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Song environment affects singing effort and vasotocin immunoreactivity in the forebrain of male Lincoln's sparrows

Kendra B. Sewall^{1,*}, Elyse C. Dankoski², and Keith W. Sockman^{1,2,*}

¹ Department of Biology, University of North Carolina, Chapel Hill, NC 27599, U.S.A

² Curriculum in Neurobiology, University of North Carolina, Chapel Hill, NC 27599, U.S.A

Abstract

Male songbirds often establish territories and attract mates by singing, and some song features can reflect the singer's condition or quality. The quality of the song environment can change, so male songbirds should benefit from assessing the competitiveness of the song environment and appropriately adjusting their own singing behavior and the neural substrates by which song is controlled. In a wide range of taxa social modulation of behavior is partly mediated by the arginine vasopressin or vasotocin (AVP/AVT) systems. To examine the modulation of singing behavior in response to the quality of the song environment we compared the song output of laboratory-housed male Lincoln's sparrows (*Melospiza lincolnii*) exposed to one week of chronic playback of songs categorized as either high or low quality, based on song length, complexity and trill performance. To explore the neural basis of any facultative shifts in behavior, we also quantified the subjects' AVT immunoreactivity (AVT-IR) in three forebrain regions that regulate socio-sexual behavior: the medial bed nucleus of the stria terminalis (BSTm), the lateral septum (LS) and the preoptic area. We found that high quality songs increased singing effort and reduced AVT-IR in the BSTm and LS, relative to low quality songs. The effect of the quality of the song environment on both singing effort and forebrain AVT-IR raises the hypothesis that AVT within these brain regions plays a role in the modulation of behavior in response to competition that individual males may assess from the prevailing song environment.

Keywords

arginine vasotocin (AVT); bird song; competition; sexual selection; social behavior network

INTRODUCTION

In many species of songbirds, males attract mates, compete for territories and respond to challenges from neighbors by singing (Catchpole and Slater, 1995; McGregor, 1991). Though females are often the intended receivers of song, neighboring males may evaluate their competitors based on their singing behavior (Cramer and Price, 2007; DuBois et al., 2009; Illes et al., 2006; McGregor and Peake, 2000; Naguib and Dietmar, 1997; Sockman et al., 2009). Ecological factors can affect male condition and singing behavior, generating

*Corresponding authors: Kendra B. Sewall, Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280, USA, ksewall@email.unc.edu, Phone: 919-843-8236. Keith W. Sockman, Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280, U.S.A., kws@unc.edu, Phone: 919-843-1989.

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variation in the quantity and quality of songs prevalent in a particular environment (Searcy and Nowicki, 2005; Sockman, 2009). Therefore, males should benefit from modulating their own singing behavior and the neural substrates that control it according to the level of competition they assess based on the quality of the song environment (DuBois et al., 2009; Schmidt et al., 2008; Sockman et al., 2009).

Male songbirds could evaluate their competitors using a number of aspects of their songs, including singing rate, repertoire size, song complexity, song length and performance of trills. Singing rate may be associated with males' physical competence and motivation (Alatalo et al., 1990; Lambrechts, 1996; Zahavi, 1975). Repertoire size (the number of distinct song types that a male can produce), song complexity (the number of distinct syllable types within a song) and song length (or total syllable count) can be correlated with males' learning abilities, age, immunocompetence, or physical condition (Catchpole, 1980; Duffy and Ball, 2002; Feare, 1984; Gentner and Hulse, 2000; Hasselquist et al., 1996; Naguib et al., 2008; Nowicki and Searcy, 2005; Wasserman and Cigliano, 1991). Trills, which are rapidly repeated syllables within a song, can be associated with male quality because the performance of trills may be constrained by a trade-off between the rate of syllable repetition and the frequency bandwidth of the signal (Podos, 1997, but see, Cardoso et al., 2007). Male songbirds that produce trills nearer the theoretical limit of performance may be older, in better condition or preferred by females (Ballentine, 2006; Ballentine et al., 2004; Ballentine, 2009; Dalziell and Cockburn, 2008; de Kort et al., 2009; Forstmeier et al., 2006; Nowicki et al., 2002; Vallet and Kreutzer, 1995). Male songbirds may not be able to modulate aspects of song that are linked to their age or learning abilities, such as repertoire size, song complexity, song length and trill performance, but they could produce songs within their repertoire that are of higher quality when they are in a more challenging competitive environment (DuBois et al., 2009). Or males could modulate the total number of songs they produce and the neural substrates that control singing effort as a means of competing within their social environment (Sockman et al., 2009).

In a wide range of species, the modulation of socio-sexual behaviors, such as singing, appears to be regulated in part by the neuropeptide, arginine vasotocin (AVT), or its mammalian homologue, arginine vasopressin (AVP, Goodson and Bass, 2001; Insel and Young, 2000). Though the AVT and AVP systems have a range of homeostatic functions, the level of peptide in a number of regions within the forebrain, some of which comprise the 'social behavior network,' has been shown to regulate the expression of some socio-sexual behaviors (Goodson, 2005; Goodson and Bass, 2001; Goodson and Kabelik, 2009; Lema, 2008; Newman, 1999). AVT and AVP activity within these brain regions is thought to facilitate socially induced changes in behavior by acting on motivational or sensory-motor processes that regulate behavioral states, such as social approach and avoidance or generalized anxiety (Goodson and Bass, 2001). Further, AVT and AVP are specifically implicated in the regulation of vocal behavior in a wide range of taxa (Boyd, 1994; Chu et al., 1998; Diakow, 1978; Goodson and Bass, 2000; Kime et al., 2007; Marler et al., 1995; Ten Eyck, 2005), including birds (Goodson, 1998a; Harding and Rowe, 2003; Maney et al., 1997; reviewed in Panzica et al., 2001).

In songbirds, testosterone can activate singing behavior (Harding, et al., 1983; Harding, et al., 1988; Walters et al., 1988; Sartor et al., 2005; Wingfield et al., 1990; but see Hau, 2007; Soma, 2006), and can elevate AVT levels in the forebrain (Plumari et al., 2004; Viglietti-Panzica et al., 2001; reviewed in Panzica et al., 2001). These findings raise the possibility that AVT may mediate the actions of factors such as seasonally and socially induced elevations of testosterone on singing behavior. Paradoxically, manipulations of AVT have demonstrated that this peptide reduces the expression of male-typical socio-sexual behaviors in males that are in reproductive condition (Castagna et al., 1998; Goodson, 1998a;

Goodson, 1998b; Goodson and Evans, 2004; Goodson et al., 2009a; Goodson et al., 2004b; Kabelik et al., 2009; Harding and Rowe, 2003; reviewed in Panzica et al., 2001; Viglietti-Panzica et al., 2001). Thus, AVT may serve as a negative feedback mechanism that inhibits the expression of male-typical behaviors promoted by seasonal increases in testosterone until appropriate stimuli trigger a reduction in AVT levels and release behavior from inhibition (Panzica et al., 2001; Viglietti-Panzica et al., 2001). This general inhibitory model of AVT action explains why AVT expression increases with circulating testosterone levels, why male sexual behavior can occur in the absence of AVT expression, and also why AVT infusions often reduce the likelihood of male socio-sexual behavior occurring (Panzica et al., 2001; Viglietti-Panzica et al., 2001). Subsequent work has suggested that AVT may also have brain region-specific effects on behavior (see below).

Because the AVT system could underlie socially induced modulation of singing behavior in birds, we measured its expression in three forebrain regions: the medial bed nucleus of the stria terminalis (BSTm), the lateral septum (LS) and the preoptic area (POA). We examined AVT levels in the BSTm because vasotocin neurons in this brain region show selective activity in response to social stimuli, including song (Goodson et al., 2009b; Goodson and Wang, 2006). We also examined vasotocin levels in a target of BSTm AVT neurons, the LS, because manipulations of the amount of AVT in this brain region have shown that AVT modulates aggressive behavior, including territoriality and song (Goodson, 1998a; Goodson, 1998b; Goodson and Evans, 2004; Goodson et al., 2009a; Goodson et al., 2004b; Kabelik et al., 2009). And we quantified AVT levels in the POA because it is correlated with the expression of male sexual behavior, including singing (Goodson and Kabelik, 2009; Panzica et al., 2001; Viglietti-Panzica et al., 2001).

To examine the modulation of singing behavior by territorial male songbirds in response to the quality of the song environment and to explore the neural basis of such facultative shifts in socio-sexual behavior, we manipulated the song environment that male Lincoln's sparrows (*Melospiza lincolnii*) were exposed to and examined their singing behavior and AVT expression in the brain. Lincoln's sparrows are migratory and breed on high-elevation or high-latitude wet meadows in North America. They are a good system for examining behavioral responses to variation in the song environment because their breeding habitat is susceptible to strong inter- and intra-annual variation in ecological resource availability, and this variation is associated with the competitiveness of the social environment and the quality of the song environment (Sockman, 2009). The repertoire of male Lincoln's sparrows consists of approximately 1 to 6 song types composed of between 6 and 31 distinct syllable types (Cicero and Benowitz-Fredericks, 2000) and Lincoln's sparrow songs almost always include trills (Sockman, 2009). Under limited resource conditions, males vary among one another in several aspects of their song that are positively correlated with one another, including song length, total syllable count, song complexity (number of distinct syllable types) and trill performance (Sockman, 2009). Environmentally induced variation in individuals' songs, which generates variation in the broader song environment, should select for the ability to evaluate the song environment and modulate singing behavior accordingly. To assess modulation of singing behavior in response to the quality of the song environment, we exposed photostimulated male Lincoln's sparrows to 7 days of persistent playback of songs that were of either higher or lower quality than the average for their natal population (see Methods). We quantified the males' song output and song quality during the 7 days of playback and on an eighth morning of no playbacks. We then measured the expression of AVT in the BSTm, LS and POA using immunohistochemistry (IHC). We compared the behavior and arginine vasotocin immunoreactivity (AVT-IR) of males in the two song environment treatments to examine (1) the effect of the song environment on male singing behavior, (2) the effect of the song environment on AVT-IR in each brain region, and (3) the relationship between AVT-IR levels in each brain region and singing behavior.

MATERIALS AND METHODS

Subjects

Permission to conduct the procedures described in this study was granted by the U.S. Department of the Interior's Fish and Wildlife Service (permit MB099926), the U.S. Department of Agriculture's Forest Service (authorization COL258), the State of Colorado's Department of Natural Resources Division of Wildlife (license 06TR1056A2), the Town of Silverton, Colorado, U.S.A., and the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (protocol 05-138.0-A). With the help of field assistants, we collected Lincoln's sparrows as approximately 8-day-old nestlings during the summers of 2006 and 2007 in an open-field meadow (elevation 3250 m) near Molas Pass, Colorado, U.S.A. (37.74°N, 107.69°W). We hand-fed the nestlings for 1–2 weeks until they were feeding *ad libitum* independently, and then, in late July, transported them to animal facilities at the University of North Carolina at Chapel Hill, U.S.A. We initially housed the young birds in large indoor cages (ca. 1 × 0.5 × 0.5 m) within a single room. From approximately the age of 2 weeks until they were about 2 months old, we exposed all subjects to song playbacks previously recorded from several free-living males from the area where they were captured. We exposed all subjects to the same set of playbacks during this period. The playbacks consisted of an iTunes (Apple Inc., Cupertino, CA, U.S.A.) playlist made of 1.4 hrs. of recordings. We played it for approximately 3 hrs. per day. In the late summer or early fall, after they had reached independence, we collected a blood sample and genetically sexed the birds following Griffiths et al. (1998) polymerase chain reaction (PCR) procedure. After sexing the birds, we moved the birds to large, sex-specific outdoor aviaries where they were exposed to a natural photoperiod and 6 live males that were taken as adults from the subjects' natal meadow. For the entire study we provided the birds with *ad libitum* food (Daily Maintenance, Roudybush, Woodland, CA, U.S.A.) and water.

Experimental procedures

On 12 May 2008 we moved the subjects from the outdoor aviaries to indoor cages and held them on a 16-hour light and 8-hour dark photoperiod (lights on at 05:00 and off at 21:00 EDT) for 2 weeks to maintain their reproductive-like physiological state (Nicholls et al., 1988). At 09:00 on 26 May 2008, we began the experiment by randomly assigning and transferring each of the first 8 subjects to 8 individual cages within each of 8 sound attenuation chambers (58 × 41 × 36 cm, Industrial Acoustics Company, New York, NY, U.S.A.). Each chamber had a fan-driven ventilation system and a light that we used to maintain the above light-dark schedule. We equipped each chamber with (1) an omnidirectional microphone (Senheiser ME 62, Old Lyme, CT, U.S.A.) plugged into an 8 line recording interface (PreSonus FP10, Baton Rouge, LA, U.S.A.) and a computer running Sound Analysis Pro II software (SAP Version 2.062, Tchernichovski and Mitra, 2001) and (2) a speaker (Pioneer TS-G1041R, Tokyo, Japan) plugged into an individual amplifier (Audiosource Amp 5.1A, Portland, OR, U.S.A.) attached to an 8 channel interface (M-Audio Delta1010, Irwindale, CA, U.S.A.) and a computer running Protools M Powered playback software (version 7.1, M-Audio, Irwindale, CA, U.S.A.). We permitted males to acclimate to the chambers until 06:00 the next day, when we began the playback of one of two song-environment treatments -- low quality or high quality (see Song Environment Treatments, below). We exposed the males to these song playbacks and collected audio recordings from these subject males for 7 days. We spatially interspersed among the chambers each replicate of the low quality song environment with each replicate of the high quality song environment. On the eighth day we provided no playback but continued collecting audio recordings of the subjects until 09:00, when we began rapidly decapitating and removing the brain of each male. All brain removal was complete by 10:30.

Using previously described protocols (Sockman et al., 2009), we fixed one hemisphere (alternating left and right between subjects within each treatment group) in 5% acrolein, saturated it with 30% sucrose for cryoprotection, froze it on dry ice and held it at -80°C for approximately 2 weeks. We repeated these procedures with the second session of 8 males, beginning on 4 June 2008. During this second session, one male from each treatment group was found dead on the second day of playbacks. Two new males were added to the study, resulting in a third session that consisted of only two subjects, one from each treatment group. We weighed the males at the beginning and end of the study. We also removed males' testes and weighed them immediately after sacrifice and again after drying them at 60°C in an oven to quantify dry mass.

Song environment treatments

For the song playbacks, we selected recordings from our 800 plus library of songs collected from the subjects' natal meadow. As mentioned previously (see Introduction), several components of Lincoln's sparrow song, including song length, total syllable count, song complexity, and trill performance, all positively correlate with one another (Sockman, 2009). Therefore, we categorized songs as being high quality or low quality based on the first-axis factor scores from a Principle Components Analysis (PCA) of three measures of song features: song length (first-axis loading=0.64), total syllable count (first-axis loading=0.57), and phrase count (first-axis loading=0.51, total proportion of variance explained by first-axis=0.75). All three song features were positively correlated with trill performance and song complexity (each pairwise comparison: $P < 0.001$; Sockman, 2009). Thus, songs selected as high quality playback stimuli were from the end of the distribution of first-axis factor scores associated with greater song length, syllable count, song complexity, and trill performance; low quality songs were from the end of the distribution associated with shorter song length and lower syllable count, song complexity, and trill performance (Fig. 1). Female Lincoln's sparrows in a reproductive-like state show greater behavioral activity in response to this same set of high quality songs than in response to the same set of low quality songs (Caro et al., 2010). Additionally, female Lincoln's sparrows preferentially phonotax toward songs with experimentally elevated trill performance over songs with experimentally reduced trill performance (Caro et al., Accepted). Given the evidence that female Lincoln's sparrows prefer songs from the end of the normal distribution associated with greater length, complexity and trill performance, and the fact that these song features have been shown to influence female choice in other species (Searcy and Nowicki, 2005), we feel that labeling these songs as high or low quality is justified. However, in future studies we aim to determine the degree to which these song features are associated with aspects of male quality and fitness in free living Lincoln's sparrows, specifically.

We played each subject 6 unique songs produced by at least 2 different free-living males. To maximize the generalizability of our study, we used the playback recordings from each free-living male for only one subject in each treatment group (i.e., in some cases a wild males' high quality songs were played to a subject in the high quality environment and his low quality songs were played to a subject in the low quality environment; Kroodsma, 1990; Kroodsma et al., 2001; Wiley, 2003). We played songs back at 70 dB 5 cm from the speaker, following a pattern of intense morning singing and intermittent afternoon/evening song (9 hr. per day at an average rate of approximately 40 songs per hr.). To ensure that the total duration of song each day was identical between treatment groups, we included additional repetitions of low quality songs, which tend to be shorter (see above), as necessary. Therefore, the treatments differed not only in their song quality, but also in their song repetition rates, with the high quality treatment having slightly lower song repetition rates.

Song recording and song analysis

We recorded the subjects in this experiment continuously over the 8 day study using automated detection settings in SAP II. We set the recording parameters so that we captured only the songs of experimental males and not the playback songs, which were much softer (peak threshold=4800, record duration=1 sec, recording cut-off=30 sec., peak comparator=262, peak gain=10, peak counter trigger=15707, recoding gain=10, threshold=4, input gain on SAP=1.1, input gain on PreSonus interface=1.0 for all channels). To estimate each male's song output, we counted the number of songs on days one, three, five and seven that were recorded during the time comparable to the morning chorus (05:00–07:00), the period when many mid-temperate songbirds including Lincoln's sparrows engage in the most territorial singing. We also counted every song file recorded from 05:00–09:00 on day 8 (one day after playbacks). Finally, we evaluated the quality of songs males produced on day 8 by quantifying the mean length, mean syllable count, mean number of distinct syllable types and mean trill performance of 10 randomly selected songs from each male.

To measure trill performance, we generated spectrograms in the program WildSpectra (<http://www.unc.edu/~rhwiley/wildspectra/index.html>) and used an automated routine that determines the high and low frequencies within 3 dB of the peak amplitude and at the start and stop times of each syllable (see Sockman, 2009 for details). We calculated the trill rate, frequency bandwidths and durations of each syllable and the gap between syllables of each trill. We used a higher threshold for measuring frequency bandwidth than in other studies (Podos, 1997), but overall, frequency bandwidth values only marginally differ between studies (see Sockman, 2009 for details). We then plotted the trill's mean syllable bandwidth in kHz against the trill rate in Hz, following Podos (1997), and calculated an upper-bound regression that represents the theoretical limit to this trill performance measure. To determine the performance value of each trill we calculated the distance between each trill's location on the bivariate plot and the nearest point on the upper-bound regression; the more negative the value, the lower the trill performance. Finally, we also determined the number of distinct song types each male produced on day 8.

Histology and immunohistochemistry

We sectioned brain tissue on a cryostat at 40 μm in the sagittal plane and stored sections in cryoprotectant (30% sucrose with ethylene glycol and polyvinylpyrrolidone) at -20°C until IHC was performed following a protocol modified from Sockman et al. (2002). Briefly, we treated every third section of one hemisphere with 0.1% sodium borohydride for 15 min., blocked endogenous peroxides with a 0.5% hydrogen peroxide solution for 30 min., and suppressed endogenous avidin and biotin binding activity with a blocking kit (15 min., Vector, Burlingame, CA, U.S.A.) before incubating for 24 hours at 4°C in the primary antibody (rabbit anti-VP diluted 1:1250, Cat. No. 64717, lot 9742, MP Biomedicals, Solon, OH, U. S. A.). The immunoreactivity of this antibody across brain nuclei has been well described in a number of species of birds and was nearly identical to the pattern of immunoreactivity that we observed (Kiss et al., 1987; Leung et al., 2009; Maney et al., 2005; Panzica et al., 2001; Panzica et al., 1999; Voorhuis and Dekloet, 1992). Preabsorption tests for this antibody have confirmed its specificity across a range of avian and non-avian species (Boyd et al., 1992; see Leung et al., 2009 for detailed explanation of antibody specificity), although no such tests have been conducted in Lincoln's sparrow tissue. Staining was visualized with anti-rabbit secondary antibody raised in goat (1 hr. incubation, Vector) to which an avidin-biotin horseradish peroxidase complex (ABC Elite kit, 30 min. incubation each, Vector) bound and which we colored with a nickel-enhanced diaminobenzidine tetrahydrachloride solution (ca. 10 min. development). We washed tissue in solutions of PBS or PBS with Triton-X-100 detergent (Cat. No. BP151-500, Thermo

Fisher Scientific, Waltham, MA, U.S.A.) between steps. We processed the tissue in two IHC batches, counterbalancing the song environment treatment between them.

Quantification

We coded all slides so that the observer quantifying AVT-IR was blind to the identity and treatment of each bird. Using a Leica DM 4000B Digital Research Microscope with a Leica DFC480 color digital camera (Bannockburn, IL, U.S.A.) connected to a Macintosh G5 dual-processor computer (Apple Inc.) running Leica Firecam software (version 3.4.1), we collected brightfield microscope images of the BSTm, LS and POA and analyzed them at 650 \times (this is the magnification of images analyzed on the computer display, after magnification by a 40 \times objective, a 10 \times ocular, and additional magnification by the camera, software, and computer hardware). We captured all images using the same exposure settings in the Leica Firecam software (exposure=9, black=11, grey=1.0, white=94, 0% saturation, 8-bit, 2 \times 2 binning).

We assessed AVT-IR in the BSTm of every third-cut section of tissue from the section immediately before the occipito-mesencephalic tract (OM) was visible, until the anterior commissure (CoA) and OM met (4 sections in total, ca. 500 to 800 μ m laterally). We based this strategy on descriptions of the location of the BSTm in coronal sections (Heimovics and Ritters, 2007; Maney et al., 2005), as the location of BSTm has not been described in sagittal sections to our knowledge. We captured images of the BSTm by placing the frame of focus immediately dorsal to the CoA (Fig. 2). Collecting images from sections in which the OM was visible likely resulted in quantification of AVT-IR in the more lateral portion of the BSTm, but ensured that we did not capture the medial septum accidentally. We assessed AVT-IR in the LS of every third-cut section of tissue from the first section in which the auditory lobule was clearly defined until the OM became visible (4 sections in total, ca. 150 to 600 μ m laterally). We placed the imaging frame at the dorsal most edge of the LS, immediately ventral to the nidopallium and in line with the CoA (Fig. 2). The location of the subdivisions of the LS have not been previously described in sagittal sections, to our knowledge. We do not attempt to describe the subdivisions of the LS in sagittal sections, but this frame placement strategy did ensure that we quantified a region well within the bounds of the LS (Goodson et al., 2004a). Finally, we assessed AVT-IR in the POA of every third-cut section of tissue from the midline until the section before the OM became visible (4 sections in total, ca. 0 to 750 μ m laterally). We placed the frame of focus ventral to the septopallio-mesencephalic tract (TSM) and at the caudal most edge of the tissue to capture images within the POA (Fig. 2). This strategy for frame placement should have ensured that we captured images dorsal to the paraventricular nucleus.

Using Image J (Rasband, 1997–2009), we used three approaches to quantify AVT-IR in the forebrain regions of interest because the extent of staining differed between brain regions. Specifically, though every subject had AVT-immunoreactive fibers in every section of the BSTm and LS, immunoreactivity often appeared as a diffuse group of fibers (Fig. 2). Unfortunately, only 2 subjects had clear AVT-immunoreactive cell bodies in the BSTm and only 5 subjects had clear AVT-immunoreactive cell bodies in the LS, making it impractical to conduct cell body counts in these brain regions. It is worth noting that AVT-immunoreactive cells in the BSTm are often difficult to visualize in *Melospiza* and *Zonotrichia* species (Goodson and Evans, 2004; Maney et al., 2005). Therefore, we overlaid photomicrographs of the BSTm and LS with a grid of forty-eight, 1849 μ m² squares. We manually scored each image by counting every square containing an AVT-immunoreactive fiber. We summed the number of squares containing AVT-immunoreactive fibers across tissue sections of a given region of interest to yield a single estimate of AVT-IR for each brain region, for each subject. In contrast, immunostaining in the POA occurred as both AVT-immunoreactive fibers and cell bodies (Fig. 2). We quantified the percent of the total

pixels in gray-scale images that were above a single threshold assigned to all specimens (determined by averaging the mean grey value of 20 squares placed over background staining) and summed the 4 area percentages measured for each subject, one for each of the 4 tissue sections, to generate an estimate of the percent of the total area in which AVT-immunoreactive staining occurred in the POA. Cell bodies were more abundant in the POA than in the other regions, so we also counted and summed the number of AVT-immunoreactive cell bodies across each of the 4 sections of POA to yield a single POA AVT-immunoreactive cell body count for each subject. Because of tissue damage and loss, we lacked one subject from each treatment group for the analysis of AVT-IR in the LS and BSTm and one subject from the high quality song environment treatment group for the analysis of AVT-IR in the POA.

Statistical analyses

Our data consisted of a hierarchically structured combination of fixed (e.g., song environment) and random (e.g., individual bird, chamber) effects, which may differ from one another in their correlation structure. Therefore we analyzed these data in a mixed, multilevel modeling framework using the software R 2.7.2 (R Development Core Team, 2008), which readily accommodates hierarchically structured combinations of fixed and random effects (see Sockman and Salvante, 2008 for details of our mixed modeling approach). We primarily used a multi-level mixed-effects linear regression (lme package in R), which uses t-tests to test the null hypothesis that a coefficient equaled 0. Because count data typically follow a Poisson distribution, we analyzed song count, count of squares with AVT-immunoreactive fibers in the BSTm and LS, and count of AVT-immunoreactive cell bodies in the POA with a mixed-effects Poisson regression (Bolker et al., 2009; Crawley, 2007, lmer package in R), which uses z-tests to test the null hypothesis. Both of these models estimated parameters with restricted maximum likelihood (REML). In most analyses, we modeled chamber as a random intercept. When the response variable was collected repeatedly from individual subjects (e.g., mass, daily song count), we modeled individual as a random intercept. The song environment treatment was coded for categorical analysis (low quality=0, high quality=1). We also conducted Pearson's correlations to examine the relationship between the two key response variables in this study, AVT-IR (in each brain region) and singing effort.

RESULTS

Male singing behavior

Each successive morning of playbacks recruited an increasing number of singing males (e.g., 11 out of 16 males sang on day one, 15 out of 16 males sang on day 8), and singers sang increasingly more songs each morning of the study (e.g., mean of 5.625 songs on day one, mean of 283.750 songs on day 8, Fig. 3). However, there was considerable individual variation in the subjects' morning song counts and song quality, despite these overall trends (e.g., day 8 song counts ranged from 0–771 and mean song length from 1.68–3.49 sec.). There was no strong effect of the song environment on subjects' repertoire sizes or any measures of song quality (Table 1). But the song environment did affect the subjects' song counts. We found that song count increased over time regardless of treatment (i.e., there was an effect of time, Table 2) but that males in the high quality song environment increased their singing output more quickly and to a greater degree than males in the low quality song environment (i.e., there was a reliable interaction between treatment and time, Table 2, Fig. 3). This difference in song output was also captured when we evaluated the effect of the song environment on the song count for day 8, the day after playback, on which we collected brains for AVT quantification (Table 2). Therefore, we used day 8 song count as a

measure of song output (hereafter singing effort) in subsequent analyses. The song environment did not influence subjects' body mass or testis mass (wet or dry, Table 3).

AVT immunoreactivity, song environment and singing effort

The song environment manipulation influenced AVT-IR in both the BSTm and LS (Table 4); males in the high quality song environment had lower AVT-IR in both the BSTm and LS, compared to males in the low quality song environment (Fig. 4). The same was true for AVT-IR in the POA (measured as percent area AVT-IR), although the effect was not statistically reliable (Table 4, Fig. 4). We found no effect of the song environment on the number of AVT-IR cell bodies in the POA (Table 4).

We wondered if the environmentally induced effects on AVT-IR levels within each brain region of interest were related to the effects that we also observed on the subjects' singing effort. Pearson's correlations suggest a negative relationship between levels of AVT-IR in the POA (measured as the percent area AVT-IR) and singing effort (Pearson's $r^2=0.261$, $p=0.074$, Fig. 4). However, neither the relationship between AVT-IR in the BSTm and singing effort (Pearson's $r^2=-0.228$, $p=0.433$), nor that between AVT-IR in the LS and singing effort (Pearson's $r^2=0.213$, $p=0.464$) was particularly reliable (Fig. 4).

DISCUSSION

This study demonstrates that the quality of the song environment influences male Lincoln's sparrows' singing effort, but not the quality of the songs that males produce. This work also reveals that AVT-IR levels in brain regions involved in responses to social stimuli (BSTm) and regulation of aggressive behavior (LS) are influenced by the quality of song cues, which likely reflect the level of competition in the environment (Fig. 4). These outcomes are consistent with previous work demonstrating that AVT neurons in the BSTm show selective responses based on the valence of social cues (Goodson et al., 2009b; Goodson and Wang, 2006). Also, though the results of this study do not indicate a causal relationship between AVT-IR and singing behavior, they are consistent with studies showing that AVT in the LS plays a role in regulating the expression of aggressive and territorial behaviors, such as singing (Goodson, 1998a; Goodson, 1998b; Goodson and Evans, 2004; Goodson et al., 2004b; Kabelik et al., 2009).

Behavioral responses to song environment

As predicted, photostimulated male Lincoln's sparrows in this experiment modulated their singing behavior in response to the quality of the song environment; males exposed to persistent playback of high quality songs increased their singing effort more quickly and to a greater degree than males exposed to low quality songs over the period of a week (Table 2, Fig. 3). Moreover, once playbacks had ended, differences between groups persisted, suggesting the formation and recall of a memory of the environment, consistent with findings from a similar experiment in male European starlings (*Sturnus vulgaris*; Salvante et al., 2009; Sockman et al., 2009). Although our treatment did not affect testis size (Table 3), it is nonetheless possible that environmentally-induced differences in male reproductive condition, such as testosterone levels, did exist and caused these differences in singing effort. However, a similar study on the long-term effects of social conditions on singing in canaries (*Serinus canaria*) found that socially modulated variation in singing behavior occurred independently of testosterone levels (Boseret et al., 2006).

It is possible that differences between treatments in the rate of song presentation, not song quality, drove response differences, but this seems unlikely given that singing effort was greatest in the high quality song environment, the treatment in which the rate of song

presentation was lower. Rather, subjects probably responded to variation in particular song features that differed between treatment groups, such as song length, song complexity, and trill performance. Any of these factors could reflect the quality of the singers and thus provide information about the nature of the competitive environment (Searcy and Nowicki, 2005).

In contrast to singing effort, we found no effect of the song environment on the subjects' own repertoire size or measures of song quality; males in the high quality song environment failed to produce more song types, longer songs or more complex songs than males in the low quality song environment (Table 1). In some songbird species, males acquire new song types and new syllable types and sing longer songs over the course of their lives, making these aspects of song quality reflective of male age and experience (Searcy and Nowicki, 2005). It has been suggested that Lincoln's sparrows learn new song components annually (Cicero and Benowitz-Fredericks, 2000), which may explain the failure of males in the more competitive song environment to produce higher quality songs than males in the less competitive song environment in this eight day study (Table 1). Similarly, trill performance may improve as males age (Ballentine, 2009) or change with the availability of environmental resources (Sockman, 2009). We controlled for both male age and resource availability in the experimental design (individuals were randomly assigned to each treatment group and males were given *ad libitum* food throughout the study). Thus, the result that males in the two song environments did not differ in their trill performance is consistent with the hypothesis that vocal performance is not immediately modifiable and could indeed be determined by and reflective of aspects of male condition or quality. However, our results do show that extrinsic song cues associated with the level of social competition in the environment influence singing effort.

Song environment, arginine vasotocin and singing effort

Although we observed treatment effects on AVT-IR, interpreting the functional significance of immunoreactivity of a neuropeptide is challenging because elevated immunoreactivity could reflect elevated production of the neuropeptide, perhaps to keep pace with elevated levels of secretion, or it could indicate an accumulation of peptide due to reduced secretion and subsequent metabolism (Goodson and Bass, 2001; Goodson and Kabelik, 2009; Panzica et al., 2001). Further, the amount of AVT is only one factor that might shift either to control or respond to behavioral states; receptor densities, for example, could also change and alter the AVT signaling system. Nonetheless, in keeping with conventions from the literature, we offer an interpretation of the present data that assumes AVT-IR reflects amounts of the peptide that are synthesized, secreted and metabolized (e.g., Panzica et al., 2001).

As described in the introduction, AVT expression in the BSTm, LS and POA may play a role in inhibiting the expression of male socio-sexual behaviors that are up-regulated by increases in plasma testosterone, thereby ensuring that behaviors are only expressed under appropriate social conditions (Panzica et al., 2001). Consistent with this hypothesis, the high quality song environment of the present study elevated singing effort but reduced AVT-IR in the BSTm and LS relative to the low quality song environment (Figs. 3,4). Thus, given the well-described effects of testosterone on singing behavior (e.g., Sartor et al., 2005), it is possible that the stimulus of the high quality song environment, acting through reduced AVT expression in the BSTm and LS, lowered the inhibition of male socio-sexual behaviors and increased the likelihood of photo-induced elevations in testosterone promoting singing.

In addition, the present findings are consistent with proposed brain region specific actions of AVT. AVT neurons in the avian BSTm respond to the valence of social stimuli, and AVT levels in the LS influence context dependent aggressive behaviors in birds, possibly including song (Goodson, 1998a; Goodson and Kabelik, 2009; Goodson and Wang, 2006).

Vasotocin neurons in the BSTm show selective activity in response to positive social stimuli, such as the presence of a female, and they show somewhat reduced activity in response to negative social stimuli, such as subjugation (Goodson and Wang, 2006). In the present study, persistent playback of high quality song may have been an aversive or possibly threatening social stimulus, and males in this environment showed lower AVT-IR in the BSTm than males in the low quality (possibly less threatening) song environment (Fig. 4). Thus, the present findings are consistent with the hypothesis that AVT levels in the BSTm change as a function of the quality or valence of social cues. Further, these findings show that AVT-IR in the BSTm is influenced by the quality or valence of song cues that persist over an extended period of time and which may reflect prevailing levels of social competition.

AVT neurons in the BSTm project to the LS, a brain region involved in regulating aggression, likely by interacting with the stress response system to modulate generalized anxiety (Goodson and Bass, 2001; Goodson and Evans, 2004; Goodson et al., 2009b). In the present study, males in the high quality song environment had lower LS AVT-IR than males in the low quality song environment, just as was found in the BSTm (Table 4, Fig. 4). It is possible that AVT neurons projecting from the BSTm explain this pattern of AVT-IR in the LS. Or the song environment could influence AVT-IR in the LS through some mechanism other than projections from the BSTm. Though this study did not evaluate the causal function of AVT, work by Goodson and colleagues (2009a, for review) suggests that septal AVT regulates the expression of aggressive behavior. Specifically, infusions of AVT and AVT antagonists into the LS have revealed that AVT decreases aggression in the context of territorial or nest defense, but increases aggression in the context of direct mate competition in a range of avian species (Goodson, 1998a; Goodson, 1998b; Goodson et al., 2004b; Kabelik et al., 2009). In this study, males were presented with cues from male competitors, not females, so differences in male singing effort likely reflect differences in general territorial aggression (though song is only one component of territoriality in songbirds, see Goodson, 1998a). Thus, the finding that males in the high quality song environment, who sang more, also had lower levels of AVT-IR in the LS, is consistent with AVT in the LS reducing territorial aggression (Figs. 3, 4).

In contrast to the apparent context dependent relationships between social conditions and AVT in the BSTm and LS, evidence from Japanese quail (*Coturnix japonica*) suggests that AVT-IR in the POA is associated almost exclusively with male sexual motivation. In Japanese quail, AVT-IR in the POA is higher in males than females, increases with circulating testosterone levels and is correlated with the expression of male appetitive sexual behavior (Panzica et al., 2001; Viglietti-Panzica et al., 2001). Consistent with the association of POA AVT-IR levels and the expression of male socio-sexual behavior, we did not find that AVT-IR in the POA was strongly affected by the quality of the song environment, but it may be associated with male singing behavior (Table 4, Fig. 4). It is possible that the level of AVT-IR in the POA directly regulates singing output. In the European starling, both neural activity in the POA and the volume of the POA are positively correlated with song output in a breeding context (Alger and Ritters, 2006; Ritters et al., 2000; Ritters et al., 2004). Also in this species, lesions of the POA inhibit the expression of sexually motivated song (Ritters and Ball, 1999). In female white crowned sparrows (*Zonotrichia leucophrys gambelii*) infusion of AVT into the third ventricle, which may result in increased AVT in the POA (Goodson, 1998a), elicits singing (Maney et al., 1997). However, the possible correlation between POA AVT-IR and male singing behavior in the present study more likely reflects an indirect relationship, such as one involving the aromatization of testosterone (Ball et al., 2004; Ball et al., 2003; Panzica et al., 2001; Viglietti-Panzica et al., 2001). Alternatively, singing behavior itself, regulated by AVT action in the BSTm and LS

or by an alternative mechanism such as the catecholamine system (Hara et al., 2007; Salvante et al., 2009), could induce changes in the level of POA AVT-IR.

Conclusion

Our results demonstrate that male Lincoln's sparrows modulate their singing effort as a function of the quality of songs in their environment, which may provide information about the relative level of male-male competition. Because the quality of competitors and prevailing songs that males experience may vary as a function of available resources (Sockman, 2009), males likely benefit from this ability to make facultative adjustments to their singing effort. The present study suggests that AVT levels in some forebrain regions, which show responsiveness to the valence of social cues (BSTm) and regulate territorial aggression (LS), could integrate information about persistent differences in the quality of the song environment. Future studies manipulating AVT levels in these brain regions while males are held in different song environments could determine if this peptide also plays a role in modulating territorial singing behavior in response to persistent cues reflective of the competitive social environment.

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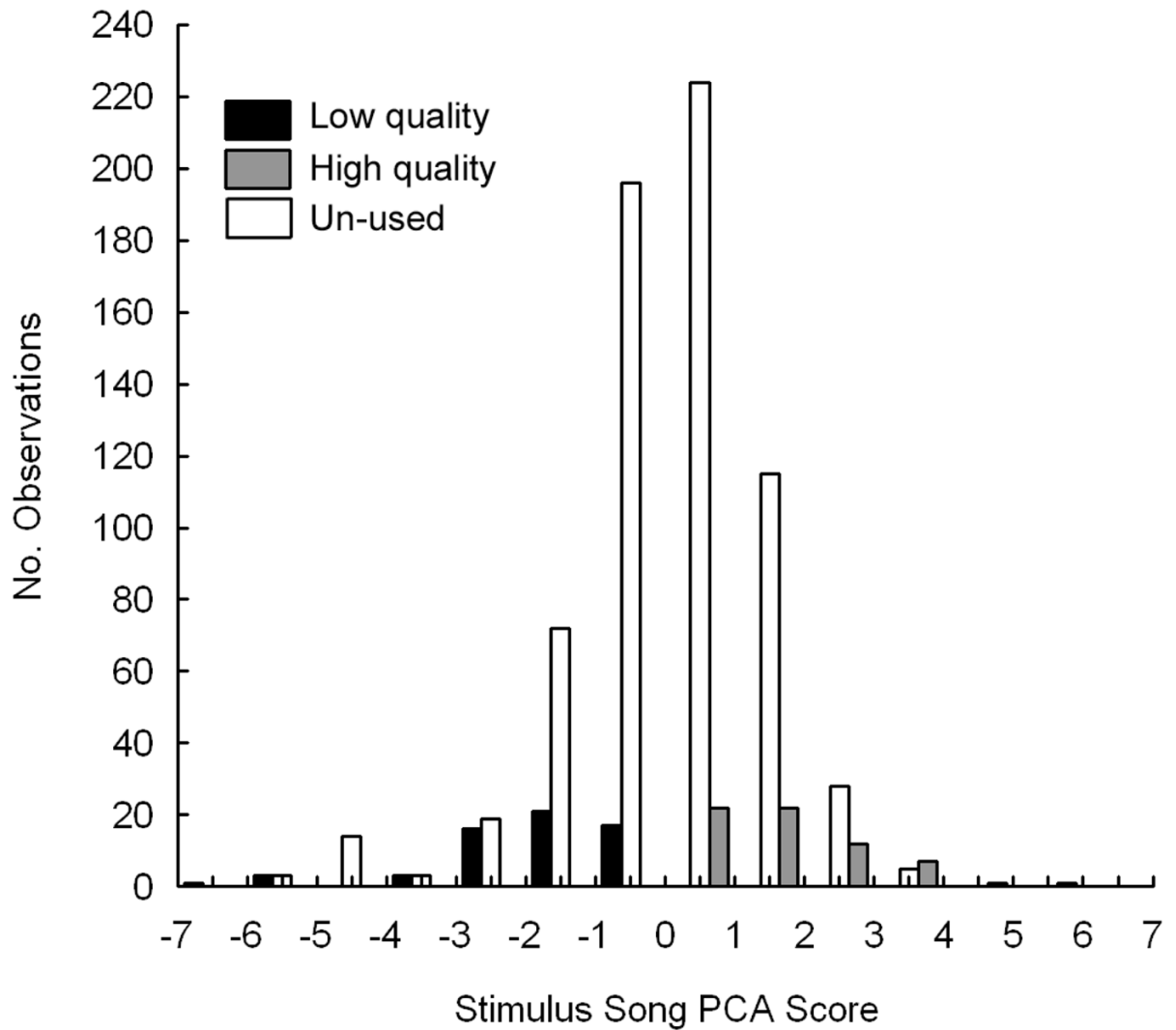


Figure 1.

Distribution of variation in the quality of songs recorded from free-living male Lincoln's sparrows in their natural environment (white bars). Song quality was quantified as the first-axis factor scores from a Principal Components Analysis (PCA) of song length and total syllable count, which are strongly, positively correlated with song complexity (unique syllable count) and trill performance. For the experimental treatment, in which the quality of the song environment was manipulated, high quality song stimuli (gray bars) were selected from the positive end of the distribution associated with longer, more complex songs containing higher performance trills, and low quality song stimuli (black bars) were from the negative end of the distribution of PCA scores.

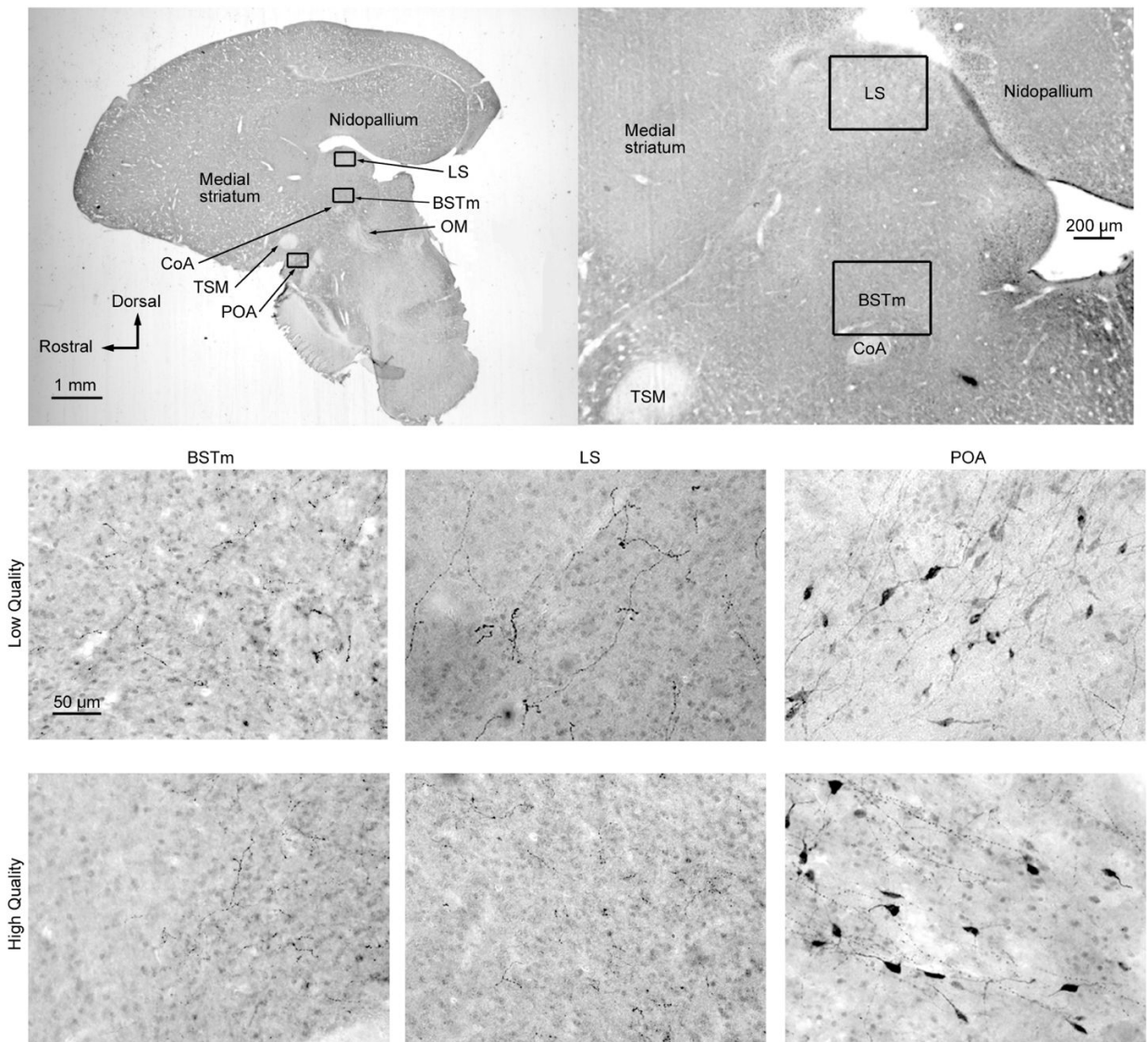


Figure 2.

Photomicrographs illustrating where frames of focus were placed (top two images) to quantify arginine vasotocin immunoreactivity (AVT-IR) in the bed nucleus of the stria terminalis (BSTm), lateral septum (LS) and preoptic area (POA). Example images from all three brain regions from male Lincoln's sparrows in the low quality and high quality song environment treatments are included (bottom two rows). Images were taken from sections approximately 600 μm from the midline but it should be noted that the POA is the most medial region and the BSTm is the most lateral region examined, making it difficult to depict in the same photomicrograph the precise locations of both regions. CoA: anterior commissure, OM: occipito-mesencephalic tract, TSM: septopallio-mesencephalic tract

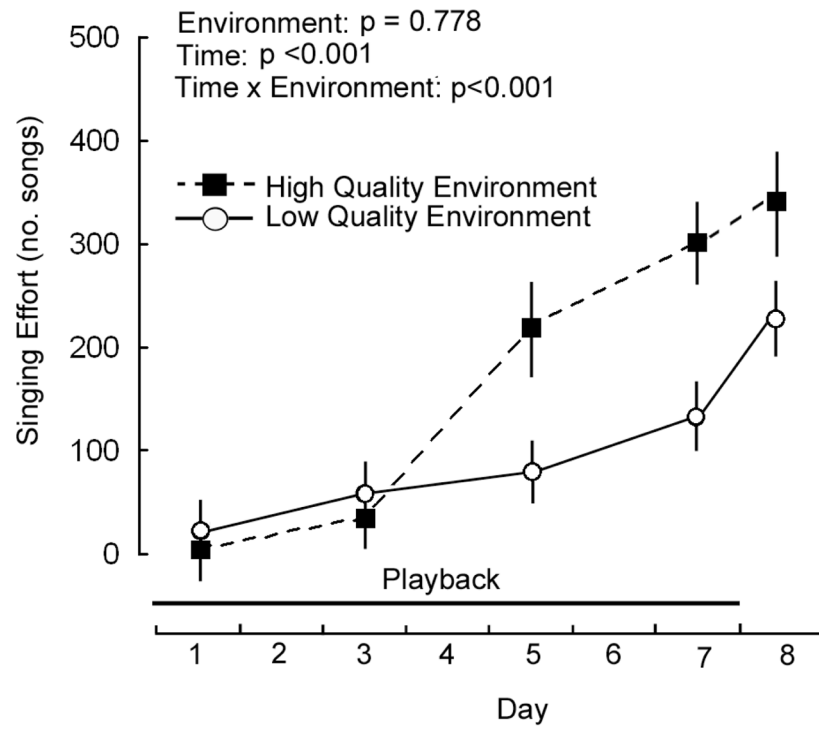


Figure 3. Effects of the quality of the song environment on singing effort (mean daily song count \pm S.E.M.) on mornings 1, 3, 5, 7 and 8 in male Lincoln's sparrows.

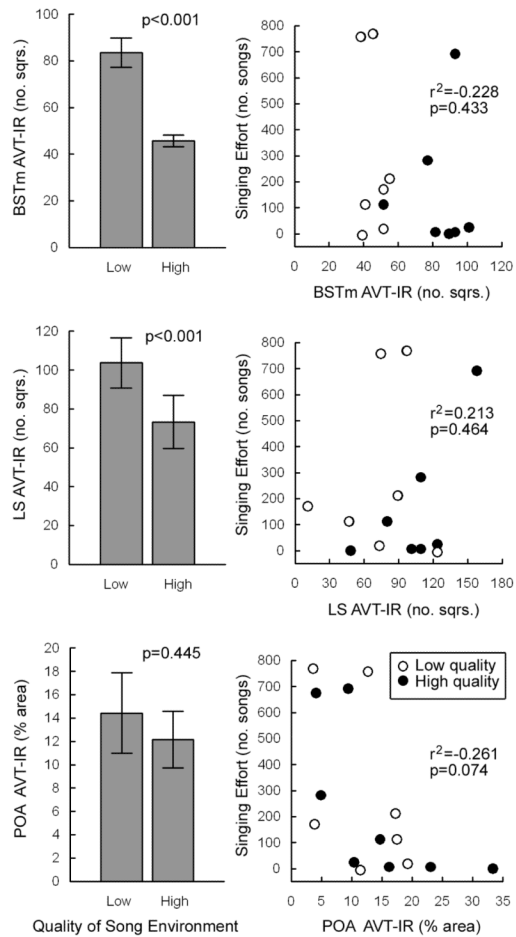


Figure 4.

(Left Column) Effect of the quality of the song environment on mean arginine vasotocin immunoreactivity (AVT-IR \pm S.E.M.) in the bed nucleus of the stria terminalis (BSTm, quantified as number of $1849 \mu\text{m}^2$ squares in digital images with AVT-immunoreactive fibers), lateral septum (LS, quantified as number of $1849 \mu\text{m}^2$ squares in digital images with AVT-immunoreactive fibers) and preoptic area (POA, quantified as the percent area with AVT-IR) for subjects in the high and low quality song environments. (Right Column) Relationship between AVT-IR in each brain region and singing effort (mean day 8 song count \pm S.E.M.; values for subjects in the high quality song environment are indicated with open circles, values for subjects in the low quality song environment with closed circles).

Table 1

Effects of the quality of the song environment (coded 0 for low and 1 for high) on the repertoire size and measures of the quality of songs produced by male Lincoln's sparrows.

Response Variable	Estimate	Standard Err.	t-score	p-value
Repertoire Size (no. song types)	-0.625	0.588	-1.063	0.323
Song Length (sec.)	0.243	0.237	1.023	0.340
Syllable No.	0.188	1.659	0.113	0.913
Song Complexity (no. syllable types)	0.449	0.467	0.961	0.369
Trill Performance*	0.144	0.122	1.183	0.275

* See text for details of how trill performance was quantified.

Table 2

Effects of the quality of the song environment (coded 0 for low and 1 for high) on singing effort (song count) in male Lincoln's sparrows.

Response Variable Predictor	Estimate	Standard Err.	z-score	p-value
Song Count (over entire experiment)				
Song Environment	0.241	0.854	0.28	0.778
Time (day)	0.344	0.008	40.75	<0.001
Environment × Time	0.037	0.011	3.49	<0.001
Day 8 Song Count				
Song Environment	0.411	0.030	13.56	<0.001

Table 3

Effects of the quality of the song environment (coded 0 for low and 1 for high) on body and testis mass in male Lincoln's sparrows.

Response Variable Predictor	Estimate	Standard Err.	t-score	p-value
Mass (g)				
Song Environment	0.513	1.425	0.360	0.725
Time (day)	0.269	0.170	1.577	0.137
Environment × Time	0.163	0.241	0.674	0.511
Testis Mass (wet, g)				
Song Environment	-0.006	0.026	-0.235	0.821

Table 4

Effects of the song environment (coded 0 for low and 1 for high) on arginine vasotocin immunoreactivity (AVT-IR) in the forebrain of male Lincoln's sparrows.

Response Variable*	Estimate	Standard Err.	t/z-score*	p-value
BSTm AVT-IR (no. sqrs.)	-0.603	0.070	-8.56	<0.001
LS AVT-IR (no. sqrs.)	-0.443	0.064	-6.96	<0.001
POA AVT (no. cell bodies)	0.088	0.117	0.750	0.453
POA AVT-IR (% area)	-3.994	4.713	-0.847	0.445

* The test statistic for the BSTm and LS AVT-IR analysis is a z-score (calculated using lmer in R) and the statistic for the POA AVT-IR analysis is a t-score (calculated using lme in R). BSTm: Medial portion of the bed nucleus of the stria terminalis, LS: lateral septum, POA: preoptic area.