

Urban and rural male song sparrows (*Melospiza melodia*) differ in territorial aggression and activation of vasotocin neurons in response to song challenge

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ABSTRACT

When living in urban habitats, ‘urban adapter’ species often show greater aggression toward conspecifics, yet we do not understand the mechanisms underlying this behavioral shift. The neuroendocrine system regulates socio-sexual behaviors including aggression and thus could mediate behavioral responses to urbanization. Indeed, urban male song sparrows (*Melospiza melodia*), which are more territorially aggressive, also have greater abundance of the neuropeptide arginine vasotocin (AVT) in nodes of the brain social behavior network. Higher abundance of AVT could reflect long-term synthesis that underlies baseline territoriality or short-term changes that regulate aggression in response to social challenge. To begin to resolve the timeframe over which the AVT system contributes to habitat differences in aggression we used immediate early gene co-expression as a measure of the activation of AVT neurons. We compared Fos induction in AVT-immunoreactive neurons of the bed nucleus of the stria terminalis (BSTm) and paraventricular nucleus of the hypothalamus (PVN) between urban and rural male song sparrows in response to a short (< 5 min.) or long (> 30 min.) song playback to simulate territorial intrusion by another male. We found that urban males had a higher proportion of Fos-positive AVT neurons in both brain regions compared to rural males, regardless of the duration of song playback. Our results suggest that AVT neurons remain activated in urban males, independently of the duration of social challenge. These findings that Fos induction in AVT neurons differs between rural and urban male song sparrows further implicate this system in regulating behavioral responses to urbanization.

1. Introduction

Anthropogenic habitat disturbance is both a conservation concern and a ‘natural experiment’ that presents an opportunity to study the mechanisms by which organisms adjust to rapid ecological change. Songbirds are well-studied in the context of one form of anthropogenic change, urbanization (Isaksson, 2018; Marzluff, 2001). Ecological studies resolving the aspects of urbanization that impact wildlife including songbirds have demonstrated that ambient noise, light at night, frequent human disturbance, temperature changes, and shifts in predation intensity and prey availability collectively generate novel ecological pressures in urban habitats (Isaksson, 2018; Marzluff, 2001). Though urbanization has been linked with local extinctions, many songbird species successfully adjust to cope with urban habitats (Blair,

1996; Bonier et al., 2007; Both et al., 2006; Marzluff, 2001; Marzluff and Ewing, 2018; McKinney, 2002; Shochat et al., 2010; Sih et al., 2010; Sol et al., 2013; Wong and Candolin, 2015). Behavioral shifts are often the first response to changing environments, and resolving how environmental conditions are transduced into neural mechanisms that regulate behavior is critical for understanding how some species are able to cope with rapid change, and for predicting the limitations of those adjustments (Charmanter et al., 2008; Lowry et al., 2013; Sih, 2013; Sih et al., 2011; Sih et al., 2010; Sol et al., 2013; Wong and Candolin, 2015). Changing environmental conditions can favor certain traits including patterns of behavior (aka. Personalities, behavioral syndromes, coping styles) (Atwell et al., 2012; Koolhaas et al., 2010; Lapiedra et al., 2017; Miranda et al., 2013; Mueller et al., 2013; Partecke and Gwinner, 2007; Sih et al., 2004; Sprau and Dingemanse, 2017). For example, wild

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animals living in suburbs and cities are often more aggressive toward conspecifics, but the mechanisms underlying this behavioral difference are unclear (Atwell et al., 2012; Bonier, 2012; Cockrem, 2005; Evans et al., 2010; Fokidis et al., 2011; Grunst et al., 2019; Riyahi et al., 2017; Wingfield, 2008).

Endocrine mechanisms are a link between organisms' perception of environmental conditions and their physiology and behavior and thus could play a key role in adjusting to urbanization (Cockrem, 2005; Lessells, 2008; Wingfield, 2008). However, few consistent patterns have been found between urbanization and circulating hormone levels (Bonier, 2023; Bonier, 2012; Deviche et al., 2023). For example, though testosterone promotes territoriality and is predicted to be higher in more aggressive urban birds, few studies have found evidence that testosterone mediates increased aggression in urban habitats (Atwell et al., 2014; Davies et al., 2018; Fokidis et al., 2011; Partecke et al., 2005). This may be because the influence of hormones on behavior is determined by interactions with regulatory systems in the brain. Such neuroendocrine mechanisms have been linked to variation in socio-sexual behaviors ranging from species differences in pair bonding and parental care to individual differences in temperament and personality and thus could contribute to stable behavioral traits (Goodson and Kingsbury, 2011; Insel and Young, 2000; Kelly and Goodson, 2014; Kelly and Wilson, 2020; Koolhaas et al., 2010). Specifically, heightened aggression in rodents, songbirds, and fish is associated with higher activity of vasoactive intestinal polypeptide and neuropeptide Y (NPY) but lower activity of vasopressin homologues (Goodson, 1998; Kelly and Wilson, 2020; Goodson et al., 2009; Quintana et al., 2021). Of these neuropeptides, vasopressin and its homologues are best studied for their roles in the regulation of territorial aggression (Kelly and Wilson, 2020). Few studies have compared neuropeptides among urban and rural birds, though, limiting understanding of their contribution to behavioral adjustments to urbanization (Fokidis and Deviche, 2012; Renthlei et al., 2021; Sewall and Davies, 2017).

Of the comparisons of neuropeptides among urban and rural birds that have been made at the time of writing, two found differences in the expression of arginine vasotocin (AVT), a nonapeptide referred to as the avian homologue of vasopressin (Fokidis and Deviche, 2012; Sewall and Davies, 2017). AVT interacts with the androgen and glucocorticoid pathways to contribute to a range of homeostatic functions, including stress responsiveness and osmoregulation mediated largely by the paraventricular nucleus of the hypothalamus (PVN; Goodson et al., 2012; Panzica et al., 2001; Plumari et al., 2004). This nonapeptide also acts within several nodes of the brain social behavior network and the abundance of AVT cell bodies in the medial bed nucleus of the stria terminalis (BSTm) is thought to maintain species and individual differences in sociality (Goodson, 2005; Goodson and Kabelik, 2009; Goodson and Wang, 2006; Newman, 1999; O'Connell and Hofmann, 2011). AVT tone (total AVT-immunoreactive cells) throughout the forebrain is sexually dimorphic, rises seasonally or in response to persistent social challenge, and is associated with an increased baseline aggressive state in territorial species (Goodson et al., 2012; Panzica et al., 2001; Sewall et al., 2010). Both prior urban-rural comparisons demonstrate differences in AVT tone between urban and rural males of territorial species though the relationships among neuropeptide expression, aggression, and urbanization differ (Fokidis and Deviche, 2012; Sewall and Davies, 2017). While urban male song sparrows are more aggressive and have more AVT-immunoreactive cells in the BSTm than rural males (Sewall and Davies, 2017), urban curved-bill thrashers are more aggressive but have less overall AVT-immunoreactivity in the BSTm (Fokidis and Deviche, 2012). It is difficult to draw conclusions from these conflicting studies because the expression of neuropeptides can reflect synthesis, sequestration, or elevated production and secretion (Panzica et al., 2001; Goodson and Kabelik, 2009). The induction of immediate early gene transcription factors such as Fos (the protein product of c-Fos) within AVT neurons can be used to estimate the recent activation of neurons (Clayton, 2000; Herdegen and Leah, 1998). Paradoxically,

though AVT tone increases with seasonal territoriality, Fos expression in AVT neurons is induced by exposure to positive social stimuli such as potential mates and is associated with an acute reduction in aggression in territorial species (Goodson, 2008; Goodson and Kabelik, 2009; Goodson and Wang, 2006). Comparisons of AVT-Fos co-expression between urban and rural birds are a step toward resolving how this system may contribute to differences in both stable baseline and short-term modulation of aggression in response to urbanization.

Our research group previously showed that urban male song sparrows, which are more territorially aggressive than rural males, also had higher AVT tone in the BSTm but not the PVN of the hypothalamus (Davies and Sewall, 2016; Sewall and Davies, 2017; also replicated in this study, Supplementary Materials Table 1). Though our prior findings of differences in the brain and behavior implicate AVT in mediating responses of song sparrows to urban habitats, a limitation of this prior work is that AVT expression can reflect synthesis or sequestration of nonapeptide over an indeterminate time period (Goodson and Bass, 2001; Panzica et al., 2001). To better determine the timeframe over which changes in the AVT system could contribute to differences in aggression in response to social challenge we compared the co-expression of AVT and the immediate early gene Fos between urban and rural males captured as close to baseline as possible (<5 min. of playback) or exposed to sustained (>30 min.) territorial challenge in the form of song playback. Rural male song sparrows are less aggressive in response to song playback, so we predicted that they would have more AVT-Fos co-expressing neurons after sustained song challenge but not near baseline (<5 min. of playback), compared to urban males.

2. Methods

2.1. Subjects

Permission to conduct the procedures described in this study was granted by the US Fish and Wildlife Service, the US Department of the Interior, the State of Virginia's Department of Game and Inland Fisheries, and Virginia Tech's Institutional Animal Care and Use Committee.

We captured a total of 40 male song sparrows in breeding condition from one urban and two rural sites within a group of six established field sites near Blacksburg, VA that are at the extreme ends of a rural-urban gradient defined by land cover but which also differ in human disturbance, ambient noise, and artificial light at night (Davies and Sewall, 2016; Seress et al., 2014). Birds in both populations are year-round residents and our fieldwork coincided with the period of territory establishment. Though the limited number of sites constrains our ability to generalize our findings, it was necessary to minimize the impact on the other long-term study populations. Territory-holding males on our established urban sites are reliably more aggressive (use higher rates of signals of aggressive intent, maintain closer approach distances, and are more likely to attack during a simulated territorial intrusion) throughout the breeding season compared to males at our rural sites (Akçay et al., 2020; Davies and Sewall, 2016) and this pattern has been found in other song sparrow populations (Evans et al., 2010; Foltz et al., 2015; Scales et al., 2011). Males were captured early in the breeding season between 8 April and 28 April 2015.

2.2. Playback treatments

We captured males in mist nets using song playback to attract them. We placed a speaker (Micro II; JBL, Northridge, CA, USA) and mist net in the center of a focal male's territory and played one of 16 conspecific song stimuli (for a description of song stimuli see Davies and Sewall, 2016; Hyman et al., 2004). We captured 10 rural and 10 urban males using as little playback as possible (mean of 0.8 ± 1.240 min.) to compare AVT-Fos co-expression among males from the two habitat types as close to baseline as feasible (hereafter the Baseline Treatment; Fig. 1). Most males heard fewer than three songs but our *a priori* limit of

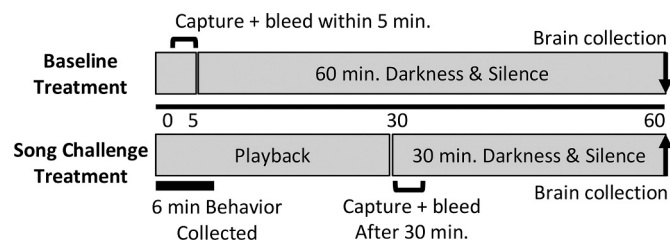


Fig. 1. Experimental design. Birds in the Baseline Treatment were captured with minimal playback (mean of 0.8 ± 1.240 min.) while those in the Sustained Song Challenge Treatment were captured after 30 min. of playback (mean of 35.05 ± 4.872 min.) and were also assayed for aggression. Blood samples were taken immediately after capture and birds were then held in darkness and silence until 60 min. had passed from the onset of playback and brains were collected. The estimated half-life of Fos protein is 60 min.

playback duration for this group was a maximum of 5 min. We captured another 10 rural and 10 urban males as soon after they were exposed to 30 min of playback as possible (mean of 35.05 ± 4.872 min.) to allow comparisons of nonapeptide responses to sustained song challenge (hereafter the Sustained Song Challenge Treatment). Following established protocols for quantifying aggression in song sparrows we collected behavioral data during the first 6 min. of playback (for methods see [Davies and Sewall, 2016](#); [Hyman et al., 2004](#)) from the 20 males in the Sustained Song Challenge Treatment to permit correlations between measures of nonapeptides and aggression ([Fig. 1](#)). We combined behavioral measures previously shown to correlate most strongly with territoriality and conspecific territorial aggression (the number of broadcast songs, soft songs, and wing waves and the average distance to the speaker) into a single ‘aggression score’ using principal components analysis (PCA, see below).

We collected all blood samples within 3 min. of capture, permitting us to quantify plasma testosterone and corticosterone. After collecting blood, we held birds in darkness and silence until we collected brains, as close to 60 min. from the start of playback as possible (mean of 64.575 ± 3.908 min.). We chose this study design to control for the duration each subject was disturbed, at the expense of controlling the duration of darkness and silence ([Fig. 1](#)). However, this period from first contact to brain collection is longer than the estimated half-life of Fos protein (60 min.; [Clayton, 2000](#); [Herdegen and Leah, 1998](#)) and should primarily reflect the experience of subjects in the Baseline Treatment just before capture and those in the Song Challenge Treatment through the song playback. All subjects necessarily experienced brief handling during the period of Fos transcription and translation. We sacrificed males by deeply anesthetizing them with isoflurane before transcardially perfusing them with wash solution (0.9 % NaCl and 0.1 % NaNO₂ in 0.1 M phosphate buffer, PB) using positive pressure from 500 mL syringes, followed by 4 % paraformaldehyde, and removing the brain from the skull. We immersion fixed the brains in paraformaldehyde in the field, transported them on ice, transferred them to 4 °C for 24 h., saturated them in sucrose, flash froze them on dry ice, and stored them at –80 °C until sectioning and immunohistochemistry (IHC) were carried out.

2.3. Plasma hormones

We collected blood samples by venipuncture of the alar wing vein and stored them on ice until they were centrifuged, the plasma separated, and frozen at –80 °C later the same day. Details regarding the quantification of testosterone and corticosterone can be found elsewhere ([Davies et al., 2018](#); [Davies and Sewall, 2016](#)). Briefly, we quantified plasma testosterone and corticosterone using validated enzyme-linked immunoassays (testosterone: ADI-900-065, corticosterone: ADI-900-097, Enzo Life Sciences, Inc., Farmingdale, NY). We ran all samples in duplicate and assigned all of the samples from a given bird to the same assay plate. The average assay sensitivity was 1.1 pg/mL. The average

intra-assay coefficient of variation were 8.1 % for the testosterone assay and 6.6 % for the corticosterone assay. Three samples, 2 from rural males and 1 from an urban male, were of insufficient volume for analysis.

2.4. Histology

We coronally sectioned brains at a thickness of 40 μ m using a cryostat at –21 °C and divided them into three series, which we stored as floating sections in cryoprotectant at –20 °C. We immuno-stained one series of brain sections using a fluorescence double-labeling protocol for Fos and AVT, in two runs, with subjects randomly assigned but treatments balance across runs ([Fig. 2](#)). Briefly, following washes in 1 $\times$ phosphate buffered saline (PBS), we incubated in 5 % normal goat serum in PBS with 0.3 % triton for 1 h to block background immunoreactivity. We then incubated sections for approximately 24 h at 4 °C in rabbit anti-AVP (EMD Millipore, cat. # PC234L at 1:8000) and mouse anti-Fos (Santa Cruz Biotech., cat. # C-10 at 1:1000). Both antibodies have been validated in avian species ([Kabelik et al., 2010](#); [Kelly and Goodson, 2014](#)) and pre-absorption of the anti-AVP antibody with AVT (Cat # V0130, Sigma) before application to brain sections abolished the staining. Following the primary incubation, we washed the tissue in PBS and incubated in secondary biotinylated goat anti-rabbit antibody at 1:250 for 1 h. We again washed the tissue in PBS before incubating in fluorescent secondary antibodies for 2 h (Alexa Fluor 555-conjugated goat anti-mouse and 488-conjugated streptavidin, Thermo Fisher Scientific at 1:200). After mounting on glass microscope slides, we affixed coverslips using ProLong Gold mounting medium containing DAPI (Life Technologies Corp., cat # P36931).

2.5. Imaging and quantification

All quantification of immunoreactivity (ir) for AVT and Fos co-expressing neurons was carried out by research assistants (T. Breeding and A. Wells) blind to the experimental condition of each subject (interscorer consistency $r = 0.93$). We captured three channel fluorescent images of each brain region in both hemispheres using an AxioCam MR camera and Apotome optical sectioning hardware attached to a Zeiss Axioimager microscope (Zeiss, USA; [Fig. 2](#)). The brain regions of interest included the BSTm and the PVN, which we located following our existing protocols ([Campbell et al., 2017](#); [Sewall and Davies, 2017](#); and with reference to [Maney et al., 2008](#)). Briefly, we located the BSTm by imaging the area just dorsal of the anterior commissure. We imaged the PVN by locating the area rostral from the ventral supraoptic decussation and caudal to the anterior commissure and occipitomesencephalic tract fuse. We captured images from every tissue section in which AVT-immunopositive cells were visible (range of 8–15 sections for PVN and 3–5 sections for BSTm) and imaged all brain regions using the 20 $\times$ objective (200 $\times$ total magnification).

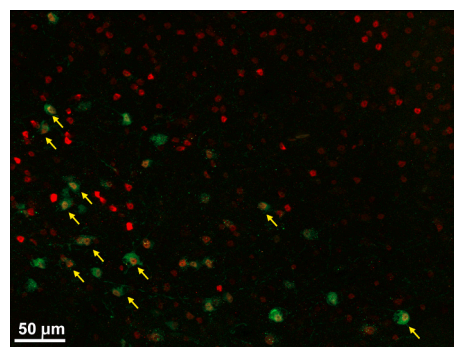


Fig. 2. Neurons in the BSTm showing Fos staining in red, AVT staining in green, and some co-localized staining indicated with arrows.

We counted every cell that was positive for both AVT and Fos in the BSTm and the PVN, after locating and imaging these brain regions as described above (Fig. 2). This strategy should have resulted in counting every AVT and Fos-positive cell within these brain regions, as AVT-expressing cells occur at relatively low density and distribution throughout the brain (Goodson and Bass, 2001; Panzica et al., 1999). We ran all statistics on AVT-expressing cells but report the AVT findings in the Supplementary Material because they replicate our previous results (Sewall and Davies, 2017). For analyses of differences in AVT-Fos co-expression we calculated the proportion of AVT neurons showing Fos induction to account for variation in AVT abundance. This approach should also minimize any contribution of variation due to differences in the number of tissue sections quantified. The BSTm of 3 urban males from the Song Challenge Treatment and the PVN from one of those males was too damaged to permit quantification, reducing our sample size for the Urban Sustained Song Challenge Treatment to 9 males total and the post-hoc comparison for the BSTm to 7 urban males (see below).

2.6. Statistics

We used R statistical software (R Development Core Team, 2008) via RStudio (RStudio Team, 2020) using the lme and lmer packages (Bates et al., 2015) for all analyses. To assess the relationships between habitat type and plasma hormones we ran general linear models (GLMs) with habitat type and playback treatment as factors. The plasma testosterone and corticosterone data did not meet assumptions of normality so we used a conventional log transformation of these data. We also compared behavior across habitat types in the subset of males in the Sustained Song Challenge Treatment using a GLM with habitat as a fixed factor by first conducting a PCA using the four measures of territorial aggression. The PCA of behavioral measures produced four principal components, with the first explaining 39.9 % of the variation in conspecific territorial aggression (Table 1, Supplementary Materials). Though this PCA explained a moderate degree of variation all variables loaded have biological significance (Hyman et al., 2004). Hereafter, we refer to this principal component as the aggression score. Higher territorial aggression scores indicate that a bird maintained a closer average distance from the speaker and performed more soft songs, wing waves, and broadcast songs, which reflects greater territorial signaling and likelihood of attack (Hyman et al., 2004).

To examine habitat differences, effects of playback treatment, and the interaction between the two, on the proportion of AVT neurons showing Fos co-expression (hereafter AVT-Fos co-expression) we ran a general linear mixed model (GLMM) with the proportion of Fos-positive AVT cells as the response variable. This approach accounts for the non-independence of brain measures made from the same individuals. Habitat type (urban or rural), brain region (PVN or BSTm), playback treatment (Baseline or Sustained Song Challenge) and the interactions among all three variables were included as fixed factors, with individuals included as a random factor. We used separate GLMs as post-hoc tests to fully explore any significant effects of habitat, playback treatment, and the interaction between the two within each brain region of interest. We report main effects from the model and all post-hoc comparisons within brain regions of interest in Table 1. We calculated effect sizes as Cohen's *d* for the contrasts evaluated by each GLM using emmeans in R and report those in Table 3 of the Supplementary Materials. To examine how testosterone and corticosterone related to aggression score and AVT-Fos co-expression we ran Pearson's correlations. We also tested for relationships among the absolute duration of playback and the proportion of Fos-positive AVT neurons, as well as AVT-Fos co-expression and aggression score using Pearson's correlations.

Table 1

Effects of habitat and playback exposure on the proportion of Fos and AVT co-expressing neurons. Effects and interactions with significance levels of $p < 0.05$ are indicated in bold text. Non-significant trends of $p < 1$ are indicated with underlining.

	Estimate	Std. Error	t value	P
Intercept	0.108	0.054	1.988	<u>0.051</u>
Habitat	0.156	0.077	2.030	0.046
Playback treatment	0.004	0.002	1.448	0.152
Brain region	0.391	0.072	5.427	<0.0001
Habitat x playback	-0.007	0.004	-1.845	<u>0.069</u>
Habitat x brain region	0.043	0.102	0.420	0.677
Playback x brain region	0.001	0.003	0.112	0.911
Habitat x playback x region	0.005	0.005	1.086	0.285

Post-hoc comparisons of effects of habitat, playback treatment, and the interaction on Fos and AVT co-expression within the PVN:

	Estimate	SE	t value	p value
Intercept	0.499	0.054	9.156	<0.0001
Habitat	0.199	0.077	2.579	0.014
Playback Treatment	0.004	0.003	1.591	0.120
Habitat x Playback	-0.002	0.004	-0.441	0.662

Post-hoc comparisons of effects of habitat, playback treatment, and the interaction on Fos and AVT co-expression within BSTm:

	Estimate	SE	t value	p value
Intercept	0.108	0.054	1.994	<u>0.054</u>
Habitat	0.156	0.076	2.037	<u>0.050</u>
Playback Treatment	0.004	0.002	1.452	0.159
Habitat x Playback	-0.007	0.004	1.859	<u>0.072</u>

3. Results

3.1. Habitat differences in aggression but not plasma hormones

Consistent with prior studies from our lab and others, urban males in the Sustained Song Challenge Treatment were more aggressive during the first 6 min. of playback than rural males (GLM, $t = 2.483$, $P = 0.024$; Fig. 3; Davies and Sewall, 2016; Evans et al., 2010; Foltz et al., 2015). We did not find an effect of habitat, playback treatment, or the

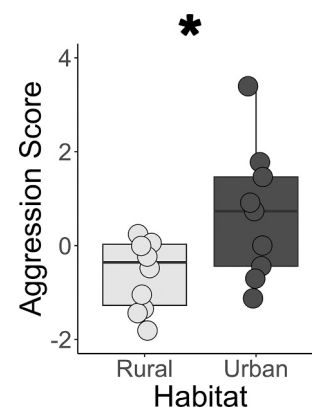


Fig. 3. The aggression score of urban and rural song sparrows from the Sustained Song Playback treatment group. Light grey indicates rural birds and darkest grey indicates urban birds. Urban male song sparrows were more territorially aggressive in response to conspecific song playback challenges than their rural counterparts. Aggression score is calculated as the first component of a PCA analysis; higher territorial aggression scores indicate that a bird maintained a closer average distance from the speaker and performed more soft songs, wing waves, and broadcast songs, which reflects greater aggression. Box and whisker plots indicate the median and first and third quartiles. Asterisks indicate statistically significant differences ($P < 0.05$).

interaction between the two on plasma corticosterone (GLM, all $P > 0.05$). Nor did we find a main effect of habitat on plasma testosterone, regardless of playback treatment (GLM, $t = -1.154$, $P = 0.2567$). However, there was an overall effect of playback treatment on plasma testosterone (GLM, $t = -2.553$, $P = 0.016$) such that all males in the Sustained Song Challenge Treatment had lower plasma testosterone than those in the Baseline Treatment (Fig. 4). Despite the effect of playback treatment on testosterone, there was no correlation between plasma testosterone and aggression score (Pearson's $R = -0.1514$, $P = 0.5756$). Nor was there a correlation between plasma corticosterone and aggression score (Pearson's $R = 0.1792$, $P = 0.4628$). There were no relationships between plasma hormones and the proportion of AVT-Fos co-expressing neurons (all $p > 0.05$).

3.2. Urban male sparrows have greater AVT and Fos Co-expression

AVT-Fos co-expression differed as a function of habitat type and brain region. Specifically, there was a higher proportion of AVT-Fos co-expressing neurons in the PVN than the BSTm, as shown in prior anatomical reports of the avian AVT system (Panzica et al., 1999). Additionally, there was a main effect of habitat on AVT-Fos co-expression in both brain regions such that rural males had a higher proportion of co-expression ($p = 0.046$; Table 1). The effect sizes of all comparisons were near or greater than $\frac{1}{2}$ of the standard deviation indicating a moderate to large effect (Table 2 of Supplementary Materials). There was also a non-significant interaction between habitat type and playback treatment on the proportion of AVT-Fos co-expressing neurons ($p = 0.069$; Table 1). Post-hoc comparisons revealed that this interaction was driven by a non-significant trend in BSTm ($p = 0.072$; Table 1; Fig. 5). Rural males exposed to Sustained Song Playback had a higher proportion of AVT-Fos co-expression than rural males exposed to brief playback, while urban males in the two treatments showed the opposite pattern; in urban males sustained playback was associated with a lower proportion AVT-Fos co-expression compared to baseline. The relationship between the absolute duration of playback (0–55 min. of exposure) and AVT-Fos co-expression was not significant ($r = 0.105$, $p = 0.366$), so the duration of playback did not explain the observed patterns. Nor did AVT-Fos co-expression correlate directly with a male's aggression score for the subset of males in the Sustained Playback Treatment ($r = 0.026$, $p = 0.879$).

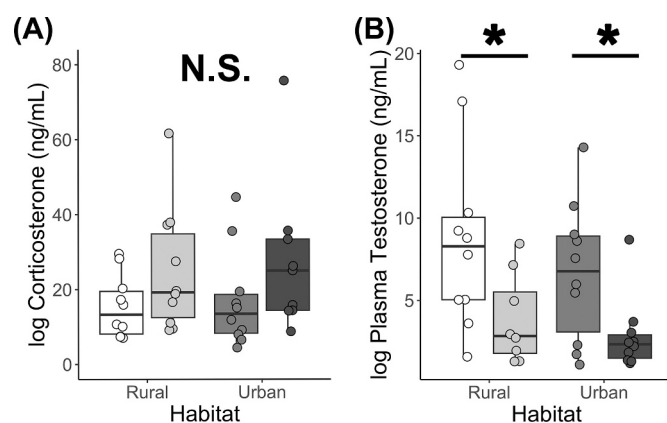


Fig. 4. Log transformed plasma hormone concentrations. White indicates rural birds in the Baseline Treatment, light grey indicates rural birds exposed to Sustained Song Playback, medium grey indicates urban birds in the Baseline Treatment, and darkest grey indicates urban birds exposed to Sustained Song Playback. There was no effect of habitat on (A) plasma corticosterone or (B) testosterone, whether birds were in the Baseline Treatment (light boxes) or the Sustained Song Playback (dark boxes) Treatment. Males exposed to sustained song challenge had lower plasma testosterone than those near baseline (GLM, $t = -2.553$, $P = 0.015$) regardless of habitat. Box and whisker plots indicate the median and first and third quartiles.

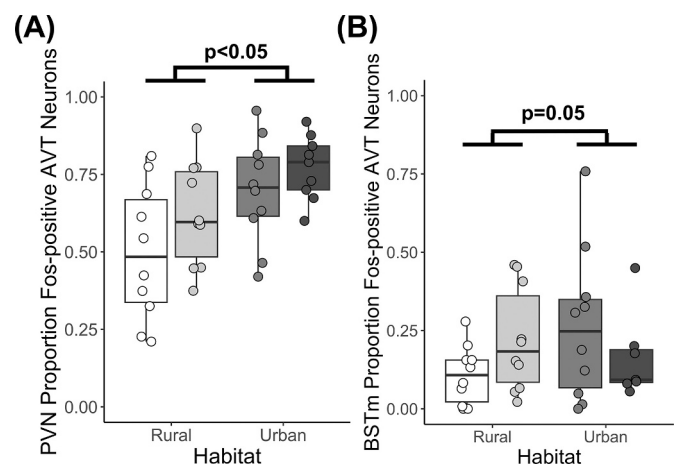


Fig. 5. The proportion of Fos-expressing AVT neurons in rural and urban birds exposed to minimal or sustained conspecific song playback. White indicates rural birds in the Baseline Treatment, light grey indicates rural birds exposed to Sustained Song Playback, medium grey indicates urban birds in the Baseline Treatment, and darkest grey indicates urban birds exposed to Sustained Song Playback. There was a main effect of habitat type on the proportion of Fos-positive AVT neurons in the brains of urban and rural males, and post-hoc tests revealed that urban males had a higher proportion of Fos-positive neurons in (A) paraventricular nucleus of the hypothalamus (PVN) and a borderline trend of a higher proportion in (B) the bed nucleus of the stria terminalis (BSTm). Box and whisker plots indicate the median and first and third quartiles.

4. Discussion

In the present study we found that urban males had a higher proportion of AVT-Fos co-expression in both PVN and BSTm than rural males (Table 1). There was no effect of the duration of song playback on AVT-Fos co-expression (Table 1, Fig. 5). This is consistent with our previous finding that urban males had more AVT tone (total AVT-immunoreactive cells) in BSTm compared to rural males (Sewall and Davies, 2017; see also Supplementary Materials Table 1). Generally, in territorial songbirds, AVT abundance increases seasonally and in response to persistent social challenge to increase stable states of aggression (Goodson et al., 2009; Panzica et al., 2001; Sewall et al., 2010). Presuming the expression of Fos within AVT neurons reflects their activation, our present results indicate that the AVT system of urban males remains activated both near baseline and in response to sustained song challenge. We cannot rule out the possibility that even the very brief playback necessary to capture birds in our Baseline Treatment could induce a neural response and thus it is possible that urban males simply respond more to any duration of song challenge than rural males. Likewise, because this was a field study, we cannot account for the experiences males had prior to playback. We discuss these possibilities below but believe these are less likely to explain our results because there is no relationship between the duration of playback and AVT-Fos co-expression (Table 1), and there is a trend for different patterns of response to Sustained Song Playback in the BSTm of urban and rural males (see below). Thus, our finding that urban males have a higher proportion of Fos-positive AVT neurons is consistent with urban males maintaining a state of heightened activation of the AVT system that may permit greater sensitivity and responsiveness to challenge (Goodson et al., 2012; Goodson and Kabelik, 2009).

The two brain regions we examined are associated with different aspects of perception, motivation, and behavioral response to social and ecological challenges. The PVN is implicated in regulating reproductive and homeostatic states because PVN contains two distinct populations of AVT-producing neurons: magnocellular neurons that are involved in maintaining homeostasis including osmotic balance, and parvocellular neurons that largely project to regions of the social behavior network to

influence behavior (Goodson, 2008; Goodson and Evans, 2004; Goodson and Kabelik, 2009; Panzica et al., 1999). Thus, though AVT neurons within the PVN play an important role in the physiological stress response, they are also sensitive to negative social stimuli (Goodson and Kabelik, 2009). Indeed, a previous study of song sparrow males found increased co-expression of AVT and the immediate early gene *Zenk* in the PVN in response to both a physical stressor (handling restraint) and simulated territorial intrusion (Goodson and Evans, 2004). Subsequent work in other territorial songbird species found that increased immediate early gene and AVT expression was negatively associated with aggression (Goodson and Evans, 2004; Goodson and Kabelik, 2009). This raises the hypothesis that Fos expression in AVT neurons in the PVN reflects the recent perception of aversive stimuli. Thus, our finding that urban males had higher AVT-Fos co-expression than rural males in both treatments could reflect that urban males experience, or are more sensitive to and perceive, more aversive social or ecological conditions prior to and during experimental playbacks. This could result in a higher proportion of their AVT neurons remaining activated across time and different ecological and social conditions (Table 1; Fig. 5). Though our study design does not allow us to rule out the effects of very brief playback or handling stress experienced in both treatments, we see at least two social and abiotic conditions that could contribute to habitat differences in neural responses of urban and rural birds. First, urban males are reliably more aggressive, so it is possible that neighbors challenge each other frequently and urban males are thus more likely to have experienced social challenges prior to our playbacks. Indeed, rural males exposed to sustained song challenge had a qualitatively higher proportion of AVT-Fos co-expressing neurons than those in the Baseline Treatment and thus were similar to urban birds, which could be consistent with greater social challenge activating the AVT system (Supplementary Materials Table 2). Additionally, urban habitats are characterized by frequent noise, light, and human disturbances, all of which could be aversive stimuli. Increased frequency of exposure to social challenges or aversive abiotic conditions in urban areas could contribute to increased activation of the AVT system in urban males. Future studies could attempt to disentangle whether differences in AVT-Fos co-expression are induced by differences in experience (e.g., frequency of disruptions) or perception (sensitivity to disruption) and to determine which population of AVT neurons in PVN are activated in urban males but from our present study design, we can conclude that differences persist across time and context, consistent with chronic activation of AVT neurons in the PVN of urban males.

In contrast to the PVN, the AVT-expressing cell bodies in the BSTm are selectively responsive to social stimuli such that they increase Fos expression in response to positive social stimuli and this activation is thought to inhibit aggression over a period of minutes to hours (Goodson and Wang, 2006). The AVT neurons of the BSTm project to the lateral septum (LS) and septal infusions of AVT inhibit aggression in territorial species (Goodson, 1998; Goodson et al., 2004). Thus, AVT may serve as a negative feedback mechanism that inhibits aggression until appropriate social stimuli reduce AVT and release behavioral inhibition (Panzica et al., 2001; Kelly and Wilson, 2020). Given this framework, it is surprising that more aggressive urban male song sparrows have a trend of a higher proportion of AVT-Fos co-expressing neurons in the BSTm than rural males, regardless of the duration of playback exposure, as this neural pattern should be associated with inhibited aggression. One explanation is that, if activation of AVT neurons in the BSTm reflects the perception of positive social stimuli, urban males may have perceived any song playback (even the very brief playback from the Baseline Treatment) as a positive social stimulus while rural males did not. Alternatively, based on the inhibitory model of AVT action, urban males may be inhibiting aggression at all times because they have higher AVT tone (Supplementary Materials Table 1; Sewall and Davies, 2017) and thus the capacity for aggression, compared to rural males.

A trend worth considering in our data is that the playback treatment may impact AVT-Fos co-expression in the BSTm of urban and rural males

differently (i.e., there was a non-significant interaction between habitat type and playback treatment; Table 1). Though we recognize that this trend can only raise hypotheses for future testing, and it is notable that urban birds showed high variance in AVT-Fos co-expression, this encourages future research. Specifically, rural males had a higher proportion AVT-Fos co-expression in BSTm after Sustained Song Challenge (> 30 min. playback) compared to Baseline (< 5 min. playback), while urban males showed a trend in the opposite direction (Table 1, Fig. 5). Though this effect of song playback duration was not significant it is consistent with other work demonstrating that the AVT system contributes to stable behavioral traits (Goodson et al., 2009; Goodson and Kabelik, 2009; Goodson and Wang, 2006; Kelly and Goodson, 2014; Koolhaas et al., 2010). For example, in violet-eared waxbills (*Uraeginthus granatinus*), less aggressive males have higher AVT-Fos co-expression in response to a challenge, while more aggressive males have equal or decreased AVT-Fos response to challenge (though these differences were found in the PVN, not BSTm; Goodson and Kabelik, 2009). Though we did not detect sufficient variation in AVT-Fos co-expression to draw conclusions, the trend suggests that variation in the activation of the AVT system, as well as differences in AVT tone, could contribute to stable behavioral traits that are suited to urban and rural habitats.

5. Conclusions

The AVT system regulates variation in aggression through at least three mechanisms. First, AVT tone increases seasonally and in response to persistent social challenge (Goodson et al., 2009; Panzica et al., 2001; Sewall et al., 2010). Second, activation of AVT neurons in the social behavior network is induced by exposure to positive social stimuli and is associated with inhibited aggression over a period of minutes to hours (Goodson et al., 2012; Goodson and Kabelik, 2009; Goodson and Wang, 2006). Third, when males of a species display distinct behavioral traits, such as dominant and subordinate roles, differences in the pattern of activation of AVT neurons emerge (Goodson et al., 2009; Goodson and Wang, 2006). Here, we show that the AVT system differs between urban and rural male song sparrows in two of these ways. First, urban male song sparrows, which are more aggressive (Fig. 3; Davies and Sewall, 2016), have higher AVT tone (Supplementary Materials Table 1; Sewall and Davies, 2017). Second, urban males show higher activation of AVT neurons (measured as Fos co-expression), consistent with the AVT system being activated to inhibit aggression (Table 1, Fig. 5). Third, though we only detected a trend, some urban males, which are more aggressive, may show a different pattern of AVT neural activation in response to the duration of song playback, compared to less aggressive rural males (Table 1, Fig. 5). This work demonstrates that urban and rural male song sparrows differ in AVT tone and activation of AVT neurons in response to song playback, and future work should pursue possible differences in the pattern of neural response to challenge that may distinguish male behavioral phenotypes aligned with urban and rural habitat.

Collectively, these findings indicate that the AVT system could play an important role in regulating behavioral responses to anthropogenic change. Though we know that behavioral plasticity is critical for species' tolerance of rapid anthropogenic change, we do not fully understand the mechanisms that permit such flexibility (Bonier, 2023; Kelly and Wilson, 2020). The present study advances this understanding by demonstrating that neuropeptide mechanisms are positioned to contribute to behavioral responses to urbanization by regulating responses to immediate social stimuli, as well as baseline behavioral traits (Cockrem, 2005; Goodson, 2008; Lessells, 2008).

Data availability

Data will be made available on request.

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Author contributions

S.D. and K.B.S. designed and supervised research; S.D., K.B.S., M.L.B., and S.J.L. performed research; S.D. and K.B.S. analyzed data; K.B.S. wrote the manuscript and provided resources; S.D., M.L.B., and S.J.L. provided manuscript feedback.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yhbeh.2023.105438>.

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