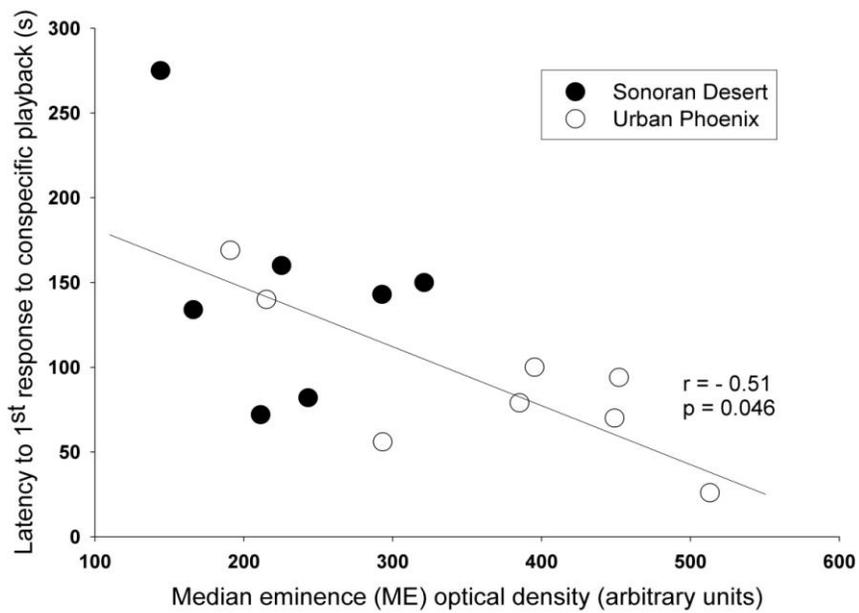
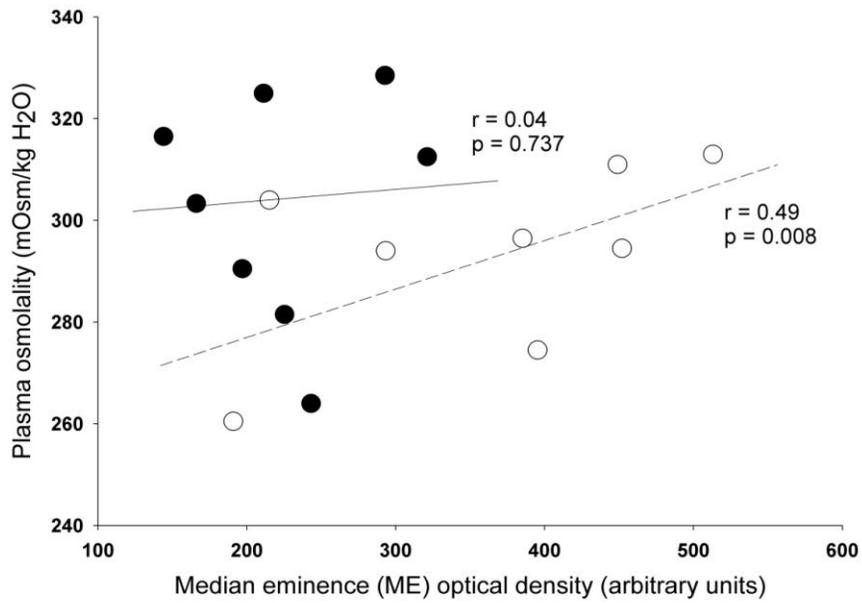


Figure 16. Relationship between AVT-like immunoreactivity at the median eminence to (above) plasma osmolality and (below) behavioral responses to simulated territorial intrusions.

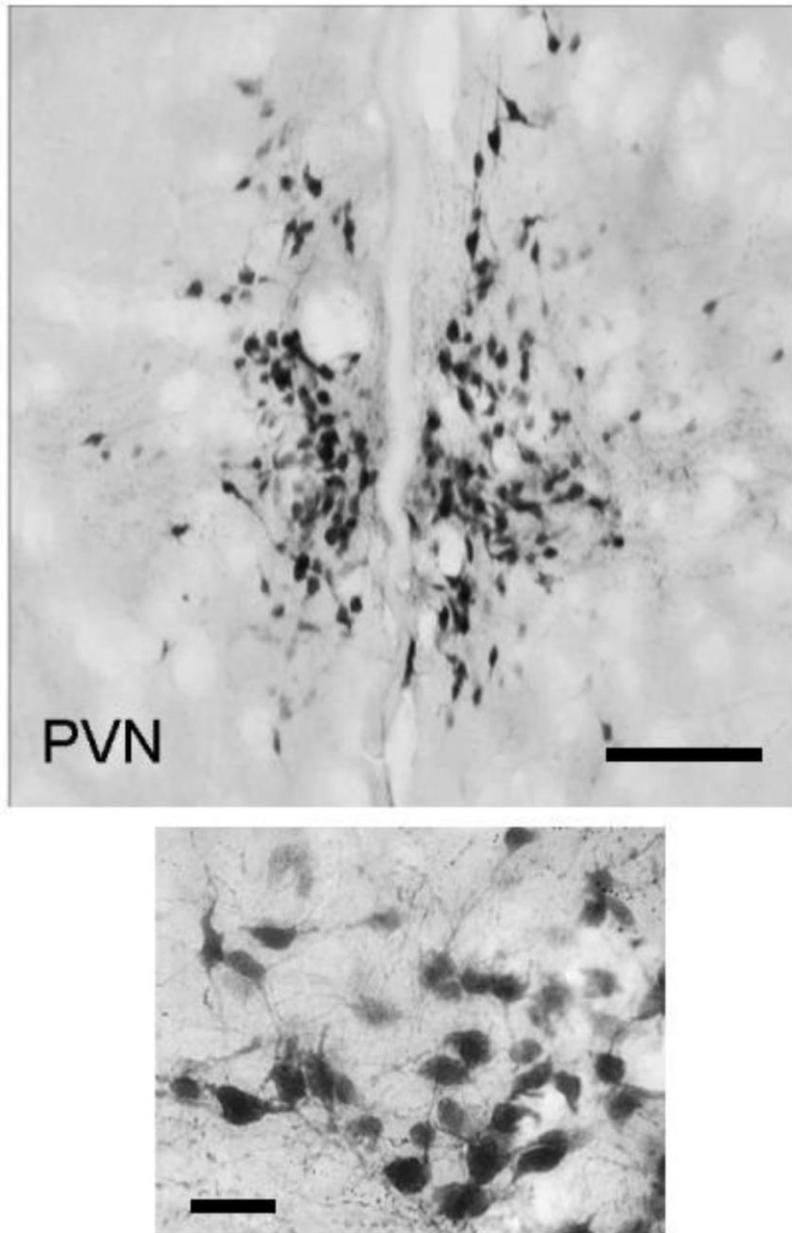


Figure 17. Image of AVT-like immunoreactive (AVT-ir) cells within of the paraventricular nucleus (PVN) taken at low and high magnification in Curve-billed Thrashers. Scale bars indicate 100 μm and 30 μm in low and magnification images respectively.

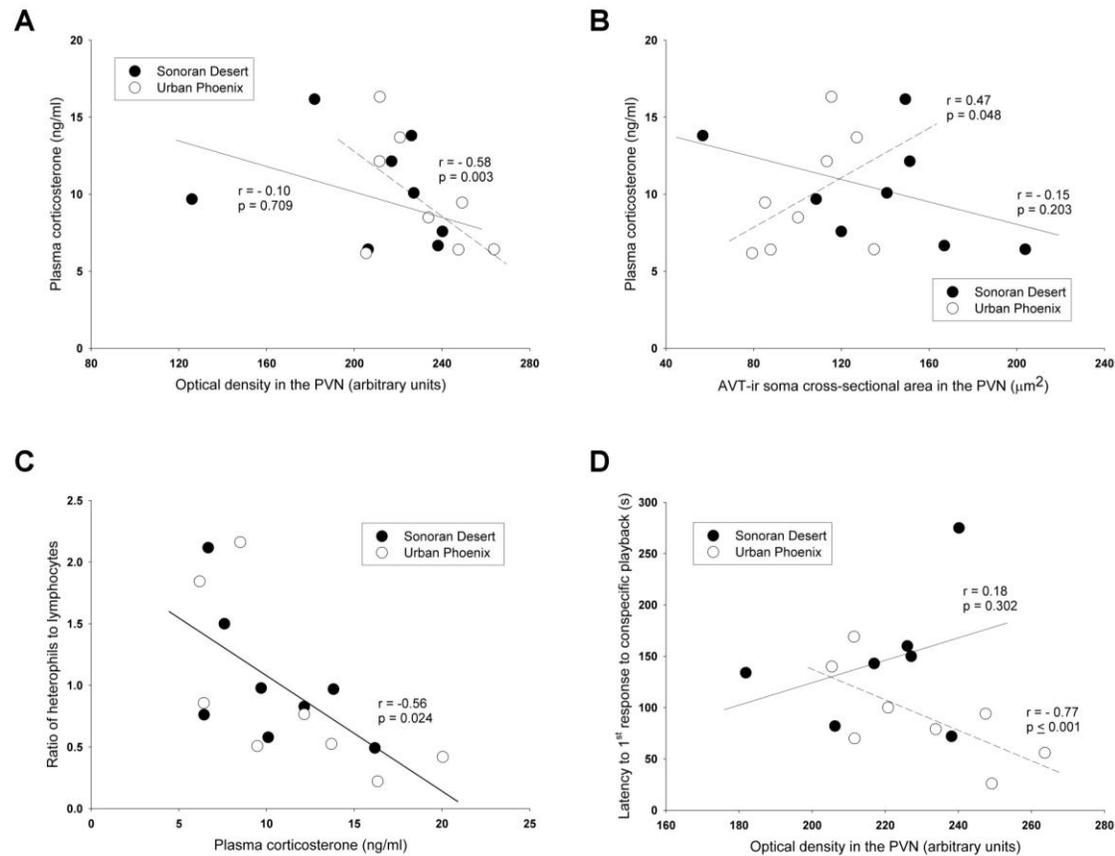


Figure 18. The relationships between plasma corticosterone and **(A)** overall optical density of AVT-ir within the PVN and **(B)** the size of AVT-ir cells within the PVN and **(C)** the ratio of heterophil and lymphocyte white blood cells (an alternate stress measure), as well as **(D)** the relationship between behavioral responses to simulated territorial intrusions and optical density within the PVN between urban and desert thrashers.

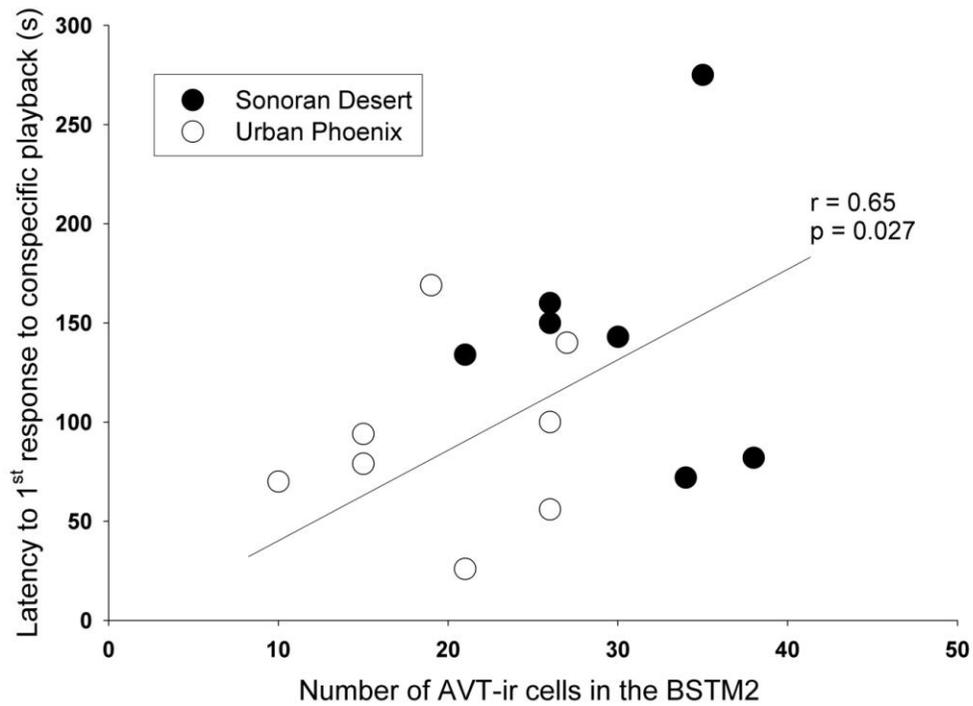
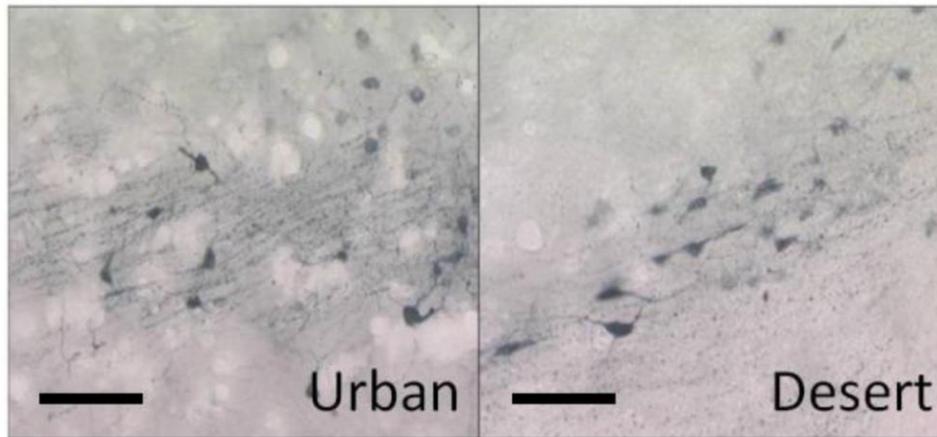


Figure 19. Images (above) depicting the average AVT-like immunoreactivity (AVT-ir) within a component of the medial bed nucleus of the stria terminalis (BSTM2) of urban and desert Curve-billed Thrashers and (below) the relationship between the AVT-ir cell density and the behavioral responses to simulated territorial intrusions. Scale bars indicate 50 μ m.

Chapter 4

MODELING METABOLIC CHANGES DURING THE AVIAN ACUTE STRESS RESPONSE USING PATH ANALYSIS

Corticosterone secretion mobilizes energy, and this energy production can involve multiple metabolic pathways, including fat and protein catabolism for gluconeogenesis. This process is well-studied in mammals but much less understood in birds, which have higher mass-specific metabolic rates, and higher plasma glucose and fatty acid concentrations, and an increased energetic demand for flight. We model changes in plasma metabolites during an acute (30 min) stress response in four songbird species using path analysis to determine how plasma metabolites change during acute stress and whether birds in better body condition are more likely to mobilize lipid and protein stores during stress. Acute stress increased plasma glucose and ketones in two and three species, respectively, and decreased glycerol, triglycerides, and uric acid in all species. Birds in better body condition showed larger decreases in triglycerides and increases in ketones suggesting greater reliance on lipid oxidation for energy during stress. Corticosterone that was not bound to plasma binding globulins explained decreases in free glycerol more than total corticosterone levels suggesting the free portion is the functional component. In all species, glucose directly effected the depletion of uric acid from plasma. Gluconeogenesis generates free radicals, necessitating the antioxidant properties of uric acid. Birds have a strong functional link between body condition and the energy mobilized during acute stress that is less evident in mammals. The high metabolic rates of birds make them highly susceptible to changes in energy balance and may prove as useful models for exploring the relationship between stress and metabolic syndromes.

1. Introduction

One outcome of the stress response in vertebrates is the mobilization of energy that can promote survival during unpredictable environmental or intrinsic challenges. Primary mediators of the acute stress response are glucocorticoids, such as corticosterone (CORT) in birds, which are secreted by the adrenal glands. During acute stress, glucocorticoids typically induce lipolysis, resulting in the release of glycerol and fatty acids (Jenni-Eiermann *et al.* 2002). Glycerol in turn acts as a substrate for hepatic gluconeogenesis and the release of glucose to cause hyperglycemia (Bizzi *et al.* 1972). Besides stimulating energy store mobilization, glucocorticoids induce hyperphagia in natural (Kitaysky *et al.* 2001; Pravosudov 2003) and laboratory settings (Bates *et al.* 2007; Foster *et al.* 2009; Warne *et al.* 2009). This hyperphagic action elevates plasma triglycerides (Seaman *et al.* 2005) and attenuates the depletion of lipid stores that results from lipolysis.

During periods of food scarcity, glucocorticoids stimulate gluconeogenesis, and organisms rely increasingly on the proteolytic action of glucocorticoids to gain energy (Dong *et al.* 2007; Braun & Sweazea 2008). Increased availability of amino acids promotes hepatic enzyme synthesis and glycogen production (Coderre *et al.* 1991). Protein catabolism also elevates plasma uric acid, the predominant nitrogenous end product of protein metabolism in birds (Fery *et al.* 1996). Sustained and severe fasting is, however, eventually associated with exhaustion of glucose levels and decreased availability of protein substrates, thus inducing hypoglycemia. This severe depletion in turn promotes the conversion of free fatty acids to ketones, such as β -hydroxybutyrate, that serve as an alternative energy source, and particularly to sustain the brain (Castellini & Costa 1990; Jenni-Eiermann & Jenni 2001). Analyzing changes in the plasma concentrations of multiple metabolites in response to a physiological challenge may enable us to infer the sources of energy (lipid, glucose, or protein) that animals preferentially use during this challenge.

This approach has been used mostly in laboratory rodents, but whether results from these animals apply to birds is uncertain. Indeed, birds have higher metabolic rates, blood glucose, and circulating fatty acid levels than similarly-sized mammals (Braun & Sweazea 2008). The higher mass-specific energetic demand of birds than mammals is associated with a greater reliance on lipid oxidation as a primary energy pathway to power muscle activity (Jenni & Jenni-Eiermann 1998). Studies on relationships between CORT and metabolism in birds have focused on seabirds, which as nestlings, normally undergo extended bouts of fasting. In King Penguins (*Aptenodytes patagonicus*), glucose infusion decreased lipid oxidation and plasma CORT in birds undergoing confinement stress, but this effect was not observed in unstressed birds (Bernard *et al.* 2003). Furthermore, inhibiting lipid oxidation did not alter plasma glucose, but elevated plasma uric acid and CORT (Bernard *et al.* 2002). Thus, in these birds plasma glucose rather than lipids may determine the metabolic substrates oxidized during stress. In some seabirds, increased CORT secretion in response to food restriction and the depletion of fat reserves may also encourage protein catabolism (Kitaysky *et al.* 2001; Walker *et al.* 2005). Metabolite profiles can change with shifts in body mass (Williams *et al.* 1999; Guglielmo *et al.* 2002), raising the question of whether body condition can predict how energy stores are used during an acute stress response.

Most plasma CORT circulates bound to corticosterone binding globulins (CBG: Deviche *et al.* 2001) and it has been suggested that only the unbound fraction of hormone (*hereafter* free CORT) can bind to intracellular receptors in target cells (Rosner *et al.* 1991; Breuner & Orchinik 2002). The relevance of distinguishing between total or free CORT is debated, in part due to the difficulty to measure a specific outcome of CORT action. Investigating associations between plasma total vs. free CORT and plasma metabolites may help elucidate the role of CBG in mediating effects of the hormone.

The intricate relationships between CORT and plasma metabolites can be modeled using a path analysis approach. This approach measures associations between variables based on a hypothetical framework of cause and effect relationships (Sinervo & DeNardo 1996). It enables the separation of direct and indirect associations between metabolites and stress, which have mostly been studied using univariate approaches (Kern *et al.* 2005; 2007). Based on current understanding of the metabolic changes occurring during acute stress, I used path analysis to: 1) examine how plasma metabolites change in response to 30 minutes of capture and handling stress in songbirds; 2) determine whether birds in good body condition are more likely to use their lipid and/or protein stores during an acute stress response than birds in poor body condition, and 3) determine whether plasma total or free CORT explains a greater amount of variation in plasma metabolites during stress.

2. Materials And Methods

I investigated five songbird species that inhabit the Sonoran Desert in and around Phoenix, Arizona, USA: two pairs of closely related species from the family Mimidae (Northern Mockingbird, *Mimus polyglottos* and Curve-billed Thrasher, *Toxostoma curvirostre*), two towhee species (Abert's Towhee, *Melospiza aberti* and Canyon Towhee, *Melospiza fuscus*), and the House Sparrow (*Passer domesticus*). The sampling locations varied between species but for each, included a mix of urban-suburban (all species), and either farmlands (House Sparrow), or natural desert habitats (mockingbird, thrasher, and towhees) with all locations no more than 25 km apart. Details concerning the study sites can be found in Fokidis *et al.* (2008; 2009). Previous research showed that birds belonging to urban populations of some species (thrasher, and mockingbird) were in better body condition and had greater plasma CORT stress responses than birds inhabiting desert areas

(Fokidis *et al.* 2008; 2009). To examine relationships between body condition, plasma metabolites, and plasma CORT, individuals from all sampling locations were analyzed together, resulting in the inclusion, for each species, of birds showing a wide range of body condition.

2.1. Field Data Collection

Birds were caught using mist nets passively (sparrows) or with conspecific song playback recordings (thrashers, mockingbirds, and towhees). All sampling occurred either between March and June 2006 or between March and May 2007. All captures took place within 5 hours of sunrise. A blood sample (~200 μ l) was taken within 3 min of capture from the right jugular vein using a heparinized 0.3 ml syringe and 29.5 gauge needle. These samples were defined as pre-stress (i.e., baseline) samples. Birds were then held in a cloth bag for 30 min, and a second blood sample (~100 μ l) was collected in the same fashion. This capture and handling protocol is commonly used to induce an acute stress response (Wingfield *et al.* 1994; 2008). Blood samples were kept on ice until centrifuged to separate plasma, which was then stored at -80 °C until assayed.

Only adult males in breeding condition were used. Age, sex, and breeding status were established using plumage characteristics (Pyle 1997) and unilateral laparotomy after the second blood sample was collected (Fokidis *et al.* 2008; 2009). Body mass (\pm 0.1 g) and wing chord length (\pm 0.1 mm) were measured to generate body condition indices. Each bird received a uniquely numbered aluminum leg band and was released at the capture location. All procedures were pre-approved by the Arizona State University Institutional Animal Care and Use Committee and were conducted with necessary permits from relevant agencies and landowners.

2.2. Corticosterone Assays

Plasma total CORT concentrations were measured using commercial competitive enzyme-linked immunoassays (ELISA; Assay Designs Inc. Ann Arbor, Michigan, USA), as described and validated by Fokidis *et al.* (2009). Samples were assayed in duplicate. Pre-stress and stress-induced samples from the same bird were assayed on the same assay plate. The sensitivity of the assay ranged from 5.8 - 18.1 pg/ml depending on the plate, and the mean intra-assay coefficient of variation was 8.58 %.

Radioligand binding assays were used to estimate plasma CBG binding capacity according to Orchinik *et al.* (2000) and Breuner and Orchinik (2001) with minor modifications specified in Fokidis *et al.* (2009). Previously described equilibrium dissociation constants for CORT binding to CBG for each species (Fokidis *et al.* 2009) were used to estimate plasma concentrations of free and CBG-bound CORT according to the equation of Barsano and Baumann (1989).

2.3. Metabolite Assays

Plasma free glycerol and triglycerides were measured using a sequential color endpoint assay (Sigma-Aldrich, reagents F6428 and T2449) described in Guglielmo *et al.* (2002a,b). Plasma glucose and β -hydroxybutyrate were measured using colorimetric enzyme endpoint assays (Cayman Chemical Co. Ann Arbor, Michigan, USA; Cat No. 10009582 and 700190, respectively). Plasma uric acid was also measured using a colorimetric assay (Biovision Research, Mountain View, California, USA; Cat No. K608-100). Previous studies have validated these assays for use on various songbird species (Williams *et al.* 1999; Guglielmo *et al.* 2002a; 2005). Samples were assayed in duplicate and in random order, and all concentrations are expressed in the same unit (mM) to facilitate comparisons. Assay sensitivities, mean intra- and inter-assay coefficients of variation are as follows: free glycerol

(0.05 – 6.3 mM, 6.5% and 13.0%); triglycerides (0.07 – 11.4 mM, 7.2% and 10.1%); glucose (2.5 – 28.36 mM, 3.3% and 11.9%); β -hydroxybutyrate (0.01 – 4.8 mM, 5.5% and 14.0%); and uric acid (0.01 – 6.1 mM, 3.7% and 9.8%).

To determine the degree to which biochemical changes associated with the acute stress response alter plasma solute concentration, plasma osmolality (mOsm/kg of plasma) was measured using a vapor pressure osmometer (Model 5500XR, Wescor Inc. Logan, Utah, USA) with 10 μ l samples assayed in duplicate. The osmometer was calibrated to known concentration standards before use.

2.4. Statistics And Modeling

Ordinary least squares regressions of body mass on wing chord length provided standardized residuals for use as body condition indices for each species. I examined the effect of sampling date, time, and site as covariates on changes in plasma CORT and metabolites (dependent variables) using repeated measures analysis of variance (ANOVA) on each individual for each species. The primary focus of the research was to determine changes in plasma metabolites in response to acute stress and w, therefore calculated the difference in concentrations of each metabolite and CORT between corresponding baseline and stress-induced concentrations. This difference was used for path analyses. When necessary, data were natural logarithm (ln) transformed to satisfy normality assumptions.

The complex physiological relationships that exist between plasma metabolites and CORT (Introduction) were investigated using path analysis (Sokal & Rohlf 1995). This method has been successfully used in other physiological and ecological studies examining variability in metabolic rate (Nespolo *et al.* 2005; Bozinovic *et al.* 2007), functional associations between brain regions (Hamm *et*

al. 2010), and ecological factors dictating reproductive effort (Sinervo & DeNardo 1996; Blums et al. 2002). Path analysis enables the testing of an *a priori* hypothesis model of relationships generated on the basis of theoretical considerations and the results of Pearson's correlation analysis between variables. I used body condition as the exogenous variable and plasma metabolites and CORT concentrations as endogenous variables. The direction and strength of relationships between variables was quantified by path (β) coefficients that are calculated using the maximum likelihood method (Sokal & Rohlf 1995). The endogenous variables are also influenced by factors outside the model. These factors could include measurement error and their potential contribution is represented by residual error terms (i.e., unexplained variation) in the model. The hypothetical model (or representation) was then compared to the observed data to determine the goodness of fit. Non-significant relationships between variables were removed from the path diagrams, except where necessary to retain connectivity.

Evaluation of alternative models involved several approaches. The model was tested using a χ^2 goodness of fit test statistic, in which a smaller value indicates better consistency with observed data. The root mean square error of approximation (RMSEA), which estimates the amount by which estimated values differ from actual values, was also used for model comparison. According to this approach a $RMSEA \leq 0.05$ is usually considered to indicate a 'close fit' to the data (Brown & Cudeck 1993). To eliminate spurious relationships, the hypothetical model was tested against a saturated model in which all variables are directly connected to each other, and an independent model (equivalent to a traditional multiple regression) in which no connection exists between variables. The Normed Fit Index (NFI) compares the model to both the saturated and independent models. Larger NFI values are preferred and values > 0.9 are generally considered adequate (Bentler & Bonett 1980). The Akaike information criterion (AIC) also distinguishes between models derived

from the maximum likelihood, with the most parsimonious model being associated with the smallest AIC value. Together, these tests provide a rigorous assessment and comparison of different model types. All statistical analyses were performed using SPSS Version 13.0 (2004) with the AMOS 7.0 extension for path analysis.

3. Results

Capture and restraint increased plasma total and free CORT in all species (Table 6). Differences in plasma baseline CORT were observed between urban and desert populations of Canyon Towhee and Curve-billed Thrasher however these differences were in opposite directions (Table 6). In addition, urban and desert populations of these two species also differed in stress CORT levels, as did urban and farmland House Sparrows (Table 6). No site difference in plasma total or free CORT was observed in Abert's Towhees or Northern Mockingbirds (Table 6). Capture and restraint decreased plasma levels of triglycerides and free glycerol in several species, but this decrease was often population-specific (Table 6). Uric acid levels decreased with stress in all species, with the exception of urban Abert's Towhees (Table 6). Stress elevated plasma glucose in Curve-billed Thrashers and Northern Mockingbirds from the desert population, and decreased plasma glucose in desert Abert's Towhee (Table 6). Plasma β -hydroxybutyrate in response to stress increased in the House Sparrow and in urban but not desert towhees of the two species, and did not change in thrashers or mockingbirds (Table 6). Stress did not influence plasma osmolality in any species (Table 6).

Plasma baseline triglycerides decreased as the day progressed in three species: Abert's Towhee ($F = 3.75$, $df = 45$, $p = 0.028$), Curve-billed Thrasher ($F = 5.82$, $df = 59$, $p = 0.006$), and Northern Mockingbird ($F = 3.17$, $df = 24$, $p = 0.031$). To account for this decrease, residuals of a

linear regression between plasma triglycerides and time of sampling were used in the path analysis for these species. Time of sampling did not influence the plasma concentrations of any other metabolite (all $p \geq 0.396$). There was no effect of sampling date on plasma CORT or any metabolite in any species (*data not shown*). Best-fit path models were successfully generated for each species with the exception of the Canyon Towhee, which failed to meet χ^2 goodness of fit, RMSEA, and NFI criteria (Table 7).

Body condition was positively associated with plasma baseline total CORT in the House Sparrow (Figure 20) and Curve-billed Thrasher (Figure 21), but not in the other species. Body condition was also associated with changes in plasma triglyceride and β -hydroxybutyrate in response to stress in the House Sparrow (Figure 20), Curve-billed Thrasher (Figure 21), and Northern Mockingbird (β -hydroxybutyrate only: Figure 22). No such association was found in the Abert's Towhee (Figure 23).

Changes in plasma CORT were negatively correlated with changes in plasma free glycerol. This association was stronger for plasma free than total CORT in all species (Figures 20-23). In no species were changes in plasma glucose directly associated with changes either in free or total CORT. The association between plasma free glycerol and plasma glucose was weakly significant only in the Curve-billed Thrasher (Figure 21) and Northern Mockingbird (Figure 22). Changes in plasma glucose were directly and negatively associated with changes in plasma uric acid in all four species (Figures 20-23).

Stress-induced changes in plasma free glycerol were positively and directly associated with changes in plasma levels of β -hydroxybutyrate in the House Sparrow (Figure 20) and the Northern Mockingbird (Figure 22). However, changes in plasma triglycerides in response to stress were

directly and negatively associated with changes in plasma β -hydroxybutyrate in the Northern Mockingbird (Figure 22) and the Abert's Towhee (Figure 23). A significant negative association between the plasma concentrations of these metabolites was also observed in the House Sparrow (Figure 20).

4. Discussion

Modeling changes in plasma metabolite profiles in response to acute stress with path analysis revealed several patterns among the songbirds studied. First, plasma glucose in response to acute stress increased only in two species (thrasher and mockingbird) and this increase was not directly related to plasma CORT; indeed in desert Abert's Towhees acute stress was associated with a decrease in plasma glucose. Instead, in all four species the stress-induced increase in plasma CORT was directly associated with a decrease in plasma free glycerol. Furthermore, the latter was directly associated with changes in plasma glucose only in the two species in which stress increased plasma glucose. Second, in all species plasma free CORT (i.e., the portion of plasma CORT that is not bound to CBG) was more closely associated with plasma free glycerol than was plasma total CORT. Third, during stress there was a direct association in all species between plasma glucose and the observed depletion of uric acid from circulation. Fourth, body condition was positively associated with plasma triglycerides and β -hydroxybutyrate in two and three species, respectively. Together, the data suggest that the avian stress response influences metabolic pathways that were not considered in previous research on this subject and that the metabolic effects of stress differ in some respects from those described in mammals.

4.1. Stress, Glycerol, And Glucose

Path analysis revealed a consistent association between plasma CORT and plasma free glycerol: as CORT secretion increases during stress, plasma free glycerol declines. This finding is consistent with previous observations in birds indicating that capture and handling decreases plasma free glycerol (Guglielmo *et al.* 2002). Other studies, however, reported either an increase (Kern *et al.* 2007) or no change (Seaman *et al.* 2005) in plasma glycerol during stress. My finding that there is a negative association between stress-induced changes in plasma CORT and glycerol, together with the uncoupling of plasma CORT and glucose, supports the hypothesis that an increase in plasma glucose during stress results, at least in part, from gluconeogenesis involving glycerol as a substrate, although the role of glycogenolysis cannot be excluded. This conclusion is consistent with results in mammals, but mobilization of lipid stores usually increases plasma glycerol in birds (Guglielmo *et al.* 2002; Seaman *et al.* 2005). The decline in free glycerol during acute stress in this study may be explained by a rapid uptake of glycerol by the liver and kidneys, the major avian gluconeogenic sites (Scanes 2009). In addition to a potential uptake of glycerol directly from circulation, liver stores of triglycerides may also be broken down to glycerols, to fuel gluconeogenesis. These additional, glycerol substrates from the liver may not be detected in plasma, unlike the glycerols from adipose tissue. However very little (if any) data are available on the contributions of liver triglycerides to gluconeogenesis in birds. Understanding the dynamics, sources, and time course of glycerol production and uptake in birds is critical to the interpretation of changes in plasma levels of this metabolite in future studies.

In mammals, plasma glucose is elevated during a stress response. This elevation is thought to result from numerous effects of CORT, including reduced glucose uptake by tissue and increased insulin resistance coupled to gluconeogenesis (reviewed in Macfarlane *et al.* 2008). Data on this

subject for birds are less consistent than for mammals. For example, continuous infusion and bolus injections of CORT to induce physiological blood levels of the hormone did not elevate plasma glucose during a 5 hour interval in the turkey (*Meleagris gallapavo*), and produced sporadic increases in plasma glucose in the chicken (*Gallus domesticus*) after several hours (Thurston *et al.* 1993). Plasma glucose in wild-caught European Starlings (*Sturnus vulgaris*) also did not change in response to repeated acute stressors over an 18 day period and despite fluctuations in plasma CORT (Cyr *et al.* 2007). By contrast, another study on this species found during molt that CORT administration induces hyperglycemia during the day but not at night (Ramage-Healey & Romero 2002). This finding contrasts with those of an earlier study demonstrating a hyperglycemic effect of acute stress during the night (when glucose is low), but not during the day (when glucose is high) in birds regardless of breeding condition (Ramage-Healey & Romero 2001).

I observed a stress-induced increase in plasma glucose only in two closely related members of the mimidae family (thrasher and mockingbird). It is possible in species not showing stress-associated hyperglycemia that glucose produced *via* gluconeogenesis had yet to rise within the 30 min time frame of acute stress administered in this study. Supporting this contention, plasma glucose in the Abert's Towhee is elevated after one hour of acute stress, but not within 30 mins (Davies, *unpublished data*). Another explanation is that gluconeogenesis and glucose utilization may occur concurrently resulting in balanced and unchanging levels of plasma glucose during an acute stress response. Thus stable plasma glucose during stress may reflect increased gluconeogenesis compensated by an increased utilization rate.

I found no effect of time of sampling on plasma glucose, CORT, or free glycerol. Due to the condensed time sampling period, diurnal variation in stress-induced hyperglycemia cannot be discounted, but to our knowledge this phenomenon has not been observed in other species. Food

intake is also a major determinant of plasma glucose levels. Birds were captured primarily in the early morning hours which coincides with the time of foraging aimed at replenishing energy reserves used up throughout the night (Guglielmo *et al.* 2002a). Thus glucose levels in plasma may reflect recent feeding events and can conceivably mask stress-induced changes in glucose levels. It is not known if birds were in a postabsorptive state when captured, but birds captured later in the day (i.e. having spent more time foraging) would presumably have higher glucose levels than birds captured earlier in the day, however no effect of time of sampling was observed in this study.

Glucose generally circulates at higher concentration in birds than mammals, and birds usually have a higher mass-specific metabolic rate than mammals, thus birds are thought to more rapidly depreciate their glucose levels during fasting (Braun & Sweazea 2008). However, the mechanisms responsible for the fact that birds show smaller or less detectable changes in plasma glucose associated with stress than mammals remain largely speculative.

4.2. Total Vs. Free CORT

Plasma free CORT is considered to represent the portion of the circulating steroid that can cross plasma membranes and bind to cytoplasmic receptors (Deviche *et al.* 2001; Breuner *et al.* 2006). There is considerable debate concerning the relative biological importance of the free, bound, and total components of CORT (Malisch & Breuner 2010). CORT bound to CBG is retained in circulation as opposed to being cleared from the plasma and thus may serve as a reservoir of CORT for potential future use. This hypothesis is supported by studies measuring the clearance of radiolabelled CORT in genetically engineered mice lacking CBG (Petersen *et al.* 2006) and by work in white-crowned sparrows (*Zonotrichia leucophrys*) treated with progesterone, which binds to CBG with higher affinity than CORT (Malisch & Breuner 2010). Other studies have reported that plasma

free, instead of plasma total CORT accounts for a greater proportion of variation in immunocompetence (Malisch *et al.* 2009) and nesting behavior (Love *et al.* 2004). These studies, however investigated the chronic (days) rather than acute (minutes) role of CORT.

In all species in the present study, path analyses revealed a direct association between total and free CORT and only one metabolite - free glycerol. Despite plasma total and free CORT being correlated, this association was consistently stronger for free than total CORT, thus supporting the free hormone hypothesis (Rosner *et al.* 1991). It has been suggested that plasma CBG decreases during acute stress and this decrease could elevate plasma free CORT levels (Breuner *et al.* 2006). However, the generality of this finding is questioned by my finding no evidence for stress-induced CBG decreases in my study species (Fokidis *et al.* 2009). Of note, during acute stress CBG-bound CORT may be utilized through several mechanisms. For example, recent research on human CBG demonstrated a reversible reduction in its binding affinity for cortisol resulting from the partial unwinding of the hormone binding site, which presumably frees a portion of the bound hormone (Zhou *et al.* 2008). Furthermore, there is evidence that the steroid-CBG complex can be taken up into cells by endocytosis (Strelchyonok & Avvakumov 1991; Maitra *et al.* 1993; Hammes *et al.* 2005). This mechanism is, however, thought to occur primarily outside the systemic circulation. The stronger association of glycerol with plasma free rather than total CORT in my study supports the hypothesis that the free CORT portion may play a more active role in the regulation of short-term changes in plasma glycerol.

4.3. Plasma Glucose And Uric Acid

Even though plasma glucose did not increase with stress in all species studied here, we found a consistent negative association between plasma glucose and uric acid. Uric acid serves

several important functions in avian circulation. It is the predominant form of nitrogenous waste excreted by birds and is derived from amino and nucleic acid metabolism. Thus, circulating uric acid levels are thought to reflect protein catabolism (Lindgard *et al.* 1992), as supported by the observation in free-living migrating birds and in captive birds that plasma uric acid increases when body mass decreases (Jenni-Eiermann & Jenni 1994; Seaman *et al.* 2005). One possible explanation for the decrease in plasma uric acid with stress is excretion during the acute stress response, however not all birds excreted during the 30 min handling period. We observed no direct relationship between body condition and uric acid, suggesting that such a breakdown of protein did not occur or was not detectable during acute stress. This observation is not surprising, however, because acute stress in birds presumably stimulates the use of amino acids for gluconeogenesis and is, therefore, likely not associated with increased plasma uric acid.

An important consideration however is that changes in uric acid levels during acute stress were accompanied by increases in plasma glucose levels in only two closely-related species (mockingbird and thrasher). In contrast, a slight decrease in glucose was seen in desert Abert's Towhee but this decline was not observed in urban birds. In combining both populations, no change in glucose was observed with stress. One explanation for these species-specific differences may be that decreases in uric acid have yet to induce a change in glucose that is detectable in circulation in some species. Thus, the decrease in uric acid observed over a 30 min time course may yield an increase in plasma glucose levels at a future time point (e.g., 1 hr). In this study Abert's Towhees did not increase glucose levels within 30 min of acute stress, but can elevate plasma glucose after one hour of handling stress (Davies, *unpublished data*), which lends some support for this hypothesis.

Uric acid also acts as an antioxidant to quench free radicals generated during oxidative stress

(Klandorf et al. 1999; Tsahar *et al.* 2006). In humans and rats, gluconeogenesis is a major generator of free radicals (Gate et al. 1999; Buffenstein *et al.* 2008). Birds may produce lower levels of free radicals than similar-sized mammals and this may be partly related to the presence of circulating uric acid (Klandorf *et al.* 1999; Braun & Sweazea 2008). A comparative study of 57 bird species demonstrated in the majority of these species that plasma uric acid declines during 1 hr of acute stress (Cohen *et al.* 2008). However, stress-induced plasma levels of uric acid in many species in that study either did not change or increased during stress. However in my study, plasma uric acid decreased in all of the species investigated. This decrease was associated only with changes in plasma glucose. A rapid stress-associated increase in plasma glucose (see above) may induce oxidative stress which demands antioxidants to prevent free radical damage to tissues. The decline in uric acid observed during acute stress may represent its mobilization from muscle protein and DNA sources, to prevent oxidative damage occurring as a byproduct of gluconeogenesis. As uric acid interacts with free radicals, its signal may be quenched in the process and thus become less detectable by the assay. However, note that changes in uric acid during the stress response were not always accompanied by changes in glucose levels in all species. The antioxidant properties of uric acid are well understood in both mammals and birds, however the significance of variability in plasma levels in response to short-term changes in other metabolites deserves further study.

4.4. Body Condition And Fat Usage

Body condition refers to the amount of energy reserves stored in both fat tissue and lean muscle and as a function of total food intake and energy expenditure (Stevenson & Woods Jr. 2006). The availability of energy stores may influence the functioning of various physiological systems including the acute stress response. Lower body condition is often associated with higher baseline

CORT levels and *vice versa*. For example, high baseline CORT levels may stimulate feeding behavior aimed at elevating body condition, meanwhile depleting lipid reserves for gluconeogenesis. Field studies have, however, demonstrated more complex relationships between plasma CORT and body condition, and they illustrate the fact that body condition can vary as a function of numerous life history, ecological, and physiological parameters. For example, body condition in marine iguanas, *Amblyrhynchus cristatus*, explained most variation in plasma CORT, but only up to a threshold level that was largely consistent across several populations of this species (Romero & Wikelski 2001).

Body condition was directly associated with a stress-induced decrease in plasma triglyceride in the House Sparrow and Curve-billed thrasher, but was not associated with plasma free glycerol and was thus removed from the final model. Triglycerides increase in plasma as they deposited into adipose tissue usually shortly after food intake (Guglielmo *et al.* 2002a,b; 2005). However, a rapid decline in plasma triglycerides as observed during stress may indicate rapid lipid metabolism.

In birds, fatty acids are important energy substrates, especially in skeletal muscles. An increased production of fatty acids during stress may explain the observed decrease in plasma triglycerides, however fatty acids are thought to be primarily derived directly from lipolysis within adipose tissue and not from circulating triglycerides. The oxidation of fatty acids for energy produces ketones including β -hydroxybutyrate. Plasma levels of this ketone increased with acute stress in three of four species, suggesting increased oxidation of fatty acids for energy. In addition, body condition had a direct positive association with β -hydroxybutyrate in three species, suggesting that increased energy reserves may induce more fatty acid oxidation during stress. Taken together, the data suggest that body condition is an important predictor of the degree to which energy reserves are used during an acute stress response.

4.5. Significance

Elevated plasma CORT with stress has similar functions in birds and mammals, but differences in the demand for energy and its subsequent effects on metabolism have altered how animals use energy in the short-term. As in mammals, gluconeogenesis in birds is an important consequence of CORT secretion. By contrast, acute stress in birds does not always increase plasma glucose. Birds generally have higher blood glucose than mammals and they show a strong resistance to insulin-mediated glucose uptake, and an increased uptake of free fatty acids into tissues (Braun & Sweazea 2008) resulting in a physiological condition resembling mammalian diabetes mellitus. Rapid gluconeogenesis during stress may result in oxidative stress that can account for a decline in plasma levels of the antioxidant uric acid with stress. During acute stress, fat reserves are quickly utilized to provide both fatty acids for muscle metabolism and glycerol for gluconeogenesis. Birds may serve as useful experimental models to investigate the role of glucocorticoids in the generation of various metabolic syndromes.

5. References

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1 Table 6. Concentrations of corticosterone (CORT), plasma metabolites, and plasma osmolality for five songbird species inhabiting urban,
 2 farm and desert habitats at baseline (*B*) and after 30 minutes of capture and handling stress (*S*). All values indicate mean \pm SEM and
 3 asterisks indicates significant differences between sites (* is $p \leq 0.05$). Numbers in parentheses indicate sample sizes and bold figures
 4 indicate significant differences between baseline and corresponding stress levels ($p \leq 0.05$).
 5

		House Sparrow		Abert's Towhee		Canyon Towhee		Curve-billed Thrasher		Northern Mockingbird	
		Urban (19)	Farm (12)	Urban (21)	Desert (25)	Urban (8)	Desert (11)	Urban (27)	Desert (34)	Urban (16)	Desert (9)
Total CORT (ng/ml)	<i>B</i>	4.9 \pm 1.9	3.4 \pm 1.3	12.7 \pm 2.8	12.2 \pm 2.2	18.1 \pm 7.1	7.9 \pm 2.0	* 8.8 \pm 2.6	15.1 \pm 2.4	* 7.6 \pm 1.2	7.6 \pm 1.3
	<i>S</i>	29.6 \pm 8.2	43.8 \pm 4.6	* 36.9 \pm 4.4	37.5 \pm 4.2	73.8 \pm 23.4	35.0 \pm 6.3	* 44.4 \pm 7.8	69.6 \pm 8.0	* 40.7 \pm 8.6	34.9 \pm 8.1
Free CORT (ng/ml)	<i>B</i>	0.2 \pm 0.1	0.1 \pm 0.0	0.9 \pm 0.2	0.8 \pm 0.1	1.0 \pm 0.3	0.4 \pm 0.1	0.4 \pm 0.1	0.4 \pm 0.0	0.9 \pm 0.1	1.0 \pm 0.2
	<i>S</i>	1.0 \pm 0.3	0.8 \pm 0.1	3.2 \pm 0.7	3.0 \pm 0.4	9.8 \pm 2.9	3.0 \pm 0.7	* 6.6 \pm 4.3	11.9 \pm 4.0	* 18.9 \pm 12.3	13.6 \pm 7.8
Triglycerides (mM)	<i>B</i>	1.7 \pm 0.1	2.2 \pm 0.1	* 2.2 \pm 0.2	1.8 \pm 0.1	1.4 \pm 0.3	1.7 \pm 0.2	3.2 \pm 0.8	2.49 \pm 0.43	2.78 \pm 0.33	3.25 \pm 0.44
	<i>S</i>	1.4 \pm 0.1	1.9 \pm 0.1	* 1.5 \pm 0.1	1.4 \pm 0.1	1.1 \pm 0.1	1.4 \pm 0.2	* 3.2 \pm 0.9	1.33 \pm 0.12	* 1.87 \pm 0.17	1.80 \pm 0.38
Free Glycerol (mM)	<i>B</i>	0.70 \pm 0.06	0.82 \pm 0.06	0.81 \pm 0.11	0.91 \pm 0.08	0.53 \pm 0.19	0.67 \pm 0.11	0.8 \pm 0.1	0.8 \pm 0.1	0.8 \pm 0.1	1.0 \pm 0.2
	<i>S</i>	0.59 \pm 0.11	0.83 \pm 0.11	0.58 \pm 0.07	0.62 \pm 0.05	0.44 \pm 0.08	0.29 \pm 0.04	* 0.6 \pm 0.3	0.3 \pm 0.3	* 0.5 \pm 0.1	0.4 \pm 0.1
Glucose (mM)	<i>B</i>	19.6 \pm 3.9	23.1 \pm 3.9	19.3 \pm 0.7	19.7 \pm 0.6	15.3 \pm 1.7	16.1 \pm 0.8	14.9 \pm 0.5	15.7 \pm 0.4	16.8 \pm 1.1	18.3 \pm 1.2
	<i>S</i>	17.9 \pm 2.6	22.3 \pm 2.6	19.2 \pm 0.6	18.1 \pm 1.0	15.4 \pm 1.6	15.5 \pm 0.9	17.8 \pm 0.8	18.4 \pm 0.5	18.3 \pm 1.1	21.8 \pm 2.0 *
Uric Acid (mM)	<i>B</i>	0.3 \pm 0.8	0.4 \pm 0.1	0.5 \pm 0.1	0.8 \pm 0.1	* 1.2 \pm 0.3	0.9 \pm 0.1	0.9 \pm 0.1	1.1 \pm 0.1	0.4 \pm 0.7	0.3 \pm 0.3 *
	<i>S</i>	0.1 \pm 0.5	0.2 \pm 0.1	0.5 \pm 0.1	0.4 \pm 0.1	0.4 \pm 0.1	0.3 \pm 0.1	0.5 \pm 0.2	0.5 \pm 0.5	0.3 \pm 0.1	0.1 \pm 0.2 *
β-hydroxybutyrate (mM)	<i>B</i>	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0	0.10 \pm 0.0	0.1 \pm 0.0	0.05 \pm 0.1 *
	<i>S</i>	0.2 \pm 0.0	0.2 \pm 0.0	0.2 \pm 0.0	1.1 \pm 0.0	0.2 \pm 0.0	0.2 \pm 0.0	0.2 \pm 0.0	0.11 \pm 0.0	* 0.1 \pm 0.0	0.04 \pm 0.1 *
Osmolality (mOsm/kg)	<i>B</i>	306.4 \pm 8.7	298.9 \pm 6.6	337.9 \pm 3.6	327.0 \pm 5.5	* 327.9 \pm 3.6	331.0 \pm 2.5	296.1 \pm 5.0	299.0 \pm 3.7	323.8 \pm 6.5	329.5 \pm 3.5
	<i>S</i>	295.3 \pm 4.8	304.0 \pm 4.7	335.4 \pm 4.9	326.7 \pm 4.4	325.4 \pm 4.9	327.7 \pm 1.4	301.0 \pm 4.4	298.8 \pm 5.5	317.1 \pm 6.0	321.2 \pm 5.2

Table 7. Model fit parameters for path analyses models (Figures 4.1-4.4) relating body condition, corticosterone and plasma metabolite levels for five songbird species. Model fits to observed data if: 1) χ^2 is not-significant, 2) RMSEA \leq 0.05; 3) NFI $>$ 0.9; and 4) has a large AIC value.

Species (N)	χ^2	Df	p	RMSEA	NFI	AIC
House Sparrow (31)	17.28	10	0.068	0.05	0.92	85.28
Abert's Towhee (46)	10.32	10	0.413	0.03	0.96	78.32
Canyon Towhee (19)	71.79	8	0.001	0.14	0.37	852.71
Curve-billed Thrasher (61)	11.35	17	0.838	0.02	0.94	65.35
Northern Mockingbird (25)	13.84	10	0.180	0.05	0.87	81.84

House Sparrow

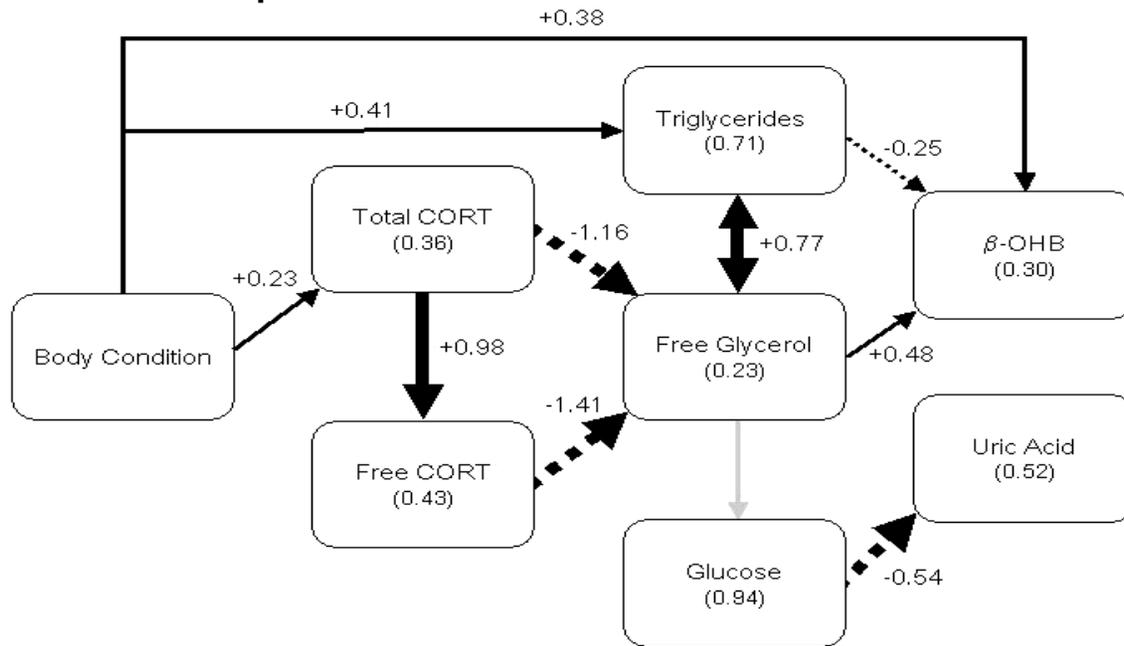


Figure 20. Path diagram relating body condition and changes in plasma corticosterone (CORT) and metabolites during 30 minutes of acute stress in 31 House Sparrows (*Passer domesticus*). Solid and dashed arrows indicate positive and negative relationships between variables, respectively. Double arrows indicate highly correlated variables ($r \geq 0.75$). Numbers next to arrows represent standardized path coefficients and numbers in parentheses indicate residual error terms (i.e., unexplained variance) for that variable. Non-significant arrows are indicated in gray. β -OHB refers to β -hydroxybutyrate.

Curve-billed Thrasher

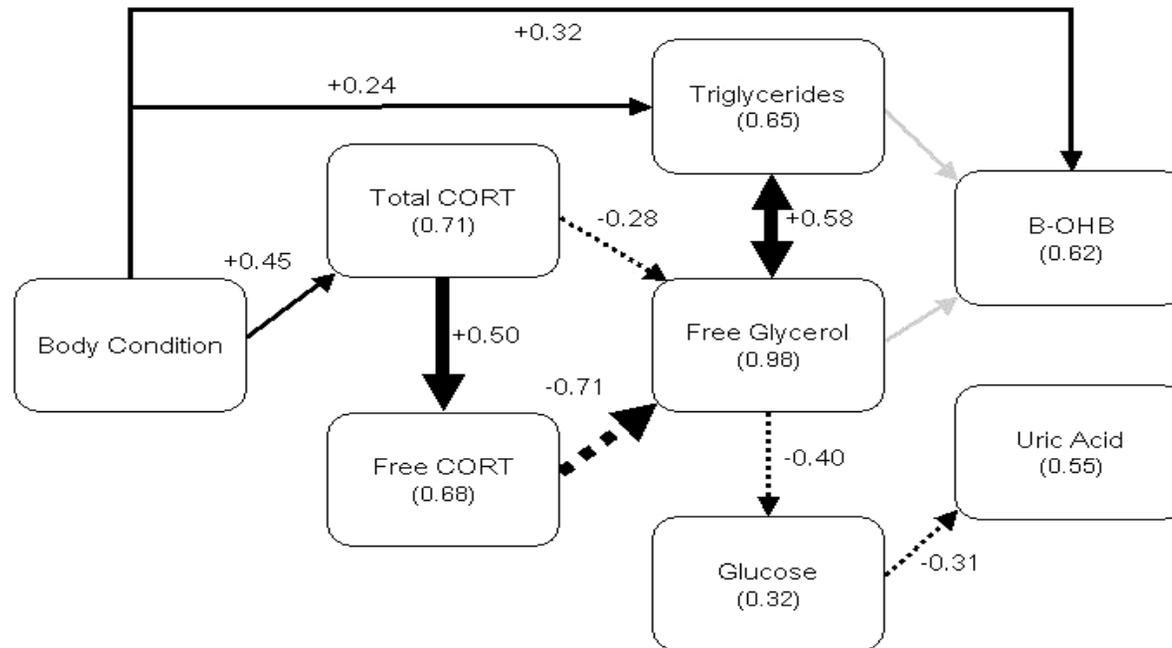


Figure 21. Path diagram relating body condition and changes in plasma corticosterone (CORT) and metabolites during 30 minutes of acute stress in 61 Curve-billed Thrashers (*Toxostoma curvirostre*). Solid and dashed arrows indicate positive and negative relationships between variables, respectively. Double arrows indicate highly correlated variables ($r \geq 0.75$). Numbers above arrows represent standardized path coefficients and numbers in parentheses indicate residual error terms (i.e. unexplained variance) for that variable. Non-significant arrows are indicated in gray. β -OHB refers to β -hydroxybutyrate.

Northern Mockingbird

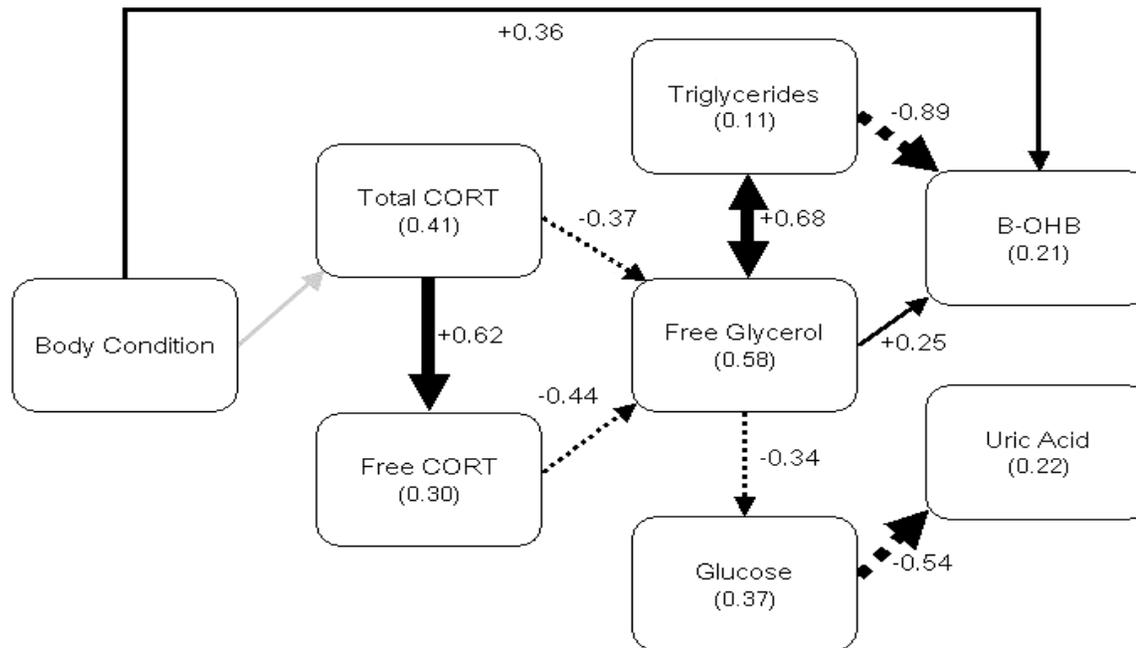


Figure 22. Path diagram relating body condition and changes in plasma corticosterone (CORT) and metabolites during 30 minutes of acute stress in 25 Northern Mockingbirds (*Mimus polyglottos*). Solid and dashed arrows indicate positive and negative relationships between variables, respectively. Double arrows indicate highly correlated variables ($r \geq 0.75$). Numbers above arrows represent standardized path coefficients and numbers in parentheses indicate residual error terms (i.e. unexplained variance) for that variable. Non-significant arrows are indicated in gray. β -OHB refers to β -hydroxybutyrate.

Abert's Towhee

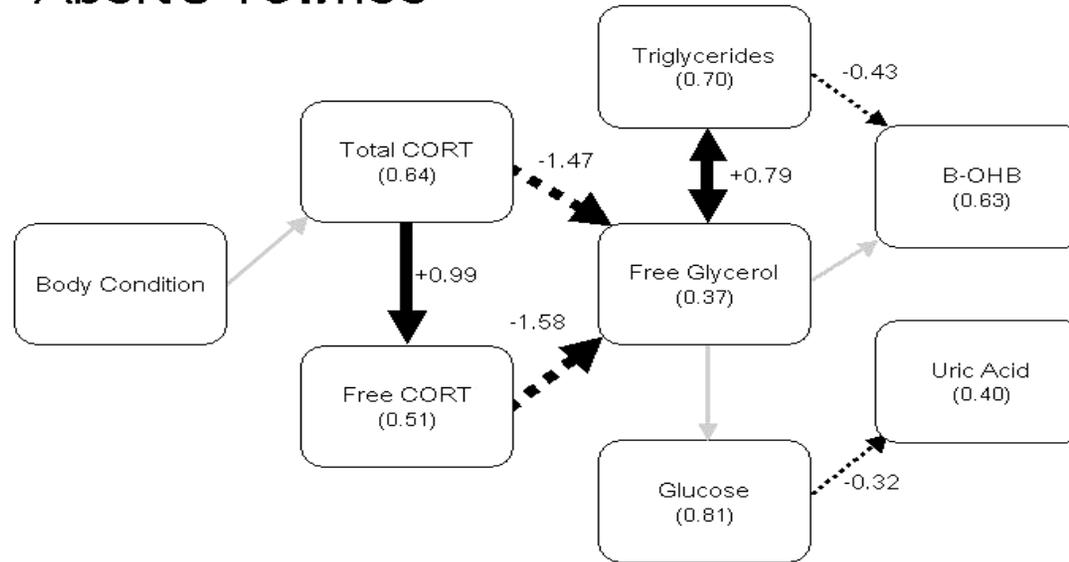


Figure 23. Path diagram relating body condition and changes in plasma corticosterone (CORT) and metabolites during 30 minutes of acute stress in 46 Abert's Towhees (*Pipilo aberti*). Solid and dashed arrows indicate positive and negative relationships between variables, respectively. Double arrows indicate highly correlated variables ($r \geq 0.75$). Numbers above arrows represent standardized path coefficients and numbers in parentheses indicate residual error terms (i.e. unexplained variance) for that variable. Non-significant arrows are indicated in gray. β -OHB refers to β -hydroxybutyrate.

Chapter 5

CAPTIVITY AND BODY CONDITION CORRELATES OF PLASMA CORTICOSTERONE AND METABOLITES AND OF LOCOMOTOR ACTIVITY IN A MALE PASSERINE

The acute stress response involves the secretion of catabolic glucocorticoids, such as corticosterone in birds, which mobilizes intrinsic energy stores primarily through a gluconeogenic pathway involving fat breakdown, thus linking body condition and stress. Yet little is understood concerning whether current energetic state influences glucocorticoid secretion and how gluconeogenic substrates are utilized during stress. I measured changes in corticosterone and gluconeogenic metabolites (triglycerides, free glycerols, glucose) during handling stress in Curve-billed Thrashers *Toxostoma curvirostre* from two habitats that differ in putative food abundance (urban vs. desert) in the wild, in captivity, and in response to food restriction and subsequent recovery. Urban thrashers were heavier and secreted more corticosterone than desert-caught birds in the field, but differences did not persist in captivity. Food restriction was associated with a diminished ability to elevate and lower plasma corticosterone and free glycerol in response to 30 min of handling stress, respectively. The opposite effect was observed as birds recovered from food restriction. No changes in plasma levels of glucose and triglycerides with stress were observed however. Food restriction also increased locomotor activity which likely further exacerbated energy loss. These observations suggest body condition and stress differences between urban and desert birds is related to food availability and current energetic state can impact both the extent to which an individual can elevate corticosterone and use free glycerol as energy during acute stress.

1. Introduction

Body condition is widely used in ecological and physiological studies that investigate the energy reserves, performance, and fitness of organisms. In free-living animals, variation in body condition has been linked to differences in food availability across habitats (Cypher & Frost 1999; Owen *et al.* 2005; Brown & Sherry 2006; Auman *et al.* 2008), reproductive effort (Korpimaki *et al.* 2000), immunocompetence (Acquarone *et al.* 2001; Ewenson *et al.* 2001; Moller & Erritzoe 2003), and survival probability (Morrison *et al.* 2007; Bender *et al.* 2008). Body condition is also thought to be reflected in circulating levels of glucocorticoids (Romero & Wikelski 2001), which are catabolic steroids produced by the adrenal glands and typically increase in circulation in response to adverse conditions. One function of glucocorticoids is to induce hyperglycemia by encouraging gluconeogenesis through lipolytic and proteolytic pathways (Bizzi *et al.* 1971; Bizzi *et al.* 1972; Macfarlane *et al.* 2008). As body condition parallels nutrient reserves, the question arises of whether the current energetic status of an individual alters how it uses these nutrients during a stress response.

During an acute stress response (i.e., over minutes of exposure to an adverse stimulus), glucocorticoids, such as corticosterone (CORT) in birds, induce the mobilization of free glycerol derived from the catabolism of triglycerides (the main lipid storage form) from fat reserves (Warne *et al.* 2009). Free glycerol can then be converted to glucose by gluconeogenesis (Warne *et al.* 2009). This metabolic pathway provides a potential mechanism that links body condition to plasma glucocorticoids. In recent research on free-living birds, (Fokidis *et al.* 2010) modeled how plasma metabolites such as glycerol and triglycerides vary relative to plasma CORT in response to acute stress in several passerine species. This study revealed a consistent association between body condition, as estimated by the residuals of a regression of body mass over body size, and CORT

secretion, and it found a strong association between the latter and free plasma glycerol.

In addition to its metabolic effects, CORT influences various behaviors including foraging (Kitaysky *et al.* 2001a; Pravosudov 2003; Bates *et al.* 2007; Warne *et al.* 2009; Foster *et al.* 2009) and locomotor activity (Lynn *et al.* 2001; 2003), that can also alter energy balance. Furthermore, CORT decreases the concentration of blood lymphocytes, which results in an increased blood heterophil to lymphocyte (H:L) ratio (Vleck *et al.* 2000). This ratio is often used to detect long-term increases in plasma CORT and as such, can serve to reliably indicate chronic stress (Vleck *et al.* 2000; Fokidis *et al.* 2008; French *et al.* 2008).

Birds are attractive models to study relationships between body condition and stress physiology for several reasons. First, free-living birds have been extensively used to investigate the life history consequences of variation in CORT, demonstrating close associations between plasma CORT and fitness (Eeva *et al.* 2003; Schoech *et al.* 2009). Second, the generally high metabolic rates and elevated glucose turnover rate of birds compared to size-matched mammals (Braun & Sweazea 2008), results in the body mass of birds being highly susceptible to factors such as short-term food restriction (Williams *et al.* 1999; Kitaysky *et al.* 2001b; Guglielmo *et al.* 2002b; Walker *et al.* 2005). Third, plasma levels of metabolites and in particular lipids, which are catabolized to provide energy during migratory flight (Williams *et al.* 1999; Guglielmo *et al.* 2002b), have been linked to changes in body mass. Finally, many bird species are common inhabitants of various habitats, including those modified by humans, that differ with respect to their putative food availability. Interest in these birds is reflected in studies demonstrating differences in body condition between habitat types (Owen *et al.* 2005; Fokidis *et al.* 2008; Geens *et al.* 2009).

The body condition of Curve-billed Thrashers, *Toxostoma curvirostre*, as estimated by body mass-size residuals, is higher in the city of Phoenix, Arizona (USA) than in surrounding native

Sonoran Desert habitats (Fokidis *et al.* 2008). Research has documented higher plant productivity in Phoenix compared to the surrounding desert (Stabler & Martin 2004). This difference is attributed to the presence of exotic mesic-type vegetation in urban environments. In addition, native and nonnative desert plants (i.e., aridity-adapted species that are not endemic to the Sonoran Desert) are more productive when growing in urban areas than the outlying desert (Martin & Stabler 2002; Stabler & Martin 2004). Furthermore, plant productivity in the urban Phoenix area is seasonally more uniform than in the surrounding desert. In the Sonoran Desert, precipitation patterns drive two distinct seasonal pulses in plant productivity: one in the spring and after the “winter rains” and the other during the “summer monsoons” (Brazel & Ellis 2003). Through watering systems and the creation of canals and residential bodies of water, human activities in Phoenix have damped the amplitude of seasonal variations in water availability and, therefore, also in plant productivity (Martin & Stabler 2002; Stabler & Martin 2004). This dampening may partly explain the decreased seasonal variation in CORT secretion in response to acute stress that is observed in urban thrashers compared to desert conspecifics. Indeed, the plasma CORT response to acute stress of desert, but not urban Curve-billed Thrashers, decreases during the summer (Fokidis *et al.* 2009).

Curve-billed Thrashers are primarily insectivorous but cactus fruits, berries, and seeds can constitute a substantial portion of their diet depending on the time of year (Tweit 1996). The diet of these birds, when they live in urban environments, may be impacted by the higher plant productivity in Phoenix compared to surrounding native habitats. In addition, experimental studies of foraging decisions indicate that relative to birds living in the outlying desert, urban birds - including thrashers - residing in Phoenix alter their foraging behavior in response to reduced predation risk and increased competition with non-native species (Shochat *et al.* 2004). Accordingly, urban thrashers may spend more time than non-urban conspecific birds foraging in an environment that is more

productive and is seasonally less variable than the surrounding desert. This difference may translate in urban thrashers having larger energy stores and being in better body condition than desert conspecifics (Fokidis *et al.* 2008; 2009).

I used captive Curve-billed Thrashers caught as adults from a local population to investigate how experimental variation in body condition induced by altering food availability and hence body mass, influences the utilization of plasma metabolites during an acute stress response. Based on the above, I predicted that 1) differences in plasma CORT between urban and desert birds result from differences in body condition; 2) during acute stress, experimentally increased body condition is associated with a more robust CORT response and increased mobilization of lipids; and 3) increased CORT secretion is associated with increased locomotor behavior. I compared the responses of birds in the field with those after acclimation to captivity to assess whether differences between urban and desert thrashers stem from a genetic or ontogenetic basis, or whether they represent phenotypic plasticity.

2. Materials And Methods

Procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and were done under scientific collecting permits from the US Fish and Wildlife Service, US Forest Service, and Arizona Game and Fish Department, and permission from local landowners.

2.1. Study Populations

Desert Curve-billed Thrashers were captured in the Four Peaks Mountains Wilderness Area, a largely unpopulated section of upland Sonoran Desert within the Tonto National Forest. This area is located about 10 km northeast of the suburban fringe development of Fountain Hills, Arizona, and

its vegetation is typical of the upland Sonoran Desert (Fokidis *et al.* 2009). Most urban thrashers were caught in East-Central Phoenix and Tempe, Arizona. Urban sites consisted primarily of small city park edges adjacent to middle income residential areas and of commercial areas with mesic style landscaping including large deciduous and palm trees. These urban habitats are representative of the types of development in the Phoenix metropolitan area.

2.2. Field Sampling And Captivity Study

Twenty-seven adult male thrashers (13 urban, 14 desert) were captured between 29 March and 23 June 2009 (mean capture date - urban: 5 June; desert: 11 June). This period coincides with the incubation and nestling stages of the reproductive cycle of the species (Fokidis *pers. obs.*). Birds were captured between 0530 and 0957 h (mean capture time - urban: 0736 h; desert: 0708 h).

Birds were lured to mist nets using conspecific song playback. Within 3 min of capture, a blood sample (200 μ l) was collected from the right jugular vein into a 0.3 ml heparinized syringe with a 29.5 gauge needle for measuring baseline plasma CORT and metabolites (glucose, free glycerol, and triglycerides). In addition, thin blood smears were made on glass microscope slides using about 5 μ l of the baseline blood sample and later used to measure H:L ratios (see below). Birds were then placed into individual cloth bags and 30 min later, a second blood sample (200 μ l) was collected as above to measure stress-induced changes in plasma CORT. This procedure was repeated after birds had been captive for 45 and also for 80 days (see below). It is widely used to induce an acute stress response (Wingfield *et al.* 1992; O'Reilly & Wingfield 2001; Arnold *et al.* 2008). Blood was stored on ice until plasma was separated by centrifugation and then stored at -80° C until assays were run.

Age and sex were determined by the presence of a developed cloacal protuberance (CP) which is associated with breeding condition. Body mass (± 0.1 g) and tarsus length (± 1 mm) were also measured and all birds received a uniquely numbered aluminum leg band.

Birds were transported to Arizona State University and individually housed in 76 x 46 x 46 cm cages within a single room maintained at 22°C, with lights on at 0700 hr and off at 2100 hr. They received an insectivorous bird diet (5MM3; PMI Nutrition Inc., St. Louis, Missouri) *ab libitum* for 80 days. This diet consists of 28% protein, 11% fat, 13% fiber, and 8% ash. The daily food consumption of each bird (*hereafter* Daily Food Intake or DFI) was monitored (± 0.01 g) daily for 45 consecutive days, beginning after 20 days in captivity. Food consumption during a 24 hour period was determined when lights came on in the morning, i.e., before birds began to feed that day. To minimize spillage, food dishes were partially covered with cardboard so as to provide only a small opening to access to food, which minimized spillage to about 0.05g. Birds were weighed weekly, shortly after lights came on and before they started feeding that day. After both 45 and 80 days in captivity with *ad libitum* feeding, baseline (< 3 mins) and stress-induced (30 mins) blood samples were again collected to measure CORT, plasma metabolites, and to produce a blood smears, such as described above. As each bird was captured on a different day I sampled individual birds, 45 and 80 days since their respective first day in captivity. This was advantageous because it allowed us to sample each bird at the same time of day to account for diurnal variation in CORT or metabolites, and before they fed for that day.

2.3. Body Condition Treatments

At the end of the 80 day period of captivity, birds received an amount of food equivalent to 80% of their individual DFI for 10 days and were then randomly assigned to a “loss-gain” (LG) or

“gain-loss” (GL) group (Figure 24). Birds (half from each habitat) in the loss-gain (LG) group were food-restricted for 5 days during which they received 60% of their individual DFI daily. Then they received *ad libitum* food until their body mass returned to that before the food restriction regime. Birds (also half from each habitat) in the gain-loss (GL) groups received *ad libitum* food for 5 days, during which time they gained weight, and were then food-restricted for 5 days during which they received an amount of food equal to 60% of their individual DFI.

During the treatment period, blood samples (< 3 & 30 mins; see above) were collected and body mass data was recorded at specific days (days 5, 12, 15, and 18) in the manner described above (Figure 24). In addition, individual food consumption was monitored daily, starting each day when lights came on. To determine locomotor activity, birds were video-recorded ($n = 4$ birds per day – one from each site and treatment) for 2 hours daily (1100 - 1300 h). This sampling time was chosen because it was well after the initial feeding for the day, and thus birds would not be primarily engaged in feeding behavior during this time. Video recordings were analyzed blind with respect to treatment to determine the number of hops (defined as movement from perch to perch or perch to the cage floor and *vice versa*) and the time spent perching (i.e., bird inactive on perch and not preening).

At the conclusion of the study, birds received *ad libitum* food for 15 days and the study was repeated, but individuals were now assigned to the alternate treatment group compared to the first part of the study. The sequence/order of treatments to which each thrasher was submitted was included in the statistical analyses of the data (see below).

2.4. Corticosterone Assays

Total plasma CORT concentrations were measured using validated commercial competitive enzyme-linked immunoassays (ELISA; Assay Designs Inc., Ann Arbor, Michigan; Fokidis *et al.* 2009). The sensitivity of the CORT assay ranged from 11.3 – 19.3 pg/ml depending on the assay plate and the mean intra-assay coefficient of variation was 14.1 % ($n = 4$ plates; 156 samples assayed in duplicate).

2.5. Heterophil To Lymphocyte Ratio

Blood smears were fixed for 10 min in absolute methanol within 3 days of collection, stained using the Giemsa method (Bennett 1970), and dehydrated for one week under partial vacuum. Stained smears were then cleared using xylene, cover-slipped, and sealed using Cytoseal 60 (VWR, San Francisco, CA).

Chronically high plasma glucocorticoids can decrease the blood concentration of lymphocyte, thereby increasing the heterophil to lymphocytes (H:L) ratio (Harmon 1998). This ratio can, therefore, serve as a marker of chronic stress (Gross & Siegel 1983; Vleck *et al.* 2000; Bonier *et al.* 2007).

Using an Olympus BX60 light microscope (Olympus Optical Co., Tokyo, Japan) I counted heterophils and lymphocytes under 400× magnification until a total of 100 cells of both types combined were counted (Fokidis *et al.* 2008; French *et al.* 2008). Cell types were identified using the criteria of (Campbell 1996). All slides were examined by a single observer (CR) without knowledge of individual, study, locality, or date of collection.

2.6. Plasma Metabolite Assays

Plasma free glycerol and triglycerides were measured using a sequential color endpoint assay (Sigma-Aldrich, reagents F6428 and T2449) described in (Guglielmo *et al.* 2002a,b; Fokidis *et al.* 2010).

Triglyceride concentration was calculated by subtracting the free glycerol component from the total triglycerides (triglycerides and glycerol) concentrations. Plasma glucose was measured using colorimetric enzyme endpoint assays (Cayman Chemical Co. Ann Arbor, Michigan, USA; Cat No. 10009582). Metabolite concentrations are expressed in mM units to facilitate comparisons.

2.7. Statistical Analyses

Body condition was defined as the standardized residual of a linear regression between body mass and tarsus length (Fokidis *et al.* 2009). I used Student's *t*-tests to compare morphological and physiological parameters between urban and desert birds in the field. To compare plasma CORT and metabolites, body condition, and H:L ratio (dependent variables) at the time of capture and during captivity, I used repeated measures analysis of variance (rmANOVA), with individual as the identifying variable, and origin (urban or desert), treatment order, and time of sampling (field, 45, or 80 days) as independent variables.

One assumption of captivity is that individual variation in traits influenced by the current environment become less noticeable with time spent in identical conditions of captivity. This can be tested by investigating differences in the amount of variation between birds captured in the field and after time spent in captivity. Here I compared changes in the values of these variables using the Levene's test of homogeneity of variances between birds in the field and in captivity.

Repeated measure ANOVA was used also to investigate changes in plasma CORT, plasma metabolites, body masses, time spent perching, and H:L ratios in response to the experimental

treatments. Origin of the birds and treatment (GL or LG) were treated as independent variables, and order of treatment (i.e., which treatment the bird underwent first) was included as a cofactor. The number of hops was analyzed using Friedman's test, a non-parametric counterpart to rmANOVA (Sokal & Rohlf 1995).

To determine how plasma metabolites and CORT changed with body condition, I calculated the individual change in body mass (g/d), during the course of food restriction and recovery from food restriction, for each study. Similarly, I calculated the change in plasma glucose, triglycerides, and free glycerol (all in mM/d), and CORT (ng/ml/d). Rates of change were assessed using linear regression analysis.

Comparisons of the change in plasma metabolites and CORT during acute stress and during the various treatments required square-root arcsine transformation of percent data to comply with statistical assumptions of normality. These transformed values were also analyzed with rmANOVA. Plasma glucose and H:L data were log- and square-root arcsine-transformed, respectively, prior to analysis.

For rmANOVA, data were tested for the sphericity assumption using Mauchly's test. Data that did not meet this assumption were further tested with X^2 analysis and the degrees of freedom were deflated using ϵ -derived Greenhouse-Geiser or Huynh-Feldt corrections (Sokal & Rohlf 1995). Comparisons between time points were made using least significant difference (LSD) post-hoc tests.

Statistical analyses were performed using SPSS Version 13.0 (2004; Chicago, Illinois, USA) with alpha levels set at 0.05. All data are presented as mean \pm s.e. and variance data are presented as percentage coefficient of variation (% cv). All graphs depict untransformed data.

3. Results

3.1. Physiological Differences Between Urban And Desert Thrashers In The Field

Urban thrashers were heavier (urban: 86.3 ± 3.72 g; desert: 81.9 ± 4.76 g; $t_{26} = 2.11$, $p = 0.021$), but did not have longer tarsi (urban: 30.9 ± 1.13 mm; desert: 31.6 ± 2.70 mm; $t_{26} = -0.62$, $p = 0.312$) than desert birds. As a result, at capture urban thrashers were in better body condition than desert birds ($t_{26} = 2.64$, $p = 0.010$; Figure 25). Urban and desert thrashers had similar plasma baseline CORT ($t_{26} = 1.01$, $p = 0.228$; Figure 26a), but plasma CORT after 30 minutes of capture and restraint was higher in urban than desert thrashers ($t_{26} = 2.05$, $p = 0.031$; Figure 26a). Plasma glucose did not differ between thrasher populations ($t_{26} = 0.50$, $p = 0.747$; Figure 26b). Urban and desert thrashers had similar plasma baseline glycerol and triglycerides, but plasma concentrations of these metabolites were higher in response to stress in urban than desert birds (glycerol: $t_{26} = 2.83$, $p = 0.007$, Figure 26c; triglycerides: $t_{26} = 2.13$, $p = 0.038$, Figure 26d).

3.2. Physiological Effects Of Captivity

After both 45 and 80 days of captivity, no differences in body condition were detected between urban and desert thrashers ($F_{2,25} = 1.22$, $p = 0.384$; Figure 25). Baseline plasma CORT was higher in urban thrashers than desert ones after 45 days in captivity, but the opposite pattern was observed after 80 days of captivity (45 days: LSD $p = 0.022$; 80 days: LSD $p = 0.041$; Figure 26a). No population differences in stress-induced plasma CORT were observed in thrashers held in captivity (both $p \geq 0.086$; Figure 26a). Similarly, in captivity urban and desert birds had similar plasma baseline and stress-induced glucose (both $p \geq 0.343$; Figure 26b) as well as free glycerol (both $p \geq 0.091$; Figure 26c). However, plasma baseline and stress triglycerides were higher in desert than urban thrashers after 45 (LSD $p = 0.019$; Figure 26d) and 80 days (LSD $p = 0.037$; Figure 26d) of

captivity, respectively. Body mass, plasma baseline and stress-induced CORT, and stress-induced triglycerides and free glycerol were individually more variable at the time of capture than during captivity (Table 8).

3.3. Body Condition Varies With Food Treatment

The order of treatments (LG, then GL vs. GL, then LG) did not influence results for any variables (all $p \geq 0.259$) and thus data for the two treatments were combined for further analysis ($n = 27$). Likewise, the origin of the bird (urban vs. desert) did not influence any variables (all $p \geq 0.163$).

Body mass decreased by 16-25% of the initial (before food restriction) body mass during the transition from 80% to 60% DFI in the LG treatment group (LSD $p = 0.007$; Figure 27). This loss was successfully recovered in response to ad libitum food (LSD $p = 0.253$; Figure 27). In contrast, the transition from 80% DFI to ad libitum access to food in the GL treatment increased the body mass above initial mass (7–12% increase) in only 23 birds (10 urban, 13 desert). After this period, food restriction (60% DFI) decreased body mass in all birds (LSD $p = 0.015$; Figure 27). Thus, I only included the 23 birds that increased their body mass in analyses of the effects of the GL treatment.

3.4. Changes In Body Condition Influences Corticosterone Secretion With Acute Stress

An increase in body mass was associated with a decrease in plasma baseline CORT and vice versa in response to food restriction as well as during recovery from restriction (Figure 28a). In contrast, stress-induced plasma CORT increased with increasing body mass (Figure 28b).

The ability to increase plasma CORT during 30 min of capture and handling stress was enhanced by the increased body mass associated with the GL treatment ($F_{2,21} = 2.14$, $p = 0.012$; Figure 29a). This effect was diminished by food restriction (LSD $p = 0.402$; Figure 29a).

Food restriction also decreased the capacity to elevate plasma CORT in response to stress during the LG treatment ($F_{2,25} = 4.77, p = 0.003$; Figure 29a), and this effect was rescued by allowing *ad libitum* access to food (LSD $p = 0.033$; Figure 29a).

3.5. Body Condition Affects Metabolic Changes During Acute Stress

Plasma glucose did not change in response to changes in body mass (Figure 28c). In contrast, as thrashers increased their body mass, they decreased their plasma baseline free glycerol (Figure 28d). An increase in body mass was associated also with increased plasma baseline triglycerides (Figure 28e).

Food treatment did not influence how plasma glucose changed during acute stress as evident by no differences in either the GL or LG treatment (GL: $F_{2,21} = 0.45, p = 0.611$; LG: $F_{2,25} = 0.92, p = 0.581$; Figure 29b). Similarly, plasma triglycerides were not affected either by the GL or by the LG treatment (GL: $F_{2,21} = 0.30, p = 0.346$; LG: $F_{2,25} = 1.02, p = 0.285$; Figure 29d).

Food restriction (LG treatment) had limited the amount by which plasma free glycerol decreased during acute stress ($F_{2,25} = 3.16, p = 0.021$; Figure 29c). In contrast, the transition from 80% DFI to *ad libitum* food availability in the GL treatment did not alter how plasma free glycerol changed during stress ($F_{2,21} = 0.73, p = 0.701$; Figure 29c). However after food restriction, providing *ad libitum* food during the LG treatment was associated with a decrease in plasma free glycerol during acute stress (LSD $p = 0.020$; Figure 29c).

3.6. Behavioral Responses To Changing Body Condition

The GL and LG treatments influenced the number of hops (GL: Friedman's $\chi^2 = 14.13, df = 3, p = 0.036$; LG: $\chi^2 = 17.09, df = 3, p = 0.048$;) and the time spent perching (GL: $F_{2,21} = 1.45, p = 0.042$;

LG: $F_{2,25} = 3.92, p = 0.008$) by the birds. During food restriction thrashers were more active, as shown by an increase in number of hops (Figure 30a) and generally less time engaged in perching behavior (Figure 30b).

3.7. No Change In Heterophil To Lymphocyte Ratio With Body Mass

The H:L ratio was individually variable and did not differ between birds sampled in the desert and in urban areas (urban: 0.24 ± 0.11 ; desert: 0.21 ± 1.3 ; $t_{26} = -0.12, p = 0.432$; Table 8). The H:L ratio increased after 45 days of captivity in urban but not desert birds (urban: 0.51 ± 0.25 ; desert: 0.18 ± 0.8 ; $t_{26} = -2.05, p = 0.023$). However, this difference was not observed after 80 days in captivity (urban: 0.31 ± 0.15 ; desert: 0.28 ± 0.9 ; $t_{26} = 0.93, p = 0.349$). The H:L ratio did not change during the course either of the GL or of the LG treatment (GL: $0.18 \pm 1.0, F_{2,21} = 1.05, p = 0.400$; LG: $0.15 \pm 0.75, F_{2,25} = 0.36, p = 0.277$).

4. Discussion

Previous research documented differences in body condition and CORT secretion between desert-dwelling Curve-billed Thrashers and those inhabiting Phoenix, Arizona (Fokidis *et al.* 2008; 2009). This study aimed to explore links between these parameters is linked by manipulating body mass through changes in food access of captive thrashers caught at urban and desert sites. Site differences in CORT secretion in response to capture and restraint and in body condition were found in field-caught birds but disappeared when birds were captive and received *ad libitum* food. Experimental manipulations of food availability during captivity revealed that associations between changes in body mass and plasma baseline and stress CORT. An increase in body mass increase was also associated with a decline in plasma baseline free glycerol, less activity, and an increase in plasma

baseline triglycerides. However, plasma glucose and the H:L ratio were not affected by mass changes. When acutely stressed, birds increased their plasma CORT and decreased their plasma free glycerol levels more when provided with *ad libitum* food than when food-restricted. Together, these findings suggest that variation in body condition between populations may partly account for differences in stress physiology between free-ranging urban and desert Curve-billed Thrashers. How energy reserves are used during a stress response may influence the ability to cope with novel urban stressors.

4.1. Local Environment Accounts For Differences Between Urban And Desert Thrashers

Captivity is a major stressor to wild animals and can alter CORT secretion (Marra *et al.* 1995; Dickens *et al.* 2009; Dickens & Romero 2009). We compared birds before and during captivity to assess whether differences in stress physiology observed between free-ranging urban and desert birds result from proximate factors (e.g., food access, different predation pressures between habitats) or from intrinsic factors causing (epi)genetic and/or ontogenetic differences between populations. Urban birds at capture were in better body condition than desert birds. During captivity, urban but not desert thrashers lost body mass, resulting in no population difference in body condition. The decrease in body condition in captive urban-caught thrashers may have resulted from the catabolic effects of CORT. This hypothesis is consistent with the observation that plasma baseline CORT and H:L ratios were higher in urban than desert birds after 45 days of captivity, even though plasma stress-induced CORT did not differ between the two groups of birds. An increase in plasma baseline CORT may have been due to prolonged exposure to stressful stimuli, i.e., to chronic stress (Sapolsky *et al.* 2000), which can depress blood lymphocyte numbers (Vleck *et al.* 2000). Urban birds may have been more stressed by captivity than their desert counterparts. The observations that

urban thrashers in captivity decreased their body mass, had higher baseline CORT levels and a higher H:L ratio unlike desert birds is consistent with this idea. Previous research suggested that variation in traits that can be directly influenced by environmental factors decreases during captivity (Calisi & Bentley 2006). Supporting this proposition, variation in body mass and plasma CORT of thrashers in this study was greater at capture than during captivity. Thus, captivity in combination with *ad libitum* food access negated population level differences in body condition and stress physiology, in turn suggesting that population differences seen in the field result from proximate environmental rather than genetic or developmental/ontogenic factors.

To assess the role of *ad libitum* food I monitored changes in plasma metabolites associated either with lipid deposition (triglycerides) or with lipid mobilization (free glycerol) (Williams *et al.* 1999; Guglielmo *et al.* 2002b; 2005), during the transition from field to captivity. Plasma glucose levels did not change with captivity or acute stress and did not differ between urban and desert caught thrashers. Plasma free glycerol decreased with acute stress in desert, but not urban birds, but levels only differed between populations in the field. In contrast, urban caught thrashers which declined sharply in body mass over the first 45 days, showed a concurrent decrease in triglyceride levels. This is consistent with decreased lipid mobilization during this time. Desert caught thrashers increased their stress-induced plasma triglycerides especially after 80 days of captivity. One interpretation is that the higher concentration of plasma triglycerides in desert thrashers with stress may indicate no inhibition of fat deposition. However this was not consistent with the lack of a change in body mass in desert thrashers during captivity. Although, fat deposition in birds is associated with an increase in body mass, actual body mass is primarily determined by muscle mass (Karasov *et al.* 2004). Therefore, catabolism of muscle protein may potentially account for a greater proportion of energy substrate during stress in desert birds.

4.2. Body Condition: Relationships To Metabolic And Hormonal Status

Changes in body condition induced by restricting or re-allowing access to food revealed that a decrease in body mass is associated with an increase in plasma baseline CORT and free glycerol and a decrease in plasma stress-induced CORT and triglycerides, and *vice versa*. Plasma glucose was not influenced by changes in body mass or captivity, suggesting that the plasma concentration of this metabolite is independent of these factors. CORT usually induces hyperglycemia (Sapolsky *et al.* 2000; Macfarlane *et al.* 2008), but in this study plasma glucose did not change in response to capture stress and despite changes in the magnitude of the plasma CORT response to stress as a function of the body condition. In most vertebrates, gluconeogenesis is the predominant mechanism by which CORT increases plasma glucose. This mechanism is associated with fat and protein degradation to provide lipid and protein-based substrates respectively, and with an increase in gluconeogenic enzyme activity (Sapolsky *et al.* 2000; Macfarlane *et al.* 2008). Birds have higher plasma glucose and mass-specific metabolic rates than mammals (Braun & Sweazea 2008). Maintaining plasma glucose constant may require a rapid uptake of energy substrates such as glycerol by the liver and kidneys, the major gluconeogenic sites in birds (Scanes 2009). This need for rapid uptake of energy substrate may also explain why many migratory bird species primarily use glycerol and fatty acids as fuel for flight muscles (Gannes 2001; Maillet & Weber 2006; Guglielmo & McFarlan 2007; Lyons *et al.* 2008), which may further assist in glucose sparing. Similarly, in some non-migratory species glucose does not increase with acute stress (Fokidis *et al.* 2010), and one possible explanation for this is a rapid replenishment of glucose used during acute stress, thereby resulting in no apparent net change in blood glucose. Some studies also report decreases in free glycerol with capture and handling stress (Guglielmo *et al.* 2002b), where as others report increases (Kern *et al.* 2007) or no changes in plasma levels (Seaman *et al.* 2005). The relationship between CORT and glycerol in birds requires further

research, particularly in the context of the acute stress response and the production of glucose.

General convention suggests high baseline CORT levels catabolize lipids to shuttle them into gluconeogenic pathways, and thus high CORT induces lower body condition. In individuals with low circulating CORT these reserves are not depleted and are free to accumulate resulting in higher body condition. However, the interplay between glucocorticoids and body condition is further complicated since prolonged stress can induce fat accumulation, through enzymatic effects at the tissue-level (reviewed in Macfarlane *et al.* 2008), and central effects inducing appetite for high-fat, high-sugars foods (Dallman *et al.* 2004; 2007). Thus it is difficult to discern if increased CORT secretion during stress is a response to greater body condition because of a greater amount of energy reserves being available.

However there is some evidence supporting this hypothesis. A comparative study of several migrating songbird species found increased handling time was associated with increased CORT levels only in birds that had large fat stores (Jenni *et al.* 2000), which supports the hypothesis that amount of energy stores influences the capacity to mount a stress response. Another study comparing CORT responses of marine iguanas (*Amblyrhynchus cristatus*) across several islands of the Galapagos found a consistent “threshold value” for body condition, below which there was a negative relationship between condition and baseline CORT secretion (Romero & Wikelski 2001). Thus, excessive CORT secretion may be mitigated by having a certain threshold level of energetic reserves.

In addition to direct metabolic effects, CORT is known to have strong impacts on behavior that can further influence energy balance. Short-term (hrs) food restriction increases perch hopping activity in white-crowned sparrows (*Zonotrichia leucophrys*) and this was mirrored by an increase in baseline total CORT levels (Lynn *et al.* 2003). Similarly, thrashers were more active during periods of

food restriction. This would appear disadvantageous as it would depreciate energy balance further, however in nature, CORT stimulates feeding behavior (Landys *et al.* 2004; Pecoraro *et al.* 2004) which acts to increase body condition.

4.3. Linking Energy Balance And The Acute Stress Response

This study adds to our growing knowledge concerning how energy in the environment translates to physiological changes in how animals respond to stress. Food supplementation studies in the field have shown that food availability can influence CORT secretion in nature (Clinchy *et al.* 2004; Schoech *et al.* 2007), and this interaction is likely a strong ecological mechanism dictating the habitats that animals occupy. However linking food availability and the physiological stress response requires us to establish how current energetic status is monitored by the body. Recent research focusing on how neural mediators (e.g. neuropeptide Y, orexin, agouti-related protein) and peripherally-secreted metabolic hormones that can act on the brain (e.g. leptin, ghrelin, insulin) interact with CORT (Heiman *et al.* 1997; Chang *et al.* 2005; Schmidt *et al.* 2008; Warne *et al.* 2009) provide a myriad of potential mechanisms that can link current energy reserves with the ability to cope with stress.

5. References

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Table 8. Coefficients of variation (%) for corticosterone and plasma metabolites in Curve-billed Thrashers *Toxostoma curvirostre* sampled in the field (Day 0) and after 45 and 80 days of captivity. Shared letters for a given parameter indicate no significant difference ($p > 0.05$, Levene's test of homogeneity of variances).

	Field	Captivity	
	Day 0	Day 45	Day 80
Body Mass	14.4 a	9.9 b	10.3 b
Baseline Corticosterone	9.2 a	4.2 a	3.3 a
Stress Corticosterone	36.8 a	12.2 b	14.8 b
Heterophil to Lymphocyte Ratio	17.2 a	29.2 a	20.3 a
Baseline Glucose	13.5 a	13.4 a	15.3 a
Stress Glucose	15.2 a	13.6 a	16.4 a
Baseline Free Glycerol	2.0 a	2.9 a	2.3 a
Stress Free Glycerol	19.5 a	9.9 b	9.3 b
Baseline Triglycerides	6.6 a	11.2 a	7.8 a
Stress Triglycerides	14.6 a	5.2 b	6.8 b

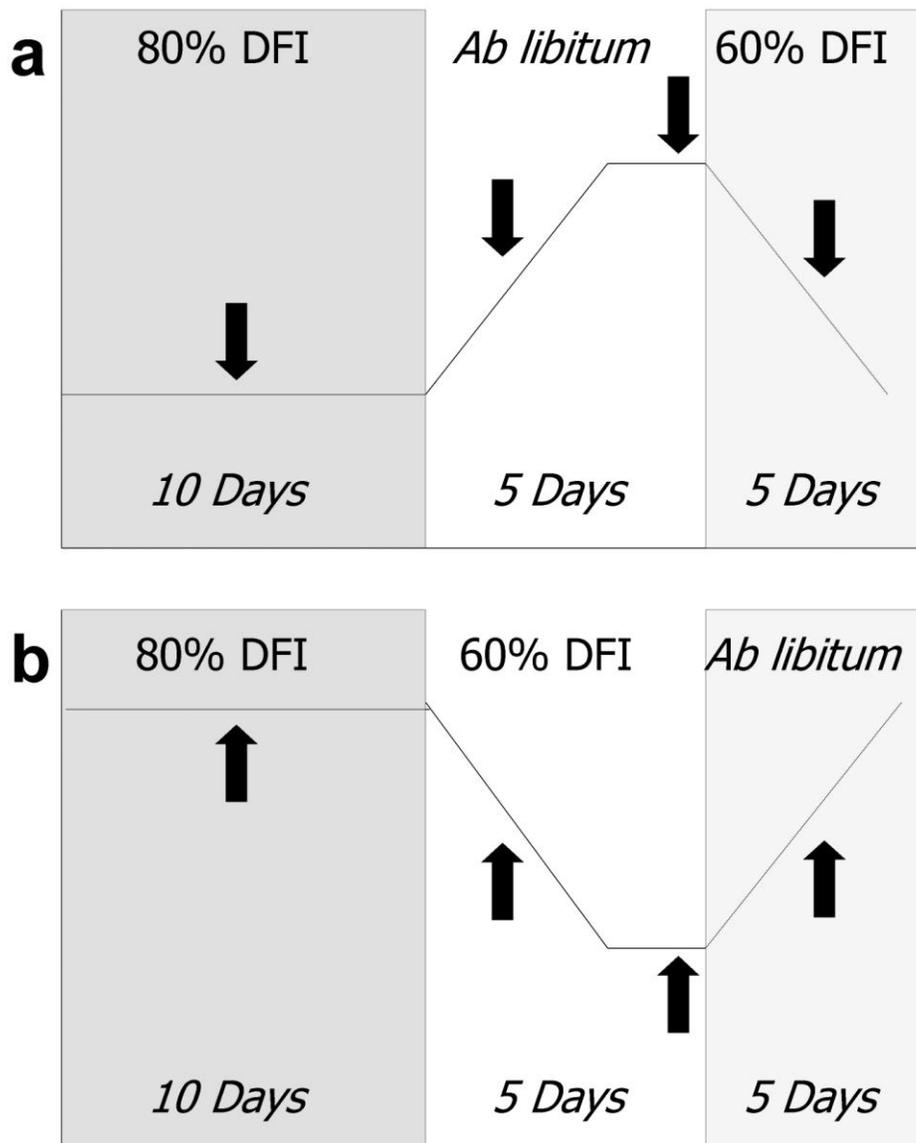


Figure 24. Schematic diagram of two experimental feeding treatments designed to manipulate body mass in Curve-billed Thrashers, *Toxostoma curvirostre*. (a) Birds first gain, then lose body mass (GL treatment) by providing *ad libitum* access to food, after a period of mild food restriction (80% daily food intake: DFI). This is followed by more severe food restriction (60% DFI). (b) Birds are first exposed to food restriction (60% DFI), followed by *ad libitum* access to food, resulting in a loss of body mass, followed by a gain (LG treatment). Daily food intake is based on the mean amount of food consumed by each bird in a 24 hr period. Arrows indicate when blood samples are taken and birds are weighed during experimental treatments.

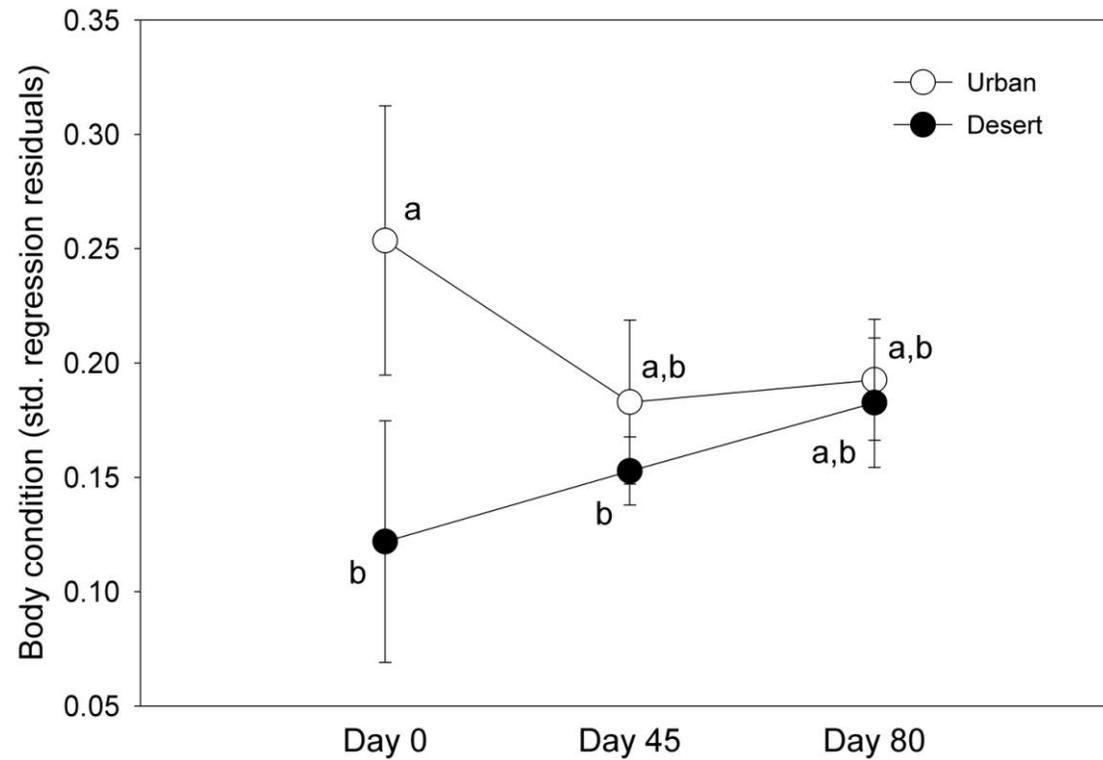


Figure 25. Changes in body condition (i.e. residuals of a body mass-size regression) in Curve-billed Thrashers, *Toxostoma curvirostre* sampled from urban (Phoenix, USA) and desert habitats from capture in the field (Day 0) and after 45 and 80 days in captivity with *ad libitum* access to food. Data points are means \pm s.e. and those that share letters are not significantly different at $p \leq 0.05$.

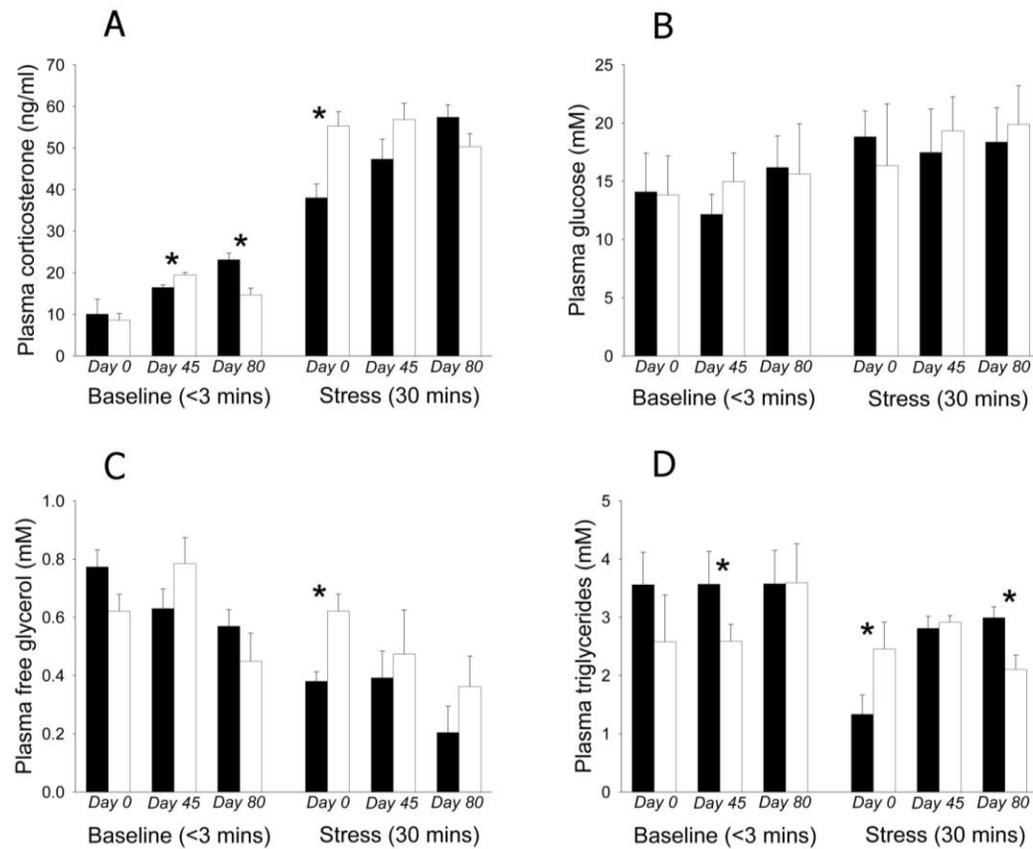


Figure 26. Differences between urban (white bars) and desert-caught (black bars) Curve-billed Thrashers, *Toxostoma curvirostre* in baseline (within 3 min of capture) and stress (30 min post-capture) plasma concentrations of (a) corticosterone, (b) glucose, (c) free glycerol, and (d) triglycerides, captured in the field (Day 0) and after 45 and 80 days in captivity with *ad libitum* access to food. Data points are means \pm s.e. and * indicates significant difference between urban and desert birds at $p \leq 0.05$.

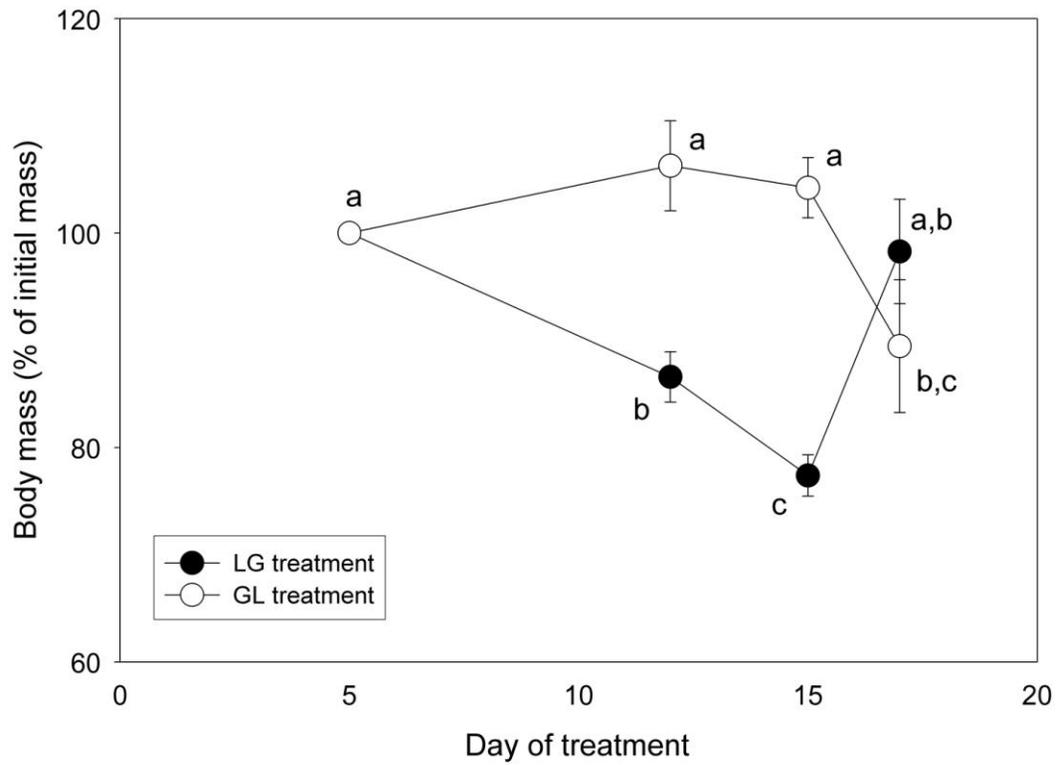


Figure 27. Percentage changes in body mass in Curve-billed Thrashers, *Toxostoma curvirostre* during the course of two feeding treatments: the LG treatment where mass is first lost then regained; and the GL treatment where body mass is first gained then lost. See Fig. 5.1 legend for details on study design. Data points are means \pm s.e. and those that share letters do not differ significantly ($p \geq 0.05$).

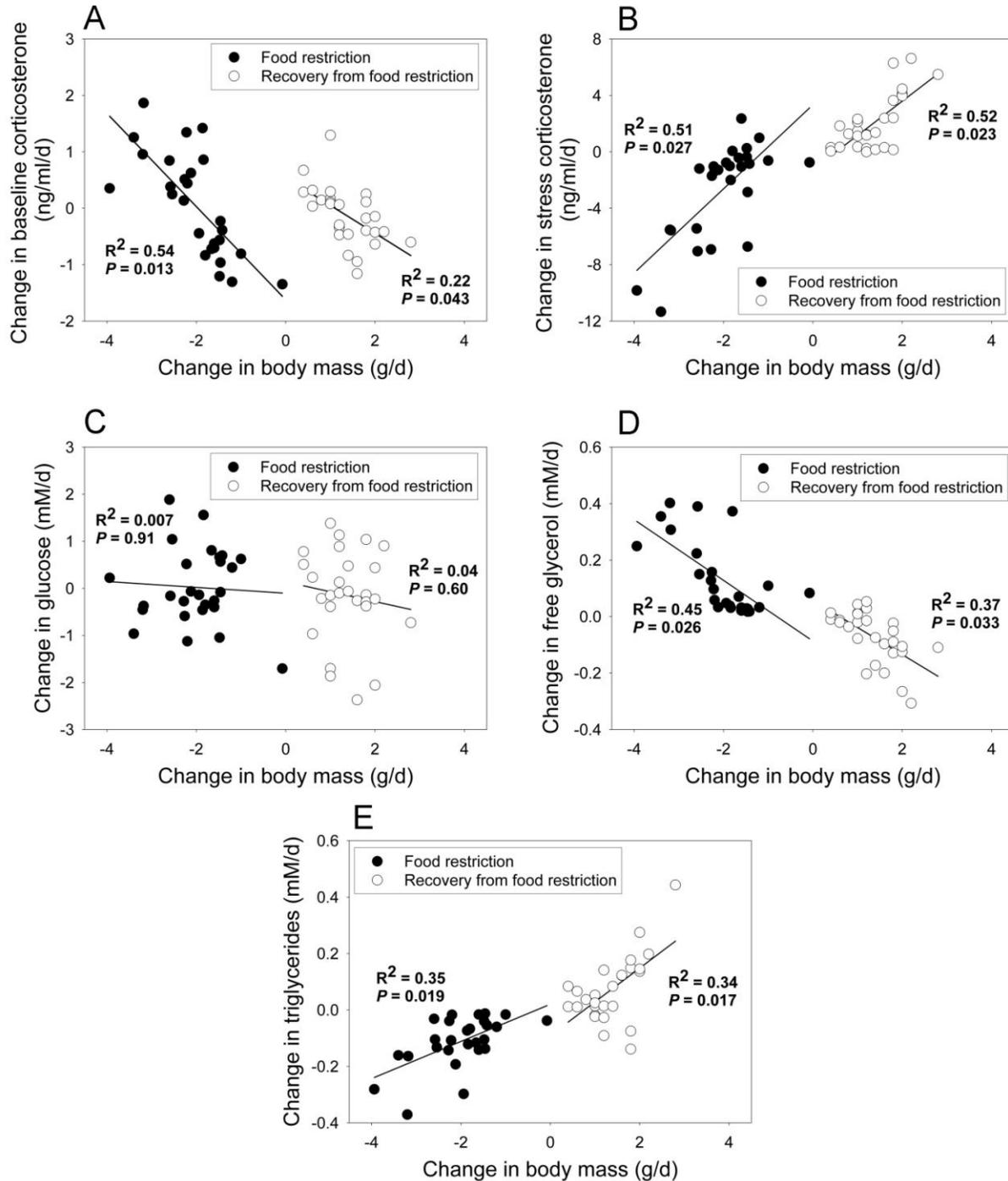


Figure 28. Daily changes in (A) baseline (≤ 3 min post-capture), and (B) stress-induced plasma corticosterone (30 min post-capture), (C) glucose, (D) free glycerol, and (E) triglycerides associated with daily changes in body mass in Curve-billed Thrashers, *Toxostoma curvirostre*, induced by periods of food restriction and recovery with *ad libitum* access to food.

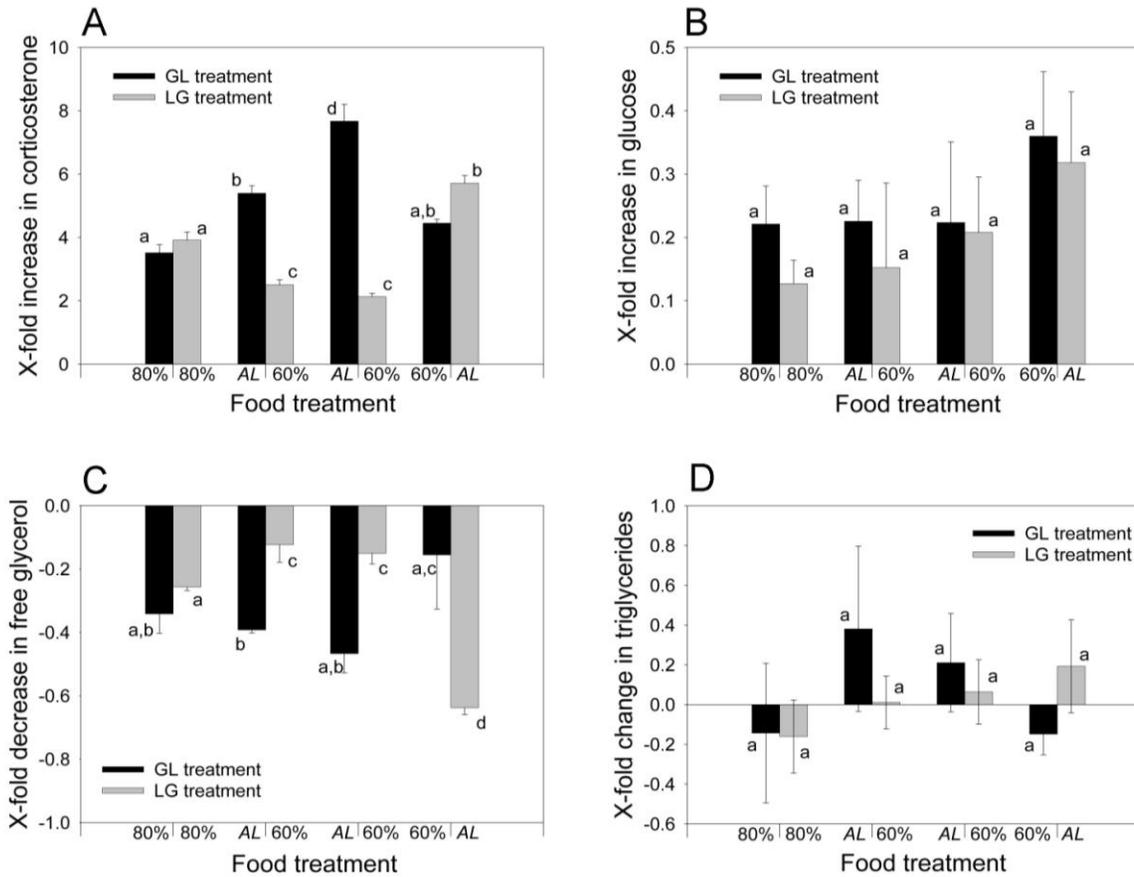


Figure 29. Changes in plasma (A) corticosterone, (B) glucose, (C) free glycerol, and (D) triglycerides before and after 30 minutes of capture and handling stress in Curve-billed Thrashers, *Toxostoma curvirostre* during the course of two feeding treatments: the LG treatment where mass is first lost then regained; and the GL treatment where body mass is first gained then lost. See Fig. 5.1 legend for details on study design. Data points are means \pm s.e. and those that share letters are not significantly different at $p \leq 0.05$.

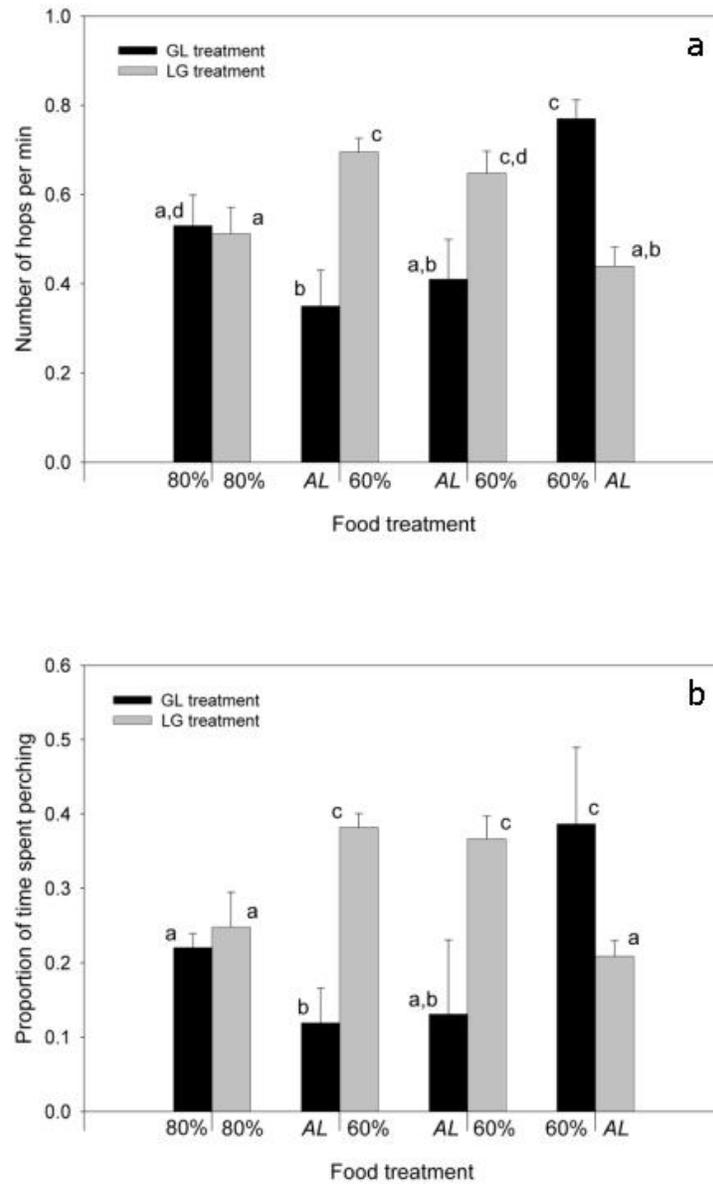


Figure 30. Variation in locomotory behavior in Curve-billed Thrashers, *Toxostoma curvirostre* during the course of two feeding treatments: the LG treatment where mass is first lost then regained; and the GL treatment where body mass is first gained then lost. See Fig. 5.1 legend for details on study design. Differences in (a) number of hops from perch to perch per minute; and (b) the proportion of time spent inactively perching. Data points are means \pm s.e. and those that share letters are not significantly different at $p \leq 0.05$.

Chapter 6

VARIABLE FOOD AVAILABILITY INDUCES STRESS AND METABOLIC CHANGE INDEPENDENT OF FOOD INTAKE IN A NON-MIGRATORY SONGBIRD

Many environments differ in their spatial and temporal resource variability, but little is known about how food predictability impacts animal physiology. The adaptive regulation hypothesis suggests that organisms maintain energetic state during periods of diminished food access by drawing on accumulated reserves from periods of greater food availability. In contrast, the chronic stress hypothesis suggests that unpredictable food access generates a prolonged stress response, resulting in maladaptive usage of energy reserves and increased behavioral activity. To distinguish between these two hypotheses, I compared behavioral, hormonal, and metabolic responses of Curve-billed Thrashers (*Toxostoma curvirostre*) fed either varying or constant amounts of food each day. Both groups consumed equivalent food amounts by the end of the study, but those in the variable group increased initial levels of the stress hormone corticosterone, used greater amounts of fat and protein energy reserves, and were more behaviorally active than those in the constant feeding group. These data provide experimental support for the chronic stress hypothesis and suggest that high temporal variation in food availability may be an important selective force.

1. Introduction

The amount of available energy in the environment (i.e. food quantity and quality) is a primary limiting factor for many physiological processes, including reproduction (Fernandez-Fernandez *et al.* 2006; dos Santos *et al.* 2010), growth (Cox *et al.* 2008; Fan *et al.* 2008), development (Corbel *et al.* 2010), and immune function (McGraw *et al.* 2006; Houston *et al.* 2007). It also acts as a proximate cue for timing reproductive activities in seasonal breeding species (Hau *et al.* 2000; Schoech & Hahn 2005). However, many vertebrates also have numerous physiological forms of energy storage to reduce their reliance on current environmental food sources (Arrington *et al.* 2006; Naya *et al.* 2008; Smith & McWilliams 2010). For example, fat reserves, in the form of triglycerides, enable organisms to meet metabolic demands of self-maintenance and activity during periods of food scarcity (Rogers & Reed 2003; Rozman *et al.* 2003). Prolonged periods of food scarcity can induce loss of body mass, due to depletion of fat (breakdown to free glycerol) and protein (breakdown to amino acids) reserves for fueling gluconeogenesis (Fery *et al.* 1996; Wingfield 2003). This is also concurrent with the general allocation of energy towards vital “life-sustaining” physiological functions (Wingfield 2003). In situations where food scarcity is followed by abundance, many organisms engage in “compensatory hyperphagia”, aimed at replenishing lost reserves (Bull & Metcalfe 1997; Jobling & Johansen 1999). The effects of food quantity and quality on birds have been well-researched (Brown & Sherry 2006; Houston *et al.* 2007; Martinez-Padilla & Fargallo 2007; Jenni-Eiermann *et al.* 2008); however much less is known about how temporal variability in these resources can influence physiology, and particularly the depletion of energy reserves. Further complications arise because food variability may constitute a significant psychosocial stressor, which can alter metabolism (Bridge *et al.* 2009).

One component of the physiological response to stress is the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Hazard *et al.* 2007). Activation of the HPA axis involves the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in mammals (or arginine vasotocin (AVT) in other vertebrates) from the hypothalamus, adrenocorticotropin hormone (ACTH) from the pituitary gland, and glucocorticoids (cortisol or corticosterone: CORT) from the adrenal glands (Hazard *et al.* 2007). A primary consequence of HPA activation is inducing hyperglycemia (i.e. elevation of blood glucose levels) *via* a gluconeogenic pathway that depletes energy reserves (Dallman *et al.* 2007). Several studies show that food restriction elevates glucocorticoid levels (Diaz-Munoz *et al.* 2000; Lynn *et al.* 2003) and impairs processes like reproduction and immunity (French *et al.* 2007; French & Moore 2008). CORT is also important in initiating daily foraging activities (Astheimer *et al.* 1992; Lohmus *et al.* 2006), and increased secretion can promote locomotory activity in captivity (Lynn *et al.* 2003; Chapter 5), which can further alter energy balance. Thus the HPA axis can mediate transitions from normal “life-history” activities to vital “emergency” functions aimed at maximizing survival, and as such is responsive to current energetic state.

In environments where food availability may be spatially or temporally variable, such as those subjected to frequent inclement weather, the relationship between maintaining energy reserves and the stress of “unpredictable” foraging has been considered in a risk foraging framework (Bednekoff & Houston 1994). In this context, periods of variable food intake due to variation in food abundance or interruptions in foraging may prompt individuals to regulate energy use and body mass (Bednekoff & Krebs 1995; Witter *et al.* 1995; Cuthill *et al.* 2000). Previous studies in birds and mammals have shown that temporal variability in food availability can be met with accumulation of energy reserves in body tissues (Bednekoff & Krebs 1995), increases in daily torpor (Munn *et al.*

2010), increased or stabilized body mass (Witter *et al.* 1995; Cuthill *et al.* 2000), and increased periods of behavioral inactivity (Dall & Witter 1998). These responses promote either the conservation of current energy supplies, or increased feeding leading to greater accumulation of energetic reserves during periods of greater food access, and collectively is called the “adaptive regulation” hypothesis (Witter *et al.* 1995; Fauchald *et al.* 2004). However, other studies have shown that variable access to food can lead to energy depletion in the body, which is signified by decreased body mass (Acquarone *et al.* 2002; Cucco *et al.* 2002), slowed growth rates (Boon *et al.* 1999), lower plasma testosterone levels (Bridge *et al.* 2009), lower haematocrit, and a weaker reaction to immune challenges (Acquarone *et al.* 2002; Cucco *et al.* 2002). Although not usually measured (but see Bridge *et al.* 2009), these effects of variable food access on resource depletion may be mediated by an overactive HPA axis (i.e. chronic stress), resulting in a depreciated health state. This “chronic stress” hypothesis may stem from an inability to dampen the HPA axis in the face of continued crises, which prolongs the catabolic actions of glucocorticoids and interferes with energy conservation. Thus the “predictability” of food resources and the ability to respond in an adaptive fashion is likely under intense natural selection in free-living individuals.

Much of the above research has been conducted in birds, which are excellent models for addressing dynamic relationships between energy and physiology. Birds have higher mass-specific metabolic rates, blood glucose levels, glucose tolerance, and insulin resistance than other vertebrates (Braun & Sweazea 2008), and yet are capable of living longer than mammals of similar size despite the apparent higher energetic demand (Holmes & Ottinger 2003). To meet this demand, birds are faster at utilizing intrinsic fat and protein stores during periods of nutritional stress (Morton *et al.* 1994; McWilliams *et al.* 2004), making them more responsive to experimental food manipulations than mammals. Previous research into the impacts of food variability on body mass mostly focused

on bird species with extensive fat reserves for enduring temperate winter nights (Bednekoff & Krebs 1995; Witter & Swaddle 1997; Pravosudov *et al.* 2001; Polo & Bautista 2002). Other studies have investigated birds known to hoard food during periods of food shortage (Hurly 1992; Acquarone *et al.* 2002; Cucco *et al.* 2002; Bridge *et al.* 2009). However, to my knowledge only a single study has attempted to relate the temporal availability of food to HPA activity; Bridge *et al.* (2009) reported no difference in CORT secretion between Western Scrub Jays (*Aphelocoma californica*) subjected to “unpredictable” and “predictable” feeding regimes. However, each of these studies differed in the methodology used to establish variable food access. These methodologies manipulated either: 1) the time available to forage (Witter *et al.* 1995; Boon *et al.* 1999); 2) the timing of food presentation (Boon *et al.* 1999); 3) the amount of food provided, but not necessarily consumed (Acquarone *et al.* 2002; Cucco *et al.* 2002; Munn *et al.* 2010); 4) the rate of food consumption, but not the absolute amount consumed (Bridge *et al.* 2009); or 5) the number of interruptions to foraging (Dall & Witter 1998). Moreover, many of these studies used control groups with access to *ad libitum* food as the basis for comparisons. However, these studies did not control for the actual amount of food consumed by the birds, and thus were unable to separate the effects of perception of variable food access from differences resulting from actual energy intake. Considering the separate effects of these two factors is important in determining whether perception of a variable food source alone is sufficient to induce changes in metabolism. Many food resources in nature can vary temporally in abundance or accessibility with respect to current weather conditions or the timing of insect emergence (Bednekoff & Krebs 1995; Dall & Witter 1998).

To address this issue, I manipulated the amount of food consumed daily by captive Curve-billed Thrashers (*Toxostoma curvirostre*) and monitored subsequent physiological and behavioral changes. These thrashers are insectivorous songbirds (~ 70 - 75 g) that reside year-round in the xeric

Sonoran Desert of the southwestern USA (Tweit 1996). They deposit little (if any) furcular fat, unlike many other songbird species (Fokidis *pers. obs.*), and there are also no known records of food hoarding in this species. Insects (a major component of their diet) are also highly susceptible to deteriorating environmental conditions, such as sudden colder temperature or heavy rainfall. In a prior experiment on thrashers, I found a strong relationship between current body mass (or condition) and CORT secretion, with birds that increased body mass (such as during recovery from food restriction) showing lower initial CORT levels (i.e. pre-stress) and a greater capacity to increase CORT levels in response to acute stress (Chapter 5). This is consistent with results from my field studies of thrashers inhabiting two habitat types, where thrashers from urban areas are heavier and have a more robust CORT response to handling-induced stress throughout the year than native desert inhabitants (Fokidis *et al.* 2009; Chapter 2).

Previous research demonstrated an increased abundance of herbivorous insects within urban Phoenix compared to outlying native Sonoran Desert being driven by the increased primary productivity within the city (Faeth *et al.* 2005). This has resulted in a potentially more predictable and abundant food resource in Phoenix compared to the desert (Faeth *et al.* 2005). Using this model species, I aim to distinguish between the adaptive regulation and chronic stress hypotheses by manipulating daily predictability but not amount of food consumed and examining associated changes in a suite of variables (Figure 31) related to the usage of intrinsic energy reserves, activity of the HPA axis, and behavioral adjustments to food variability. The adaptive regulation hypothesis predicts that birds will maintain or up-regulate metabolic and hormonal state and/or minimize daily activity to either conserve available energy or increase body stores, respectively (Figure 31). However note that the ability to engage in adaptive regulation may be dependent on the current energetic state of the bird prior to the experiment. In contrast, the chronic stress hypothesis predicts excessive

activity of the HPA axis will deplete intrinsic energy reserves and promote energetic expenditure (i.e. increased activity), resulting in the loss of body mass and deteriorating health (Figure 31). This study provides a vital assessment of the physiological consequences of variation in food availability, which is considered to be an important proximate factor influencing life-history evolution across a variety of taxa and habitats worldwide.

2. Material And Methods

All procedures in this study followed guidelines approved by the Arizona State University Institutional Animal Care and Use Committee and were done under scientific collecting permits from the US Fish and Wildlife Service, US Forest Service, and Arizona Game and Fish Department, and permission from local landowners.

2.1. *Field Sampling And Housing*

Adult male Curve-billed Thrashers were captured from one of two locations: either an unpopulated Four Peaks mountains wilderness area within the Tonto National Forest (Arizona, USA) or urban areas in East-Central Phoenix and Tempe, Arizona. Birds from these two populations differ with respect to both their body condition and stress physiology (Fokidis *et al.* 2009; Chapter 2), but a previous common-garden experiment on the same individual birds as those used in this study determined that housing in captivity was sufficient to negate all population differences in these parameters (Chapter 5).

In the field, I captured 27 thrashers (13 urban, 14 desert) actively holding territories using mist nets and conspecific song playback. Sex was confirmed in hand by the presence of a developed cloacal protuberance (CP), which signifies breeding condition. Birds were captured between 0530

and 0957 h (mean capture time - urban: 0736 h; desert: 0708 h) and between 29 March and 23 Jun 2009, (mean capture date - urban: 5 Jun 09; desert: 11 Jun 09). This time period coincides with the incubation and nestling stages of the reproductive cycle of this species (Tweit 1996).

Birds were then transported to the Arizona State University Animal Care Facility and housed in 76 x 46 x 46 cm dove cages within a single room maintained at 22°C and a 14:10 h (light:dark) lighting regime. Birds were fed *ad libitum* with a Mazuri insectivore diet (5MM3; PMI Nutrition Inc. St. Louis, Missouri, USA), consisting of 28% protein, 11% fat, 13% fiber, and 8% ash. To determine the daily food intake (DFI) of individuals, I weighed the food provided to each bird at lights on each morning (0700 h) before birds began their morning feeding. Feeding dishes were covered with cardboard, which provided only a small opening for accessing food and minimized spillage. The amount of food consumed over a 24 hour period was assessed for 45 days, which provided a customized mean DFI for each animal (mean = 11.7 g, range 10.6 - 12.4 g). During this period, body mass in all animals was also monitored weekly prior to morning feeding shortly after lights on.

2.2. *Variable Vs. Constant Feeding Treatments*

I randomly divided birds into control (constant) and treatment (variable) groups. Those in the constant group received an amount of food corresponding to its own DFI. Thrashers in the variable group received an amount of daily food that fluctuated daily between 30% and 200% of the DFI amount by weight (Figure 32). The sequence of the treatment was determined at random, but the specific amounts were corrected so that they equaled the total amount of food provided to the control group over the course of the study. The first trial was first conducted for 18 days using 13 and 14 birds in the treatment and constant groups, respectively. At the completion of the study, all birds were provided access to food *ad libitum* for 15 days, during which time body mass was

monitored on three occasions throughout this period. Then the study was repeated but with each bird switched to the other experimental group (i.e. 14 and 13 birds in treatment and constant groups, respectively) for this 18-day trial.

On the first day of each study trial (day 1), an (“initial”) blood sample (300 μ l) was collected from each bird within 3 min of entry into the room and removal from the housing cage from the right jugular vein using a heparinized 0.3 cc syringe with a 29.5 gauge needle. Birds were held in a cloth bag for 30 mins, after which time a second (“stress”) blood sample (200 μ l) was collected. This 30 min capture and handling technique is widely used to induce an acute stress response (Wingfield *et al.* 1992; Fokidis *et al.* 2009), here described as a rapid (mins) increase in plasma CORT concentration. Blood samples were also taken on days 9 (mid-point) and 18 (final) to determine changes in response to experimental treatment (Figure 32). On days 8 and 17, all birds were provided food at the DFI rate so that blood sampling on days 9 and 18 reflected the treatment integrated over more than only a single day (Figure 32). All blood samples were taken just after sunrise, before birds had an opportunity to feed. Blood samples were stored on ice until plasma was separated by centrifugation and then stored at -80°C until the various assays were run.

2.3. Monitoring Behavioral Activity

To estimate behavioral activity (as a proxy for energy expenditure), I digitally video recorded birds ($n = 4$ birds per day) for two hours each day (1100-1300 h) from both treatment groups concurrently. The recording times were chosen because they coincided with a period of time after the initial feeding for the day. Digital video recordings were then examined and two variables were recorded: 1) number of hops per minute, here defined as either hops from perch to perch, perch to cage floor, or *vice versa*; and 2) time spent perching, defined as sitting perched inactive and without preening.

2.4. *Inducing Feather Molt And Fault Bar Analysis*

To determine if variability in food access influences feather growth, I induced molt in all birds five days after the termination of the first trial. A left retrix (tail) feather (third from the right = R4: Pyle 1997) was removed, and allowed to grow for 10 days prior to the start of the second trial. At the conclusion of the study, after birds were euthanized (*see below*), the re-grown tail feathers were removed, measured (from tip to base) and the number of fault bars on each feather was manually counted. Fault bars are obvious translucent bands across the feathers resulting from stressful conditions that impact barbule formation (Prentice *et al.* 2008). Fault bars often coincide with points of feather breakage, and thus are viewed as a great handicap to a bird (Serrano & Jovani 2005; Prentice *et al.* 2008).

2.5. *Plasma Corticosterone*

Total plasma CORT concentrations were measured using validated commercial competitive enzyme-linked immunoassays (ELISA; Assay Designs Inc., Ann Arbor, Michigan, USA) as previously described and validated in Fokidis *et al.* (2009). All samples from the same individual were run on the same plate to minimize interplate variation. The sensitivity of the CORT assay ranged from 9.9 – 15.1 pg/ml depending on the plate, and the mean intra-assay coefficient of variation was 16.2 % ($n = 5$ plates; 162 samples assayed in duplicate).

2.6. *Triglycerides, Glycerol And Glucose*

Free glycerol and triglycerides in plasma were measured using a sequential color endpoint assay (reagents F6428 and T2449, Sigma-Aldrich Inc. St. Louis, Missouri, USA) described in Guglielmo *et al.* (2002, 2005) and Fokidis *et al.* (2010). “True” triglyceride concentration was

calculated by subtracting the free glycerol component from the total triglycerides (i.e. both triglycerides and free glycerol). Plasma glucose was also measured using colorimetric enzyme endpoint assays (# 10009582, Cayman Chemical Co. Ann Arbor, Michigan, USA). All metabolite concentrations were converted to mM units to facilitate comparison. Assay sensitivities, mean intra- and inter-assay coefficients of variation are as follows: free glycerol (0.06 – 6.4 mM, 6.7% and 14.0%); triglycerides (0.09 – 15.1 mM, 8.1% and 11.3%); and glucose (2.5 – 27.01 mM, 2.9% and 13.1%).

2.7. Plasma Osmolality And Total Protein

Plasma osmolality, (mOsm/kg of plasma), defined as the amount of solute within the plasma, was measured using a vapor pressure osmometer (Model 5500XR, Wescor Inc. Logan, Utah, USA) with 50 μ l samples assayed in duplicate. The osmometer was calibrated to known concentration standards before use and has been previously used in this species (Fokidis *et al.* 2010). Total plasma protein concentration was determined using a hand-held clinical refractometer with temperature compensation (Model RHC-200; Huake Instrument Co., Shenzhen, China). A 20 μ l drop of plasma was placed on the refractometer surface, and the specific gravity was read according to manufacturer's instructions. This provides a measure of the degree to which light passing through the sample is "bent" or refracted by the presence of protein solutes (Haller 2003). Before each reading, the refractometer was calibrated using distilled water, which has a specific gravity of 1.0. Previous research in a wide range of domestic and wild avian species has demonstrated that this technique provides a reliable and highly accurate measurement of total plasma proteins and is comparable to direct protein measurements, such as the Biuret method (Dawson & Bortolotti 1997; Cray *et al.* 2008; Santos Schmidt *et al.* 2008).

2.8. *Heterophil To Lymphocyte Ratio*

From the initial blood sample, about 5 μ l was used to make a thin blood smear on a glass microscope slide for measuring the ratio of heterophils to lymphocytes (H/L ratio). These smears were air-dried at ambient temperature and stored until fixation. These blood smears were then fixed for 10 min in absolute methanol within 3 days of collection and subsequently Giemsa-stained (Bennett 1970). Stained smears were then dehydrated for one week under partial vacuum. Stained slides were then cleared using xylene, cover-slipped, and sealed using Cytoseal 60 (VWR, San Francisco, CA) to facilitate long-term storage.

Chronic elevations in CORT can suppress lymphocyte numbers, thereby increasing the H/L ratio (Harmon 1998); thus the H/L ratio has been used as an alternative marker of stress (Gross & Siegel 1983; Vleck *et al.* 2000). The number of heterophils and lymphocytes were counted under 400x magnification using an Olympus BX60 light microscope (Olympus Optical Co., Tokyo, Japan) until a total of 100 cells (both types combined) was reached (Fokidis *et al.* 2008; French *et al.* 2008). Cell types were identified using the criteria of Campbell (1996), and all slides were examined without knowledge of individual, treatment, locality, or date of collection.

2.9. *Organ Weights And AVT Immunocytochemistry*

At the conclusion of the second study trial (Day 18), birds were weighed and then deeply anesthetized using metofane (methoxyflurane: Mallinckrodt, Mundelein, IL, USA) inhalation and euthanized by decapitation. Four birds were used for a separate study, and thus 12 treatment and 11 control birds were sacrificed for this study. Brains were removed from the skull (3-6 minutes post-capture) and placed into 5% acrolein solution in 0.1 M phosphate buffer (PB) overnight at 4 °C (King *et al.* 1983; Luquin *et al.* 2010). In addition, the left and right pectoralis muscles, liver, spleen

and gizzard were dissected out and weighed to the nearest 0.01 g on an electronic scale. Brains were post-fixed and gelatin-embedded following a modified protocol outlined in Saldanha *et al.* (1994) and chapter 3. Briefly, brains were rinsed three times with 0.1 M PB (30 min each), immersed in 4% gelatin solution for 30 minutes and embedded in an 8% gelatin solution-filled mold. Gelatin was allowed to solidify overnight at 4 °C and then embedded brains were post-fixed in 4% paraformaldehyde for 48 hrs and then immersed in 10%, 20% and then 30% sucrose solutions for 48 hours each. Brains were frozen on dry ice and stored at - 80 °C until sectioned.

Brains were coronally sectioned at 30 µm and every 3rd section (i.e., at least 60 µm apart) was collected into cryoprotectant solution (Watson *et al.* 1986). Free-floating sections were stained for AVT using an indirect immunohistochemistry procedure described in chapter 3. Sections were washed three times with 0.1 M PB for 30 min, incubated with 0.36% H₂O₂ for 15 min, washed three times with 0.1 M PB (5 min each), incubated with normal horse serum (1:30 in PBT: PB with 0.3% Sigma Triton X-100; Sigma-Aldrich Co., St. Louis, MO, USA) for one hour, and then incubated overnight in 0.3% PBT containing anti-AVT polyclonal antibody (1:15,000; raised in rabbit and generously provided by Dr. M.S. Grober (Georgia State University, Atlanta, GA, USA). The specificity of the AVT antibody has been previously validated in this species and is described in chapter 3. Sections were washed five times (10 min each) in 0.1 M PB, incubated for one hour in 1:100 biotinylated horse anti-rabbit IgG in 0.3% PBT (Vector Laboratories Inc. Burlingame, CA, USA), washed three times for 10 min each in 0.1 M PB, incubated in Vectastain ABC solution (Vector Laboratories Inc.) for one hour, washed three times (15 min each) in 0.1 PB, and then incubated for 3 min in Vector SG peroxidase chromagen (Vector Laboratories Inc.), and washed twice for 5 min in 0.1 PB. Immunolabelled sections were mounted onto gelatin-coated glass microscope slides, air dried at room temperature for 24 hours, dehydrated with ethanol, cleared in

xylene, and coverslipped using Cytoseal 60 (Stephens Scientific, Kalamazoo, MI, USA).

The production of AVT during stress occurs primarily within the paraventricular nucleus (PVN) of the hypothalamus, which is then released into the median eminence (ME), to eventually act on the pituitary gland. Patterns of AVT immunolabelling in this species have been previously described in chapter 3 and this information was used to locate the PVN and ME in this study. Images of the PVN and ME were digitized using a camera (Olympus DEI-750D, Olympus Optical Co. Ltd., Tokyo, Japan) attached to a light microscope (Olympus BX60) at 40× magnification and using constant microscope, camera, and computer settings. Images were analyzed blind with respect to treatment. Also for each brain section, an additional “out of focus” image was taken of an area devoid of immunolabelling to correct for background staining as described in chapter 3 using Image-Pro Plus version 4.0 (Media Cybernetics, Silver Springs, MD, USA). Briefly, all images were converted to black and white (Gray Scale 16 function) and flattened (Filter enhancement function) and then the “out of focus” image was “subtracted” from the image of interest (background correction function).

Four separate measurements were used to quantify differences in AVT immunolabelling in the PVN between treatments: 1) the *staining intensity* of AVT within a 100 µm diameter circular area of interest (AOI) centered over the PVN; 2) the *cell density*, or the number of AVT immunoreactive cells within the 100 µm AOI; 3) the *cell size*, or the cross-sectional area of a subset ($n = 7$ to 20 cells per section) of clearly delineated and non-overlapping AVT-ir cells; and 4) the *cell staining*, the optical density of the above individual cells. For each measurement, means from multiple sections including both left and right hemispheres (mean within hemisphere coefficient of variation = 15%; mean between hemisphere coefficient of variation = 8%) from each bird were used in analyses. To quantify the staining within the ME (*ME staining*), the optical density of ten circular AOIs (each 25

μm diameter) placed along the entire length of each representative image of the ME was determined and means were generated for each bird and used in further analyses. Further details on quantifying these variables can be found in chapter 3.

2.10. Statistical Analysis

To ensure that total food intake (summed throughout the duration of the study) did not differ between treatment and control groups for both trials of the study, two-sample Student t-tests were used. All data were tested for normality prior to analysis, and both H/L ratio and the proportion of time spent perching required arcsine square-root transformation prior to further analysis. Changes of body mass, plasma CORT, triglycerides, free glycerol, glucose, total protein, osmolality, and the H/L ratio in response to treatment was assessed using repeated measures analysis of variance (rmANOVA). The rmANOVA models included treatment groups (variable vs. constant) as the fixed factor, and study trial (1 vs. 2), bird origin (urban vs. desert), and body mass at the onset of the experiment as covariates. Post-hoc comparisons were assessed using Fisher's least-significant difference (LSD) tests.

Comparisons of organ and muscle masses, AVT immunolabelling (staining intensity, cell size, cell and ME staining), and behavioral data were made using 2-way analysis of variance (ANOVA) with treatment group, bird origin, and trial number as between-subject factors. Body mass was entered into the model as a random factor, but was subsequently removed if not significant at $p \leq 0.05$. Tail feather length was compared between treatment and control groups using two-sample Student t-tests. However, the number of fault bars and AVT cell density were ordinal data and thus were analyzed using non-parametric Mann-Whitney U tests across treatment groups. All data are presented as means \pm standard errors (s.e.) and the critical alpha level for all

tests was set at $p \leq 0.05$. Where multiple univariate comparisons were made, a Bonferroni-corrected alpha value of $p \leq 0.016$ was used.

3. Results

In both trials, birds in the variable group consumed an equivalent amount of food as the constant controls (trial 1: $t_{25} = 0.825$, $p = 0.371$; trial 2: $t_{25} = 0.506$, $p = 0.749$; pooled: $t_{52} = 0.676$, $p = 0.571$).

At the onset of the experiments, variable and constant birds did not differ in body mass or any plasma variable (Figure 33; all $p \geq 0.092$). However, body mass decreased significantly more in birds receiving variable amounts of food each day compared to those fed constant food amounts daily ($F_{2,25} = 5.110$, $p = 0.002$; Figure 33). Birds that received variable amounts of food each day also had elevated initial CORT levels, but was only significantly different from controls at the end (day 18) of the experiment ($F_{2,25} = 3.018$, $p = 0.046$; Figure 33). In contrast, treatment birds decreased in CORT levels in response to 30 min of capture and handling, which was evident on both days 9 and 18 ($F_{2,25} = 7.225$, $p \leq 0.001$; Figure 33). However, the H/L ratio did not change in response to feeding treatment ($F_{2,25} = 1.920$, $p = 0.067$; Figure 33); also, plasma osmolality did not change in response to feeding regime and was similar between treatment and control thrashers ($F_{2,25} = 0.483$, $p = 0.395$; Figure 33).

Treatment birds also had significantly lower and higher levels of plasma concentrations of triglycerides ($F_{2,25} = 2.099$, $p = 0.048$; Figure 33), and free glycerol ($F_{2,25} = 2.444$, $p = 0.037$; Figure 33), respectively. In both cases post-hoc tests revealed significant differences between treatment and control animals only on the last day of the experiment (Figure 33). By contrast plasma glucose levels did not change during the studies nor differ between variable and constant groups ($F_{2,25} = 0.099$, $p =$

0.612; Figure 33). Total plasma protein concentration declined in the variable treatment group and differed significantly from controls on day 18 ($F_{2,25} = 6.037, p = 0.008$; Figure 33). Body mass at the onset of the experiment did not affect any of the measure variables (all $p \geq 0.065$). The origin of the bird (urban vs. desert) did not significantly influence any of the above variables measured during the study (all $p \geq 0.071$). Responses in all measured variables were similar between experimental trials (all $p \geq 0.103$).

In response to the variable feeding regime, thrashers spent 81% less time perched without engaging in activity compared to control birds ($F_{1,26} = 5.141, p = 0.007$; Figure 34). They were also more active, as they hopped in their cages 32% more often than birds fed a constant amount of food ($F_{1,26} = 3.005, p = 0.026$; Figure 34).

All birds had completely regrown their plucked tail feathers by the time of resampling, and regrown feathers did not differ in length between treatment and control birds (constant: 12.8 ± 1.4 mm; variable: 13.1 ± 2.6 mm; $t_{25} = 1.025, p = 0.178$). However, feathers regrown by birds in the variable feeding group were in obviously worse condition, especially along their proximal ends. This is reflected in the 594% increase in the prevalence of fault bars in this group compared to controls (Mann-Whitney $U = 73.50, p = 0.018$; Figure 35).

Variable daily food amounts also induced a significant decrease in mass of the pectoralis muscles ($F_{2,21} = 4.179, p = 0.002$; Figure 36), with no differences in response to treatment between left and right muscles (both $p \geq 0.306$). In contrast, variable feeding did not alter the mass of the liver ($F_{2,21} = 0.561, p = 0.622$), gizzard ($F_{2,21} = 0.937, p = 0.307$), or spleen ($F_{2,21} = 0.490, p = 0.781$). None of the responses of the above variables to treatment were associated with body mass of the bird at the end of the study (all $p \geq 0.081$).

Variable and constant groups also differed in expression of AVT immunoreactivity. Birds in the variable group had 24% decreased staining intensity in the PVN ($F_{2,21} = 7.115, p \leq 0.001$; Figure 37), 16% smaller AVT cells in the PVN ($F_{2,21} = 2.662, p = 0.047$; Figure 37), and 97% higher ME staining ($F_{2,21} = 8.931, p \leq 0.001$; Figure 37) than birds in the constant group. However, the number (Mann-Whitney $U = 18.00, p = 0.603$) and staining ($F_{2,21} = 1.079, p = 0.062$) of AVT cells in the PVN did not differ between groups.

4. Discussion

An oft-cited factor influencing life-history traits is the temporal variation in food supply (Reznick & Yang 1993; Anderies et al. 2007; Mikolajewski et al. 2007), but few empirical studies have attempted to test the consequences and adaptations associated with food unpredictability in animals. I subjected captive Curve-billed Thrashers to either a constant (same amount of food each day) or variable (differing amounts of food daily) feeding regime for 18 days and measured a number of behavioral and physiological responses to test alternative predictions of the “adaptive regulation” and “chronic stress” hypotheses. I found that, compared to controls birds, variable food treatment resulted in mass loss, energy- reserve depletion (e.g. protein and lipids), increased activity, altered hypothalamic AVT immunoreactivity, and increased initial CORT secretion in thrashers, but a down-regulated CORT response to capture-and-handling. These observations are largely consistent with the predictions of the chronic stress hypothesis, providing only limited support for the adaptive regulation hypothesis

4.1. *Stress Physiology*

Previous empirical studies aimed at testing the effect of a variable food supply on body condition, each of them using different “variability” paradigms, have produced mixed results. Great

tits (*Parus major*) subjected to variable lengths of food deprivation increased in body mass to a greater extent than birds deprived of food for constant periods of time (Bednekoff & Krebs 1995). Studies manipulating the time available to feed have revealed increased asymmetry in primary feather growth in European Starlings, *Sturnus vulgaris* (Swaddle & Witter 1994), but no effect on either CORT or reproductive hormones in Western Scrub-Jays, *Aphelocoma californica* (Bridge *et al.* 2009).

Interruptions of feeding resulted in increases in body mass of great tits (MacLeod & Gosler 2006), but had no effect on body mass in zebra finches (*Taeniopygia guttata*; Dall & Witter 1998). Based on these studies, it is difficult to ascertain whether the perception of a food supply as being variable (i.e. unpredictable) constitutes a stressful environmental factor capable of imparting a selective pressure on an individual. The current energetic state of an individual may also influence how it responds to variation in food supply. For example, an individual with adequate energy reserves may be capable of maintaining its body mass, whereas another bird with less reserves may have to expend greater energy to forage, which in turn can further depreciate body mass. However, these studies were unable to separate metabolic effects of eating different amounts of food from perceptual effects, because they did not monitor actual food consumption. Thrashers from both treatment and control groups in my study consumed an equivalent amount of food by the end of each experimental trial. This was possible because birds consumed more food than their daily average when it became available (i.e. compensatory feeding).

Despite the similar energetic intake, thrashers given a variable food source showed increases in initial CORT and a suppressed CORT response to handling stress. Increases in baseline (i.e. initial) CORT can reflect chronic stimulation of the HPA axis (Sapolsky *et al.* 2000; Romero & Romero 2002), but some evidence suggests that chronic stress can attenuate HPA activity, thus decreasing CORT secretion (Rich & Romero 2005). Baseline (initial) and stress levels of CORT are

involved in different physiological functions, due to their interaction with different receptor types (Sapolsky *et al.* 2000). Higher initial CORT levels in treatment birds may enable the mobilization of intrinsic energy reserves, which is consistent with the overall decline in body mass observed in these birds. Changes in baseline CORT levels are regularly seen as organisms transition into energetically demanding activities like reproduction (Wingfield & Sapolsky 2003). The decrease in stress levels may be explained by the limits placed on adrenal production of CORT, in that high baseline CORT secretion limits the ability to increase CORT levels further during stress, and *vice versa*. This relationship has received little research attention and warrants further investigation. Another explanation may be that declining stress levels may result from a negative feedback effect imparted by the higher levels of initial CORT.

To further investigate how stress physiology reacted to unpredictable food sources, I also considered an upstream component of the HPA axis, the production and secretion of AVT from the hypothalamus. Previous research in thrashers identified the neural AVT system and its action on the pituitary glands as a source of variation in the HPA axis of thrashers from urban and desert areas (Chapters 2 and 3). Thrashers from these two habitat types show distinctive differences in CORT secretion, with urban birds having consistently lower initial levels and a higher CORT response (Fokidis *et al.* 2009), as well as increased ME staining intensity (Chapter 3), than desert birds.

Thrashers subjected to variable food treatment also had higher staining intensity at the median eminence. Although the interpretation of immunohistochemistry data can be troublesome (see Panzica *et al.* 2001; Goodson & Kabelik 2009), greater ME staining may indicate an increased release of AVT into the hypophyseal portal system (Chapter 3). This would be consistent with the higher initial CORT levels observed in variable treatment birds. Within the PVN, treatment birds had smaller AVT-producing cells and decreased AVT-staining in the PVN. The former may be

indicative of the recent “dumping” of AVT into the ME during capture-and-handling stress (Dawson & Goldsmith 1997; Clerens *et al.* 2003), which is consistent with greater ME staining. Based on this framework, these observations suggest that with the higher initial CORT levels of birds exposed to a variable food supply may be partly associated with changes in neural AVT. The secretion of CORT also impacts immune physiology, including the H/L ratio. Increased stress is thought to increase this ratio (Gross & Siegel 1983; Vleck *et al.* 2000), but in thrashers initial CORT is negatively associated with this ratio. Although H/L ratio did not significantly change during the study, the variable treatment tended to show a decrease compared to controls, which relates to my observations of increased initial CORT with treatment. The H/L ratio is an integrated measure of immune function, and immune activity can be further assessed by examining spleen size (Møller & Erritzøe 2003). However, I did not observed differences in spleen size in response to food variability. Whether variability in food abundance can impact the immune system is unclear.

4.2. Energy Balance

Thrashers exposed to a variable feeding regime decreased in body mass, plasma protein and triglyceride concentration, and mass of their pectoralis muscle. These results suggest that variable food access increases energy usage. This is further supported by the increased plasma concentrations of free glycerol, which may indicate triglyceride (i.e. lipid) breakdown (Guglielmo *et al.* 2002, 2005), and by the increased occurrence of fault bars during feather regrowth. The catabolic nature of CORT provides an explanation for these observations, since CORT encourages hepatic gluconeogenesis, which involves both lipid and protein breakdown to provide substrates for glucose production (Rahman & Clayton 1981; Warne *et al.* 2009). However glucose did not change during the course of the experiment, although free glycerol did increase with treatment. One interpretation

is that glucose levels are tightly regulated (Braun & Sweazea 2008) and thus gluconeogenesis was sufficient to just keep pace with metabolic usage of glucose, resulting in stable (i.e. non-increasing) levels.

Varying the daily amount of food had a large effect on the occurrence of fault bars in the tail feathers. Free-living thrashers often have fault bars on retrice feathers but not to the extent observed in this study. This may suggest that birds in the variable group were subjected to an unnatural feeding regime that may have induced a nutritional stress that is not observed in nature. Although this cannot be entirely ruled out, treatment birds did consume an equivalent amount of food as controls, and thus the occurrence of fault bars is unlikely due to nutritional stress *per se*. One explanation may be that birds in the treatment group also decreased their body mass, which may have limited energy allocated to feather regrowth. However, a study of food restricted white-crowned sparrows, *Zonotrichia leucophrys*, suggested nutritional stress is likely not the sole cause of fault bars (Murphy *et al.* 1989). Another explanation is that the increase in initial CORT levels with treatment may have interfered with feather regrowth. Several studies have shown that CORT administration can limit rates of feather growth (Romero *et al.* 2005) and feather integrity (DesRocher *et al.* 2009). An important point here is that length of the regrown feathers did not differ between treatment and controls, indicating equal rates of feather regrowth.

The majority of the decrease in body mass seen in the thrashers exposed to a variable food source is likely the result of muscle and not fat breakdown, since this species, unlike others routinely studied, likely have limited capacity for fat storage (Bednekoff & Krebs 1995; Witter & Swaddle 1997; Pravosudov *et al.* 2001; Polo & Bautista 2002). This is exemplified by two observations: 1) the decrease in mass of the pectoralis muscle, a predominant “power” muscle involved in flight (Driedzic *et al.* 1993); and 2) no treatment effect on liver mass, which is used as a measure of hepatic

lipid storage (Frayn *et al.* 2006). During flight, avian muscles often rely on fatty acid catabolism for energy (Maillet & Weber 2006), which can be further supplied by triglyceride breakdown. However thrashers in the variable food treatment also exhibited a decline in plasma protein concentration compared to controls. This protein metric is thought to largely reflect plasma albumin and globulin levels (Cray *et al.* 2008; Roman *et al.* 2009). High glucose levels in birds may interfere with refractometry readings (Harr 2002), but because my food treatment did not affect plasma glucose levels, the decrease is unlikely attributable to changes in any glucose fraction of a refractometry reading.

Previous research in starlings demonstrated that albumin can decrease in response to chronic stress (Awerman & Romero 2010). Among the functions of albumin is its maintenance of osmotic pressure (Harr 2002), but despite the decrease in plasma proteins I observed no change in osmolality with treatment. Decreases in plasma albumin are typically due to metabolic clearance (i.e. loss to urine) or severe blood loss, such as hemorrhage (Harr 2002). These are unlikely to be occurring in this study, since birds excrete very little protein (Harr 2002), and all birds remained in good health throughout the study. Previous studies have identified that nutritional stress can deplete circulating globulins (Lynn *et al.* 2003, 2010), which may account for the decrease in total protein observed in treatment birds. Understanding how avian blood proteins respond during stress and energy restriction is important to gain a clearer perspective on how energy reserves are utilized. Although the changes in plasma metabolites observed in this study suggest a direct effect of CORT on energy-reserve usage, both experimental feeding regimes resulted in an equivalent energy intake. The equal energy intake was likely due to compensatory feeding by birds in the treatment group. Despite this apparent difference in daily food intake, no differences in mass of the gizzard were observed. Many species exhibit changes in the size of the gizzard in response to seasonal changes in food type

(Walsberg & Thompson 1990; Piersma *et al.* 2004) and food intake rate (van Gils *et al.* 2006). The lack of change in size of the gizzard may simply reflect the massive energetic cost associated with restructuring this large abdominal organ (Piersma *et al.* 2004; van Gils *et al.* 2005) or perhaps the lack of sufficient food deprivation to induce such change.

An alternative explanation for the increased usage of energy reserves in response to variable feeding is an increase in energy expenditure. Thrashers exposed to a variable food source showed an increase in behavioral activity compared to controls. Here CORT may play an indirect role in mediating the usage of energy reserves by encouraging locomotory behavior. Several studies have identified that CORT promotes foraging behavior (Lynn *et al.* 2003; Lohmus *et al.* 2006; Angelier *et al.* 2007; Vaanholt *et al.* 2007), and in this captive study may have mediated the compensatory feeding and increased hopping rate of thrashers in the treatment group. Although many studies have examined how body mass responds to variability of a food source, few have considered this behavioral component, which is also a strong determinant of energy balance and hence body mass. Indeed, species that gain body mass with variable feeding may in part attain this by decreasing their activity and thus conserving energy.

4.3. Conclusion

I experimentally tested the adaptive regulation and chronic stress hypotheses for explaining songbird hormonal, metabolic and behavioral responses to a variable (i.e. unpredictable) food source. I largely found support for the latter hypothesis by showing an increased usage of intrinsic energy reserves and changes in stress physiology. However, instead of a direct effect of stress on energy reserves (since food consumption was controlled for), I saw an indirect effect mediated by increased energy expenditure. Many studies have considered adaptive regulation as a mechanism for

persisting in unpredictable habitats that can vary in their abundance and accessibility to food resources in space or time. In the Sonoran Desert excessively hot and dry conditions minimize plant productivity, which in turn impacts herbivorous insect abundance (Faeth *et al.* 2005). Precipitation patterns especially during the summer monsoon period can revive plant and animal communities, but can be very unpredictable in timing and extent (Faeth *et al.* 2005). Inclement weather can induce cold snaps that further decrease insect availability. Thus Curve-billed Thrashers, which are lean birds with little fat reserves, may be susceptible to short-term changes in the abundance of insects. This research demonstrates that behavioral variables, which are rarely considered in studies on this topic, may drive energy use patterns in response to variable food supplies. It also provides evidence that differences in the predictability of a food source between habitat types, across seasons, or even in response to landscape change, may be sufficient to have a negative physiological impact, even without altering the amount of food available. Studies that aim to investigate the role of food availability in establishing ecological patterns in free-living species should account for this temporal component as well.

5. References

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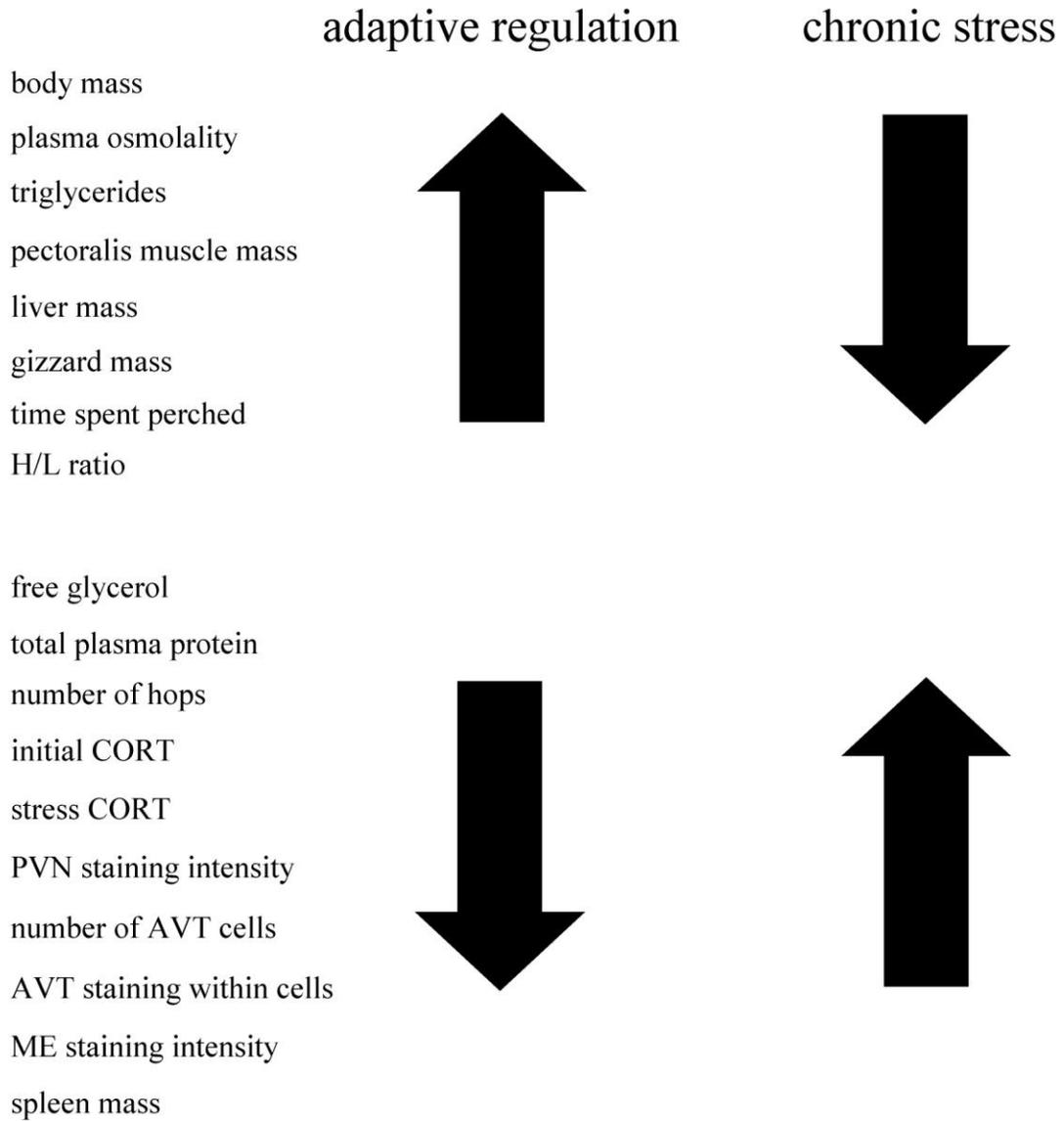


Figure 31. Predictions concerning how physiological and behavioral measurements in captive Curve-billed Thrashers, *Toxostoma curvirostre*, will respond to a feeding source varies in the amount of food available each day.

constant (control)

100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	--

variable (treatment)

100	30	60	200	180	70	60	100	180	90	130	40	130	70	90	70	100	
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days

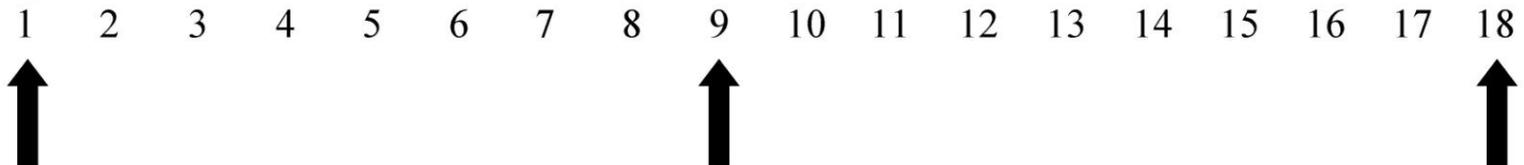


Figure 32. Schematic representation of constant (control) and variable (treatment) feeding regimes over the course of 18 days. Numbers within boxes indicate the amount of food provided as a percentage of each individual bird's daily food intake. Arrows indicate days when birds were weighed and blood was collected.

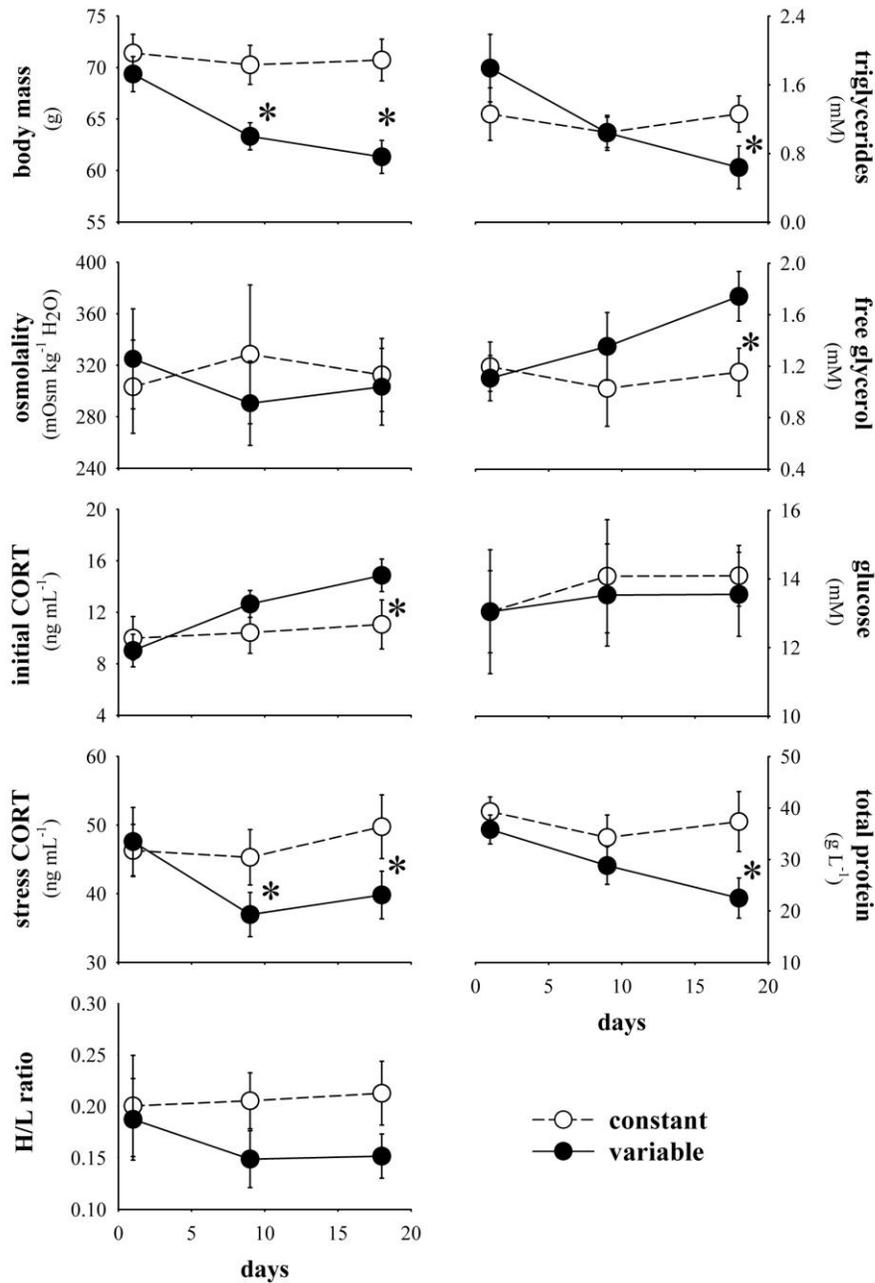


Figure 33. Changes in body mass, plasma corticosterone (CORT), heterophil to lymphocyte (H/L) ratio, and plasma metabolites in Curve-billed Thrashers, *Toxostoma curvirostre* ($n = 27$), in response to either a constant or variable feeding treatments. * indicates a significant difference between treatment and control groups at $p \leq 0.05$.

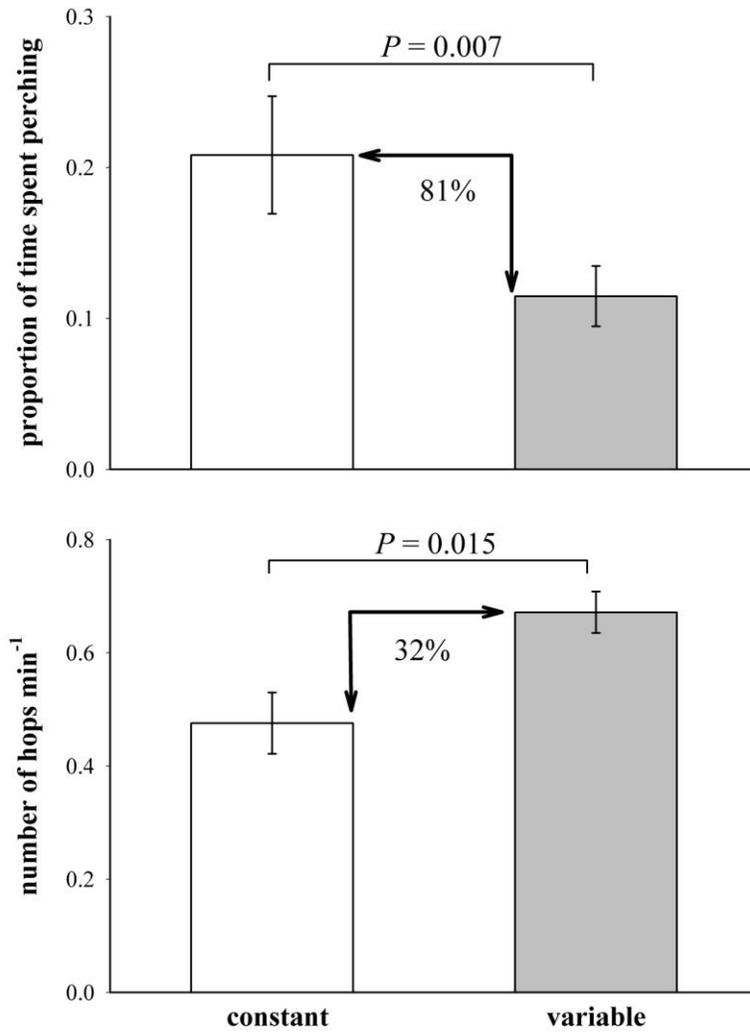


Figure 34. Percentage differences in two measures of behavioral activity between Curve-billed Thrashers, *Toxostoma curvirostre* ($n = 27$), in response to constant or variable feeding treatment. Critical value is set at $p \leq 0.05$.

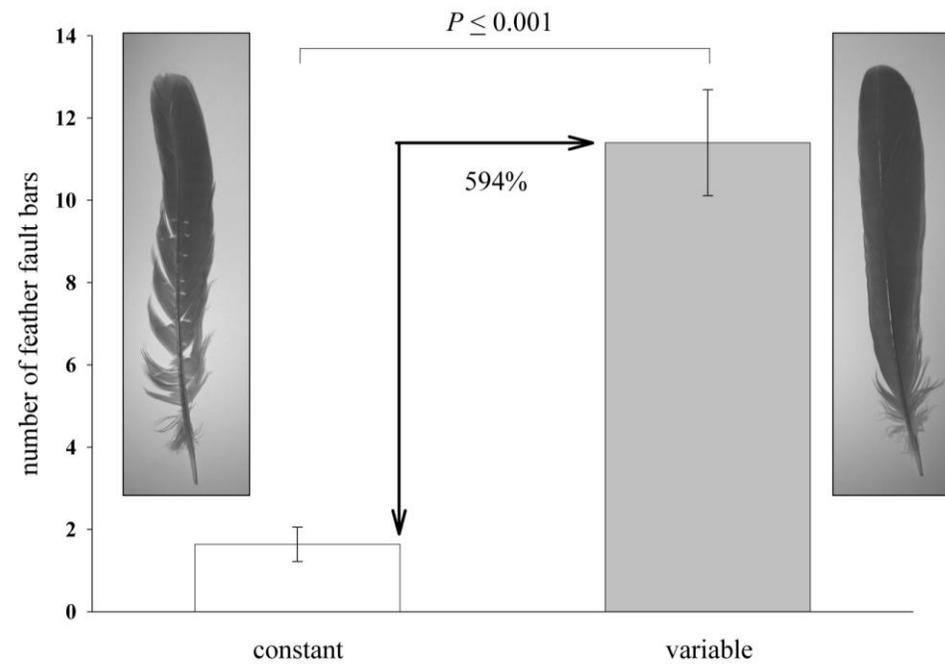


Figure 35. Differences in the presence of fault bars in re-grown tail feathers between Curve-billed Thrashers, *Toxostoma curvirostre* ($n = 27$), in response to constant or variable feeding treatments. Representative photos indicate variation in feather integrity with treatment. Critical value is set at $p \leq 0.05$.

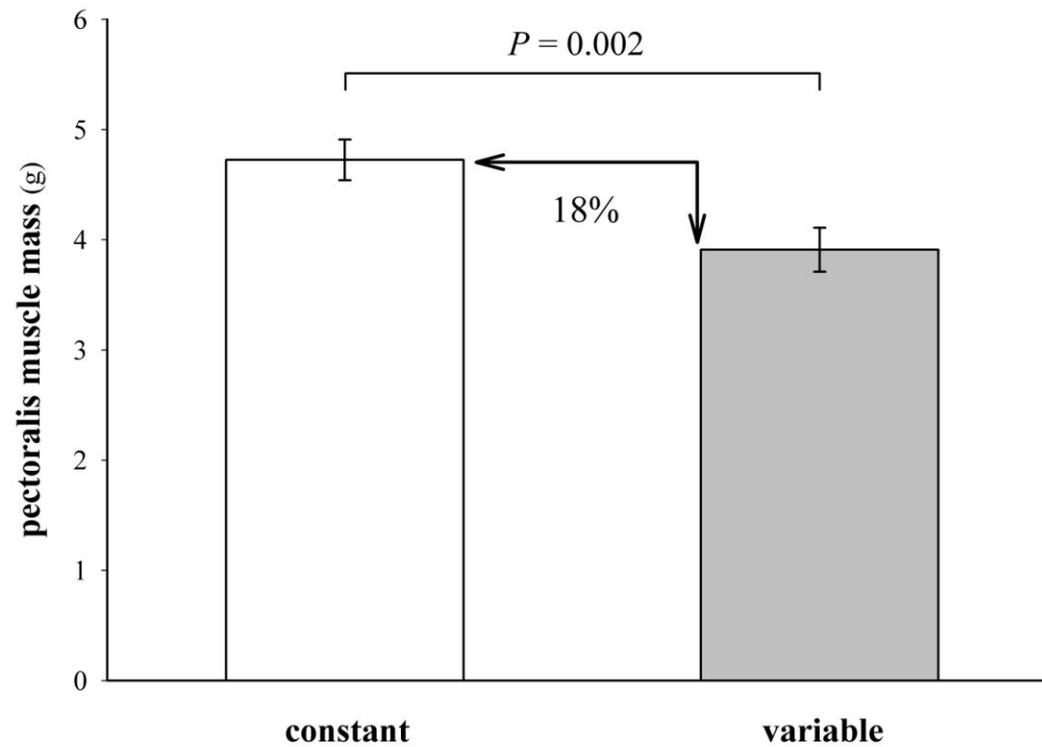


Figure 36. Differences in mass of the pectoralis muscle between Curve-billed Thrashers, *Toxostoma curvirostre*, fed a constant ($n = 13$) or variable ($n = 14$) amount of daily food. Critical value is set at $p \leq 0.05$.

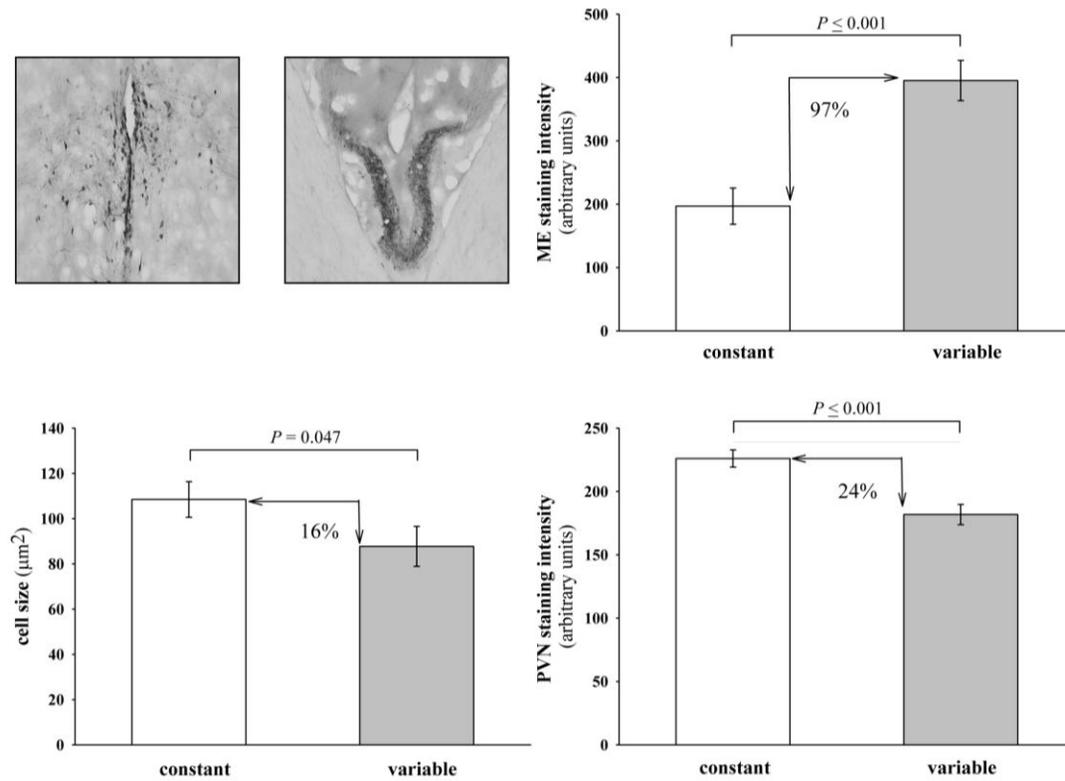


Figure 37. Differences in immunoreactivity of arginine vasotocin (AVT) within the paraventricular nucleus (PVN: *left photo*) and the median eminence (ME: *right photo*) of Curve-billed Thrashers, *Toxostoma curvirostre*, fed a constant ($n = 13$) or variable ($n = 14$) amount of daily food. For detailed description of AVT measurements see methods. Critical value is set at $p \leq 0.05$.

CONTEXT-SPECIFIC TERRITORIAL BEHAVIOR IN URBAN BIRDS: NO EVIDENCE FOR INVOLVEMENT OF TESTOSTERONE OR CORTICOSTERONE

Testosterone produced by the gonads is a primary mediator of seasonal patterns of territoriality and may directly facilitate territorial behavior during an encounter with a potential intruder. Costs and benefits associated with territoriality can vary as a function of habitat, for example through differences in resource distribution between areas occupied by different individuals. I investigated behaviors in response to simulated territorial intrusions (*hereafter* territorial behaviors) in urban (Phoenix, Arizona) and nearby desert populations of two Sonoran Desert birds (Curve-billed Thrasher and Abert's Towhee). I also examined the degree to which these behaviors are mediated by testosterone (T) and the adrenal steroid, corticosterone (CORT), which can interact with T in territorial contexts. In both species, urban birds displayed more territorial behaviors than their desert conspecifics, but this difference was not associated with variation in either plasma total or free (i.e., unbound to binding globulins) T or CORT. In addition, neither plasma T nor CORT changed as a function of duration of the simulated territorial intrusion. Urban Abert's Towhees displayed more territorial behaviors in areas where their population densities were high than in areas of low population densities. Urban Curve-billed Thrashers displayed more territorial behaviors in areas with a high proportion of desert-type vegetation, particularly in areas that differed in vegetation composition from nearby randomly sampled areas, than in areas with a high proportion of exotic or non-desert type vegetation. Associations between territorial behavior and habitat characteristics were not related to plasma T or CORT. Understanding the mechanistic basis of such associations may provide insight into how free-ranging animals assess territorial quality and alter their defensive behavior accordingly.

1. Introduction

In many bird species, the onset of breeding is marked by the male establishment of territories and its subsequent defense from conspecific intrusion (Beletsky & Orians 1987a,b; Wingfield 1984). The advantage that birds gain from defending a territory presumably is species-specific (Maher & Lott 1995) and may include nest protection (Burger & Beer 1975; Larsen *et al.* 1996), mate-guarding (Moore 1984; Bodily & Neudorf 2004; Komdeur *et al.* 2007), or monopolization of spatially limited resources (Sasvari 1991; Gorrell *et al.* 2005). Available resources (e.g., food and nest sites) may vary between habitats and may in turn influence the necessity to engage in territorial behaviors. In environments where resources are clumped, such as remnant woodlands or natural habitats in urban areas, selection may favor increased territorial behavior and vigilance during agonistic encounters as compared to the situation where resources are uniformly distributed. This may be the case particularly during the energetically costly reproductive period (reviewed in Harshman & Zera 2006).

The conversion of native to urbanized landscapes can result in a mosaic of habitat types that differ in their suitability for use by native birds (Brennan & Schnell 2005; Turner 2006; Hodgson *et al.* 2007; Palomino & Carrascal 2007). This habitat “patchwork” can alter territorial behavior that is driven by the need for resource defense. Studies on the impacts of urbanization found increased population densities in urban compared to adjacent wild lands (Green & Baker 2003; Chace & Walsh 2006; McKinney 2006; Van Turnhout *et al.* 2007). Increased population densities may be associated with a parallel increase in vigilance and territorial behavior resulting from more frequent intrusion by conspecifics (Sasvari 1991). In addition, predation pressure is often lower in urban than native environments due either to decreased predator numbers or to lower predator hunting success in urban areas (reviewed in Chace & Walsh 2006; Anderies *et al.* 2007). This difference may reflect a

decreased fitness cost of engaging in territorial behavior, which may involve making an individual more conspicuous to ward off territorial intruders.

Antagonistic encounters between males involve the expression of behaviors that are often regulated by testosterone (T), particularly during reproductive periods (Farner & Wingfield 1980; Wingfield 1984; Wingfield *et al.* 1987; Jawor 2007). Furthermore, persistent or frequent territorial intrusion can elevate plasma T, which is thought to, in turn, facilitate the persistence of territorial behavior (“challenge hypothesis”: Wingfield *et al.* 1990; Wikelski *et al.* 1999; Ramos-Fernandez *et al.* 2000; Ros *et al.* 2002; Goymann *et al.* 2007; Goymann 2009). In many monogamous species, plasma T is elevated in males during territory establishment and decreases with progression towards nestling and fledging stages (Wingfield 1984; Wingfield *et al.* 1990; Van Duyse *et al.* 2003). In contrast, in species exhibiting frequent extra-pair copulations, plasma T often remains elevated throughout the breeding season (Wingfield 1984; Wingfield *et al.* 1990).

Many species, including those inhabiting hot deserts and tropical regions, show year-round territorial behavior. In these species, territoriality outside the breeding season may be independent of circulating T (Hau *et al.* 2000; 2004; Hau 2001; Moore *et al.* 2004). Several hypotheses have been proposed to explain this seasonal uncoupling of territorial behavior and plasma T (Hau *et al.* 2004; Wacker *et al.* 2008). One hypothesis is that fluctuations in secretion of glucocorticoids, such as corticosterone (CORT), in response to the stress of territorial intrusion influence T levels, with CORT generally suppressing T secretion (reviewed in Wingfield & Sapolsky 2003) by acting directly on the hypothalamus, pituitary gland, and/or gonads (Dobson & Smith 2000; Wingfield & Sapolsky 2003). In avian plasma, most T and CORT are thought to bind competitively to "corticosterone binding globulins" (CBG), resulting in only a small fraction (free hormone) of these hormones circulating unbound (Deviche *et al.* 2001; Breuner *et al.* 2006). According to the “free hormone”

hypothesis, only unbound steroids can diffuse into cells and influence intracellular receptors or be cleared from circulation (Rosner *et al.* 1991). An increase in plasma CORT presumably displaces a portion of globulin-bound T and as a result, increases plasma free T even though T secretion may not be affected (Deviche *et al.* 2001). Although increased CORT occupation of CBG may elevate free T levels during territorial intrusion in the short-term, this greater circulating free T may also induce a negative feedback effect of T on the hypothalamus and pituitary gland, resulting in decreased overall T secretion in the long-term. Thus increased CORT occupation can conceivably increase or decrease overall T in circulation depending on time-course, however these hypotheses remain untested. Indeed, few studies have focused on how the interaction of T and CORT with CBG can influence territorial behavior. However, no differences in total or free plasma T, CORT or CBG was observed in male Mountain White-crowned Sparrows (*Zonotrichia leucophrys oriantha*) exposed to simulated territorial intrusion relative to passively captured controls (Lynn *et al.* 2007). Similarly, in Blue Tits (*Cyanistes caeruleus*) increases in occupation of CBG by CORT during challenge, was not accompanied by significant increases in free T, thus suggesting this mechanism is not responsible for changes in circulating T levels (Landys *et al.* 2007). However there is still considerable debate concerning the interrelationships of CBG, CORT and T in free-living birds and particularly in the context of behavioral regulation.

I investigated differences in territorial behavior in two Sonoran Desert bird species, the Curve-billed Thrasher (*Toxostoma curvirostre*) and the Abert's Towhee (*Pipilio aberti*), inhabiting the city of Phoenix, Arizona and adjacent native habitat. These species are sedentary and exhibit year-round territorial behavior towards conspecific intrusion (Finch 1984; Vleck 1984; Fokidis *et al.* 2008) unlike both the Mountain White-crowned Sparrows and Blue Tits. The present study tested the hypothesis that urban birds are more responsive to simulated territorial intrusions (i.e., displayed more territorial

behaviors) than desert birds and that this difference in responsiveness is mediated by interactions between plasma T, CORT, and CBG. I also hypothesized that urban-desert differences in behavior are related to the need to defend spatially clumped resources, particularly native vegetation that these birds use as nest sites, which can be limited in an urban environment (Dunford & Freemark 2005).

Urban thrashers and towhees show decreased seasonal variation in body condition than their desert counterparts suggesting urban habitats may provide stable food resources for individuals capable of maintaining territories in the city (Fokidis *et al.* 2008). In this study I determined whether: 1) urban birds displayed more territorial behaviors than desert conspecifics; 2) territorial behaviors were related to plasma total or free T and CORT; 3) plasma T or CORT increases in response to duration of simulated territorial intrusion (i.e. song playback); and 4) territorial behaviors were related to differences in population density or suitable nesting sites. I predicted that compared to desert birds, urban birds would display more territorial behaviors during a territorial intrusion and would have higher total T levels (i.e. more T secretion) and/or higher levels of free T (i.e. increased CORT occupancy of CBG). I also predicted that the levels of free or total T would increase more in urban than desert birds in response to prolonged territorial intrusion, particularly in areas of high density or more vegetation for nesting than in areas of low bird density or low nesting site availability.

2. Materials and Methods

2.1. Study Sites And Populations

The study was conducted at three desert and three urban sites. Desert sites are largely unpopulated but differ with regard to their proximity to urban housing developments. The Sierra Estrella Wilderness Area (14,400 acres) is located at the base of the Sierra Estrella Mountain Range

about 26 km southwest of Phoenix. The McDowell Mountain Sonoran Preserve and Regional Park (940 acres and 21,099 acres, respectively) is located at the northern periphery of Phoenix, 2 km from the fringe suburban developments of Troon, Fountain Hills, Ancala, and Pinnacle Peaks. The vegetation at both desert sites is typical of upland Sonoran Desert and includes columnar cacti, cholla (*Opuntia* spp.), ocotillo (*Fouquieria splendens*), mesquite (*Prosopis* spp.), and palo verde (*Prosopis* spp.). A third desert site was the Robbin's Butte Wildlife area (1,511 acres) located 48 km west of Phoenix. This area borders the Gila River and vegetation primarily consists of mesquite, saltbush (*Atriplex* spp.), creosote (*Larrea tridentata*), bursage (*Ambrosia* spp.), and willows (*Salix* spp.). This area also contains dense thickets of invasive salt cedar (*Tamarix* spp.) and retired agricultural lands. Urban sites included high- and low-income residential housing tracts, commercial areas, business districts, and city parks of varying sizes in Phoenix, Scottsdale, and Tempe. Most urban sampling occurred in three areas: South-Central Phoenix, East Phoenix and Tempe, and North Scottsdale and North Phoenix. Each site was sampled weekly between 15 March to 2 June 2007, which coincides with the early (i.e., nest-building and territory establishment) and mid-reproductive periods (i.e., incubation and nestling stages) of the two study species. Birds were caught between 0500 and 1100 h.

2.2. Simulated Territorial Intrusions And Bird Sampling

To simulate territorial intrusions, I used recordings of songs and calls of thrashers and towhees compiled from independent recordings of several individual birds from sites in and around Phoenix, Arizona. Studies involving simulated territorial intrusions often couple song playback recordings from a speaker with the presence of either a stuffed or live conspecific decoy. Although this technique provides a more natural simulation of a territorial intrusion, logistic and permitting issues prevented my use of live decoys or my obtaining new taxidermy specimens. However use of a

taxidermy specimen of an Abert's Towhee did not reveal significant differences in territorial behavior between the presence of a stuffed decoy coupled to a speaker and a speaker alone in this species (all $p > 0.26$; $n = 9$). In addition, responses by individuals by both species tended to be rapid and focused on the area around the speaker. Thus, I felt the presence of the speaker alone was sufficient to elicit representative territorial responses in these two bird species, and this method has been successfully used in other passerine species including: Spotted Antbirds (*Hylophylax naevioides*: Wikelski *et al.* 1999), and Great Tits (*Parus major*: Franco & Slabbekoorn 2009). Recordings were played on a Sony MD Walkman minidisk player through a handheld speaker at constant volume (74 dB at 2 m). Birds in urban areas were sampled during times of minimal human disturbance (early mornings and weekends). All males were exposed to the same compilation of songs and calls for their respective species. Upon locating a male bird visually without use of song playback, a mist net was erected and the bird was exposed to the recording with the observer (HBF) located 40-80 m away and having an unobstructed view of the mist net and surrounding area. Additional birds (4 thrashers and 3 towhees) were captured by passive netting (no playback) near known nest sites.

The observer, dressed in camouflage and remaining quiet and still, recorded the occurrence of several stereotyped behaviors associated with responses to playback: Latency to first approach to playback speaker; number of approaches from the air and ground; number of times the bird flew over the net; number of times the bird sang and/or called; number of calls made in direct response to calls from the speaker (match calls); number of times the bird wiped its beak on vegetation; number of times the bird raised its wings (wing flash) and/or spread its tail feathers (tail fan); and number of times the bird was observed feeding on the ground. In addition to these behaviors, for the towhees I also recorded the number of times a squeal duet was heard. This vocalization is produced by birds of a mated pair (Tweit & Finch 1994) and was frequently heard in response to playback.

Birds under study were naïve and, in some cases, were lost sight of during experimental trials. The period during which a focal bird was not in view was recorded, thus enabling us to correct behavioral observations as a function of the actual time the bird was observed (*hereafter* number of behaviors per minute observed). The behavioral response of two birds seen at the same time was also noted, as was the presence of another individual, which likely indicated a mated pair, since one individual would sing in response to playback (presumably the male), but would be closely accompanied by a non-singing adult bird (likely the female). In such cases, male birds that were captured were identified either via observations of singing behavior (as in thrashers) or by the persistent presence of the bird around the net until it was captured and identified by hand. All birds were observed displaying these behaviors just before capture, thus allowing us to identify them in hand.

During preliminary research, Brown-headed Cowbirds (*Molothrus ater*) were observed during 20-30 % of the playback trials in both urban and desert settings. Brown-headed Cowbirds are brood parasites and are known to use Abert's Towhees (Finch 1983) and thrasher species as hosts (Finch 1982; Haas & Haas 1998). Their presence was noted as it may alter the behavior of nesting parents wanting to deflect attention from a nest site. The amount of time that a bird was exposed to the playback recording before capture was also recorded. Behavioral data for birds that I was unable to capture ($n = 4$ towhees, 5 thrashers) were used to supplement the behavioral data.

Upon capture, birds were removed from the net within 3 min and about 200 μ l of blood was collected from the right jugular vein using a heparinized 0.3 cc syringe with a 29.5 gauge needle for measuring baseline plasma CORT and T. An acute stress response was then induced by holding the bird in a cloth bag for 30 minutes as previously described (Wingfield *et al.* 1994a,b; chapters 1,2,4). Thirty min later, a second 200 μ l blood sample was collected to measure stress-associated changes in

plasma CORT and T. Blood samples were kept on ice until plasma was separated by centrifugation and then stored at -80°C until the assays were run. Adult males were identified by the presence of a developed cloacal protuberance (CP). Body mass ($\pm 0.1\text{ g}$), tarsus and culmen length ($\pm 1\text{ mm}$), and wing chord ($\pm 1\text{ mm}$) were also measured. All birds received a uniquely numbered aluminum US Geological Survey leg band and released at the site of capture. All procedures followed guidelines approved by the Arizona State University Institutional Animal Care and Use Committee and were done under scientific collecting permits from the US Fish and Wildlife Service and Arizona Game and Fish Department.

2.3. CORT And T Assays

Total plasma T and CORT concentrations were measured using validated commercial competitive enzyme-linked immunoassays (ELISA; Assay Designs Inc. Ann Arbor, Michigan, USA) as described by Fokidis *et al.* (2009). Additional validations included the successful recovery of known T and CORT amounts added to samples ($n = 3$ for each steroid) before assay (96 % and 93 % recovery, respectively). Overnight incubation of plasma with dextran-coated charcoal resulted in undetectable plasma T or CORT. Plasma samples from the two study species were assayed independently. The sensitivity of the CORT assay ranged from 7.3 - 18.1 pg/ml depending on the plate and the mean intra-assay coefficient of variation was 9.19 % ($n = 4$ plates; 156 samples assayed in duplicate). The sensitivity of the T assay ranged from 4.93 – 9.65 pg/ml depending on the plate and the mean intra-assay coefficient of variation was 7.14 % ($n = 4$ plates; 156 samples in duplicate).

2.4. CBG Assay And Estimation Of Free Hormone Concentration

Radioligand binding assays to determine plasma CBG binding capacity were performed

using $^3\text{H-CORT}$ according to Breuner and Orchinik (2001) and Orchinik *et al.* (2000) with minor modifications specified in Fokidis *et al.* (2009). To determine CBG binding capacity (B_{\max}) I incubated samples with a saturating concentration of $^3\text{H-CORT}$, which was equivalent to 9 times the equilibrium dissociation constant (K_d) for each species. The K_d values for CORT binding by CBG in the two study species have been previously described (Abert's Towhee: $K_d = 5.97 \pm 0.89$ nM; Curve-billed Thrasher: $K_d = 6.13 \pm 0.78$ nM; Fokidis *et al.* 2009). I also used previously calculated inhibition constants for T (K_i ; Fokidis *et al.* 2009), determined from IC_{50} values using the Cheng-Prusoff equation (Cheng & Prusoff 1973). The K_i values for T in the Abert's Towhee and Curve-billed Thrasher were 22.88 ± 1.07 nM and 15.97 ± 1.05 nM, respectively (Fokidis *et al.* 2009). The B_{\max} and K_i values were used to estimate plasma concentrations of free and CBG-bound CORT and T for each sample using the equation of Barsano and Baumann (1989). For this, I assumed that T and CORT bind to CBG competitively and first estimated the amount of plasma CBG occupied by empirically determined basal and stress-induced CORT concentrations. I then estimated how much T binds to the remaining (free or unsaturated) portion of CBG, thus providing an estimate of both free and bound T concentrations (see Deviche *et al.* 2001 for details).

2.5. Estimating Population Density And Nest Site Availability

To estimate population densities, I surveyed birds along 800 m transects where the midpoint was located at the site of a captured bird. Transects in urban areas were oriented east-west to coincide with main roads and alleyways. At one desert site (Sierra Estrella wilderness area), transects were oriented in a north-south direction to avoid sampling steep mountain slopes with different plant communities. Transects were surveyed twice, once in September 2007 and once in October 2007, and only birds found within 50 m of either side of a transect were counted. I was interested

only in sampling densities for the species captured at the midpoint site, and thus the survey was conducted by playing species-specific songs every 50 m along transects and tallying the number of individuals that sang in response to playback (indicating an adult male bird). When two or more individuals appeared to respond together (likely a breeding pair and fledglings) they were counted as one individual. The survey was conducted in Fall for logistical reasons. As adult Curve-billed Thrashers and Abert's Towhees are sedentary and philopatric (Tweit & Finch 1994; Tweit 1996), I assumed that estimated Fall densities of both species were similar to densities during the breeding season earlier in the year.

Assessing nest site availability involved conducting vegetation surveys where the site of capture represented the mid-point of north-south and east-west oriented transects that were 200 m in length. This length was chosen as representative of the vegetation present in the average home range of a Curve-billed Thrasher or Abert's Towhee (Tweit & Finch 1994; Tweit 1996). I compared vegetation data collected from the native desert habitat with those from urban sites using a protocol modified from Daniels and Kirkpatrick (2006). Specifically, I recorded the number of tree types, cacti, succulents, and artificial perching structures (e.g., electric wires, roof edges, TV antennas, fences, and electric poles) as well as the height of ground-covering vegetation, and the number of natural shrubs and hedges, the latter being defined as shrubs that have been actively pruned (Table 9). For each vegetation category, I recorded the number of each plant type with thorns. I only recorded vegetation within 10 m of the transect on either side. At urban sites this method only measured vegetation located in the front yards of residential homes. Urban mid-points were for the most part located near street intersections. I also assessed vegetation structure at sites where birds were captured and relative to the surrounding landscape. To do this I conducted transect surveys at three randomly selected points 1 km from each site of capture (*hereafter* random sites). In urban

areas, these points were also selected to coincide with street intersections and were conducted in a north-south and east-west direction.

2.6. Statistical Analyses

Behavioral variables were corrected for the amount of time a bird was observed by determining the number of behaviors performed per minute that the bird was directly observed (see above). These time-corrected data were then reduced using principal components analysis (PCA) to generate a single score, with mean standardized to zero, which represented territorial responses to playback. PCA analyses included all birds exposed to playback, captured and not captured (thrashers: $n = 44$, towhees: $n = 25$). I assume that these individuals were males because in both species, males displayed more territorial behaviors in response to playback than females and the latter rarely respond alone (Fokidis *pers. obs.*). Most behavioral variables associated with territorial displays (e.g., approaches towards speaker) loaded on the first component (PC1) in a unidirectional manner (Table 10) and thus the larger the score's value, the more responsive the individual was to playback. Other non-territorial behaviors (e.g., feeding, bill-wiping) loaded primarily on the second component (PC2), but directionality differed between variables (Table 10). This statistical technique has been used in similar studies to reduce behavioral variables to single score values representing most of the variation in behavior (see Landys *et al.* 2010). However univariate approaches were also used to compare the most common behaviors between urban and desert birds to determine whether these specific behaviors were different between habitats.

I used two-tailed Student's t-tests to compare latencies to first response (i.e. approaches to the speaker) and individual component behaviors between urban and desert birds, using data from all the birds for which these data were available. Student's t-tests were also used to compare PC

scores between birds that were captured and those that were not captured to ensure that my study sample was not biased towards more territorial individuals. Further analyses focused only on captured birds. I used Student's paired t-tests to compare plasma hormone levels in samples collected 3 vs. 30 min after capture. Student's t-tests were used to compare hormone levels of passively captured birds with those of birds captured using song playback. Differences in behaviors were then assessed using analysis of covariance (ANCOVA) with PC1 and PC2 as the dependent variables, site (urban or desert) as the fixed independent variable, and baseline total T and CORT, capture date, time (minutes after sunrise), cowbird presence, and body mass as covariates. This analysis was repeated, substituting baseline free plasma T and CORT for total plasma hormone concentrations. Separate analyses were necessary because these variables were correlated and not doing so would violate statistical assumptions of data independence. To assess differences in hormone concentrations between urban and desert birds, I used multivariate analysis of covariance (MANCOVA), with plasma hormone concentrations as the dependent variables, site as fixed factor, and date, time of day, playback duration, wing chord length and body mass as covariates. I used linear regression to determine whether duration of playback exposure elevated plasma T or CORT.

Regression analysis was used to determine if mean population density was associated with territoriality. To identify the habitat variables that significantly explained variation ($p \leq 0.05$ – see below) in territorial behavior, I used canonical correspondence analysis (CCA; XLSTAT 4.03 software) with a stepwise forward selection model and a Monte Carlo permutation test (500 random permutations; ter Braak & Schaffers 2004). An analysis was performed separately for both urban and desert sites and for each species, and was repeated with the inclusion of capture sites as well as random sites to generate scores for habitat structure for each site. This approach was used to assess habitat similarity between capture and random sites by calculating the Euclidean distance between

sites. The mean from these comparisons for each site provided a general index of similarity in vegetation structure, with a smaller value indicating greater similarity between sites. I eliminated sites, such as parking lots, where vegetation was completely lacking ($n = 3$). These “zero” data points did not, therefore, contribute to the overall similarity index. Relationships between habitat variables and similarities with individual behaviors (see above) were assessed using Pearson correlation coefficients.

Where necessary, data were tested for the presence of outliers using Chauvenet’s criterion outlier test (at $p < 0.05$) which identifies data points beyond two standard deviations from the mean, which were then excluded from subsequent analysis. All data are presented as means + standard errors (s.e.) and the critical alpha level was set at $p \leq 0.05$. For the sake of brevity only the p -values are presented for results that were not statistically significant.

3. Results

3.1. Behavioral Differences Between Urban And Desert Birds

There was no difference in sampling between urban and desert birds in terms of capture date (thrashers: $p = 0.672$; towhees: $p = 0.490$) or time of capture (thrashers: $p = 0.781$; towhees: $p = 0.901$). There were also no significant differences in the proportion of time that birds were observed before being captured between sites (Table 11). Urban birds responded to song playbacks more quickly (thrashers: $t_{38} = 2.007$, $p = 0.032$; towhees: $t_{21} = 3.501$, $p = 0.019$; Figure 38); with a greater number of approaches to the speaker, and by making more calls (see statistics in Table 11) than desert conspecifics. Confirming this difference, urban birds displayed more territorial behaviors (i.e., greater PC1 scores) than desert conspecifics (thrashers: $F_{1,38} = 8.510$, $p = 0.007$; towhees: $F_{1,21} = 13.177$, $p = 0.003$; Figure 39). In contrast PC2 scores, consisting of behaviors not usually associated

with territoriality (see Table 10), did not differ between urban and desert birds of either species (both $p \geq 0.083$; Figure 39).

Thrasher behavioral scores were not influenced by date ($p \geq 0.081$), time ($p \geq 0.357$) or the presence of cowbirds ($p \geq 0.230$). In towhees, territorial behavior (PC1) was not influenced by date ($p = 0.148$), time after sunrise ($p = 0.380$), or cowbird presence ($p = 0.412$). However, non-territorial behavior (PC2) in this species was influenced by date ($F_{1,21} = 3.547, p = 0.034$), but not time after sunrise ($p = 0.147$) or presence of cowbirds ($p = 0.110$). Body mass and wing chord was not associated with behavior in either thrashers (both $p \geq 0.401$) or towhees (both $p \geq 0.104$).

3.2. Is Territorial Behavior Related To Plasma T Or CORT?

3.2.1 Thrashers

Urban and desert thrashers did not differ with respect to their plasma total baseline T or CORT concentrations (Table 12). The CBG binding capacity did not differ between urban and desert birds. Additionally, neither baseline total nor free plasma levels of either hormone were associated with capture date, suggesting that these levels did not fluctuate during the thrasher reproductive period sampled (all $p \geq 0.681$). Estimated free CORT concentrations were higher in urban birds than desert birds, but free T concentrations were similar (Table 12, Figure 40). I estimated that, at baseline levels, $98.1 \pm 0.1\%$ of CORT and $94.9 \pm 0.2\%$ of T were bound to CBG. Birds caught without or with the assistance of song playback had comparable baseline levels of T and CORT ($p \geq 0.992$), but firm conclusions on this topic are limited by the small number of birds captured (urban: $n = 2$; desert: $n = 2$). Total and free baseline T or CORT were not associated either with territorial or with non-territorial behavior (PC1 or PC2, respectively (all $p \geq 0.216$).

Plasma total CORT and free CORT ($t = 2.833, p = 0.036$) increased in response to 30 minutes of capture and restraint ($t = 7.408, p < 0.0001$ and $t = 2.833, p = 0.036$, respectively). After 30 minutes of capture and restraint, plasma total CORT was higher in urban than desert birds; however there was no difference in plasma total T between sites (Table 12, Figure 40). I estimated that, after 30 min of restraint, $96.2 \pm 0.9 \%$ and $91.6 \pm 1.9 \%$ of CORT and T were bound to CBG, respectively. Plasma total T decreased after 30 min of capture and restraint ($t = -2.264, p = 0.029$) but plasma free T did not change significantly ($p = 0.957$). Urban and desert thrashers had similar plasma T in response to restraint (Figure 40).

3.2.2. Towhees

Baseline plasma hormone (free and total) and CBG concentrations did not differ between urban and desert birds (Table 12). Similarly, hormone levels did not differ between urban and desert towhees after 30 minutes of capture and restraint (Table 12, Figure 40). Capture date was not associated either with baseline total or with free levels of CORT or T, thus hormone levels did not change during the study period (all $p \geq 0.443$). Plasma baseline T and CORT were also similar in passively captured towhees and in birds captured using playback (desert: $n = 3$; both hormones $p = 0.981$). I estimated that at baseline levels, $98.7 \pm 0.1 \%$ and $95.0 \pm 0.2 \%$ of plasma CORT and T were bound to CBG, respectively.

Similar to thrashers, plasma total T decreased after 30 min of capture and restraint ($t = -2.426, p = 0.024$), and this decrease was more pronounced in urban than desert towhees (Figure 40), but plasma free T did not change ($p = 0.222$). After 30 minutes of capture and handling, $98.3 \pm 0.1 \%$ and $93.6 \pm 0.6 \%$ of plasma CORT and T were bound to CBG, respectively. Plasma total CORT ($t = 6.709, p < 0.0001$), but not plasma free CORT ($p = 0.064$), increased following capture and

restraint (Figure 40). As for thrashers, neither behavioral score (PC1 nor PC2) was associated with plasma total or free baseline T or CORT (all $p \geq 0.136$).

3.3. Does Playback Exposure Elevate Plasma T Or CORT?

There was no statistical association between the duration of exposure to playback (from start of playback until bird capture; 8.3 ± 19.1 mins: range 13 secs to 34 mins) and plasma total or free baseline total plasma T or CORT (all $p \geq 0.272$) in thrashers. Likewise, playback duration (4.6 ± 11.3 mins: range: 27 secs to 26 mins) did not alter plasma total or free baseline total T or CORT (all $p \geq 0.073$) concentrations in towhees.

3.4. Is Territorial Behavior Related To Population Density Or Nest Site Availability?

In thrashers, population density (2.3 ± 1.4 and 3.4 ± 1.1 birds/transect for urban and desert, respectively) was not associated with behavior (PC1 or PC2 scores) either in urban or in desert birds (site x population density: both $p \geq 0.452$). In towhees, territorial behavior (PC1) was positively associated with population density (3.6 ± 0.9 and 1.8 ± 0.5 birds/transect for urban and desert, respectively) in urban (site x population density: $F_{3,12} = 7.787$, $p = 0.020$), but not desert areas. Towhees in high density urban areas showed more territorial behavior to playback ($p = 0.009$) than birds in low density urban areas. In contrast, non-territorial behavior (PC2) was not associated with population density in urban or desert areas (site x population density: both $p = 0.499$). There were no associations between population density and any hormonal parameter measured in either species (all $p \geq 0.064$).

Variation in territorial behavior was linearly related to habitat composition in both urban ($F = 4.219$; $p = 0.014$) and desert ($F = 2.361$; $p = 0.043$) thrashers according to CCA analysis with 500 random permutations. For urban thrashers, thin-leaved evergreens containing thorns, shrubs, prickly pear, teddy bear cholla, and canebrake were significant predictors of variation in territorial behavior (Table 13). However, some desert-type vegetation (TEt, PP, TbC) were associated primarily with territorial behaviors (PC1), and non-territorial behavior (PC2) was associated with non-desert (Sh, Cb) vegetation (Figure 41).

Habitat explained less variation in behavior in desert than urban birds. The best predictor of this behavior in desert thrashers was the presence of teddy bear cholla, but other predictors included other cacti, creosote, and thorny thin-leaved evergreens (Table 13). All significant habitat predictors were associated with territorial behavior (Figure 41). Thrashers captured at urban sites with larger Euclidean distances (i.e., distances between two points) than nearby random sites were also more territorial (i.e., higher PC1 scores: $r = 0.73$; $F = 2.383$; $p = 0.007$) but this was not the case in desert thrashers ($r = 0.36$; $F = 0.042$; $p = 0.64$; Figure 42).

Habitat variables were linearly related to territorial behavior in towhees sampled in both urban and desert areas (urban: $F = 1.145$; $p = 0.010$, desert: $F = 1.012$; $p = 0.026$). Over 50% of the variation in territorial behavior in urban towhees was explained by the presence of shrubs, but various tree types were also related to territorial behavior (Table 13). By contrast, territorial behavior in desert towhees was primarily associated with creosote and thin-leaved evergreens (Table 13). Unlike in thrashers, there were no distinct associations between behavior and vegetation associated either with non-desert or desert-type vegetation in either urban or desert towhees (Figure 43). Territorial behavior in both urban and desert towhees did not show associations between the Euclidean distance between sites of capture and nearby random sites (both $p \geq 0.10$; Figure 42).

4. Discussion

I compared territorial behavior and its association with plasma steroids, population density, and potential nest site availability, in urban and desert populations of two Sonoran Desert breeding bird species. Urban birds of both species responded with more territorial behaviors during a simulated territorial intrusion than their desert counterparts, but this difference was apparently not related to plasma T or CORT. Contrary to the predictions of the “challenge hypothesis”, prolonged exposure to simulated territorial intrusion did not alter plasma hormone levels in either species (Wingfield *et al.* 1990; Goymann *et al.* 2007; Moore 2007). Territoriality during the breeding season is related to plasma T in some (Lynn *et al.* 2000; Wingfield *et al.* 2001; Ferree *et al.* 2004; Goymann *et al.* 2007; Moore 2007) but not other species (Lynn & Wingfield 2005; Lynn *et al.* 2005; 2007). In my study, neither thrashers nor towhees showed an association between territorial behavior and circulating total or free T levels. Experimentally elevating plasma T often decreases paternal care (Lynn *et al.* 2000; 2008), and Lynn (2008) proposed that the relationship between male territorial behavior and T may be uncoupled in species showing a high degree of male parental investment. The two species that I studied are monogamous and exhibit biparental care, as seems to be the case of many desert-dwelling birds (Tweitt & Finch 1994; Tweitt 1996). As deserts often offer less food resource availability, biparental care may be needed to successfully fledge young of desert-dwelling birds (Vleck 1993). Plasma T in many monogamous birds is elevated only at the beginning of the breeding season. This seasonal pattern has been reported in thrashers (Vleck 1993), but not towhees. In thrashers, Vleck (1993) reported high plasma T levels from January to March and lower hormone levels during the rest of the year. In this study, I did not detect any change in plasma T in either species during the sampling period (March to July), which is consistent with the above observations. Despite seemingly low T levels through most of the year, thrashers and towhees exhibit territorial responses to playback year round.

4.1. *The Relationship Between Plasma T And CORT*

An objective of the present work was to research relationships between steroids and the expression of territorial behavior. I found no relationship between plasma total T and behavior. One explanation may be that my study design, which did not include a live decoy, may have been sufficient to elicit a behavioral response, but was not sufficient to elicit a hormonal response to territorial intrusion. There is very little information concerning how the presence/absence of a live decoy can alter territorial behavior, however decoy type altered both behavioral and hormonal responses of European Robins, *Erithacus rubecula*, (Scriba & Goymann 2008). Although this may be an important consideration, the observation in my two study species that territorial behavior in response to solely a speaker was maintained without elevating plasma T levels suggests increasing total concentrations of this hormone is not directly required for territorial behavior during a short-term intrusion. Changes in the plasma free T (i.e., T not bound to CBG) may be more critical for the regulation of behavior than total plasma T (Deviche *et al.* 2001; Breuner & Orchinik 2002; Lynn *et al.* 2007). I investigated this hypothesis by also measuring plasma CBG and CORT and estimating how an elevation in plasma CORT, such as resulting from 30 minutes of capture and restraint, alters free T in plasma. In thrashers and towhees, CORT and T bind competitively to “CBG” with high affinity (Fokidis *et al.* 2009). Therefore, an increase in plasma CORT presumably increases the number of binding sites on CBG occupied by CORT and thus decreases the number of binding sites for T, in turn increasing plasma free T. Thus, if territorial behavior is modulated by short-term changes in plasma T, an increase in plasma CORT induced by territorial intrusion, as observed in other studies (Van Duyse *et al.* 2004; Landys *et al.* 2007), may increase territorial behavior. However, I found no association between territorial behavior and total or free plasma CORT. Furthermore, song playback

did not elevate plasma total or free CORT or T levels and plasma hormone levels did not differ between birds that I caught passively or using song playbacks. Collectively these data suggest that territory holders do not perceive simulated territorial intrusion as stressful, at least in terms of stimulating glucocorticoid secretion.

The acute 30 minutes of capture and handling protocol that I used in the present study increased total plasma CORT and decreased total plasma T in thrashers and towhees. These results are consistent with those of other studies (Quillfeldt *et al.* 2006; Baird & Hews 2007; Husak *et al.* 2007). The mechanism by which acute stress decreases plasma T in birds is not entirely understood and likely involves multiple pathways acting at various levels of the reproductive endocrine axis (see review in Wingfield & Sapolsky 2003). In a recent study on male Rufous-winged Sparrows, *Aimophila carpalis*, suggests that this decrease partly results from a direct impairment on endocrine testicular function (Deviche *et al.* 2010). In my study 30 minutes of capture and restraint led to elevated free CORT in towhees but not thrashers and did not affect free T levels in either species. The lack of effect of this acute stress on plasma free T suggests that CBG is not directly involved in the control of territorial behavior.

Thrashers and towhees maintain territorial behavior year-round, even during the non-breeding season when gonadal steroid secretion is presumably low. Territorial behavior outside the breeding season in birds may be mediated either by steroids other than T and such as the androgen precursor dehydroepiandrosterone (DHEA: Hau *et al.* 2004; Soma *et al.* 2004; Soma 2006) or through changes in brain levels of aromatase, which may alter territorial behavior independent of plasma T (Schlinger & Callard 1989; Balthazart & Foidart 1993; Soma *et al.* 2003). Another possibility is that differences in the brain levels of arginine vasotocin (AVT), a neuropeptide known to influence aggression in birds (Goodson 1998a,b; 2009), explain the differences in territorial

behavior between urban and desert birds. Administration of AVT to thrashers elevated plasma CORT more in urban than desert birds, suggesting higher sensitivity of pituitary gland corticotropic cells in urban than desert thrashers (Chapter 2). Testosterone administration to rats increases AVT immunoreactivity in several brain regions associated with aggression (e.g., amygdala). Similarly, in Dark-eyed Juncos, *Junco hyemalis*, castration reduced AVT immunoreactivity in several areas of the brain associated with social behavior and this reduction was reversed by T administration that resulted in high physiological circulating levels of this hormone (Plumari *et al.* 2004). How changes in AVT brain expression regulate territorial behavior during competitive bouts remains, however, unclear (reviewed in De Vries & Panzica 2006). Together, these data suggest the central AVT system involved in territorial behavior responds to changes in androgens levels, but how habitat type may impact this physiological system is unknown. Future studies should compare levels of these endocrine mediators of territorial behavior between or within species that occupy different habitat types and should incorporate hormone inhibition and replacement studies to elucidate mechanisms.

4.2. *Why Would Urban Birds Be More Territorial?*

Few studies have investigated the mechanisms that control context-specific territorial behavior in free-ranging animals. Understanding the specific factors that potentially contributed to the evolution of territoriality in birds may assist in determining its regulation. I measured two ecological parameters that differ between urban and desert populations of my study species: Population density and vegetation composition. Among urban towhees, higher urban population densities were associated with increased territorial behavior. This association was not observed in thrashers or in towhees inhabiting desert areas. Towhees in Phoenix are found at similar or higher densities than in appropriate desert habitat, whereas the opposite is seen in thrashers. Increased

territorial behavior by urban towhees may facilitate their establishment of breeding territories in areas where intrusions by conspecific birds is more common due to higher densities. Previous studies also found a positive association between territorial behavior level and population density (Marvin 1998; Vervust *et al.* 2009), but this hypothesis cannot explain all my behavioral data. Habitat-related differences in territorial behavior in towhees were not related to variation in plasma T or CORT and their physiological basis is presently unclear.

Urban thrashers showed a positive association between territorial behavior and the degree of difference in vegetation composition between the capture site and surrounding randomly sampled areas (i.e., habitat similarity). This observation suggests that higher levels of territorial behavior are exhibited by urban thrashers that defend “unique” territories, relative to surrounding areas, compared to birds that defend more homogeneous territories. In urban and desert thrashers, the more territorial behaviors of thrashers were associated with “thorny” desert-type vegetation such as cholla, prickly pear cacti, and mesquites. These plants types, particularly the cholla, serve as important nest sites for this species (Tweit 1996), suggesting that increased territorial behavior is associated with defense of high quality nesting grounds (i.e., sites with extensive thorny vegetation). In towhees, this type of association was less prominent in both urban and desert habitats. Towhees are largely riparian birds and nest primarily in mesquite (*Prosopis* spp.), coyote bush (*Baccharis* spp.), and wolfberry (*Lycium* spp.; Tweit & Finch 1994). In urban areas, Abert’s Towhees can nest in any dense shrub available (Fokidis *pers. obs.*). Collectively these results can be interpreted as evidence for habitat-dependent territorial behavior that is regulated independently of plasma T or CORT. I propose that birds alter their territorial responses based on perceptions of habitat quality (as in thrashers) or cues associated with population density (as in towhees), and not as a function of differences in plasma T or CORT.

Another factor that may explain differences in territorial behavior between urban and desert birds is variation in signal transmission between noisy urban and quieter rural habitats (Brumm 2006; Slabbekoorn & den Boer-Visser 2006; Warren *et al.* 2006; Slabbekoorn & Ripmeester 2008). In noisier (urban) habitats, birds may need to rely more increasingly on territorial displays in close proximity, as vocal communication from a distance may not be sufficient to ward off a potential intruder.

Intraspecific studies comparing levels of territorial behavior between populations of free-living animals are few. A study comparing Dark-eyed Juncos from a natural population with birds living in a recently colonized urban area (San Diego, California) showed that territorial responses to playback were decreased in the urban population (Newman *et al.* 2006). The decreased territorial behavior may be associated with a decrease in sexual selection due, at least in part, to a lengthened breeding season in the milder urban climate (Price *et al.* 2008). However, another study found no correlation in territorial responses of Noisy Miners, *Manorina melanocephala*, with proximity to road edges (Maron 2009). These studies highlight how territorial behavior can be modulated by local environmental factors and differences between species can vary depending on what the territorial behavior is meant to defend.

Urban birds may cope with the fragmentation of nesting habitat in cities by promoting territorial behaviors that ultimately may increase fitness. Historically, territorial behavior in birds has been largely thought to be T-mediated. However my research suggests that territorial behavior by urban birds may be mediated by neural mechanisms or hormones not examined in this study. Further studies are warranted to explore possible mechanisms of territorial behavior in urban animals, and particularly the role of local ecological factors in shaping context-specific territorial behavior.

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Table 9. Habitat variables for assessing habitat structure in urban and desert bird populations.

Habitat Categories	Abbreviations	Inclusion Criteria
<i>Trees</i>		
Broad-leaved Evergreen	BE	<i>Citrus</i> spp.
Thin-leaved Evergreen (no thorns)	TEnt	<i>Prosopis</i> spp. no thorns
Thin-leaved Evergreen (thorns)	TEt	<i>Prosopis</i> spp. with thorns
Coniferous	C	<i>Pinus</i> , <i>Abies</i> , <i>Picea</i> spp.
Monocot	M	Arecaceae or Palmae
Deciduous	D	Seasonal shedding of leaves
<i>Shrubs</i>		
	Sh	unmanaged
<i>Hedges</i>		
	Hg	actively pruned
<i>Cacti</i>		
Saguaro	Sag	<i>Carnegiea gigantea</i>
Teddy Bear Cholla	TbC	<i>Cylindropuntia bigelovii</i>
Chain-Fruit Cholla	ChC	<i>Cylindropuntia fulgida</i>
Buckhorn Cholla	BuC	<i>Cylindropuntia acanthocarpa</i>
Prickly Pear	PP	<i>Opuntia</i> spp.
Barrel Cacti	BC	<i>Ferocactus</i> spp.
<i>Desert Shrubs & Succulents</i>		
Yucca	Yuc	<i>Yucca</i> spp.
Agave	Ag	<i>Agave</i> spp.
Ocotillo	Oco	<i>Fouquieria splendens</i>
Creosote	Cr	<i>Larrea tridentata</i>
Bamboo or Canebrake	Cb	Poaceae, or <i>Saccharum</i> spp.
<i>Washes</i>		
	W	Wash crosses transect
<i>Artificial Perching Structures</i>		
Telephone Poles	TP	
Telephone Wires	TW	
Transformers	T	
Fences, Walls	Fen	
Television Antennae	TV	
Street Signs	Sig	
Street Lights	Lig	

Table 10. Territorial behaviors (calculated as number of observations/min the bird was observed) and associated principal component (PC) scores, in response to simulated territorial intrusions in Curve-billed Thrashers ($n = 44$) and Abert's Towhees ($n = 25$).

Component Behaviors	Abert's Towhee			Curve-billed Thrasher		
	PC1	PC2	PC3	PC1	PC2	PC3
Air approach	0.775	-0.225	-0.680	0.857	-0.141	-0.166
Ground approach	0.662	-0.243	0.200	0.807	0.103	-0.030
Fly over	0.718	-0.273	0.146	0.776	0.213	0.033
Song	-0.345	0.232	-0.960	-0.576	-0.039	-0.376
Calls	0.595	-0.064	0.024	0.571	-0.301	0.008
Match calls	0.083	-0.140	0.038	-0.065	-0.129	-0.006
Squeal duet	-0.027	0.587	0.076	----	----	----
Bill wiping	-0.358	0.629	-0.041	-0.329	0.745	-0.402
Feeding	0.012	0.015	0.157	-0.342	0.569	-0.269
Tail fan	-0.082	-0.820	0.004	0.100	0.275	0.582
Wing flash	0.172	0.374	-0.401	0.283	0.129	0.039
% Cumulative Variance Explained	48.11	58.53	67.15	60.89	72.34	81.77

Table 11. Behavioral responses (here as occurrences per minute of direct observation) to simulated territorial intrusion in two Sonoran Desert songbird species inhabiting both urban and desert habitats. The amount of time that birds were directly observed is expressed as “proportion of time observed”, and all data are means + s.e. * indicates significant differences between urban and desert birds at $p \leq 0.05$.

	Urban	Desert	<i>t</i>	<i>p</i>	
Abert's Towhee	<i>n</i> = 10	<i>n</i> = 11			
<i>Proportion of time observed</i>	0.88 ± 0.15	0.82 ± 0.23	0.613	0.520	
Air approach	4.27 ± 0.742	1.75 ± 0.629	6.14	< 0.001	*
Ground approach	7.24 ± 0.633	1.91 ± 0.679	7.29	< 0.001	*
Fly over	4.01 ± 0.568	2.08 ± 0.519	2.17	0.016	*
Song	0.11 ± 0.116	0.08 ± 0.083	0.410	0.180	
Calls	7.72 ± 0.925	2.91 ± 1.395	2.30	0.026	*
Match calls	0.33 ± 0.213	-----			
Squeal duet	3.91 ± 0.795	3.08 ± 0.668	0.073	0.314	
Bill wiping	3.57 ± 1.157	5.75 ± 1.508	0.137	0.714	
Feeding	0.31 ± 0.213	0.58 ± 0.287	0.638	0.202	
Tail fan	1.12 ± 0.822	0.83 ± 0.505	1.001	0.079	
Wing flash	4.86 ± 0.853	4.83 ± 1.429	0.456	0.090	
Curve-billed Thrasher	<i>n</i> = 18	<i>n</i> = 16			
<i>Proportion of time observed</i>	0.83 ± 0.23	0.91 ± 0.11	0.382	0.711	
Air approach	7.56 ± 0.549	2.31 ± 0.497	6.83	< 0.001	*
Ground approach	5.63 ± 0.530	1.56 ± 0.67	3.01	< 0.001	*
Fly over	6.38 ± 0.555	1.19 ± 1.17	1.99	0.035	*
Song	3.53 ± 0.444	5.25 ± 0.392	0.044	0.384	
Calls	6.11 ± 1.137	3.62 ± 0.789	1.98	0.041	*
Match calls	0.81 ± 0.301	0.75 ± 0.381	0.059	0.414	
Bill wiping	0.40 ± 0.831	1.43 ± 0.24	1.720	0.039	*
Feeding	0.44 ± 0.258	0.18 ± 0.135	0.177	0.679	
Wing flash	1.28 ± 0.348	0.56 ± 0.24	1.51	0.046	*

Table 12. Baseline and stress-induced (30 mins) concentrations (mean \pm s.e.) of total testosterone (T), total corticosterone (CORT), corticosterone-binding globulin (CBG), and free (unbound to CBG) fractions of T and CORT in both urban and desert populations of two Sonoran Desert bird species. Hormone (T and CORT) data are presented in ng/ml of plasma, and CBG data are shown in nM. * indicates significant differences ($p \leq 0.05$) between populations based on analysis of covariance.

	Urban	Desert	<i>F</i>	df	<i>p</i>
Curve-billed Thrasher	<i>n</i> = 18	<i>n</i> = 16			
Total Baseline T	5.54 \pm 1.284	4.49 \pm 1.404	0.263	1, 39	0.612
Total Stress T	3.11 \pm 0.730	3.76 \pm 0.799	0.310	1, 39	0.581
Total Baseline CORT	9.57 \pm 2.632	15.00 \pm 2.878	1.680	1, 39	0.084
Total Stress CORT	48.09 \pm 6.882	36.23 \pm 7.523	2.170	1, 39	0.007*
CBG	368.28 \pm 19.632	377.48 \pm 21.463	0.870	1, 39	0.770
Free Baseline T	0.31 \pm 0.094	0.26 \pm 0.103	0.137	1, 39	0.714
Free Stress T	0.35 \pm 0.102	0.21 \pm 0.112	0.721	1, 39	0.402
Free Baseline CORT	0.19 \pm 0.068	0.33 \pm 0.074	1.084	1, 39	0.179
Free Stress CORT	4.18 \pm 2.000	1.28 \pm 2.186	1.830	1, 39	0.039*
Abert's Towhee	<i>n</i> = 10	<i>n</i> = 11			
Total Baseline T	7.48 \pm 3.604	5.82 \pm 1.651	0.180	1, 22	0.677
Total Stress T	3.77 \pm 0.924	2.08 \pm 0.272	2.860	1, 22	0.109
Total Baseline CORT	10.37 \pm 3.711	10.02 \pm 3.572	0.005	1, 22	0.947
Total Stress CORT	38.27 \pm 5.253	40.23 \pm 7.031	0.033	1, 22	0.858
CBG	486.18 \pm 31.523	435.41 \pm 72.392	0.311	1, 22	0.584
Free Baseline T	0.42 \pm 0.126	0.31 \pm 0.092	0.177	1, 22	0.679
Free Stress T	0.32 \pm 0.125	0.14 \pm 0.116	1.101	1, 22	0.309
Free Baseline CORT	0.13 \pm 0.081	0.14 \pm 0.048	0.016	1, 22	0.900
Free Stress CORT	0.81 \pm 0.104	0.73 \pm 0.253	0.045	1, 22	0.835

Table 13. Percentage of variance in territorial behaviors explained by habitat variables in urban and desert populations of two Sonoran Desert bird species. **Bold** indicates significant ($p \leq 0.05$) association with behavioral data. See Table 9 for definitions of variable abbreviations.

Variable	Curve-billed Thrasher		Abert's Towhee	
	Urban	Desert	Urban	Desert
BE	---	---	5.3	0.4
TEnt	3.9	1.1	10.7	24.9
TEt	37.4	6.4	5.6	0.0
Con	---	---	0.2	0.9
M	0.8	0.0	5.9	2.6
D	1.9	0.0	4.3	6.0
Sh	16.5	0.9	51.2	17.4
Hg	0.5	0.0	0.6	0.2
Sag	0.3	11.6	---	---
TbC	6.6	19.4	0.0	0.2
ChC	0.5	0.6	---	---
BuC	0.6	7.9	0.5	0.0
PP	14.0	13.3	1.4	0.0
BC	0.3	4.5	0.2	0.3
Cr	3.4	13.9	3.8	25.6
Yuc	0.0	0.6	---	---
Ag	2.1	1.8	3.6	0.9
Cb	3.8	0.0	3.0	0.0
Oco	0.3	8.7	0.1	0.2
W	0.2	0.2	0.0	8.3
TP	1.1	0.0	0.0	1.2
TW	0.8	0.0	0.6	1.7
T	---	---	0.0	0.2
Fen	2.4	0.0	1.2	1.0
Sig	1.8	0.0	0.6	1.3
Lig	0.8	0.0	0.4	1.9
Cow	0.0	0.2	0.9	3.0

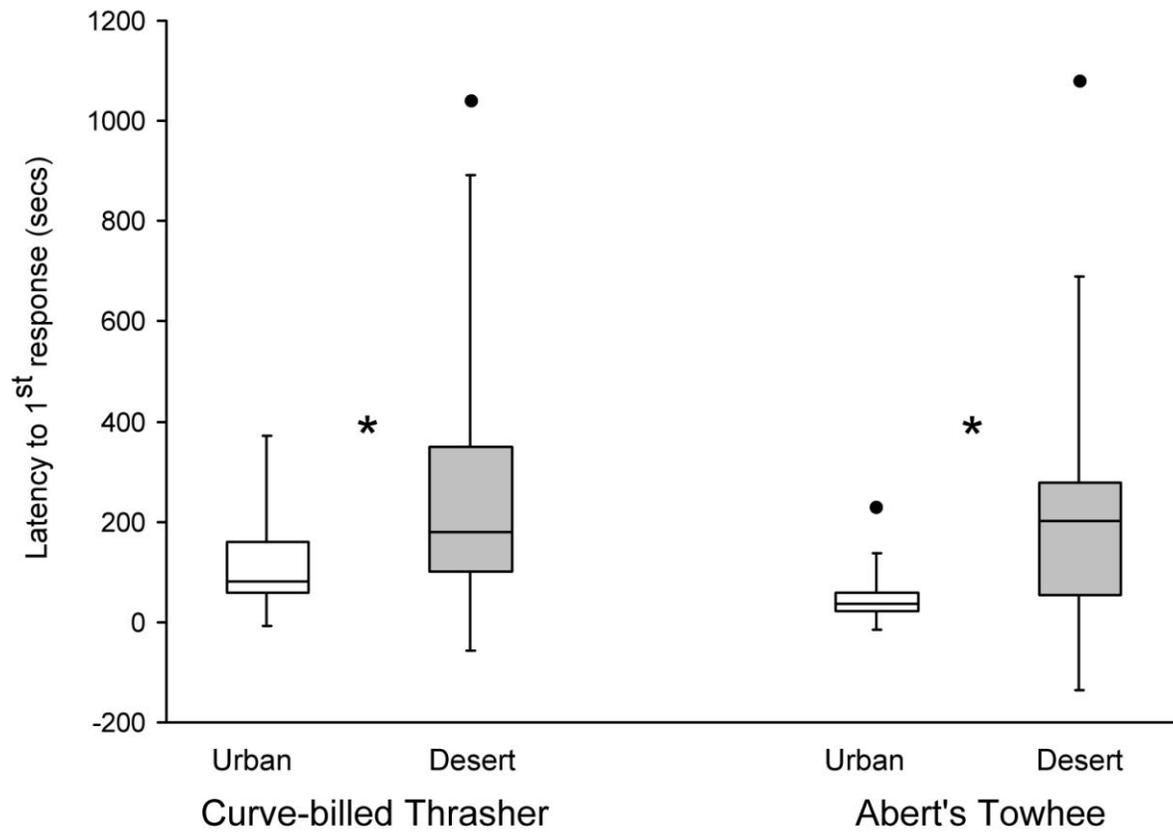


Figure 38. Differences in latency to respond to simulated territorial intrusion between urban and desert populations of Curve-billed Thrasher and Abert's Towhee. Boxplot illustrates median, 10th and 90th percentiles, bars indicate range, and circles indicate outliers. Sample sizes: Urban thrashers $n = 19$, desert thrashers $n = 20$, urban towhees $n = 12$, desert towhees $n = 13$.

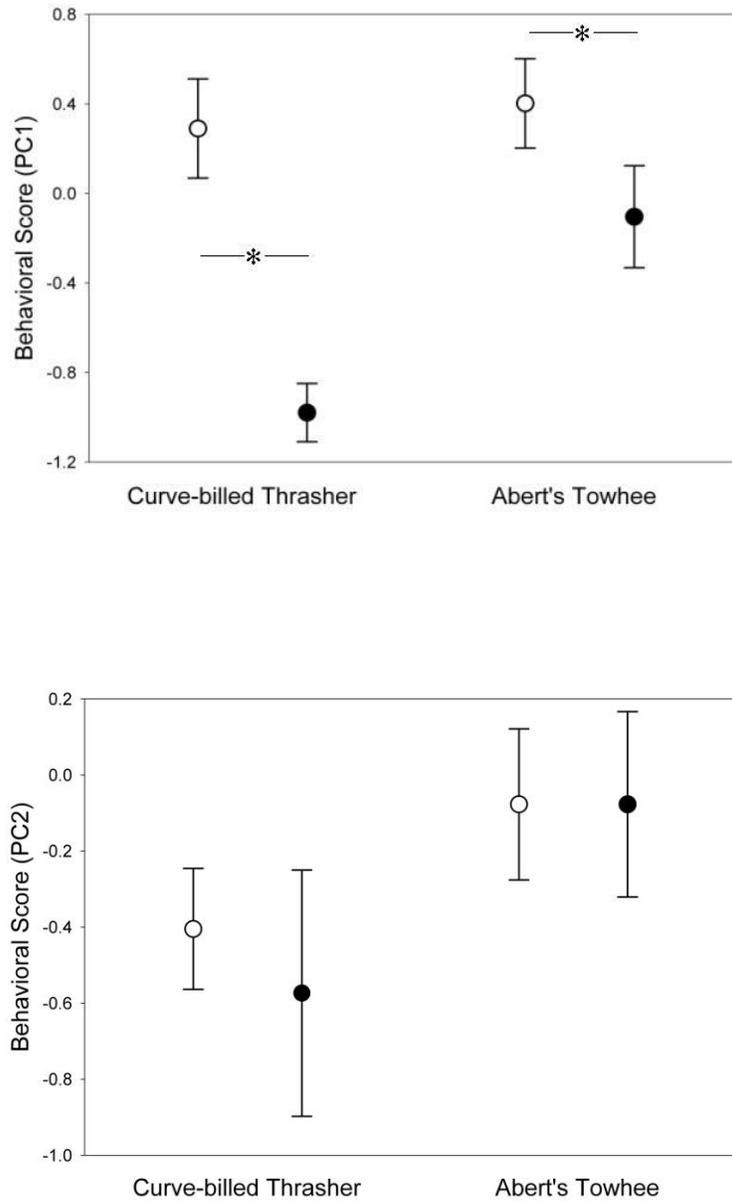


Figure 39. Mean principal component (PC) scores based on behavioral responses to simulated territorial intrusions in urban (unfilled) and desert (filled) populations of Curve-billed Thrashers and Abert's Towhees. See methods for explanations of PC analysis and Table 10 for component behaviors. Bars indicate standard errors of the mean. Sample sizes: Urban thrashers $n = 19$, desert thrashers $n = 20$, urban towhees $n = 12$, desert towhees $n = 13$. * indicates significant differences ($p \leq 0.05$) between populations.

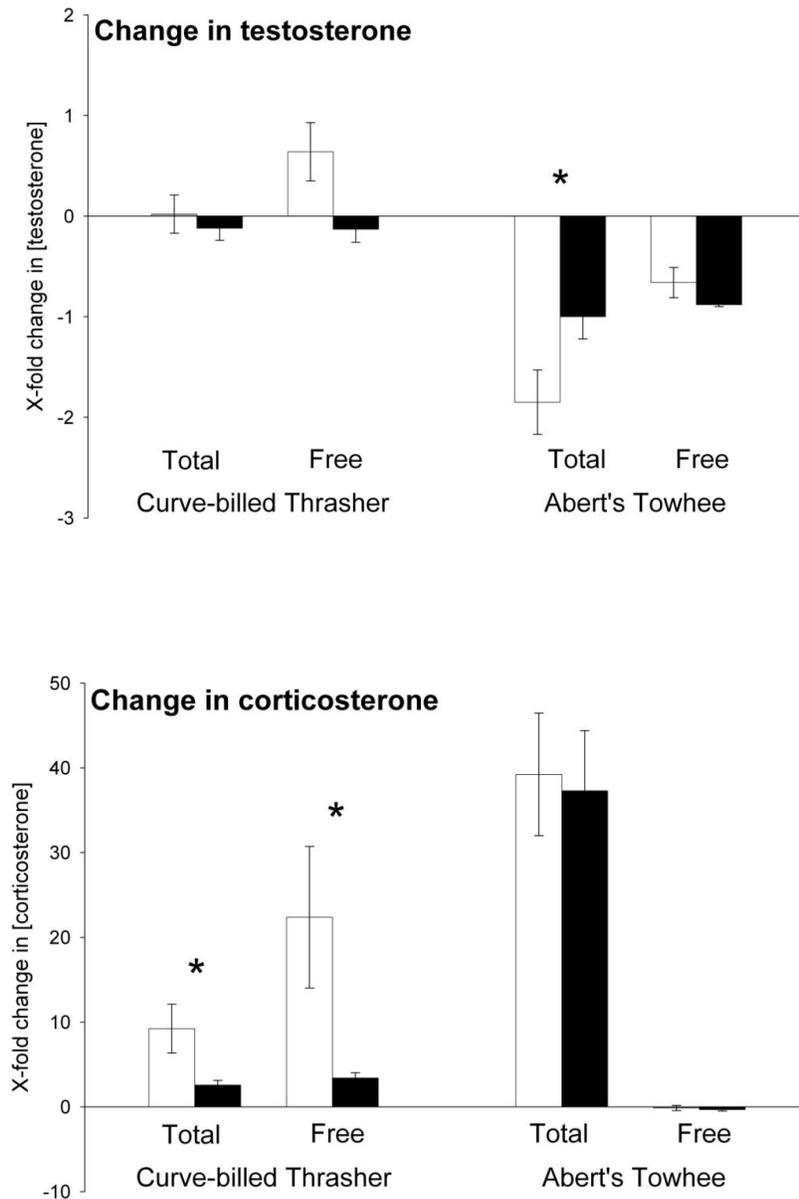


Figure 40. Effect of capture and handling on steroid hormone levels *via* X-fold changes relative to baseline levels in both total and free (unbound to binding globulins) testosterone and corticosterone with 30 minutes of capture and handling in two bird species: Curve-billed Thrasher (urban: $n = 18$; desert: $n = 16$) and Abert's Towhee (urban: $n = 10$; desert: $n = 11$). White and black bars indicate urban and desert populations, respectively. * indicates significant differences ($p \leq 0.05$) between populations based on analysis of covariance.

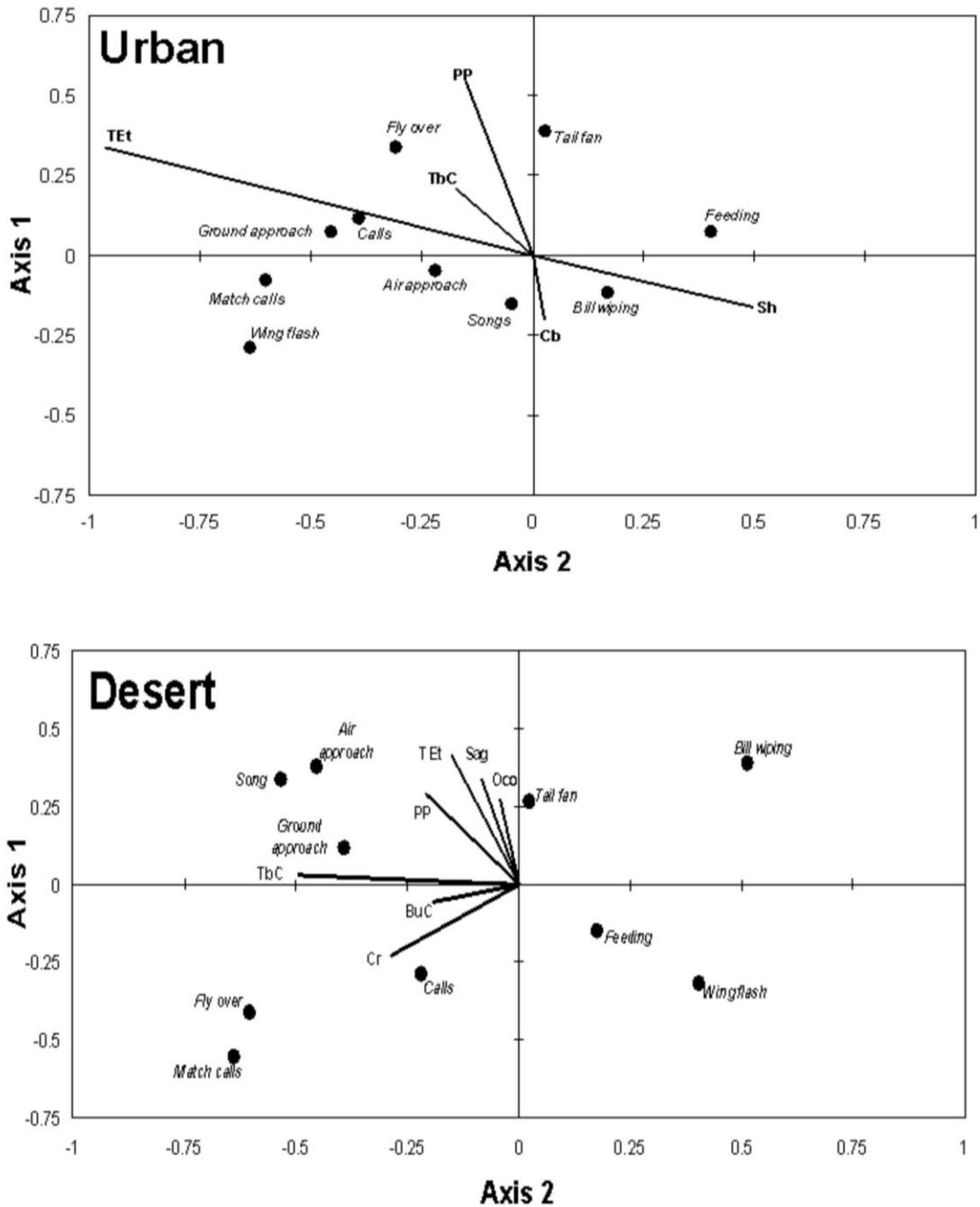


Figure 41. Canonical correspondence analysis plot relating habitat variables to territorial behaviors demonstrated by urban and desert populations of Curve-billed Thrashers in response to simulated territorial intrusion. Length and direction of vectors correspond to the degree of association between habitat and behavior variables. See Table 9 for abbreviations to habitat variables.

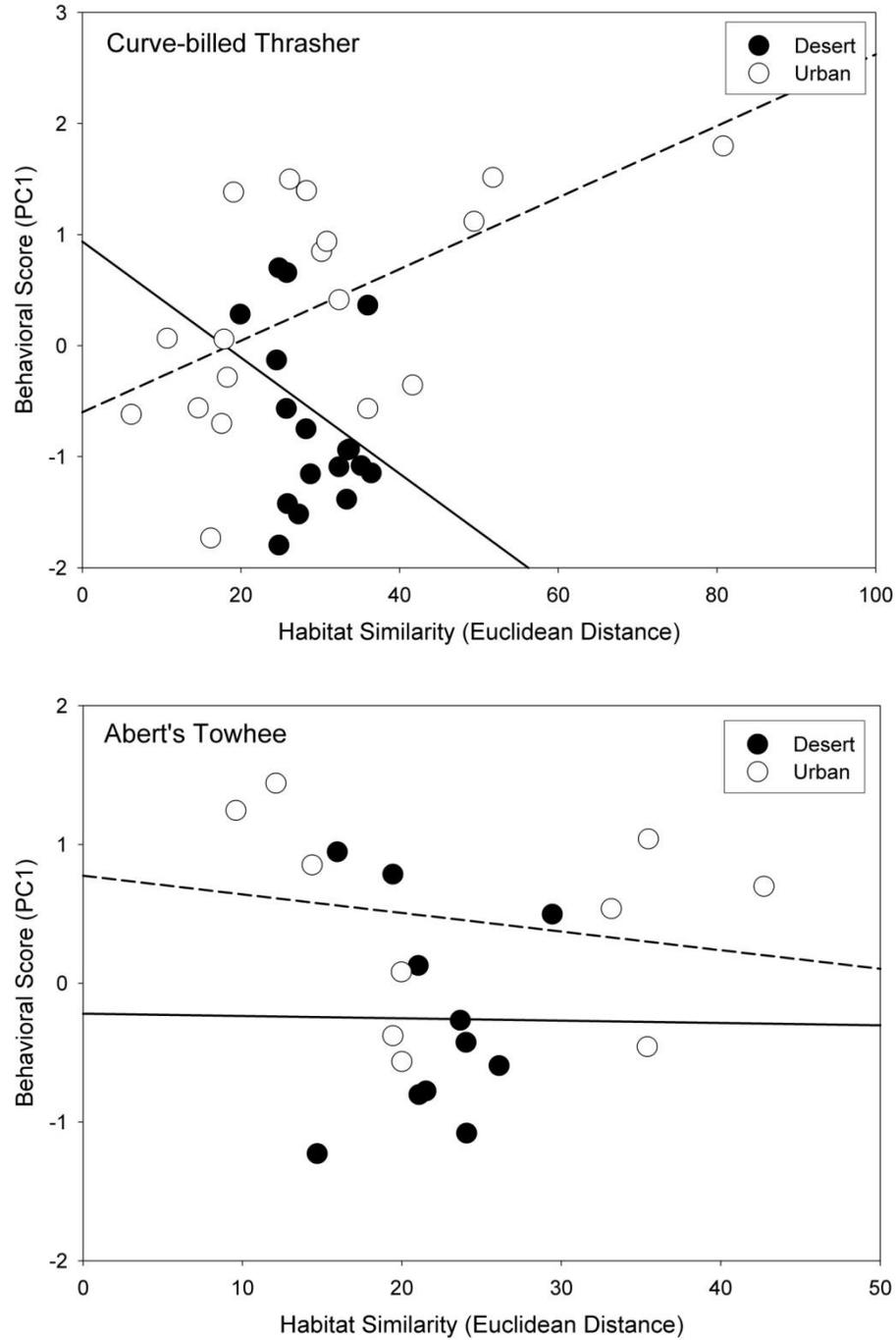


Figure 42. Relationship between territorial behaviors (principal component “PC” score) to simulated territorial intrusion and the degree of similarity in habitat composition between sites of capture and randomly sampled sites as assessed by Euclidean distances between points. Solid and dashed lines indicate relationships in desert and urban populations, respectively.

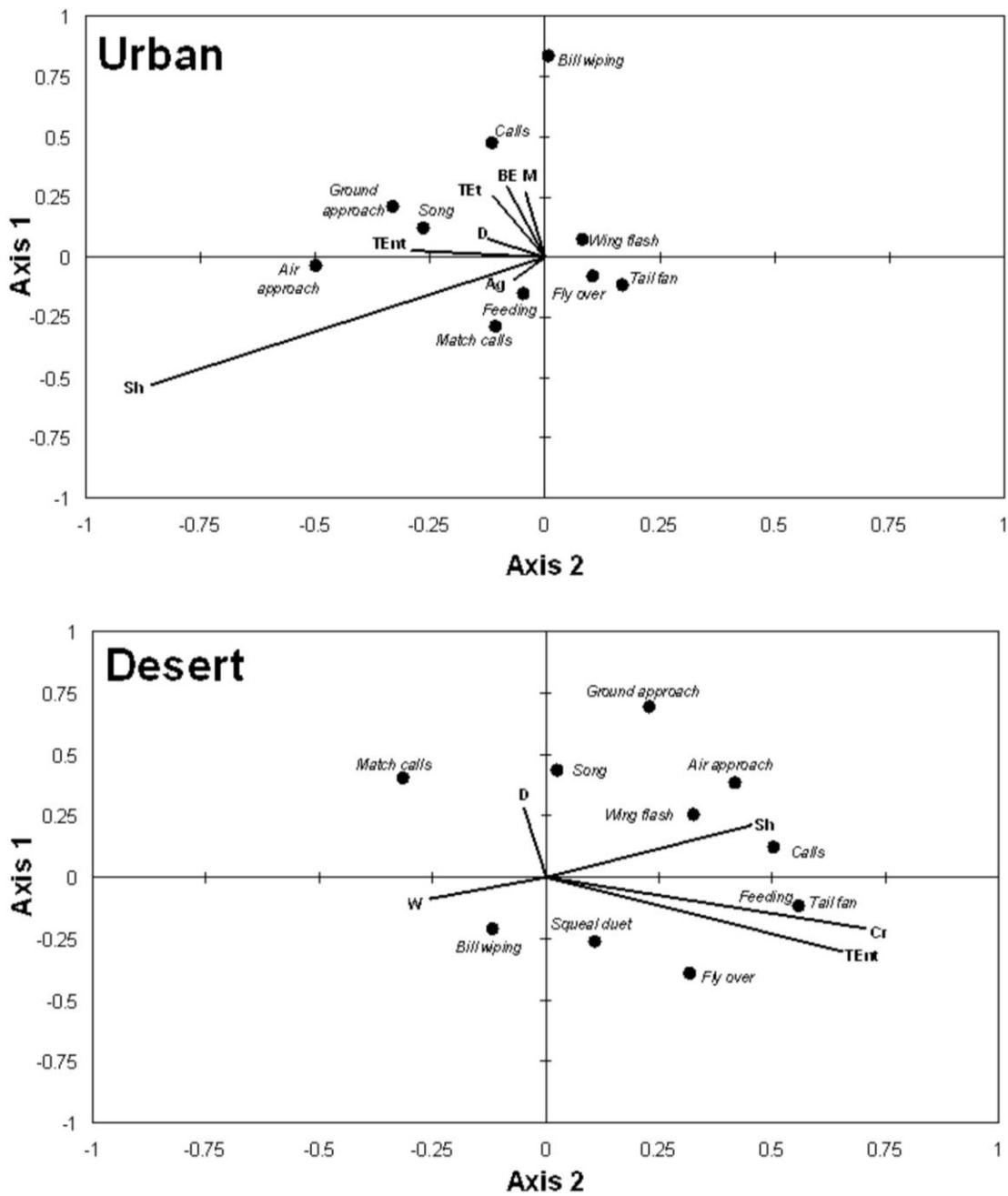


Figure 43. Canonical correspondence analysis plot relating habitat variables to territorial behaviors demonstrated by urban and desert populations of Abert's Towhees in response to simulated territorial intrusion. Length and direction of vectors correspond to the degree of association between habitat and behavior variables. See Table 9 for abbreviations to habitat variables.

Chapter 8 CONCLUDING STATEMENTS

This dissertation revealed differences in stress physiology and body condition between two habitats in close proximity to one another. Within the 45 km between the various research sites there were major shifts in the CORT stress response, territorial behaviors, body condition, and even the neural vasotocin system. Such intraspecific variation in these traits has been previously documented in populations of various taxa living in different areas of their geographic range. But this research has shown that studies comparing individuals living in urban and native habitats offers substantial promise for investigations of how physiology adapts in response to local conditions. This dissertation work, although not the first to explore physiology in response to urbanization, is the first to test a specific mechanism that can account for variation in stress physiology seen in urban animals.

Contributions To Understanding Of Endocrine Functioning

The endocrinology literature is littered with references to the “condition-dependence” of the acute stress response. This is grounded in observations that individuals in poor condition (i.e. low body mass or few energy reserves) often secrete higher amounts of glucocorticoids, and *vice versa*, and this served as an impetus for using CORT levels as a proxy measure of stress/condition in studies with an ecological and conservation focus. Surprisingly though there is very little empirical research investigating how short-term changes in energy levels, such as what occurs during food manipulation, alters how glucocorticoids are secreted. The captive studies in this dissertation research address how glucocorticoid physiology

directly responds to changes in body condition. Specifically, increases in body condition result in a more robust ability to secrete CORT during acute stress. This is important for two reasons: 1) it supports the idea that CORT secretion is partly dependent on current energetic state; and more importantly; 2) suggests that some mechanism in place that can detect and assess current energetic state, communicate this information to the HPA axis, and mediate CORT secretion accordingly. The latter is interesting because chapters 4 and 5 revealed how changes in the degree of CORT secretion during food manipulations, are also associated with the degree of change in free glycerol, a major substrate for the production of new glucose. Thus not only does CORT secretion differ with body condition but so does the source of energy that is mobilized during stress. The energy available to an individual is likely a major determinant in functioning of many endocrine systems and yet the specific “sensing” mechanisms in place to detect current energetic status and relay this information are complicated and poorly understood.

Although the source of variation in the HPA axis was not completely identified, the combination of a field-based study involving injecting birds with various HPA hormones (chapter 2), and an immunohistochemical investigation (chapter 3) suggested a role for the neuropeptide AVT. Specifically, higher AVT-like immunoreactivity in a predominant area of the hypothalamus associated with the stress response, and increased sensitivity to AVT in urban compared to desert thrashers suggests it may drive some of the differences in CORT secretion observed in birds in the field. This is bolstered by the observation that a similar change in neural AVT was observed in birds subjected to a food variability experiment, where

the treatment also induced birds to lose body mass (chapter 6). In mammals, AVT can maintain the HPA axis, even after a negative feedback decreased the secretion of CRF (the other main stress neuropeptide). Thus even during chronic stress (which presumably induces a negative feedback) an individual can still retain the capacity to respond to an acute stressor, which may be advantageous. The CRF data obtained in chapter 2 is equivocal, and the relative importance of CRF and AVT in birds is poorly understood. However, this research adds to the growing knowledge that AVT is a neuropeptide that is responsive to environmental influences, such as urbanization. The consequences of this is unclear, but one possible implication is changes in behavior. Research in chapter 7 revealed a profound difference in territorial behavior between urban and desert birds. But this difference was not related to levels of CORT or T, or the interaction of these two with CBG in circulation. However a link between neural AVT immunoreactivity in a region of the brain associated with sociality and territorial behavior is suggested based on data from chapter 3. This “link” is at best tenuous and whether this is related to variation in AVT in stress responsive areas of the brain is an avenue for future study. Nonetheless this dissertation highlights the possibilities that studies of the urbanization of animals have for answering basic questions about the plastic nature of the endocrine system and how environment can influence physiological function.

Contributions To Urbanization Studies And Conservation

Most of the world’s people now live in cities and they are without doubt the most rapidly expanding habitats on earth. This revelation has prompted

conservationists and conservation biologists alike (not necessarily the same thing) to consider how wildlife responds to city encroachment on native landscapes. This dissertation research makes a strong point that some species can have the capacity to alter their physiology and behavior in a way that may enable them to persist in a changing environment. Indeed, the endocrine system is a prime physiological adaptation for assessing change in the environment (such as a stressful stimulus, a photoperiodic shift, or a change in temperature), facilitating an appropriate body-wide physiological response, and coordinating a new physiological set point (allostasis) if necessary. In fact, the endocrine system frequently does this during transitions from one life history stage to another. The Curve-billed Thrasher numbers have remained stable based on nationwide bird survey data (CBC and BBS), in contrast to most other Sonoran Desert bird species. Urbanization is the most pressing regional environmental issue in central Arizona and the stable numbers of Curve-billed Thrashers is likely due in part to their ability to use these new urban habitats.

Chapter 1 compared the CORT responses to acute stress of five bird species found in Phoenix and outlying areas. These species differed in their capacity to use habitats within Phoenix (based on previous demographic studies). One central observation in this study was that seasonal variation in CORT secretion is lessened in city populations of birds compared to those living outside the city. This is the first study to report differences in the seasonal profile of a physiological variable between a man-made and natural habitat, and provides support for previous theoretical models that propose cities are “aseasonal” habitats with a more constant (i.e. invariable) resource availability through time.

However beyond simply describing how urbanization changes the numbers of animals, we know very little about specific physiological or even ecological mechanisms that enable some species to thrive and others to vanish. This dissertation proposes that accessing food resources in the city provides the intrinsic energetic resources necessary to allow a robust stress response to be maintained. This dissertation in turn demonstrated that food quantity and “predictability” can alter both the magnitude of the acute stress response and the means by which intrinsic energy resources are used during stress. This model sounds elegant, but I must outline a couple of limitations in my understanding. First, I assume that (for a thrasher) there is more food or it is more predictable in the city. This is unknown but studies by the CAP LTER have shown increased plant biomass, greater presence of fruits year-round and more insects in urban areas, so this combined with the generalist diet of the thrasher and higher body condition of urban birds suggests this is possible.

Secondly, this model assumes that a robust stress response is adaptive to a bird in the city. Other research in Europe indeed showed that urban blackbirds have a dampened stress response compared to forest birds. Many papers also cite the increased approachability or “tameness” of urban birds, which may also stem from a dampened stress response. This is a difficult question to answer, but one possibility is that these responses could be simply species specific, as was shown in chapter 1 for birds inhabiting the same areas of a city. Curve-billed Thrashers can still increase CORT in response to stress, yet have a robust negative feedback in place (chapter 2) suggesting they are not in a state of HPA dysregulation as with chronic stress. A

situation such as this could interfere with reproduction (which does occur in Phoenix) and would likely result in declines in body condition, which was not observed in this species. Thus there doesn't appear to be a detriment to having an intact stress response. In contrast, the capacity to elevate CORT levels can enable birds to flee from and cope with the various acute stresses that can present themselves in an urban environment (e.g. people, dogs, cats, vehicles, loud noises, etc). This may be especially important since the thrasher is a resident bird that is highly-territorial throughout the year. This means when a stressor presents itself, the bird is unlikely to completely flee from the area whereas a non-territorial species may have this option. Indeed, blackbirds and the majority of "tame" urban species mentioned above are gregarious species that do not establish territories.

The biology of urban ecosystems is still in its infancy, but the need to understand the mechanisms that operate in this environment is urgent. Cities offer a myriad of opportunities for understanding questions of phenotypic plasticity, micro-evolutionary change in genetic structure, source-sink dynamics and even the evolutionary role of epigenetic mechanisms. Cities and their colonization by native species are also potential models for understanding questions pertaining to the biology of invasive species and how organisms will respond to climate change, since most cities come equipped with their own heat island effect. These conservation issues are very important and although most biologists love their untouched landscapes, the truth is that cities are not going away and figuring out how to minimize their effects on wildlife will (or has) become the forefront of animal conservation.

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APPENDIX A

APPROVAL LETTER FROM INSTITUTE FOR ANIMAL CARE AND USE

COMMITTEE

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
Tempe, Arizona 85287-1103
Phone: (480) 965-2179 FAX: (480) 965-7772

Animal Protocol Review

ASU Protocol Number: 09-1022R
Protocol Title: Neuroendocrine and Nutrition-Based Mechanisms of Adaptive Plasticity Underlying Urbanization of Native Birds
Principal Investigator: Pierre Deviche
Date of Action: 9/29/2008

The animal protocol review was considered by the Committee and the following decisions were made:

- The original protocol was APPROVED as presented.
- The revised protocol was APPROVED as presented.
- The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be considered when these issues are clarified and the revised protocol is submitted.
- The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- The Committee requests you to contact _____ to discuss this proposal.
- A copy of this correspondence has been sent to the Vice President for Research.
- Amendment was approved as presented.

RESTRICTIONS, CHANGES OR WAIVER REQUIREMENTS:

Total # of Animals: 140 **Pain Level:** C **Species:** Bird, Curve-billed Thrasher
Sponsor: National Science Foundation
Title: Neuroendocrine and Nutrition-Based Adaptive Plasticity Underlying Urbanization of Native Birds
Approval Period: 10/1/2008 – 9/30/2011

Additional Investigator: Bobby Fokidis, Ph.D. Student

Signature:  Date: 10-26-10
IACUC Chair or Designee

Original: Principal Investigator
Cc: IACUC Office
IACUC Chair