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Brain Arginine Vasotocin Immunoreactivity Differs between Urban and Desert Curve-Billed Thrashers, *Toxostoma curvirostre*: Relationships with Territoriality and Stress Physiology

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Key Words
Curve-billed thrasher · Urban versus desert birds · Arginine vasotocin · Corticosterone · Aggression · Stress · Water balance · Territoriality

Abstract
The neuropeptide arginine vasotocin (AVT: the avian homolog of vasopressin) has numerous functional roles including mediating social behaviors, coregulating the adrenocortical stress response and maintaining water balance. 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, AVT immunoreactivity was compared between male curve-billed thrashers, *Toxostoma curvirostre*, from native Sonoran Desert locations and those within the city of Phoenix, Ariz. Previous research found that urban thrashers are more responsive to territorial intrusion, secrete more corticosterone (CORT) during capture stress, and they may also have greater access to water than desert counterparts. Variation in AVT immunoreactivity was also related to levels of plasma CORT and osmolality, and with behavioral responses to a simulated territorial intrusion. Birds from these two habitats showed different AVT immunoreactive patterns in two brain regions: the paraventricular nucleus of the hypothalamus and the medial bed nucleus of the stria terminalis (BSTM), 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 no such association with osmolality was observed in birds from either habitat. The total number of BSTM AVT-immunoreactive cells was related to a decreased responsiveness to territorial intrusion. These data suggest that divergence in the AVT system between urban and desert thrashers may help explain observed differences in both the adrenocortical stress response and territorial behavior between populations. Whether differences in water availability between habitats contribute to population differences in the brain AVT system is unknown.

Introduction

The nonapeptide arginine vasotocin (AVT) and its mammalian homolog arginine vasopressin (AVP) have a conserved distribution in the vertebrate brain [Moore and Lowry, 1998; De Vries, 2006]. The wide neuroanatomical distribution of these nonapeptides reflects their multiple roles in the control of peripheral and central physiological processes. These functions include: maintenance of hydromineral balance by increasing the water

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permeability of the renal collecting ducts [reviewed in Goldstein, 2006; Lau et al., 2009], acting as a vasoconstrictor [Nakano, 1974; Conklin et al., 1996; Donald and Trajanovska, 2006] and stimulating the secretion of corticosterone (CORT) during stress [Madison et al., 2008; Fokidis and Deviche, 2011]. In addition, AVT can act centrally to modulate social behaviors, such as aggression [Goodson, 1998a; Kabelik et al., 2009]. The brain distribution of the AVT immunoreactivity of several avian species has been described [Kiss et al., 1987; Voorhuis and de Kloet, 1992; Panzica et al., 1999; Fabris et al., 2004; Klein et al., 2006] and studies have linked behavior and physiological processes to specific AVT immunoreactive (AVT-ir) cell populations in the diencephalon and forebrain [reviewed in Goodson and Kabelik, 2009].

In birds, much like in mammals, the paraventricular nucleus (PVN) contains AVT-producing magnocellular and parvocellular neurons [Kiss et al., 1987; Voorhuis and de Kloet, 1992; Panzica et al., 1999; Fabris et al., 2004]. Magnocellular PVN neurons project axons to the neurohypophysis, where they can secrete AVT into general circulation [Mikami et al., 1978; Barth and Grossmann, 2000]. In contrast, parvocellular PVN neurons can secrete AVT to a number of neural sites, including the median eminence (ME) [Mikami et al., 1978] where they stimulate VT2 receptors on the anterior pituitary gland and induce the secretion of adrenocorticotropic hormone by corticotropic cells in response to stressors [Jurkevich et al., 2008]. Early studies also demonstrated that magnocellular neurons within the PVN project axons to the median eminence [Oksche et al., 1963; Mikami et al., 1978]. The specific function of these projections and whether they transport AVT remains unclear.

In addition, AVT can function synergistically with corticotropin-releasing hormone (CRH), as secretagogues of CORT, the main glucocorticoid produced by the avian adrenal glands, as evidenced by studies using peripheral AVT and CRH administration [Romero, 2006; Fokidis and Deviche, 2011]. In humans, rats and poultry, CRH and AVP/AVT are often colocalized within the parvocellular division of the PVN, where these peptides are presumably secreted into the ME [Sawchenko et al., 1984; Whitnall, 1989; Mouri et al., 1993; Shibata et al., 2007; reviewed in Kuenzel and Jurkevich, 2010].

Parvocellular AVT/AVP-secreting neurons are also found in forebrain structures, including the medial bed nucleus of the stria terminalis (BSTM). Studies in birds have focused on the role of AVT in the control of social and aggressive behaviors [Goodson, 2008; Goodson et al., 2009]. AVT from the BSTM and the PVN is thought to be released into several brain areas including the lateral septum [DeVries and Buijs, 1983; DeVries et al., 1985], where it works within the social behavior network to modulate aggressive behavior [reviewed in Goodson and Kabelik, 2009]. Simulated territorial intrusions did not alter the colocalization of AVT and the immediate early gene expression cell marker c-fos in the BSTM of song sparrows, *Melospiza melodia*, but AVT-fos colocalization in the PVN was reduced in the more aggressive birds compared to less aggressive ones [Goodson and Kabelik, 2009]. Similar results have been obtained in laboratory mice [Ho et al., 2010]. These data suggest that AVT regulates aggressive behavior through actions mediated by the BSTM and the PVN.

Bilateral lesions to the septum in both field sparrows, *Spizella pusilla*, and zebra finches, *Taeniopygia guttata*, produced the predicted behavioral effects supporting a role for the lateral septum as a neural site involved in mediating aggressive behaviors [Goodson et al., 1999]. Infusion of AVT into the lateral septum inhibits aggression in territorial species such as the field sparrow [Goodson, 1998a] and the violet-eared waxbill, *Uraginthus granatina* [Goodson, 1998b], but has the opposite effect in gregarious species such as the zebra finch [Goodson and Adkins-Regan, 1999]. However, such species-specific differences in the effects of AVT on aggression likely result from the use of different behavioral testing methods and not opposing responses to AVT in the lateral septum [Goodson et al., 2009]. Varying relationships between neural AVT and aggression (or specific dominant vs. subordinate phenotypes) have also been reported in fish and reptiles [Larson et al., 2005; Lema, 2006; Ohya and Hayashi, 2006; Santangelo and Bass, 2006; Dewan et al., 2008; Greenwood et al., 2008; Hattori and Wilczynski, 2009; Iwata et al., 2010]. Collectively, these studies highlight the complexity of AVP/AVT's role in aggression and how this evolutionarily conserved system can contribute to context-specific behavioral responses.

Animals inhabiting urban areas often differ from their nonurban counterparts physiologically and behaviorally, e.g. in terms of their adrenocortical stress response [Partecke et al., 2006; Schoech et al., 2007; French et al., 2008; Fokidis et al., 2009; Fokidis et al., in press] and territoriality [Newman et al., 2006; Fokidis et al., 2011]. The curve-billed thrasher, *Toxostoma curvirostre*, is a native passerine (family: Mimidae) of the southwest USA that inhabits native Sonoran Desert habitats (hereafter referred to as 'desert birds') as well as the city of Phoenix, Ariz. (hereafter 'urban birds'). Compared to urban birds, desert birds have higher baseline ('prestress')

plasma CORT, particularly during molt, and a seasonally more variable plasma CORT response to 30 min of acute stress induced by capture and restraint [Fokidis et al., 2009]. Desert thrashers also have a lower ratio of blood heterophils (the avian equivalent of mammalian neutrophils) to lymphocytes (H:L ratio) than urban birds [Fokidis et al., 2008]. This ratio is often used as an indicator of prolonged CORT secretion in desert birds [Vleck et al., 2000; Fokidis et al., 2008; French et al., 2008] as opposed to urban birds [Fokidis et al., 2008]. In addition, desert birds 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 [Fokidis and Deviche, 2011]. It is currently unknown whether the putatively lower pituitary gland sensitivity to AVT of desert versus urban birds reflects a population difference in AVT secretion. Finally, 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 [Fokidis et al., 2011]. This difference is not related to population differences in plasma testosterone or CORT [Fokidis et al., 2011], suggesting that it is centrally mediated.

The 'mesic' landscaping that is common in the Phoenix metropolitan area involves extensive water supplementation through irrigation. This supplemental water alters the local ecosystem [Martin and Stabler, 2002; Keys et al., 2007; Shen et al., 2008] and produces a habitat with essentially unlimited water availability [Cook and Faeth, 2006; Shen et al., 2008]. By contrast, in the surrounding Sonoran Desert water availability is intermittent, resulting primarily from irregular precipitation 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 and Bradshaw, 1998]. The Death Valley pupfish, *Cyprinodon nevadensis*, showed differences in neural AVT-ir between isolated populations that differ with respect to their habitat temperatures and social organization [Lema and Nevitt, 2004]. Thus, the brain AVT system may differ as a function of environmental conditions.

We compared the brain AVT-ir distribution of thrashers inhabiting an urban or a native desert habitat to test three hypotheses relating to the potential roles of the peptide. The first hypothesis was that differences in the adrenocortical stress response between urban and desert birds are reflected in differences in brain AVT-ir between these populations. According to this hypothesis, we predicted that AVT-ir, and specifically AVT from the PVN parvocellular neurons, is positively associated with plasma CORT. The second hypothesis was that AVT-ir in PVN magnocellular neurons is positively associated with plasma osmolality. The third hypothesis was that behavioral differences between urban and desert thrashers reflect differences in AVT-ir within the BSTM, and are negatively correlated with aggressive behavior in response to simulated territorial intrusion. To our knowledge, this is the first investigation to describe the neuroendocrine differences associated with urbanization in a free-living vertebrate species.

Materials and Methods

All procedures 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.

Study Locations

The study was conducted at a desert and at an urban location. The desert location was the largely unpopulated Hummingbird Springs (12,626 ha) and the adjoining Bighorn Mountains Wilderness Area (8,498 ha) located about 80 km west of Phoenix. The vegetation at this location 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 September 11 and October 22, 2008, after the species' breeding season and during the prebasic molt period [Pyle, 1997]. Captures took place between 06:04 and 10:35 h.

Behavior Recording

Thrashers (8 from both the urban and desert locations) were captured by luring them to mist nets using conspecific playback recordings, which simulate a territorial intrusion. We 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 locations in and around Phoenix. Recordings were played on a Sony MD walkman minidisk player through a handheld speaker at constant amplitude (74 dB at 2 m from the speaker). Birds in the urban habitat were sampled during times of minimal human disturbance (early mornings and weekends).

When a male was located, a mist net was erected and the bird was exposed to the recording with the observer (H.B.F.) located 40–80 m away, with an unobstructed view of the mist net and sur-

rounding area. The observer, remaining quiet and still, recorded the occurrence of several stereotyped behaviors previously reported as being associated with responses to playback [Fokidis et al., 2011]. These included: (1) the latency to first approach the playback speaker, (2) the number of approaches from the air or ground and (3) the number of 'whit-whit' calls, the predominant nonsong vocalization of this species [Tweit, 1996; Fokidis et al., 2011]. 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 an examination of gonads after euthanasia (see below).

Bird Collection

Thrashers were removed from the net and approximately 300 μl of blood was collected from the right jugular vein within 3 min of capture, using a heparinized 0.3-ml syringe with a 29.5-gauge needle. A short delay between capture and bleeding avoided the increase in plasma CORT that is associated with the stress of capture and handling [Fokidis et al., 2008]. In addition, a 5-μl 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; Fokidis et al., in press]. Blood samples were kept on ice until the plasma was separated by centrifugation and then stored at –80°C until assayed. We 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 (±1 mm) of each bird prior to euthanasia. Thrashers in the study area begin to replace their primary feathers in middle-to-late summer (mid-July to early August) and the longest primary feathers (p7–p9) are usually fully grown by late September to early October [Fokidis, unpubl. data]. At the time of the study, these feathers had recently been replaced and were fully grown, so wing chord measurements were unaffected by error associated with abraded or underdeveloped feathers.

Tissue Processing

After the above measurements were taken, birds were deeply anesthetized using Metofane (methoxyflurane, Mallinckrodt, Mundelein, Ill., USA) inhalation and euthanized by decapitation. Brains were removed from the skull (6–11 min postcapture) 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]. Brains were weighed (±0.01 g) after fixation and were then postfixed and gelatin-embedded prior to cryostat-sectioning following a modified protocol described by Saldanha et al. [1994]. Briefly, they were rinsed 3 times with 0.1 M PB (30 min each) and immersed in 4% gelatin solution in water for 30 min. Brains were then placed into 8% gelatin solution-filled plastic molds and the gelatin was allowed to solidify overnight at 4°C. Embedded brains were postfixed in 4% paraformaldehyde solution for 48 h and then immersed in 10, 20 and 30% sucrose solutions in PB for 48 h each. Brains were frozen on dry ice and stored at –80°C until sectioned.

AGV Immunohistochemistry

Brains were coronally sectioned at 30 μm and every 3rd section was collected into cryoprotectant solution [Watson et al., 1986]. In addition, sections were also directly mounted onto gelatin-coated slides and stained for Nissl substance using thionine. These sections were used to confirm the neuroanatomy of AVT-ir-stained tissues. Free-floating sections were stained for AVT-ir using an indirect immunohistochemical procedure modified from Small et al. [2008]. Sections were washed 3 times in 0.1 M PB solution for 30 min, incubated with 0.36% H_2O_2 in 0.1 M PB for 15 min, washed 3 times with 0.1 M PB (5 min each), incubated with normal horse serum (1:30 in PBT, i.e. PB containing 0.3% Triton X-100; Sigma-Aldrich Co., St. Louis, Mo., USA) for 1 h, 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 5 times (10 min each) in 0.1 M PB, incubated for 1 h in 1:100 biotinylated horse anti-rabbit IgG in 0.3% PBT (Vector Laboratories Inc., Burlingame, Calif., USA), washed 3 times for 10 min each in 0.1 M PB, incubated in Vectastain ABC solution (Vector Laboratories) for 1 h, washed 3 times (15 min each) in 0.1 M PB, incubated for 3 min in Vector SG peroxidase chromagen (Vector Laboratories), and washed twice for 5 min in 0.1 M PB. Immunostained sections were mounted onto Vectabond-coated (Vector Laboratories) glass microscope slides, allowed to dry for 24 h at room temperature, dehydrated with ethanol, cleared in xylene, and coverslipped using Cytoseal 60 (Stephens Scientific, Kalamazoo, Mich., USA).

The antibody specificity was tested by preabsorption with 200 μg/ml of AVT (V0130, Sigma-Aldrich), which eliminated the staining. In contrast, staining was not affected by preabsorption with 200 μg/ml of mesotocin (H-2505, Bachem lnc., Torrance, Calif., USA). Sections were also incubated as above but either omitting the primary or secondary antiserum or replacing the ABC solution with buffer. Sections incubated in these conditions also showed no staining.

Image Analyses

Images of brain sections were digitized using a camera (Olympus DEI-750D, Olympus Optical Ltd., Tokyo, Japan) attached to a light microscope (Olympus BX60) at 40× magnification and using constant (i.e., equivalent contrast, hue and brightness) microscope, camera and software settings. For each brain section photographed, an 'out of focus' image was also taken of an area devoid of immunolabeling 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 (Grayscale 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 defined on the basis of easily recognizable neuroanatomical landmarks defined in Stokes et al. [1974] and Balthazart et al. [1996]. The location of the bed nucleus of the BSTM 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] for the avian telencephalon.

We used 4 measurements to quantify AVT-ir staining in the BSTM and PVN. First, we measured the overall optical density of AVT-ir within a 100-μm-diameter circular area of interest centered over the brain region of interest. This intensity (hereafter referred to as relative optical density, ROD) was defined as the optical density (0 = all black, 256 = all white) and not the staining hue or saturation [see Kabelik et al., 2008].

Second, we counted the number of AVT-ir perikarya in each section for each brain region and added them to provide a total cell number, 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. cells that were not highlighted), including cells that were partially overlapping (less than half their soma area visible), were counted manually. This 'total cell number' value, although based on measurements made on every 3rd cross section of the brain, was more informative than using the number of cells counted per section, which was highly variable depending on the specific cross section examined (middle vs. edge of brain region). Values were corrected for the total number of brain sections examined (range 4–6 sections for PVN and 3–6 for BSTM) and included both hemispheres.

Third, the cross-sectional area of a subset (7–20 cells per section and 28–47 cells in total per bird) of clearly delineated and nonoverlapping AVT-ir perikarya within each brain region (hereafter referred to as 'cell size') was determined using a manual tracing function. These cells were selected as those that intersected the lines on a grid pattern.

Fourth, we measured the OD of individual AVT-ir perikarya for which a cross-sectional area was available. For both the cell size and OD, means from multiple sections including the left and right hemispheres of each brain were used for statistical analyses. As consecutive sections used for analyses were 90 μm apart, no cell was counted twice. Cell size and OD data were not collected for cells within brain areas where the presence of numerous AVT-ir fibers made individual perikarya difficult to distinguish.

We also quantified AVT-ir within the ME, using the OD of 10 nonoverlapping circular areas of interest (diameter: 25 μm) located along the entire length of 2 adjacent images taken from sections of the middle of the ME from each bird. Mean ODs of staining obtained for both images of the ME for each bird were used for statistical analyses.

CORT Assay and Plasma Osmolality

Total plasma CORT concentration was measured using a validated commercial competitive enzyme-linked immunoassay (ELISA; Assay Designs Inc., Ann Arbor, Mich., USA) as described by Fokidis et al. [2009]. The assay sensitivity was 11.9 pg/ml and the mean intra-assay coefficient of variation was 6.23%. Samples were assayed in duplicate.

Plasma osmolality 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. Its use to determine the osmolality of thrasher plasma has been previously validated [Fokidis et al., 2010].

H:L 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, coverslipped, and sealed using Cytoseal 60 (VWR, San Francisco, Calif., USA). Using an Olympus BX60 light microscope, heterophils and lymphocytes were counted at 400× magnification until a total of 100 cells of both cell types together were counted [Fokidis et al., 2008; French et al., 2008; Fokidis et al., in press]. Cell types were identified using the criteria of Campbell [1996].

Statistical Analyses

All microscope slides and digitized images were analyzed without knowledge of the bird identity, collection habitat, or collection date. We used Student's t tests to compare the sampling date and time, morphological and physiological data, and the latency to first response to playback between urban and desert populations. 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]. This measure is thought to indicate the relative amount of energy stored as either lean muscle mass or fat reserves. Body molt differences between urban and desert birds were assessed using a χ^2 test. Data for AVT-ir ROD, total cell number, and ME staining were normally distributed, thus allowing for comparisons using analysis of variance (ANOVA), with habitat as a fixed factor and body condition, molt intensity and sampling date and time as covariates. The effect of habitat on ME staining was analyzed using a 2-sample Student t test. Cell size and cell OD data followed a Poisson distribution and were compared using nonparametric Kruskal-Wallis tests with brain region and habitat tested as the main-effect variables separately. Post hoc comparisons of data that were significant with the Kruskal-Wallis test were done by first conducting a pair-wise Mann-Whitney U test and then comparing the resulting statistic to a studentized range (Q) in a manner similar to a Tukey test, using a critical value equal to $p = 0.05$ [Sokal and Rohlf, 1995].

Pearson product-moment correlations were used to relate variations in AVT-ir within different brain regions with ME staining, as well as to examine the relationship between AVT-ir and plasma osmolality, CORT, the H:L ratio and territorial behaviors. All data were tested for the presence of outliers using the Chauvenet criterion outlier test ($p < 0.05$), which identifies data points beyond two standard deviations from the corresponding group mean. A single outlying point [latency to first response (desert bird) = 1,043 s] was identified and was excluded from subsequent analysis. Whether habitat (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 habitats, separate correlations were depicted for each habitat. All data are presented either as means ± standard errors (SE) for normally distributed data or as medians ± 95% confidence intervals for nonnormal data. The critical α-level for all tests was set at $p \leq 0.05$, except in the case of multiple univariate comparisons where a Bonferroni-corrected α-value of $p \leq 0.014$ is also presented.

Results

Morphology, Physiology and Behavior of Urban and Desert Thrashers

Neither sampling date nor time of sampling differed between urban and desert birds (table 1). Urban thrashers were heavier, but not structurally larger (i.e. no difference in wing chord or tarsus length) than desert conspecifics (table 1). The mean brain weight of birds from the 2 study

Table 1. Mean sampling date and time, as well as morphometrics, reproductive status, and variables associated with the adrenocortical stress response, water balance, and territorial behavior in adult male curve-billed thrashers sampled from urban and desert habitats (n = 8 each)

	Desert	Urban	t_{14}	p
Sampling date	7-Oct-08	7-Oct-08	0.372	0.291
Time of sampling	7:40 AM	6:28 AM	1.008	0.104
Body mass, g	72.8 ± 1.77	78.6 ± 1.58	2.161	0.007*
Wing chord, mm	107 ± 1	104 ± 2	1.372	0.173
Tarsus length, mm	34.5 ± 0.4	33.8 ± 0.4	1.308	0.191
Brain weight, g	6.06 ± 0.61	6.01 ± 0.47	1.139	0.247
Cloacal protuberance width, mm	4.2 ± 0.32	4.3 ± 0.27	1.067	0.274
Testis width, mm	5.7 ± 0.72	5.5 ± 0.61	1.092	0.623
Plasma corticosterone, ng/ml	15.3 ± 1.24	10.1 ± 2.11	1.649	0.048*
Heterophil to lymphocyte ratio	1.1 ± 0.22	0.9 ± 0.31	0.205	0.759
Plasma osmolality, mOsm/l	302.7 ± 7.96	293.5 ± 6.35	0.907	0.343
Latency to first response, s	920 ± 16	197 ± 56	2.438	0.026*
	Desert	Urban	Z	p
Number of approaches to speaker	6 ± 1	11 ± 2	–2.515	0.011*
Number of calls	3 ± 1	7 ± 2	–0.095	0.933

* Indicates significant differences between habitats ($p \leq 0.05$) based on either Student t (degrees of freedom = 14) or Mann-Whitney U tests.

populations was similar and regressed testes and undeveloped cloacal protuberances were evidence that all birds were in a nonreproductive condition (table 1). Desert birds had higher plasma CORT than urban conspecifics (table 1). Plasma osmolality did not differ between urban and desert thrashers (table 1). 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, urban birds responded to simulated territorial intrusion more quickly and with more approaches toward the speaker than desert birds (table 1). However, the number of calls given during playback did not differ between habitats (table 1).

Neuroanatomical Distribution of AVT-ir in the Thrasher Brain

Discrete cell clusters were observed throughout the preoptic area with fibers primarily in the most rostral portion of this region. In some brain sections from each bird, preoptic area perikarya were confined to a narrow strip of overlapping cells bordering the 3rd ventricle. Diffuse immunoreactive perikarya and fibers were also detected in the supraoptic nucleus medial to the nucleus geniculatus lateralis ventriculatus. Extensive immunostained fibers and few lightly stained perikarya were present in the lateral hypothalamus and extended laterally towards the lateral forebrain bundle (fasciculus prosencephali lateralis) and along the edge of the tractus thalamomedialis dorsal to the nucleus rotundus. Discrete populations of cells with few fibers were seen in the PVN (fig. 1) from which fibers apparently extended to the ME. No clear differences in cell size or ODs within perikarya in the PVN were apparent; thus, we were unable to distinguish between 'larger' and 'darker' magnocellular neurons that secrete AVT into the neurohypophysis and 'smaller' and 'lighter' parvocellular neurons that secrete into the ME. The bed nucleus of the BSTM was identified, based on the presence of perikarya below the lateral septa and ventral to the caudal section of the anterior commissure and dorsal to the tractus occipitomesencephalicus and just dorsolateral to the PVN (fig. 1). Staining of the BSTM was confined primarily to the caudal portion of the anterior commissure (fig. 1). Another isolated population of perikarya was located dorsal to the tractus occipitomesencephalicus and ventral to the fasciculus prosencephali lateralis. In contrast to previous studies [Aste et al., 1998; Jurkevich et al., 1999], no immunoreactive fibers or perikarya were observed dorsal to the anterior commissure or along the lateral septa. Extensive immunoreactive fibers were present both dorsal of and within the ME.

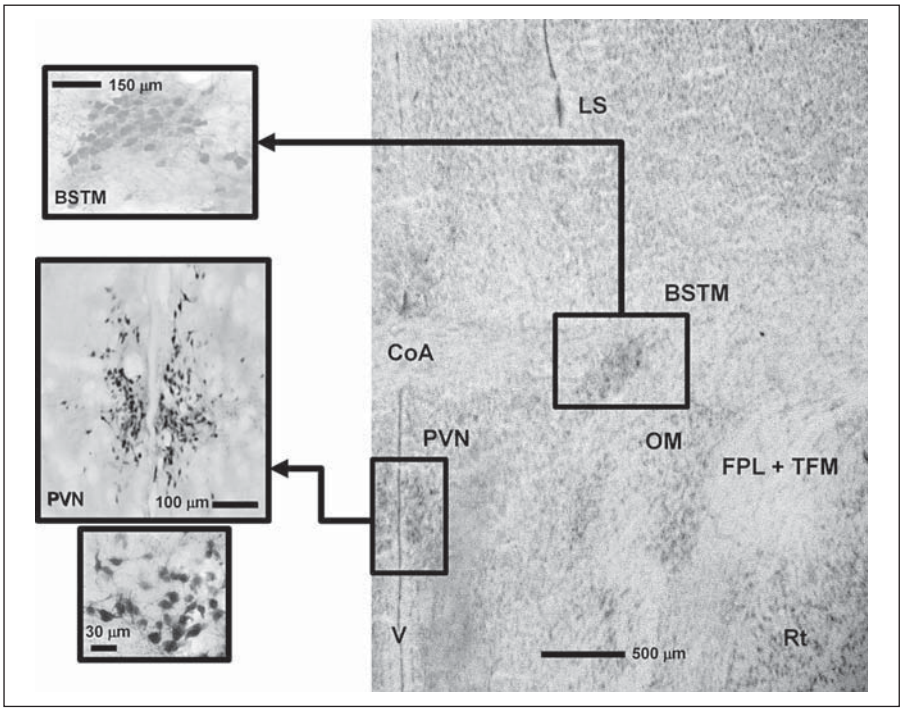


Fig. 1. On the right is Nissl substance staining of a curve-billed thrasher brain coronal section showing the localization of the PVN and medial bed nucleus of the BSTM. Brain areas are shown in relation to several neuroanatomical landmarks: anterior commissure (CoA), occipitomesencephalic tract (OM), tractus thalamo-frontalis and frontalis-thalamicus medialis (TFM), ventricle (V), fasciculus prosencephalicus lateralis (FPL), lateral septum (LS), and nucleus rotundus (Rt). On the left are AVT-immunoreactive cell clusters in the BSTM (top) and the PVN, the latter at both high and low magnification (bottom).

Brain AVT-ir in Urban and Desert Thrashers

In the PVN, cell ODs were higher ($Q = 3.91$, $p \leq 0.05$) and cell size smaller ($Q = 3.62$, $p \leq 0.05$) in urban birds than in desert birds. No differences between habitats were seen for either variable in the BSTM (all $p \geq 0.262$). No relationships were observed between any of these measures and sampling date or time, body condition or molt intensity (all $p \geq 0.092$).

The AVT-ir ROD differed between urban and desert thrashers (ANOVA: $F = 4.810$, d.f. = 2, 14, $p = 0.049$), but this difference was brain-region-specific (ANOVA: $F = 3.077$, d.f. = 2, 8, $p = 0.045$) with AVT-ir ROD in the BSTM being 19% higher in desert than urban birds (LSD: $p = 0.026$; fig. 2a). With regard to the 2 habitats, no differences in ROD were observed in the PVN (LSD: all $p \geq 0.361$; fig. 2a), nor in total AVT-ir cell number (ANOVA: $F = 1.061$, d.f. = 2, 14, $p = 0.275$; fig. 2b). Within the PVN, the AVT-ir perikarya of urban birds had 11% more immunostaining (cell OD: $Q = 4.87$, $p \leq 0.05$; fig. 2c) and were 21% smaller (cell size: $Q = 3.50$, $p \leq 0.05$; fig. 2d) than those of desert birds.

Urban thrashers also had 73% more ME staining than desert conspecifics (2-sample t test: $t = 4.021$, d.f. = 15, $p \leq 0.001$; fig. 3). There were no significant associations between ME staining and the various measures of AVT-ir within the PVN (all $p \geq 0.083$).

Stress, AVT-ir and the PVN

Plasma CORT was negatively correlated with PVN AVT-ir ROD (fig. 4a) and positively correlated with AVT-ir cell size (fig. 4b) in urban but not in desert birds. Thus urban birds with higher plasma CORT had less AVT-ir staining within the PVN and AVT-ir cells in urban birds were smaller than in desert birds. Plasma CORT was negatively correlated with the H:L ratio (fig. 4c); however, none of the measures of AVT-ir within the PVN were correlated to the H:L ratio (all $p \geq 0.061$). Neither plasma CORT nor H:L ratio was associated with ME staining (both $p \geq 0.316$).

Plasma Osmolality and AVT Immunoreactivity

Plasma osmolality was positively associated with ME staining in urban but not desert thrashers (fig. 5). There were no other associations between plasma osmolality and any variables (all $p \geq 0.069$).

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 BSTM AVT-ir cell number was correlated with the latency to first response to playback (fig. 6), but neither with the number of approaches toward the speaker nor with the number of calls (both $p \geq 0.577$).

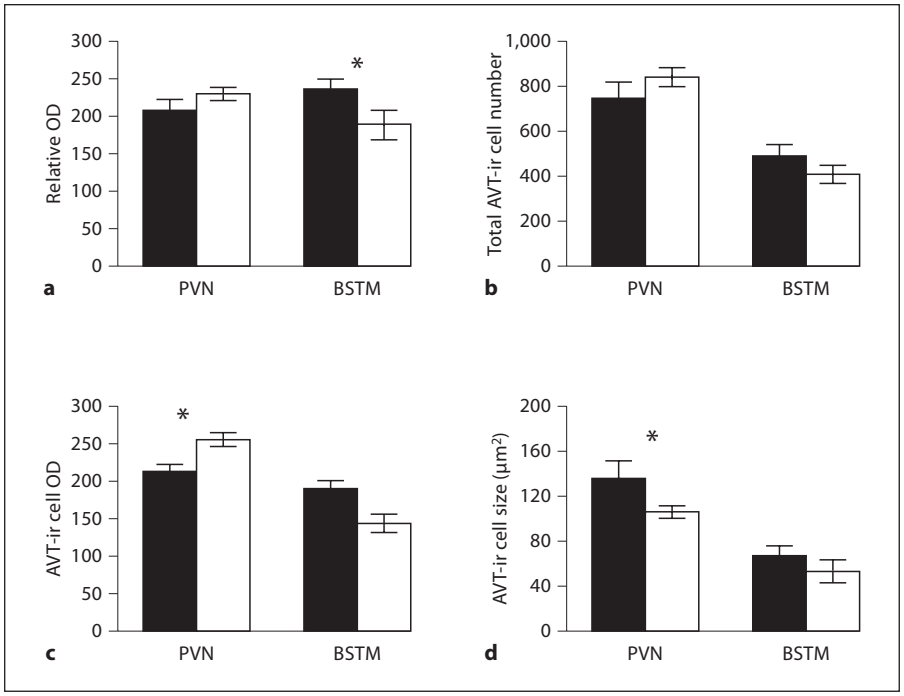


Fig. 2. Differences in AVT-ir in the PVN and BSTM between urban (□; n = 8) and desert (■; n = 8) populations of curve-billed thrashers. The graph compares the 2 bird populations with respect to: the relative ODs (arbitrary units) of AVT-ir (**a**), the total number of AVT-ir cells (**b**), the AVT-ir cell OD (arbitrary units) (**c**), and the sizes of individual AVT-ir cells (μm^2) (**d**). * $p < 0.05$ indicates significant differences between urban and desert birds for each brain region.

The PVN ROD was negatively correlated with latency to first responses to playback, but only in urban thrashers (ROD: $r = -0.528$, $p = 0.018$). The PVN ROD was also correlated with the number of approaches toward the speaker ($r = 0.521$, $p = 0.038$): birds with greater AVT-ir staining within the PVN were behaviorally more responsive to conspecific playback.

The ME staining was negatively correlated with the latency to first response to playback ($r = -0.51$, $p = 0.046$). The number of calls was not associated with measures taken from any brain region (all $p \geq 0.092$).

Discussion

We found differences in AVT-ir in specific brain regions of free-living songbirds sampled from a native Sonoran Desert location and a nearby urban habitat, the city of Phoenix. The distribution of AVT-ir perikarya and fibers in the thrasher brain is consistent with that in other birds [Kiss et al., 1987; Voorhuis and de Kloet, 1992; Panzica et al., 1999; Fabris et al., 2004; Klein et al., 2006] and also with the general distribution of AVP in the brain of laboratory rodents [Sofroniew et al., 1979; Vandesande 1980; Hawthorn et al., 1984; Vanzwieten et al., 1991, 1993] and primates [Wang et al., 1997]. The PVN plays a func-

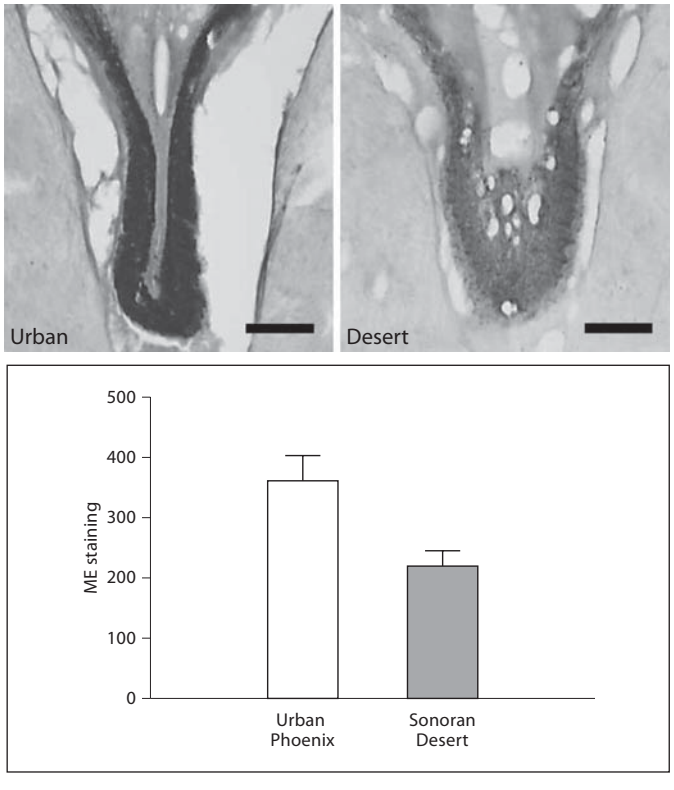
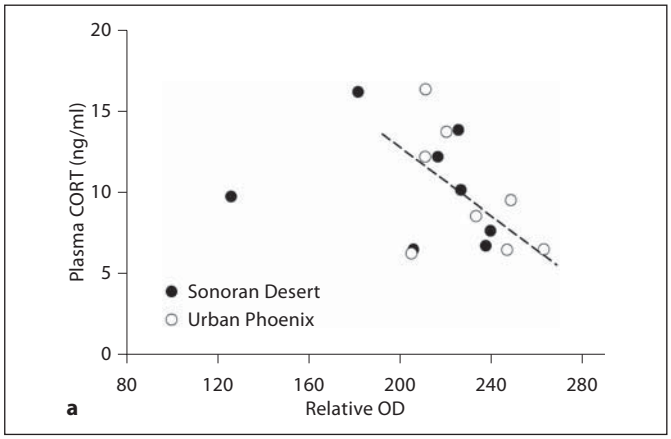
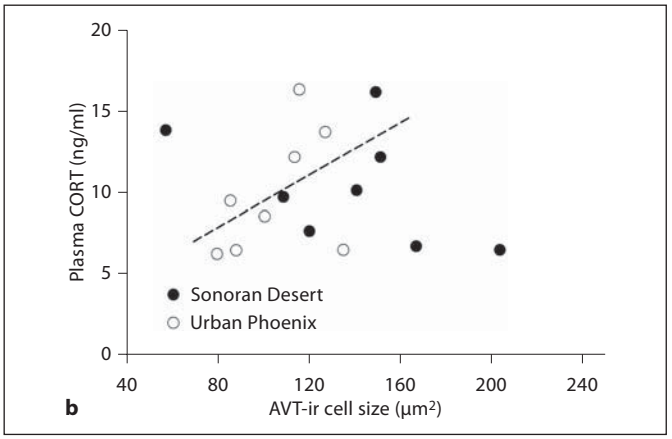


Fig. 3. a Distribution of AVT-ir in the ME of urban and desert birds. Scale bars indicate 200 μm. **b** OD (in arbitrary units) of ME AVT-ir staining in birds belonging to the 2 study populations. * $p \leq 0.001$.





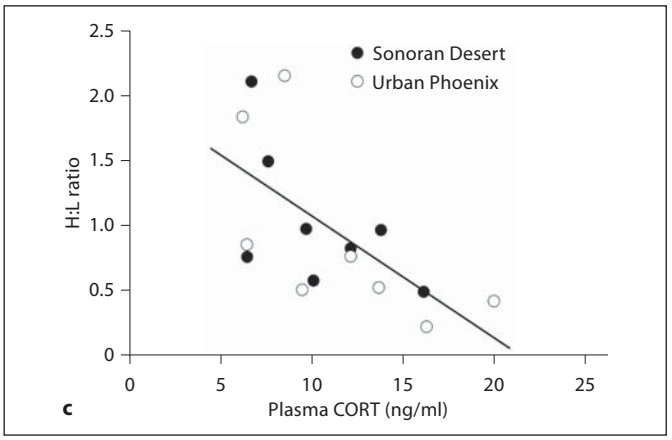


Fig. 4. The correlations between: plasma CORT and ROD of AVT-ir within the PVN ($r = -0.58$, $p = 0.003$) (**a**), the size of AVT-ir cells within the PVN between urban and desert thrashers ($r = 0.47$, $p = 0.048$) (**b**), and white blood cell H:L ratio (an alternate measure of chronically elevated CORT levels) ($r = -0.56$, $p = 0.024$) (**c**). Solid lines indicate pooled correlations for both habitats and dashed lines indicate correlations only for urban birds.

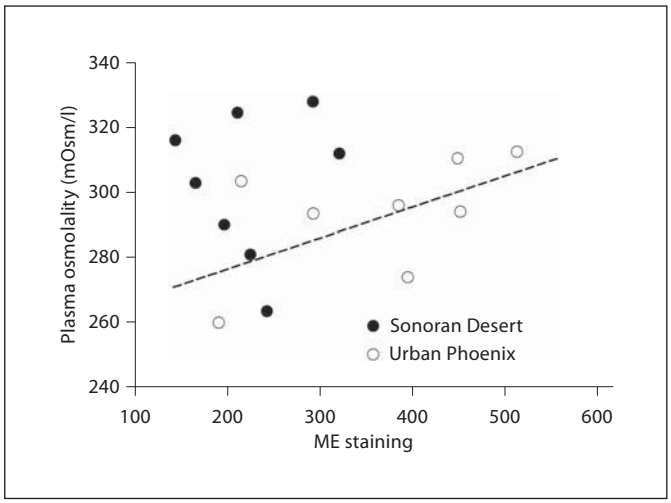


Fig. 5. Relationship between AVT-ir at the ME and plasma osmolality in both urban and desert thrashers. Only the correlation for urban birds is shown (dashed line). $r = 0.49$, $p = 0.008$.

tional role in stress responses [reviewed in Kuenzel and Jurkevich, 2010] and in the regulation of water balance [reviewed in Bisset and Chowdrey, 1988]. Along with the PVN, the BSTM is also involved in mediating social behaviors, including intermale aggression [Goodson and Kabelik, 2009]. Consistent with these roles, we report associations between AVT-ir in these two brain regions and plasma CORT and territorial behaviors. These associations are consistent with previous field observations showing differences in the adrenocortical stress response and territoriality between urban and desert populations of the study species.

Hypothalamic AVT, Stress and Water Balance

Both CRH and AVT are secretagogues of adrenocorticotropic hormone during an acute stress response [Romero and Rich, 2007; Madison et al., 2008; Fokidis and Deviche, 2011]. In birds, both central and peripheral AVT administration increase plasma CORT [Romero and Rich, 2007; Jurkevich et al., 2008; Madison et al., 2008]. This increase is greater in urban than in desert

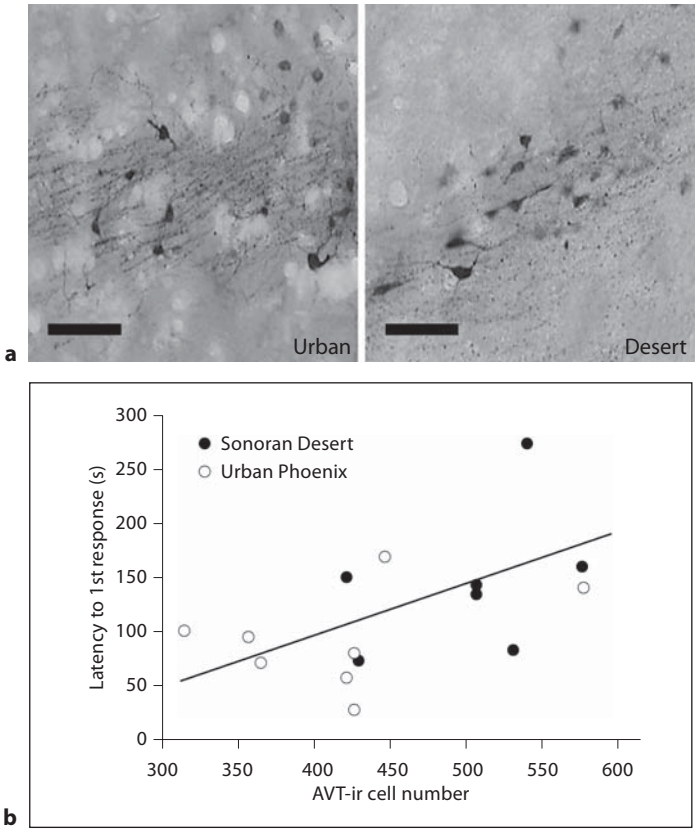


Fig. 6. a Images depicting the average AVT-ir within a component of the medial bed nucleus of the BSTM of urban and desert curve-billed thrashers. **b** Correlation (both habitats pooled) between the total AVT-ir cell number and the behavioral responses to simulated territorial intrusions. Scale bars indicate 50 μm. r = 0.48, p = 0.035.

thrashers, suggesting that the pituitary gland of urban birds is more sensitive to AVT than that of desert birds [Fokidis and Deviche, 2011]. The current observations also suggest that the PVN perikarya of urban birds contain more AVT than those of desert birds. An increase in AVT-ir in the PVN may reflect cellular accumulation of the peptide resulting from a reduced processing rate or transport [see Panzica et al., 2001; Goodson and Kabelik, 2009]. However, this increase is generally thought to reflect elevated peptide production in response to higher levels of neuropeptide secretion [Kabelik et al., 2008; Sewall et al., 2010]. The hypothesis that this may be the case in our study is complicated by two observations. First, urban birds had smaller AVT-ir perikarya than desert birds and convention predicts that increased production of AVT is associated with larger perikarya [Panzica et al., 2001; Goodson and Kabelik, 2009]. Second, we found no difference in PVN ROD between urban and desert birds. Both PVN AVT-ir cell size and ROD were positively related to plasma CORT, but only in urban birds. However, no relationship between AVT-ir in the PVN and H:L ratio, a putative marker of chronic activation of the adrenocortical stress response, was observed.

Birds with more AVT-ir staining in the PVN also showed more immunostaining in the ME. This observation is consistent with the hypothesis that PVN neurons secrete AVT into the ME and that these neurons are a major source of AVT in the ME [Panzica et al., 2001]. Furthermore, the intensity of AVT-ir staining in the ME was higher in urban than in desert birds. The observed differences in AVT-ir within PVN perikarya and ME staining between urban and desert thrashers suggest that urban thrashers secrete AVT into the hypophyseal portal system either in larger amounts or faster than is the case in desert thrashers. Consistent with this contention, the acute stress of capture and handling stimulates CORT secretion more in urban than in desert birds [Fokidis et al., 2009], and we proposed that AVT has a greater stimulatory effect on the pituitary gland of urban than of desert birds [Fokidis and Deviche, 2011]. This difference may account for urban thrashers exhibiting a more robust secretion of CORT than desert thrashers under acute stress. It may also reflect a coping strategy for dealing with prolonged stressful challenges, such as pollution, in an urban setting. Interestingly, CORT responses to exogenous CRH injection did not differ between urban and desert thrashers [Fokidis and Deviche, 2011]. Thus, AVT may provide a means to respond to acute 'urban' stress, while restricting the 'chronic' stress of urbanization. Future work is warranted to compare the CORT responses of urban and desert birds exposed to acute and chronic stress.

The secretion of AVT from magnocellular neurons of the PVN is thought to play an important role in the control of water balance, in a manner comparable to AVP in mammals [Bisset and Chowdrey, 1988]. In this study, we were unable to distinguish between magnocellular and parvocellular AVT-ir cell populations within the PVN. Baseline plasma osmolality in this study was similar to that measured in other bird species [Gray and Erasmus, 1989; Gray and Brown, 1995; Goecke and Goldstein, 1997; Goldstein and Bradshaw, 1998; Saito and Grossmann, 1998; Sharma et al., 2009] and in curve-billed thrashers [Vleck, 1993], and we found neither an association between AVT-ir in the PVN and plasma osmolality nor a population difference in plasma osmolality. However, plasma osmolality was positively correlated with AVT-ir ME staining in urban but not in desert birds. Previous studies in the Japanese quail, *Coturnix coturnix japonica*,

have demonstrated that water deprivation alters the AVT system, including increasing AVT-ir in the PVN [Chaturvedi et al., 1997; Singh and Chaturvedi, 2006], as well as hypothalamic [Chaturvedi et al., 2000] and more specifically, PVN AVT gene expression [Seth et al., 2004].

Peptides secreted into the ME are thought to act on the adenohypophysis and not the neurohypophysis and the significance of this correlation is, therefore, unclear. The lack of a significant difference in plasma osmolality suggests that urban and desert birds did not vary with respect to their water balance. Urban thrashers have access to numerous water sources that persist even during the hot and dry summer, and thus can rely on current water intake as opposed to a water conservation strategy. By contrast, desert birds either rely on often unpredictable precipitation patterns or derive water from dietary sources, such as fruits. These birds will likely require an effective water conservation strategy, at least at certain times of the year. Although there was little difference in plasma osmolality between habitats and no association with AVT-ir in the PVN, it is important to note that plasma osmolality was measured at baseline, and not during osmotic stress. Whether urban and desert thrashers alter osmolality differently during water deprivation warrants further investigation.

Effects of AVT on Territorial Behavior

Thrashers in Phoenix are more responsive to conspecific playback songs than desert birds throughout the year [this study, Fokidis et al., 2011]. Previous research on thrashers found that territorial breeding behavior was not directly associated with acute changes in plasma testosterone or CORT [Fokidis et al., 2011]. Here we report that urban thrashers had less AVT-ir in the BSTM than desert thrashers, and birds that responded quicker to playback had fewer AVT-ir cells in the BSTM than birds that took longer to respond. These data suggest a negative relationship between AVT-ir in the BSTM and aggression. However, we did not observe a 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 and we also did not observe AVT-ir staining in the lateral septum.

The BSTM is considered part of the social behavior network, a collection of interacting basal forebrain and midbrain regions that together function to coordinate behavioral responses to social stimuli [Goodson, 2005]. However, establishing the specific role of the BSTM and of AVT/AVP in aggressive behavior appears complex, as these neuropeptides can inhibit or facilitate aggression depending upon which specific neurons are stimulated [Goodson and Kabelik, 2009; Goodson and Wang, 2006]. Early studies in rats demonstrated that lesions of the BSTM, but not the PVN, decreased AVP fiber staining in the lateral septum, suggesting this was a major site of action for AVP within the BSTM [De Vries and Buijs, 1983]. Recently, microdialysis studies in rats demonstrated that endogenous AVP secretion within the lateral septum correlated with intermale aggression in a resident-intruder paradigm, and this was likely mediated through a V1a receptor [Veenema et al., 2010]. They also report, however, that endogenous AVP release from the BSTM was negatively correlated to this type of aggression. Another recent study in birds, administering a V1a antagonist centrally to block endogenous AVT function in violet-billed waxbills, demonstrated the context-specific nature of AVT's effect on aggression [Goodson et al., 2009]. Antagonist treatment did not alter aggressive behavior during a resident-intruder paradigm, although a possible effect of phenotype (subordinate vs. dominant) was detected [Goodson et al., 2009]. However, similar treatment of birds tested using a mate competition paradigm showed an inhibitory effect of the V1a antagonist on aggression [Goodson et al., 2009].

This study employed a simulated territorial intrusion which is the field equivalent of a resident-intruder test, and our observation of decreased AVT-ir within the BSTM in urban (i.e. more aggressive) thrashers supports an inhibitory effect of AVT on aggression within this brain region. However, our study used solely an auditory stimulus and not a live decoy. The decoy type used in a simulated territorial intrusion may influence the behavioral response of challenged birds [Scriba and Goymann, 2008]. In Lincoln's sparrows, *Melospiza lincolnii*, playback song quality (high vs. low, based on song length, complexity and trill performance) altered AVT-ir in the forebrain, namely the BSTM and lateral septum [Sewall et al., 2010]. Future studies could benefit from further investigating the activity of the central AVT system in different behavioral contexts.

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 the physiological basis of these characteristics. We found differences in the brain distribution of AVT and in the behavior between urban and nonurban birds of the same species. The study adds to a growing body of literature that shows differences in physiology as-

sociated with urbanization. Urbanization can alter the stress physiology and particularly the CORT secretion of vertebrates [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 neural AVT. Other studies have shown the effects of urbanization on behavior. In gregarious species, these effects include increased tameness and approachability [Moller, 2008; Valcarcel and Fernandez-Juricic, 2009], increased vigilance [Partan et al., 2010], changes in singing behavior [Fuller et al., 2007; Slabbekoorn and Ripmeester, 2008] and altered migratory behavior [Partecke and Gwinner, 2007]. Thus, urbanization constitutes an exciting 'natural' experiment to investigate shifts in behavioral phenotypes and the underlying processes that mediate them.

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