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Two Neural Measures Differ between Urban and Rural Song Sparrows after Conspecific Song Playback

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Urbanization is a critical form of environmental change that can affect the physiology and behavior of wild animals and, notably, birds. One behavioral difference between birds living in urban and rural habitats is that urban males show elevated boldness or territorial aggression in response to simulated social challenge. This pattern has been described in several populations of song sparrow, *Melospiza melodia*. Such behavioral differences must be underpinned by differences in the brain, yet little work has explored how urbanization and neural function may be interrelated. We explored the relationship between urbanization and neural activation within a network of brain regions, collectively called the social behavior network, which contributes to the regulation of territorial aggression. Specifically, we captured free-living, territorial male song sparrows by playing them conspecific songs for 6–11 min, and then collected their brains. We estimated recent neural activation, as indicated by the immediate early gene FOS, and measured levels of a neuropeptide, arginine vasotocin (AVT), which is involved in the regulation of social behavior. Based on previous studies we expected urban males, which are generally more territorially aggressive, to have lower FOS expression in a node of the social behavior network implicated in regulating territoriality, the lateral septum (LS). Additionally, we expected urban males to have lower AVT expression in a brain region involved in the regulation of sociality, the medial bed nucleus of the stria terminalis (BSTm). We found that, compared to rural males, urban male song sparrows did have lower FOS expression in the LS. This pattern suggests that lower neural activation in the LS could contribute to behavioral adjustments to urbanization in male song sparrows. Additionally, counter to our predictions, urban male song sparrows had higher AVT-like immunoreactivity in the BSTm. Future work building upon these findings is needed to determine the causal role of such neural differences across rural and urban habitats. Understanding the mechanisms impacted by urbanization will inform our understanding of the reversibility and consequences of this form of habitat change.

Keywords: urbanization, social behavior network, arginine vasotocin, immediate early gene, territorial aggression, song sparrow

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INTRODUCTION

Anthropogenic habitat disturbance is now recognized as impacting the phenotypes of wild animals and is a particular concern for wild birds (Crick, 2004; Both et al., 2006; Caro, 2007; Visser, 2008; Wingfield, 2008; Bonier, 2012; Sol et al., 2013; Wong and Candolin, 2015). Though some species are threatened by anthropogenic habitat disturbance, many animals adjust their behavior

and physiology through phenotypic plasticity to cope with such environmental change (Vitousek et al., 1997; Wingfield, 2008; Bonier, 2012; Sol et al., 2013; Wong and Candolin, 2015). Endocrine mechanisms are a major link between organisms' perception of environmental conditions and behavioral and physiological responses, and thus play a central role in mediating phenotypic plasticity or acclimation to urbanization (Lessells, 2008). There is an urgent need to understand the endocrine and neuroendocrine mechanisms that permit animals to cope with changing environments because elucidating how animals adjust their physiology to urbanization will shed light on why some species persist and others decline when faced with a changing environment (Cockrem, 2005; Visser, 2008; Wingfield, 2008; Whitman and Agrawal, 2009; Engel et al., 2011; Hoffmann and Sgrò, 2011; Wong and Candolin, 2015). Reciprocally, determining how novel urban environments alter neuroendocrine and behavioral phenotypes provides insight into the function and evolution of these traits.

Several species of birds and mammals living in urban habitats are more bold or aggressive toward conspecifics than those living in undisturbed habitats (Warren et al., 2006; Parker and Nilon, 2008; Evans et al., 2010; Fokidis et al., 2011; Atwell et al., 2012). Song sparrows are an excellent illustration of this, as multiple research groups have shown that urban male song sparrows are more aggressive toward conspecifics than their rural counterparts (Evans et al., 2010; Foltz et al., 2015b; Davies and Sewall, 2016). Further, male song sparrows living in urban habitats are more bold in response to heterospecific alarm calls and human approach than are rural males, though only response to alarm calls is correlated with conspecific aggression (Evans et al., 2010; Scales et al., 2011; Myers and Hyman, 2016). Understanding the origins of such differences across habitat types requires addressing both ultimate explanations and proximate mechanisms of behavior. Increased boldness or aggression in urban male song sparrows could be a response to resource availability (Foltz et al., 2015b) or differences in conspecific density (though see Davies and Sewall, 2016). However, regardless of the fitness benefit of the behavior, proximate mechanisms must mediate behavioral differences. Despite repeated demonstrations of behavioral differences in song sparrows living along urban-rural gradients, the neural and physiological basis of differences in aggression are not fully understood (Evans et al., 2010; Foltz et al., 2015a; Davies and Sewall, 2016).

Understanding the mechanisms underlying reliable differences in conspecific territorial aggression across habitats is important for predicting the reversibility and fitness consequences of behavioral responses to urbanization. A first step in this process is to compare measures of the physiological processes that underlie behavior in animals living in different habitats to determine which traits vary and therefore could regulate behavioral changes. Prior studies have failed to find reliable differences in levels of the avian stress hormone, corticosterone, between rural and urban songbirds despite predictions that urban habitats could impact stress reactivity (Partecke et al., 2006; Bonier et al., 2007; Schoech et al., 2007; Fokidis et al., 2009, 2011; Atwell et al., 2012; Bonier, 2012; Foltz et al., 2015a). Nor have consistent differences been reported in levels of testosterone, a hormone traditionally thought to promote aggression in vertebrates (Partecke et al., 2005; Fokidis et al., 2011; Deviche and Davies, 2013; Atwell et al., 2014; Davies and Sewall, 2016; Davies et al., 2016). Failure to find differences in circulating hormone levels suggest that reliable behavioral differences may be underpinned by deeper brain mechanisms, yet little work has explored how urbanization and neural function may be interrelated. Therefore, we explored possible relationships between urbanization and neural activation within brain regions involved in the regulation of social behaviors, namely territoriality and aggression, to identify mechanisms that could be impacted by, or reflect behavioral adjustment to, urbanization.

To identify brain mechanisms that could be impacted by urbanization, or underpin adjustments to novel urban environments, we first measured the expression of the immediate early gene (IEG) FOS, a rapidly inducible transcription factor that is a proxy for recent neural activity (Clayton, 2000), within regions of the brain social behavior network. The social behavior network is a taxonomically conserved, reciprocally connected, network of brain regions that play a central role in regulating patterns of social behavior, including regulating male territorial behavior and aggression, across a wide range of taxa (Newman, 1999; Goodson, 2005; O'Connell and Hofmann, 2011). Despite the central role of the social behavior network in regulating conspecific territorial aggression, it remains unclear whether activation of these brain regions in response to social challenge differs across urban and rural habitats. Therefore, we compared FOS expression in the social behavior network of male song sparrows from urban and rural habitats exposed to conspecific song playback. This commonly employed experimental approach (Jarvis et al., 1997; Clayton, 2000) is a first step toward identifying the brain regions underpinning behavioral adjustments to urbanization. Based on previous studies examining territorial aggression and FOS expression in male song sparrows, we expected to find lower FOS-ir in the lateral septum (LS) and paraventricular nucleus (PVN) of urban sparrows (Goodson et al., 2005b).

Additionally, the neuropeptide arginine vasotocin (AVT), the avian homolog of vasopressin (AVP), plays a central role in the modulation of sociosexual behaviors across taxa (Goodson and Bass, 2001; Insel and Young, 2000). Although the AVT and AVP systems have a range of homeostatic functions, including stress responsiveness and osmoregulation (Simon-Oppermann et al., 1980; Madison et al., 2008), this peptide acts within several nodes of the social behavior network to regulate sex and species-typical behaviors (Santangelo and Bass, 2006; Goodson et al., 2009a; Kabelik et al., 2009). In birds, AVT expressing cell bodies in the bed nucleus of the stria terminalis (BSTm) are selectively responsive to social stimuli and are implicated in regulating male territorial aggression, while AVT in the PVN is involved in homeostatic function (Kiss et al., 1987; Panzica et al., 1999; Plumari et al., 2004; Goodson and Wang, 2006; Goodson et al., 2009b; Fokidis and Deviche, 2012). Therefore, we also examined whether AVT expression differed within the BSTm and PVN of male song sparrows living in rural and urban habitats.

Collectively, our approach was designed to determine how neural activation within the brain related to urbanization and thus which regions could be impacted by habitat. Future work can then assess how activity within these brain regions may be involved in regulating persistent differences in territorial aggression between urban and rural male song sparrows (Evans et al., 2010; Foltz et al., 2015b; Davies and Sewall, 2016). Additionally, this work evaluated whether AVT could be a mechanism impacted by urbanization, as it is known to contribute to the regulation of aggression. Based on previous studies, we expected urban birds to have different patterns of neural expression in the social behavior network compared to rural birds, particularly within the BSTm and lateral septum (LS), regions centrally involved in conspecific aggression and territoriality (Kollack-Walker et al., 1997; Goodson, 1998; Goodson et al., 2009b; Motta et al., 2009; Goodson and Kingsbury, 2011). Additionally, based on previous studies, we expected rural birds to have less AVT within the BSTm, but not the PVN (Fokidis and Deviche, 2012). This work moves us toward understanding how "urban adapters" are able to cope with human-impacted habitats and why some species may be limited in such physiological or neural acclimation.

MATERIALS AND METHODS

Subjects

Permission to conduct the procedures described in this study was granted by the US Fish and Wildlife Service (permit MB08005B-0), the US Department of the Interior (permit 23818), the State of Virginia's Department of Game and Inland Fisheries (permit 053668), and Virginia Tech's Institutional Animal Care and Use Committee (protocol 13-074).

Male song sparrows in breeding condition were captured in the wild from one urban ($N = 9$) and one rural ($N = 7$) site within a group of 9 field sites near Blacksburg, VA that are along a rural urban gradient. We calculated urbanization scores using a technique validated by Seress and colleagues, which uses aerial images to quantify land-cover in a 1 km^2 area around each study site (Seress et al., 2014; Davies and Sewall, 2016; **Figure 1**). Larger PC scores from this analysis indicate higher abundance of buildings and paved surfaces, and lower abundance of vegetation. Though the limited number of sites constrains our ability to generalize our findings, it was necessary to collect birds from only two sites and limit impact on the other long-term study populations (each population consists of ∼20–30 breeding pairs). Males from these urban sites are reliably more territorial throughout the breeding season compared to rural male song sparrows (Davies and Sewall, 2016) and this pattern has been found in other rural and urban populations (Evans et al., 2010; Scales et al., 2011). Males were captured between 11 June and 19 June 2014 by placing a speaker (Micro II; JBL, Northbridge, CA, USA) and mist net in the center of a focal male's territory and playing one of 16 conspecific song playback stimuli to simulate a social challenge to the territory holder. Each playback track consisted of two song types recorded from one of 16 males from a population in Durham, NC, USA. Songs were presented at a rate of 1 song per 10 s at an amplitude of 80 dB, 1 m from the speaker. An average of 8.5 (±2) min of playback was required to capture each male; we did not present longer playback to avoid habituation and to maximize our chance of capturing the territory holder. We were unable to collect behavioral data because of insufficient field assistance, but collected blood samples within 3 min. of capture, permitting us to later quantify plasma testosterone. After collecting blood, we held birds in darkness and silence until we collected brains (ca. 40 min) to allow time for FOS protein translation, while minimizing the contribution of additional stimuli to patterns of neural expression (Herdegen and Leah, 1998). We sacrificed males by deeply anesthetizing them with isoflurane before rapidly decapitating them and removing the brain from the skull. We collected brains ca. 50 min from the start of playback, which is shorter than the experimental timeline of previous studies in song sparrows (e.g., 90 min; Goodson et al., 2005b) but longer than the half life of FOS protein (45 min; Herdegen and Leah, 1998). Thus, though the FOS expression we quantified was at least partially induced by hearing and responding to conspecific song, comparison with other FOS studies in song sparrows may be limited. We fixed the brains by immersion in acrolein for 4 h, saturated them in sucrose, flash froze them on dry ice, and stored them at −80°C until sectioning and immunohistochemistry (IHC) was carried out.

Plasma Testosterone

We collected blood samples by veinipuncture 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. We measured plasma testosterone using enzyme-linked immunoassay kits (ADI-900-065, Enzo Life Sciences, Farmingdale, NY, USA), which we have optimized and validated for use with song sparrow plasma (Davies and Sewall, 2016). Briefly, we diluted samples 30 times, added steroid displacement reagent at 0.5% of plasma volume, randomly assigned samples to

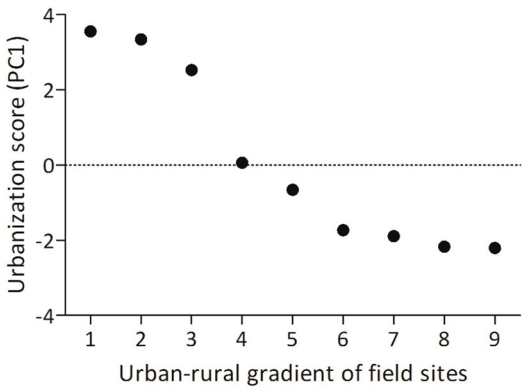

FIGURE 1 | Birds in this study were collected from one urban site (number 1) and one rural site (number 9, above) along an urban rural gradient within 15 km of Blacksburg, VA, USA. The urbanization index for each site was calculated following Seress et al. (2014); larger PC scores on the y-axis indicate higher abundance of buildings and paved surfaces, and lower abundance of vegetation.

assay plates ($N = 3$ plates), and assayed all samples in duplicate. The average assay sensitivity was 1.1 pg/mL. The average intra- and inter-assay coefficients of variation were 8.1 and 5.7%, respectively.

Histology

We coronally sectioned brains at a thickness of 40 μm 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 two series of brain sections separately, one for FOS and one for AVT, in two runs per antigen, with subjects randomly assigned to runs. For both AVT and FOS, following washes in 1× phosphate buffered saline (PBS), we incubated in 1% sodium borohydride for 30 min to unmask antigens. In the case of AVT, but not FOS, we then incubated three times, each for 5 min, in freshly boiled citrate buffer (10 mM citric acid, 0.05% tween 20, pH 6.0). We then incubated both AVT and FOS sections in 0.3% hydrogen peroxide for 30 min to quench endogenous peroxidase activity, and then 5% normal goat serum for 1 h to block background immunoreactivity. We then incubated sections for ∼24 h at 4°C in either rabbit anti-FOS (Santa Cruz Biotechnology, Dallas, TX, USA, cat. # sc-253 at 1:5,000) or guinea pig anti-AVP (Penisula Laboratories International, San Carlos, CA, USA, cat. # T-5048 at 1:5,000, previously distributed by Bachem, Torrence, CA). Following the primary incubation, we blocked endogenous aviden and biotin by incubating for 15 min in avidin/biotin blocking reagent (Vector Laboratories, Burlingame, CA, USA), then incubated in biotinylated secondary antibody for 1 h (FOS: biotinylated goat anti-rabbit at 1:500; AVT: goat anti-guinea pig at 1:250; Vector Laboratories, Burlingame, CA, USA). We next incubated sections for 1 h in avidin-biotin complex (ABC Vectastain Elite kit at 1:200; Vector Laboratories, Burlingame, CA, USA), then visualized labeling by incubating for 1 min in 3, 3–diaminobenzidine chromagen (Vector Laboratories, Burlingame, CA, USA). Between each incubation described above, we washed in PBS. After mounting on glass microscope slides, we allowed immunolabeled sections to dry at room temperature for 24 h before dehydrating through a graded ethanol series, clearing in xylenes, and affixing coverslips using Permount mounting medium (Fisher Scientific, Pittsburg, PA, USA).

Imaging and Quantification

All quantification of immunoreactivity (ir) for FOS and AVT was carried out by research assistants (T. Breeding and A. Wells) blind to the experimental condition of each subject. We captured gray scale images of each brain region using an AxioCam MR camera attached to a Zeiss Axioimager microscope (Zeiss, USA). The brain regions of interest included the anterior hypothalamus (AH); medial preoptic area (POM); medial extended amygdala, which includes the medial bed nucleus of the stria terminalis (BSTm) and nucleus taeniae (TnA); lateral septum, specifically the caudal ventrolateral portion (LSc.vl; Goodson et al., 2004); ventral tegmental area (VTA); central gray (CG); ventromedial hypothalamus (VMH), which consists of both a lateral and medial portion (Goodson, 2005; Maney et al., 2008); and the paraventricular nucleus of the hypothalamus (PVN). To quantify FOS immunoreactivity (ir) we imaged all brain regions using the 20× objective (200× total magnification).

We located the social behavior network regions following Maney et al. (2008) and references therein. Specifically, we located the AH following Kuenzel and van Tienhoven (1982) and quantified FOS-ir in the region dorsal to the dorsal supraoptic decussation. We located the POM with reference to Alger and Riters (2006) and quantified the region medial to the septomesencephalic tract. To identify the BSTm, we followed Aste et al. (1998) and Maney et al. (2008) and we placed the counting circle dorsal to the anterior commissure. We used Cheng et al. (1999) and Stokes et al. (1974) to locate the TnA. For the caudal ventrolateral portion of the LS, we referenced Goodson et al. (2004) and placed the counting circle medial to the lateral ventricle and beginning rostrally at the level of the anterior commissure. To identify the VTA and CG, we followed Heimovics and Riters (2005) and LeBlanc et al. (2007). For the VTA, we measured the region lateral to the rostral extent of the oculomotor nerve, and for the CG we measured FOS-ir within this region when it resembled the shape of a dolphin's tail. We identified the lateral and medial portions of the VMH at the level of the median eminence following Goodson et al. (2005a) and Maney et al. (2008). We quantified FOS-ir in the PVN starting rostrally from the ventral supraoptic decussation and caudally until the anterior commissure and occipitomesencephalic tract fuse following Davies et al. (2015). We made cell counts within a 0.20 mm^2 circle placed within the AM, BSTm, VTA, and PVN; a 0.15 mm^2 circle in CG and TnA; a 0.13 mm^2 circle inside VMH-m and VMH-l; a 0.05 mm^2 circle placed within the LSc.vl; and a 0.03 mm^2 circle placed in POM. We made counts in the medial most tissue sections of each brain region of interest for a total of 2 sections in AM; 3 sections in CG, POM, VTA, VMH-l, and VMH-m; 4 sections in LSc.vl; 5 sections in BSTm and TnA; and 6 sections in PVN. We used Image J software (ver. 3.1, National Institutes of Health) to view and manually count immunoreactive cells that were visible within the counting frame. All FOS data were calculated as the total number of immunoreactive cells per mm^2.

To quantify AVT-containing cells we counted every immunopositive cell in the BSTm and the PVN, after locating and imaging these brain regions as described above. These data are reported as total cell counts per brain region for each bird. The number of sections in which the brain region of interest was observed is included in analyses (see below) because the value differed across subjects. Though the antibody we used has been validated in avian species (e.g., Kabelik et al., 2010; Kelly and Goodson, 2014) staining in the BSTm was light (**Figure 2**). This could be because of how we prepared the tissue, the ephemeral nature of this cluster of AVT neurons (reviewed in Goodson and Kingsbury, 2011), or cross-reactivity with other non-apeptides. To be conservative, we refer to this hereafter as AVT-like immunoreactivity.

Statistics

We used R statistical software (R Development Core Team, 2008) for all analyses. First, to assess the relationship between habitat type and plasma testosterone we ran a general linear model (GLM) with habitat as a factor and playback duration and the

day of year as covariates. We also examined possible relationships between testosterone and AVT cell numbers in the BSTm and PVN using two separate Pearson's correlations.

Because our data are hierarchically structured and to minimize the number of comparisons, we ran one general linear mixed model (GLMM) and one generalized linear mixed model using the lme and lmer package in R (Bates et al., 2015). To examine habitat differences in FOS-ir within the social behavior network we ran a GLMM with habitat type and brain region as fixed factors. We included individual as a random factor to account for the non-independence of samples and nested the number of sections measured per brain region within individual because different numbers of sections were quantified for different brain regions. We used a default Gaussian distribution. The covariates in this model were the day of year, plasma testosterone, and playback duration because reproductive condition, exposure to playback, and hormonal status can influence IEG response (Goodson and Evans, 2004; Goodson et al., 2005b; Maney et al., 2008).

We ran a generalized linear mixed model for AVT in which we tested whether total counts of all AVT cells within a brain region of interest were explained by habitat type, brain region, or the interaction between the two. We specified individual as a random factor to account for non-independence of cell counts. We specified a Poisson distribution for count data (which was supported by reduced residual deviance; Crawley, 2007). We included plasma testosterone and the day of the year as covariates because of the interaction between the testosterone and AVT systems and the effects of breeding state on AVT expression (Panzica et al., 2001). Additionally, we coded the number of tissue sections in which the brain region of interested was found as a covariate because this value differed both between subjects and across brain regions and therefore was not hierarchically structured.

Lastly, we ran separate general or generalized linear models as *post-hoc* tests for each neural marker and brain region of interest, specifying the same covariates and distributions as the main models. We report only main effects from initial models and the main effects of habitat type on measures within each brain region in **Tables 1, 2** but include all model outputs in the Supplementary Materials.

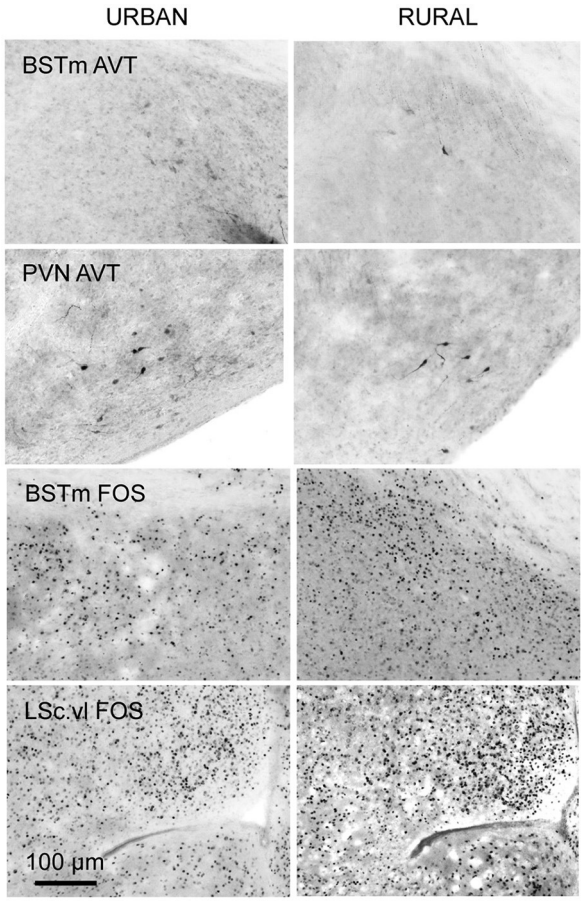

FIGURE 2 | Coronal sections showing examples of AVT-like (top two rows) and FOS induction (bottom two rows) in the BSTm, PVN, and LSc.vl from urban (left column) and rural (right column) subjects. Urban birds had greater AVT-like staining in the BSTm than rural birds but less FOS-ir in the BSTm and LSc.vl than rural birds.

RESULTS

Plasma Testosterone

Though there was a trend for the day of sampling to impact plasma testosterone such that testosterone was counter-intuitively higher later in the season (GLM, effect of day of year, $t = 2.093$, $P = 0.058$), there were no overall differences in testosterone levels as a function of habitat type when day of year was taken into account (mean ± SEM: rural 2.36 ± 0.717 ng/mL;

TABLE 1 | Effects of habitat on FOS-ir quantified as cells per mm^2.

	Estimate	Std. Error	t-value	*P*
Intercept	−2,096.411	928.705	−2.257	**0.028**
Habitat	−2.477	52.105	−0.048	0.963
Brain region	116.402	39.359	2.957	**0.005**
Day of year	14.283	5.684	2.512	**0.029**
Playback duration	2.618	2.797	0.936	0.354
Testosterone	−5.932	12.847	−0.462	0.653
Post-hoc comparisons of effects of habitat on FOS-ir within brain regions (habitat × brain region):				
x AM	26.540	67.801	0.391	0.707
x BSTm	−126.506	58.189	−2.174	0.058
x CG	25.747	90.741	0.284	0.784
x LSc.vl	−126.194	40.638	−3.105	**0.013**
x POM	−64.151	39.246	−1.635	0.137
x PVN	−47.491	96.123	−0.494	0.635
x VMH-l	−102.423	62.235	−1.646	0.151
x VMH-m	−106.808	76.223	−1.401	0.211
x VTA	−41.776	58.080	−0.719	0.495
x TnA	40.998	34.179	1.200	0.258

AM was coded as brain region 1, rural habitat was coded as 0 and urban as 1. Bold text indicates significant ($p < 0.05$) effects and underlined text indicates trends ($p < 0.06$).

TABLE 2 | Effects of habitat on AVT-ir quantified in the PVN and BSTm.

	Estimate	Std. Error	Z-value	*P*
Intercept	−4.188	5.394	−0.776	0.437
Habitat	1.041	0.284	3.665	**<0.001**
Brain region	3.814	0.190	20.072	**<0.001**
Day of year	0.037	0.033	1.115	0.265
Testosterone	−0.207	0.094	−2.205	**0.027**
Number of tissue sections	−0.028	0.052	−0.545	0.586
Region x Habitat	−1.020	0.216	−4.714	**<0.001**
Post-hoc comparisons of effects of habitat on AVT-ir within brain regions:				
x BSTm	1.355	0.243	5.572	**<0.001**
x PVN	0.2059	0.039	1.478	0.139

The log of plasma testosterone was included as a covariate in the main model and post-hoc GLMs. Rural habitat was coded as 0 and urban as 1; BSTm was coded as brain region 1 and PVN as brain region 2. All cells within each brain region of interest were counted and a Poisson distribution was specified for count data. Bold text indicates significant ($p < 0.05$) effects.

urban 1.35 ± 0.198 ng/mL; GLM effect of habitat, $t = -0.892$, $P = 0.389$). Nor was testosterone correlated with the number of cells showing AVT-like immunoreactivity in either the BSTm ($R = -0.306$, $p = 0.287$) or PVN ($R = -0.325$, $p = 0.279$).

FOS Immunoreactivity

We found a significant effect of habitat type on FOS-ir in the LSc.vl (GLMM, habitat x LSc.vl, $t = -3.105$, $P = 0.013$) and, nearly, in the BSTm (GLMM, habitat x BSTm, $t = -2.174$, $P = 0.058$; **Table 1**; **Figure 3**). Relative to urban birds, rural birds had more FOS-ir neurons in the LSc.vl and a trend of more in the BSTm. Though there was no overall effect of habitat on the number of FOS-ir neurons, the date of the sampling contributed to FOS-ir throughout the brain such that birds collected later in the season had higher FOS-ir (GLMM, effect of day of year, $t = 2.512$, $P = 0.029$; **Table 1**) and there were overall differences in expression across brain regions (GLMM, effect of brain region, $t = 2.957$, $P = 0.005$; **Table 1**). Neither the duration of playback nor plasma testosterone levels significantly explained the number of FOS-ir neurons in the main model or *post-hoc* tests (**Table 1**; Supplemental Materials).

AVT Immunoreactivity

We found a significant effect of habitat on AVT-like immunoreactivity (GLMM, effect of habitat, $z = 3.665$, $p < 0.001$; **Table 2**). Additionally, AVT-like immunoreactivity differed across brain regions (GLMM, effect of brain region, $z = 20.072$, $p < 0.001$), with far higher cell numbers in the PVN than the BSTm (**Figure 4**). Of most importance, there was an interaction between habitat and brain region such that urban male song sparrows had more cells showing AVT-like immunoreactivity in BSTm than rural males (GLMM, habitat x BSTm, $z = 5.572$, $p < 0.001$) though this did not hold in the PVN (**Table 2**; **Figure 4**). Finally, testosterone levels contributed to differences in AVT-like immunoreactivity (GLMM, effect of testosterone, $z = -2.205$, $p = 0.027$).

DISCUSSION

We found that male song sparrows living in urban habitats of Blacksburg, VA have different patterns of immediate early gene and neuropeptide levels in their brains in response to hearing conspecific song, compared to birds living in rural habitats. This work demonstrates that urbanization has impacts on neural processes, and is an important first step toward understanding how habitat change and neural function are interrelated; it is among only a handful of studies exploring how urbanization impacts the brains of wild birds (Maklakov et al., 2011; Fokidis and Deviche, 2012; Davies et al., 2016). The present findings raise two specific hypotheses about how urbanization and neural processes may be interrelated. First, associations between FOS expression and urbanization implicate the social behavior network in the regulation of persistent differences in territorial behavior between urban and rural male song sparrows (Evans et al., 2010; Foltz et al., 2015b; Davies and Sewall, 2016). Second, the finding of elevated AVT-like immunoreactivity in the BSTm of urban male song sparrows supports the hypothesis that these socially sensitive AVT neurons (Goodson et al., 2009b) could also contribute to maintaining behavioral differences between urban and rural song sparrow populations.

Habitat Differences in Neural Response to Conspecific Song

Patterns of IEG expression in the social behavioral network are associated with both perception of social stimuli and behavioral response to those stimuli, with the result that measures differ across social contexts and also as a function of a focal animals' phenotype (Goodson and Wang, 2006; Maney et al., 2008; Kabelik et al., 2009; Goodson and Kingsbury, 2011). Previous studies have found that simulated territory intrusions increase IEG expression in the BSTm, LS, PVN, AH, VMH, CG, and VTA of territorial birds (Maney and Ball, 2003; Goodson and Evans, 2004; Goodson et al., 2005b). However, aggressive response to social challenge is negatively correlated with IEG responses in the AH, PVN, and multiples zones of the LS (Goodson et al., 2005b; Goodson and Kingsbury, 2011) in territorial male song sparrows both during and outside of the breeding season, implicating these regions in the maintenance of persistent differences in territorial behavior (Goodson and Evans, 2004; Goodson et al., 2005b; Goodson and Kingsbury, 2011).

The primary finding in the present study was that urban male song sparrows, who are reliably more territorially aggressive (Evans et al., 2010; Foltz et al., 2015b; Davies and Sewall, 2016), had fewer FOS-ir neurons in the LSc.vl than their rural counterparts (**Table 1**; **Figure 4**). Thus, our finding is consistent with patterns of lower FOS expression in the LSc.vl of animals that show elevated territorial aggression and suggest this region is involved in behavioral adjustments to living in urban areas. Though we also expected to find this pattern of FOS expression across other nodes of the social behavior network (e.g., the AH and PVN; Goodson et al., 2005b) we may not have seen this pattern because our playback was shorter than prior studies in song sparrows (10 min instead of 30 min; Goodson and Evans, 2004; Goodson et al., 2005b; though see Maney and Ball, 2003

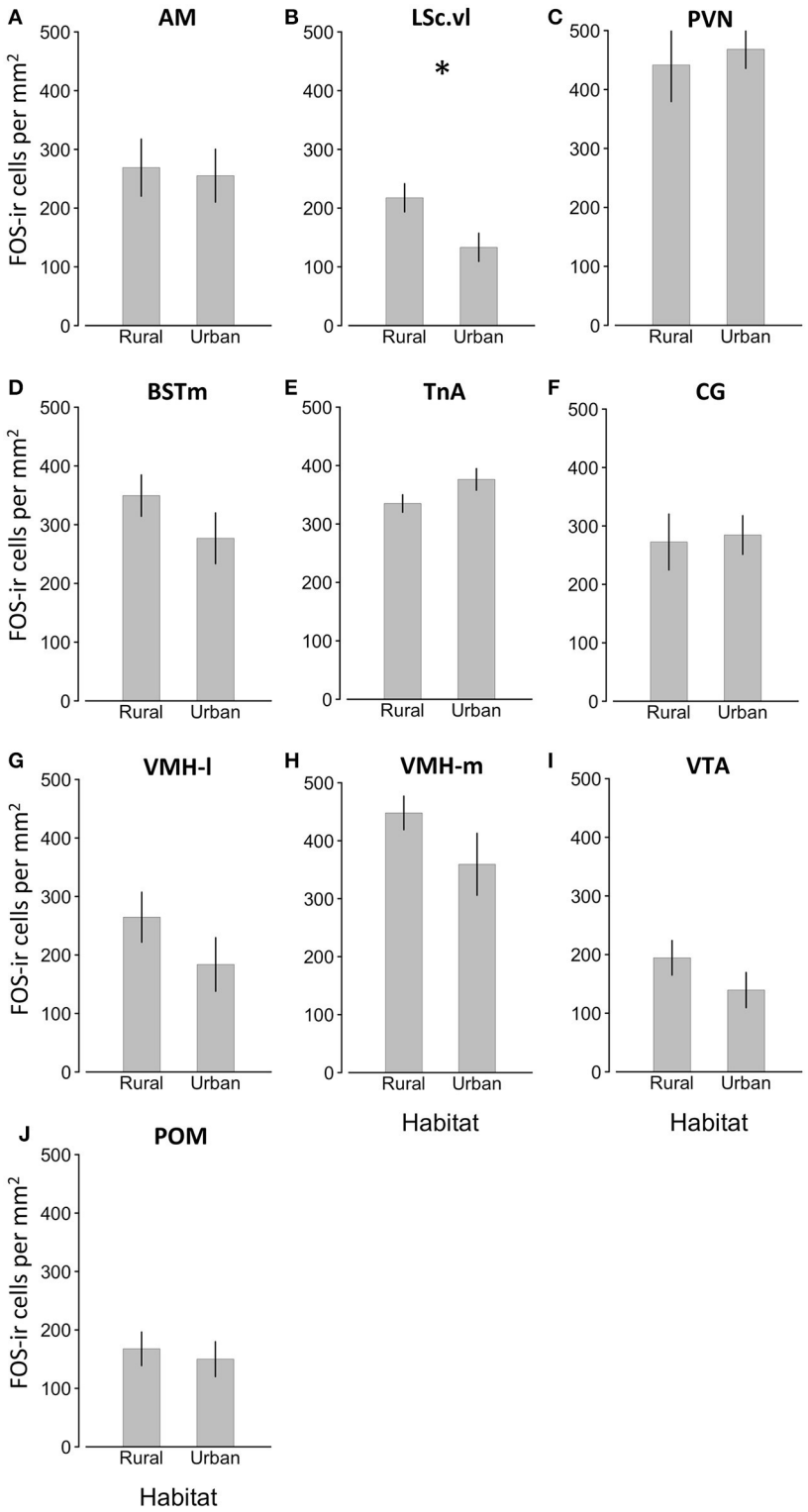

FIGURE 3 | Main effects of habitat type on FOS-ir ±SEM within nine brain regions of interest (A–J) Effects of day of year, duration of playback, relationship with plasma testosterone, and controls for the number of tissue sections in which we found the brain regions of interest were included in the model, but are not represented. Asterisk denotes significant difference between groups.

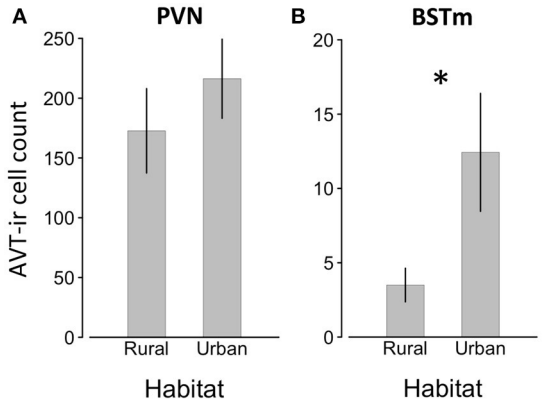

FIGURE 4 | Number of AVT positive cells ±SEM in the PVN (A) and BSTSm **(B)** in male song sparrows collected in rural and urban habitats. Asterisk denotes significant difference between groups.

who also used 10 min) or because capturing males in the wild using playback may unavoidably select the most aggressive birds from each population, minimizing our chances of detecting differences (though thoroughly sampling only two populations should have minimized this risk). Future studies should test the hypothesis that neural activity in the LSc.vl contributes to the modulation of territoriality across rural and urban habitats by comparing FOS expression within this brain region in passively caught subjects from each habitat, as well as by correlating expression with experimental subjects' behavioral responses to song challenge.

While FOS-ir in the LSc.vl is associated with differences in aggressive behavior, FOS-ir in the BSTm may reflect the perception of social challenge, but not the regulation of behavioral responses. It is difficult to disentangle neural responses induced by the perception of a social challenge from those induced by behavioral response to challenge, but previous studies in song sparrows have found correlations between IEG expression in the BSTm and exposure to song playback, but not behavioral response to challenge (Goodson and Evans, 2004; Goodson et al., 2005b). Working from the hypothesis that FOS-ir in the BSTm is positively associated with the perception of social challenge, our results suggest that rural and urban birds perceived playback differently. Specifically, the duration of playback did not impact FOS-ir in the BSTm (**Table 1**; Supplemental materials), yet there was a trend of elevated FOS-ir in the BSTm of rural birds relative to urban birds. This could be interpreted as rural birds perceiving the playback as a greater social challenge than did urban birds. This hypothesis could be tested by presenting birds from different habitats with social challenges of varying intensity such as familiar and unfamiliar conspecifics (Stoddard et al., 1990).

Habitat Differences in AVT-Like Immunoreactivity

Like FOS, the relationship between AVT within the BSTm and aggression can depend upon context and phenotype (Goodson et al., 2009a,b; Kabelik et al., 2009). However, generally, elevated AVT is associated with decreased territorial aggression in breeding, territorial male songbirds. Septal infusions of AVT reduce male-male aggression (Goodson, 1998; Kabelik et al., 2009). Further, studies using double labeling of cells with IEGs and AVT show that AVT neurons in the BSTm are activated by positive social stimuli, such as a potential mate (Goodson and Wang, 2006; Goodson et al., 2009a; Goodson and Kingsbury, 2011). Here, we found that urban birds, which are persistently more territorially aggressive, had more AVT-like immunoreactive neurons in the BSTm, but not the PVN. While AVT levels in the BSTm are associated with territoriality and aggression, AVT in the PVN is involved in maintaining homeostasis (Simon-Oppermann et al., 1980; Madison et al., 2008; Goodson et al., 2009b; Fokidis and Deviche, 2012). Thus, our finding is seemingly counter to studies that used septal manipulations of AVT and found decreased aggression (Goodson, 1998). However, immunoreactivity can reflect elevated production and secretion of the neuropeptide, or it can indicate an accumulation of peptide due to reduced secretion, making it difficult to interpret the functional significance of greater staining (Panzica et al., 2001; Goodson and Kabelik, 2009; Sewall et al., 2010). One interpretation is that urban male song sparrows were sequestering AVT within cell bodies in the BSTm, reducing bioavailable AVT, which would be consistent with lower AVT levels increasing territorial aggression. However, the present finding is counter to that of Fokidis and Deviche (2012), who reported that urban curve-billed thrashers, *Toxostoma curvirostre*, had significantly less AVT staining in the BSTm than desert thrashers and that AVT staining was also inversely related to territorial aggression. Overall, greater AVT-like staining in the BSTm of urban birds is counter to our predictions and the findings of a prior study, so resolving the contribution of the AVT system to behavioral differences associated with urbanization will require double-labeling cells for AVT and IEGs. The present results implicate AVT within socially-sensitive neurons of the BSTm in regulating well-described differences in territorial behavior between rural and urban male song sparrows, but more work is needed to resolve the direction of this relationship. Moreover, additional mechanisms, such as serotonin could contribute to the regulation of territorial aggression (Nelson and Trainor, 2007). Future work is needed to understand how multiple regulatory pathways are integrated to yield differences in territorial aggression in urban and rural song sparrows (Cohen et al., 2012). Further, given that species differ in their behavioral responses to urbanization and multiple physiological pathways could yield similar behavioral outcomes, our understanding of the proximate mechanisms that permit animals to cope with changing habitats is only in its infancy.

CONCLUSIONS

Urbanization impacts the physiology and behavior of free-living animals and is a particular concern for birds (Wingfield, 2008; Bonier, 2012; Sol et al., 2013; Wong and Candolin, 2015). Determining the mechanistic basis of behavioral responses to urbanization is essential to predicting the consequences and limitations of organismal reactions to habitat disturbance and

will ultimately help us understand why some species adjust and others decline in the face of environmental change. Here, we found that, compared to rural male song sparrows, urban song sparrows had reduced expression of the IEG FOS within a node of the social behavior network of the brain (the LS), and also more AVT-like immunoreactive neurons within the BSTm. This study adds to the growing evidence that urbanization impacts the brain to influence behavior. We expect future work in this area will identify a vast number of behavioral responses to urbanization and also considerable diversity in the proximate mechanisms that permit such adjustments.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the US Geological Service's bird banding lab and the National Research Council's Guide for the care and use of laboratory animals. The protocol was approved by the Virginia Tech's Institutional Animal Care and Use Committee (protocol 13-074), the US Fish and Wildlife Service (permit MB08005B-0), the US Department of the Interior (permit 23818), the State of Virginia's Department of Game and Inland Fisheries (permit 053668).

AUTHOR CONTRIBUTIONS

KS conducted field work, sample collection, data analysis, and wrote the majority of the paper. SD completed all histology, enzyme immunoassay, immunohistochemistry, quantification, and assisted with editing the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00046/full#supplementary-material

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