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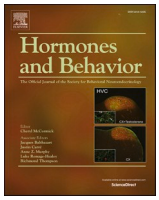

Androgen signaling in LMAN regulates song stereotypy in male canaries

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ABSTRACT

During breeding when testosterone concentrations are high, male songbirds that are open-ended vocal learners like canaries (*Serinus canaria*) tend to produce a stable, stereotyped song that facilitates mate attraction or territory defense. Outside breeding contexts, song becomes more variable. The neuroendocrine mechanisms controlling this vocal variability across seasons are not entirely clear. We tested whether androgen signaling within the lateral magnocellular nucleus of the anterior nidopallium (LMAN), a cortical-like brain region of the vocal control system known as a vocal variability generator, plays a role in seasonal vocal variability. We first characterized song in birds housed alone on a short day (SD) photoperiod, which simulates non-breeding conditions. Then, cannulae filled with the androgen receptor (AR) blocker flutamide or left empty as control were implanted bilaterally in LMAN. Birds were then transferred to long days (LD) to simulate the breeding season and song was analyzed again. Blocking AR in LMAN increased acoustic variability of song and the acoustic variability of syllables. However, blocking AR in LMAN did not impact the variability of syllable usage nor their sequencing in LD birds, song features that are controlled by androgen signaling in a somatosensory brain region of the vocal control system called HVC. These findings highlight the multifactorial, non-redundant actions of steroid hormones in controlling complex social behaviors such as birdsong. They also support the hypothesis that LMAN is a key brain area for the effects of testosterone on song plasticity both seasonally in adults and during the song crystallization process at sexual maturity.

1. Introduction

Singing behavior in oscines or songbirds is controlled by a dedicated network of interconnected brain nuclei (Brainard, 2008; Nottebohm, 1980; Nottebohm et al., 1976; Wild, 2008). Two sub-circuits have been distinguished in this so-called vocal control system. The motor pathway connects HVC (Initially an acronym, now used as a proper name; Reiner et al., 2004) to RA (nucleus robustus of the arcopallium) to the motor neurons directly controlling the muscles of syrinx and directly mediates song production (Schmidt and Wild, 2014; Wild, 2008). The rostral forebrain pathway also connects HVC to RA but via other nodes including via Area X of the basal ganglia, the dorsolateral nucleus of the thalamus (DLM) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN). This more rostral circuit plays a key role in song learning and song stability. Indeed, electrophysiological and lesion studies have clearly demonstrated that LMAN plays a key role as a variability generator to facilitate song learning (Bottjer and Johnson, 1997; Fee and Scharff, 2010; Jarvis, 2008).

One peculiar feature of the vocal control system is that several of its nodes express a high density of sex steroid receptors, including androgen receptors (AR) and estrogen receptors of the alpha sub-type (ERα), which represents quite an exception among vertebrates (Arnold et al., 1976; Gahr et al., 1993); for review: (Ball and Balthazart, 2007). Indeed, high densities of androgen receptors are usually observed only in hypothalamic and limbic nuclei (Morrell et al., 1975; Morrell and Pfaff, 1978). Within the vocal control system of canaries, AR and ERα are expressed in HVC while ARs are expressed in LMAN and RA (for review: Ball, 1990; Brenowitz, 1991).

Testosterone has been shown to reduce vocal variability during adulthood (Brenowitz et al., 1998; Cornez et al., 2020a; Cornez et al., 2020b; Smith et al., 1995; Whaling et al., 1995) and during vocal ontogeny testosterone is thought to be the key driver of song

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crystallization (Marler et al., 1988). Recent work has begun to disentangle the sites of action of testosterone via precise pharmacological manipulations in HVC, RA and the preoptic area, specifically the medial preoptic nucleus (POM), revealing where testosterone may act to modulate vocal variability (for review: Alward et al., 2017b; Ball et al., 2020). For instance, testosterone in the POM modulates the motivation to sing without altering song variability (Alward et al., 2013). Follow-up work determined that activation of ARs in HVC reduces the variability of syllable usage and of syllable sequencing, while activation of ARs in RA reduces the acoustic variability of syllables and songs (Alward et al., 2017a). It is not fully understood what function is served by AR activation in LMAN a nucleus that is well-known for being implicated in song learning during ontogeny (Bottjer et al., 1984; Scharff and Nottebohm, 1991) and in the maintenance of song stability in adulthood (Fee and Scharff, 2010; Kojima et al., 2018; Moorman et al., 2021; Woolley and Kao, 2015). One way that LMAN does this is by introducing variability into song production that is required for song learning during ontogeny and song plasticity in adulthood (Fee and Scharff, 2010). It has been hypothesized that T acting in LMAN induces song stability that is critical for song to function in contexts such as mate choice and territorial defense (Rouse Jr. and Ball, 2016).

In the present study we analyzed the effects of flutamide (an AR blocker) implanted in LMAN upon the acoustic characteristics as well as the usage and sequencing of syllables in canary song. We had previously found, using a similar approach, that blocking the effects of androgens in HVC affected sequencing and usage of syllables, while blocking the effects of androgens in RA affected syllable phonology (Alward et al., 2017a). We predicted that blocking androgen effects in LMAN would increase the variability of the acoustic structure of syllables.

2. Materials and methods

2.1. Animals used and pre-experimental manipulations

This experiment was performed with male canaries (*Serinus canaria*) of the Border strain. These birds were purchased from a local breeder (Maryland Exotic Birds) and were one or two years old. They all had experienced at least one breeding season before being included in the present experiment. When they arrived in the laboratory, males were first kept on a short-day (SD) photoperiod (8 L:16D) for six weeks to induce photosensitivity (Hurley et al., 2008; Nicholls and Storey, 1977) and housed in mixed-sex groups. All protocols and procedures were approved by the University of Maryland at College Park and followed the ASAB/ABS Guidelines for the use of animals in research.

2.2. Song recording and housing

After six weeks of SD housing in mixed-sex groups, males were transferred to individual sound-attenuating recording chambers (41 cm × 48 cm × 51 cm) still under a SD photoperiod (8 L:16D). Chambers contained a wooden perch and birds could move freely and perform all maintenance activities. Birds were housed alone given our previous work demonstrating that male canaries sing when housed alone but reduce singing substantially and sometimes do not sing at all when housed with a female (Alward et al., 2014; Boseret et al., 2006). Songs recorded and analyzed here can thus be considered as "undirected" songs but a similar study of songs directed at a female would be difficult given the low singing rate of canaries in these conditions, contrary to what is observed in zebra finches (Jarvis et al., 1998; Sakata et al., 2008).

After seven days in these conditions, males were removed from their chambers and underwent surgical procedures for peripheral implantation of testosterone and bilateral implantation of flutamide directed towards LMAN (Fig. 1; see next section for details). They were then returned to their isolation chamber where the photoperiod was switched to long days (LD, 16 L:8D).

Each isolation chamber was equipped with a microphone (BT-MP8087 Mini microphone; B&H Photo and Electronics Corp, New York, NY) and camera (KPC-600 Pinhole Camera 3.6 mm; B&H Photo and Electronics Corp, New York, NY) connected to computer running DVRserver (V6.33b; Mammoth Technologies, Austin, TX) designed for real-time video and audio surveillance recording. Each day, the DVRserver captured song behavior from 0800 h to 1030 h (lights on at 0800 h) in .wav files sampled at 22,050 Hz which translated to a frequency range of 0–11 kHz. Recordings were collected each day but were only analyzed in detail on SD 7 and LD 7, 14 and 21.

2.3. Bilateral implantation of flutamide targeting LMAN

At the beginning of the experiment while birds were still in SD, birds were implanted with a Silastic™ capsule filled with testosterone to ensure high rates of singing. They were anesthetized with isoflurane gas

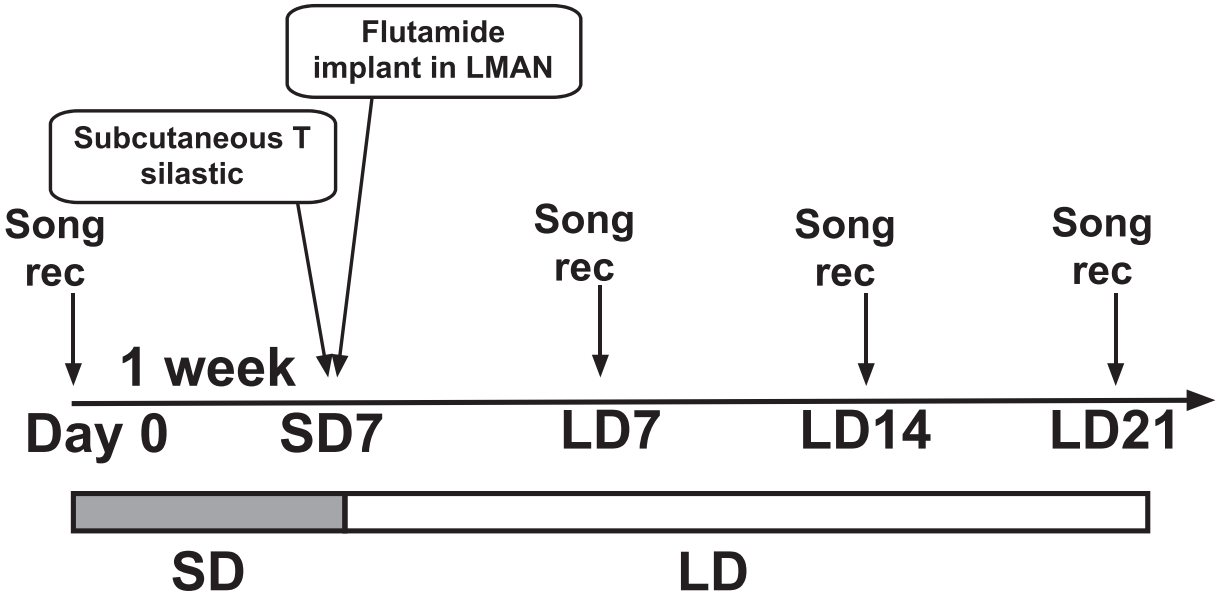

Fig. 1. Schematic representation of the time schedule of the experiment.

(3–4 %) and implanted subcutaneously with testosterone-filled Silastic™ implants (Dow Corning, Midland, MI, USA, outside diameter = 1.65 mm and inside diameter = 0.76 mm; 12 mm in length filled with 10 mm of testosterone) as described in previous studies (Alward et al., 2013, 2017a). These implants were previously shown to establish circulating testosterone concentrations in the upper range of reproductively active male canaries (Alward et al., 2013; Alward et al., 2016b).

Immediately afterwards, while still under anesthesia, birds were placed in a stereotaxic apparatus modified for use with small birds such as canaries with the beak holder placed 45° below the horizontal axis of the apparatus. Isoflurane anesthesia was maintained throughout the procedure. Birds were bilaterally implanted with 27-gauge cannulae targeting LMAN. Cannulae were filled with crystalline flutamide (Sigma Flutamide, F9397) or left empty as a control. Cannulae were filled over a length of about 2 mm with flutamide by tapping them repeatedly in the flutamide powder as described in Balthazart and Surlemont (Balthazart and Surlemont, 1990b). They were cleaned using acetone and a Kimwipe™ to remove any flutamide that stuck to the outside of the cannula.

Flutamide is a potent non-steroidal androgen receptor antagonist that has been used extensively in songbirds and other species as a global and a local antagonist for androgen signaling, with no reports of apparent toxicity or off-target effects (Balthazart and Surlemont, 1990a; Bottjer and Hewer, 1992; Fuxjager et al., 2012; Grisham et al., 2007; Meitzen et al., 2007; Soma et al., 1999; Sperry et al., 2010). Our goal was to place each cannula just dorsal to LMAN without entering into the nucleus to avoid damage to the structure of interest (Alward et al., 2013, 2017a; Meitzen et al., 2007). This technique has been used previously and shown to be effective in modifying specific aspects of singing behavior without confounding effects that could be induced by microlesions (Alward et al., 2013, 2017a; Meitzen et al., 2007). Our previous work demonstrated that the effects on song structure of similar flutamide implants near HVC or RA linearly decrease as a function of the distance from the target. Effects have essentially disappeared (data at control level) when the implant was 200–300 μm from its target.

Based on recent work lesioning LMAN in canaries (Rouse and Ball, 2016), we used the following stereotaxic coordinates for targeting the dorsal edge of LMAN: dorsoventral: −2.35 mm from the dorsal surface of the brain; anterior–posterior: 4.3 mm from the rostral tip of the cerebellum, and medial–lateral: ±1.48 mm from midline (Fig. 2).

A total of 11 male canaries were implanted with bilateral cannulae targeting LMAN, 7 with cannulae filled with flutamide and 4 with empty cannulae. Based on our past experience with similar studies (Alward et al., 2017a), we anticipated that in a number of flutamide-implanted birds the cannulae would miss the target and be too distant to exert any effect on LMAN. ARs are expressed in a discrete manner specifically in LMAN but not in the surrounding nidopallium (Balthazart et al., 1992; Bernard et al., 1999; Gahr and Metzdorf, 1997; Smith et al., 1996) and the stereotaxic implant procedure used here consequently produces highly localized effects.

2.4. Song processing and analysis

The daily 2.5 h-long song files were run through a high-pass filter set to a threshold of 900 Hz to remove low-frequency noise and converted to a digital format using Goldwave™ (Version 5.55; GoldWave, St. John's, NF, Canada) before they were visualized as sound spectrograms using Avisoft (SASlab Pro, Berlin, Germany), a Windows application for sound analysis. For the spectrograms, the fast Fourier transform length was set to 512 with an overlap of 75 % to increase temporal resolution. Songs were defined as vocalizations having a duration longer than 1 s with gaps no longer than 500 milliseconds (Alward et al., 2013, 2017a; Alward et al., 2014; Voigt and Leitner, 2008). Spectrograms were

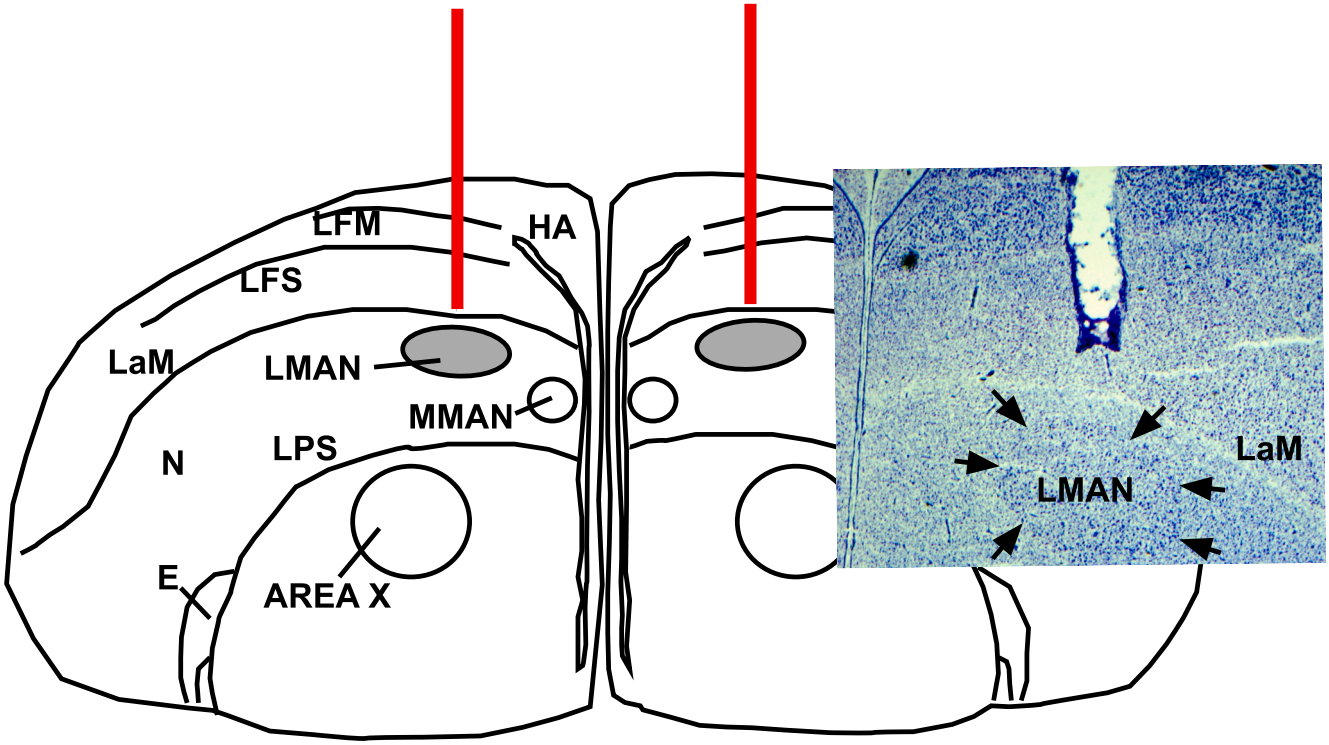

Fig. 2. Schematic representation of the location of flutamide-filled cannulae at the level of LMAN. The figure shows the path used to implant flutamide just dorsal to LMAN (red lines) and a representative photomicrograph of a Nissl-stained section including the cannula implantation track. The boundaries of LMAN are outlined by arrows.

Abbreviations–E: Entopallium; HA: Hyperpallium Apicale; LMAN: Nucleus Lateralis Magnocellularis Nidopallii Anterioris; LaM: Lamina Mesopallialis; LFM: Lamina Frontalis Suprema; LFS: Lamina Frontalis Superior; LPS:Lamina Pallio-Subpallialis; MMAN: Nucleus Medialis Magnocellularis Nidopallii Anterioris; N: Nidopallium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

visually inspected to further eliminate parts of recordings that corresponded to noise that escaped the filter.

We showed previously that the variability of song bandwidth (i.e., the difference between the minimum and maximum frequency) is substantially modified by testosterone action in canaries (Alward et al., 2013, 2017a). Avisoft was used here to measure the bandwidth of each song on each analysis day. We then computed the coefficient of variation (CV) of these data, i.e. the standard deviation of bandwidth across individual songs produced by a given bird divided by the average bandwidth across those songs multiplied by 100. CV is a measure of consistency of an acoustic feature over renditions of songs and has been used extensively in birdsong studies as a measure of song variability or stereotypy (Alward et al., 2013, 2017a; Alward et al., 2016b; Meitzen et al., 2007; Sakata et al., 2008). A higher value of CV means that across song renditions the feature of interest is more variable or less stereotyped. The number of songs produced, their duration and the total time spent singing were also measured to control for general non-specific effects.

Canaries sing a variety of syllable types (made of one or a few notes or elements always combined in the same way). Phrases are sequences of repeated syllables; songs consist of sequences of phrases that are sung in an order that may or may not vary between songs (Catchpole and Slater, 2008; Leitner et al., 2001; Nottebohm et al., 1986). In canaries, how those syllables are used and how they are sequentially arranged has been shown to change seasonally (Nottebohm et al., 1986).

We also quantified the acoustic stereotypy of syllables as performed previously (Alward et al., 2017a). In the present recordings as well as in birds previously studied (Alward et al., 2017a), all subjects sang a common syllable type with distinct acoustic and temporal features that permit an automatic analysis of this particular syllable type for all subjects. This presumably resulted from the fact that all subjects came from the same breeder and they probably heard and copied the same or similar songs during ontogeny. Therefore, as before, we quantified this particular syllable type in all birds to determine syllable bandwidth stereotypy. To conduct an unbiased analysis of the stereotypy of these syllables, all syllables were collected by the Avisoft software and, based on a random number generator, 40 of these syllables were selected for each bird on each day from the total number of syllables detected. The same features (syllable duration and bandwidth CV) as those used for overall songs were then quantified.

2.5. Quantification of syllable usage and syllable sequence variability

Syllables were manually labeled in Avisoft over 10–20 songs corresponding to 180–300 syllables for each bird on each day on which song was analyzed. Different syllables can be identified visually from sound spectrograms and we and others have used manual labeling methods to designate and tabulate different syllable types in canary song (Alward et al., 2016b; Iserbyt et al., 2017; Leitner et al., 2001; Nottebohm et al., 1986).

To quantify syllable usage variability, we calculated the zero order entropy of syllable usage, which describes the distribution of syllable usage:

$$\text{Zero order entropy} = -\Sigma\, p_i \text{ x } log_2(p_i)$$

where p_i is the probability of each syllable occurrence and the sum adds up probabilities for all different syllables used. Male canaries can sing anywhere from 15 to 40 different syllable types and this varies highly among birds. Therefore, zero order entropy values were standardized to the maximum amount of entropy possible (Parker, 2009):

$$\text{Syllable usage variability} = \text{zeroorderentropy}/log_2(\text{y})$$

where y is the number of different syllable types produced by each bird. This measure has been used in previous work (Parker, 2009). Syllable usage variability values varied between 0 and 1, where 1 is maximum possible entropy (i.e., exactly equal usage of all syllable types) and lower values correspond to a more skewed distribution, with some syllables being used more frequently and other being used more rarely. A higher syllable usage variability reflects a more random syllable usage and a more variable syllable usage.

To measure syllable sequence variability, we used methods similar to others and to our recent work (Nottebohm et al., 1986; Sakata et al., 2008). Given the large number of syllable types produced by individual canaries and the heterogeneity of the number of different syllable types produced between individual birds, we analyzed for each bird transitions from its dominant (i.e., most frequently used) syllable type to all other types as in Nottebohm et al. (Nottebohm et al., 1986). We repeated this analysis for the next four most frequently sung syllable types, to determine a sequence variability average for the birds. We first determined the dominant syllable type from the labeled syllables that were used for quantifications of syllable usage variability and then quantified first order entropy for the dominant syllable type:

$$\text{First order entropy} = -\Sigma\, p_i \text{ x } log_2(p_i)$$

where the sum is over all over possible transitions and p_i is the probability of the i^{th} transition from the dominant syllable type. As for syllable usage variability, we standardized this measure (Parker, 2009):

$$\text{Syllable sequence variability} = \text{Firstorderentropy}/log_2(\text{z})$$

where z is the number of different syllables that followed the dominant syllable type. This yielded syllable sequence variability values between 0 and 1, where 1 is the maximum entropy (i.e., maximum syllable sequence randomness) possible. In some cases, the dominant syllable type was followed by the same syllable sequence >95 % of the time. These types of transitions are considered to be fully stereotyped sequences (e.g., similar to motifs in zebra finch songs). For these situations, we treated the transitions from these stereotyped sequences as "branch points" (e.g., in the fully stereotyped sequence A-B, the transitions from B were used for calculating first order entropy) as done in previous studies (Hampton et al., 2009; Matheson et al., 2016; Sakata et al., 2008; Tchernichovski and Marcus, 2014).

2.6. Brain collection and verification of implant site

After 21 days of treatment, birds were deeply anesthetized (4 % Isoflurane), weighed, rapidly decapitated All brains were then extracted and fixed in acrolein. Brains were agitated in 5 % acrolein for 2 h, then washed for 15 min four times in phosphate buffered saline and cryoprotected in 30 % sucrose overnight. Brains were flash frozen in dry ice for 5 min, and then placed into a − 70 °C freezer until used.

We also measured at that time the length and width of the cloacal protrusion area (CPA) to compare with measures collected before the beginning of the treatments. The CP is an androgen-sensitive organ (Alward et al., 2017a; Alward et al., 2016b; Meitzen et al., 2007) that was expected to grow following exposure to exogenous testosterone and to the long day photoperiod. This provided a functional test of whether flutamide had or had not leaked from the brain and entered the general circulation. At brain collection we additionally measured the mass of each brain and the length (L) and width (W) of the left testis. These two values were used to compute an estimate of the testis volume based on the formula of an ellipsoid ($V = (4/3) * \pi * (L/2) * (W/2)^2$). Body mass was measured at the beginning and at the end of the experiment. We also confirmed that the testosterone implant was still present in all birds and was still filled with testosterone.

Brains were sectioned on a cryostat in four series of 30-μm-thick coronal sections that were stored in cryoprotectant at −20 °C. One series was later mounted on gelatin-coated slides, air-dried for one day, Nissl stained and coverslipped with Permount (Fisher Scientific).

Photomicrographs were taken at low magnification (2.5× objective) in all Nissl-stained sections that contained LMAN where the implant

tract was located using an Axiocam attached to a Zeiss Axioskop. If the implant track was not present in the section where LMAN was, adjacent sections were scanned to identify where it was and this was denoted as being out of LMAN for treatment grouping purposes. LMAN is easily identifiable in Nissl-stained sections in dorsal position relative to Area X and the cannula tracts are also readily apparent (Alward et al., 2013, 2017a). Implant locations and tracts were identified in these images and under the microscope. The goal was to place the cannula tips adjacent (dorsal) to the nucleus to minimize damage to the target nucleus (Meitzen et al., 2007). Based on these analyses, in 2 of the 7 flutamide-implanted subjects one or both implants were too distant from LMAN (cannula tracts located 120 μm or more rostrally to last section where LMAN was present) and data from these males were pooled with those of the control empty-implanted group. We later confirmed that the two birds moved from the flutamide to the control group had values for all song measures that fell in the range of control values. The final sample size for this experiment was therefore 5 Flutamide (FLUT) implanted males and 6 controls (CTRL) for all song analyses, but remained at 7 FLUT and 4 CTRL for the morphological measures assessing potential leakage of Flutamide from the brain into the general circulation.

2.7. Statistical analyses

Mixed-design ANOVAs were used to determine the effects of AR antagonism on song measures, using day (SD, LD7, LD14, and LD21) as the within-subjects factor and treatment (FLUT versus CTRL) as the between-subjects factor. Following significant interactions in the omnibus ANOVA, post-hoc Sidak tests (as recommended by Prism software and more powerful than the Bonferroni procedure) were used to determine the differences driving the interaction effects. A mixed-design ANOVA was also used to assess the effects of treatment on CP size, where time (pre-treatment versus post-treatment) was the within-subjects factor and treatment (flutamide versus control) was the between-subjects factor. Brain mass and estimated testis volume were compared between control and flutamide birds by Student *t*-tests. Effects were considered significant for $p \leq 0.05$. All analyses were done in SPSS and Graphpad Prism (version 8.4).

Effect sizes were calculated as Cohen d for comparisons of two groups (computed with the software available at http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD1.php) and partial eta square (η_p^2) based on the sums of squares in the two way ANOVA.

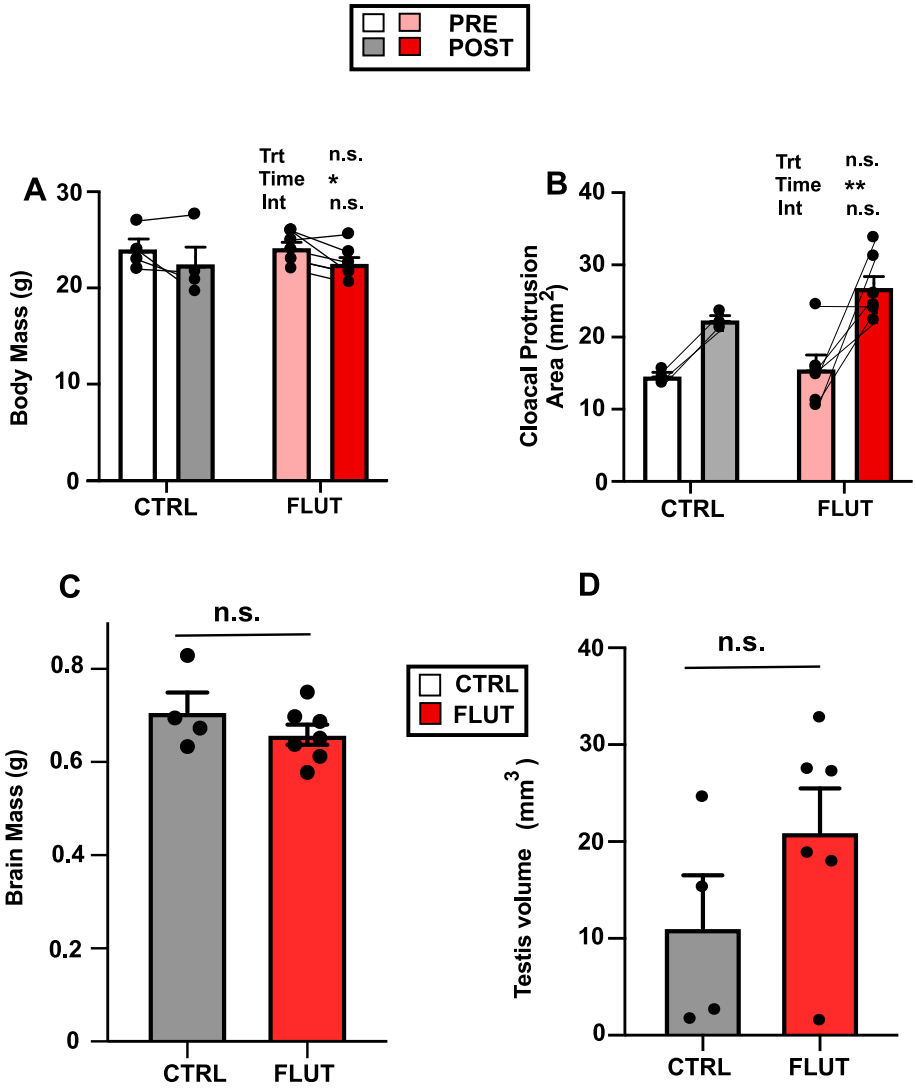

Fig. 3. Morphological measures from male canaries that had received an implant filled with flutamide (FLUT) or a control (CTRL) implant at the level of nucleus LMAN. In panels A-B, the insert summarizes the results of the two-way ANOVA (Trt: treatment, Int: interaction, *: $p < 0.05$, **: $p < 0.01$, n.s.: not significant). Pre/Post = before or after flutamide implantation.

3. Results

We first analyzed a number of morphological measures to assess whether the flutamide implants had affected the general condition of the birds and/or had leaked from the brain into the general circulation. For these analyses, all birds that had received empty implants ($N = 4$) were compared to all birds that had a flutamide implant irrespective of its position in the brain ($N = 7$).

Body mass was not affected by the treatment ($F_{1,9} = 0.007$, $p = 0.937$, $\eta_p^2 = 0.001$) nor by the interaction of treatment with time ($F_{1,9} = 0.006$, $p = 0.941$, $\eta_p^2 = 0.001$). Birds however lost around 6 % weight during the experiment ($F_{1,9} = 8.319$, $p = 0.018$, $\eta_p^2 = 0.480$; decrease of 1.5 g in controls and 1.6 g in Flutamide birds i.e., respectively 6.4 and 6.7 %) for reasons that are not understood and could potentially relate to the stress related to manipulations or to the isolation in sound-attenuating chambers (Ketterson et al., 1991) (see Fig. 3A).

As expected, the size of the cloacal protrusion increased after birds were transferred to a long day photoperiod and received a subcutaneous testosterone implant (effect of time: $F_{1,8} = 20.20$, $p = 0.002$, $\eta_p^2 = 0.716$). This increase was not affected by the FLUT implants ($F_{1,8} = 3.091$, $p = 0.117$, $\eta_p^2 = 0.279$) nor by the interaction of the treatment with time ($F_{1,8} = 0.703$, $p = 0.426$, $\eta_p^2 = 0.081$; Fig. 3B). Flutamide affected neither brain mass ($t_9 = 1.136$, $p = 0.285$, d = 0.715.; Fig. 3C) nor testis volume ($t_9 = 1.131$, $p = 0.287$, d = 0.846; Fig. 3D).

3.1. Song stereotypy

The measurements of overall singing activity (number of songs produced during 2.5 h on each day, average song duration and total time spent singing during 2.5 h on each day) were not affected by the flutamide treatment even if some aspects (number of songs and time spent singing) unexpectedly decreased with time after the transfer to long days and subcutaneous implantation of testosterone (see Fig. 4A-C and Table 1 for detail of statistical analyses). In contrast, the reproducibility of these songs across multiple renditions, as reflected by the bandwidth coefficient of variation (CV; Fig. 4D) changed with time and was also

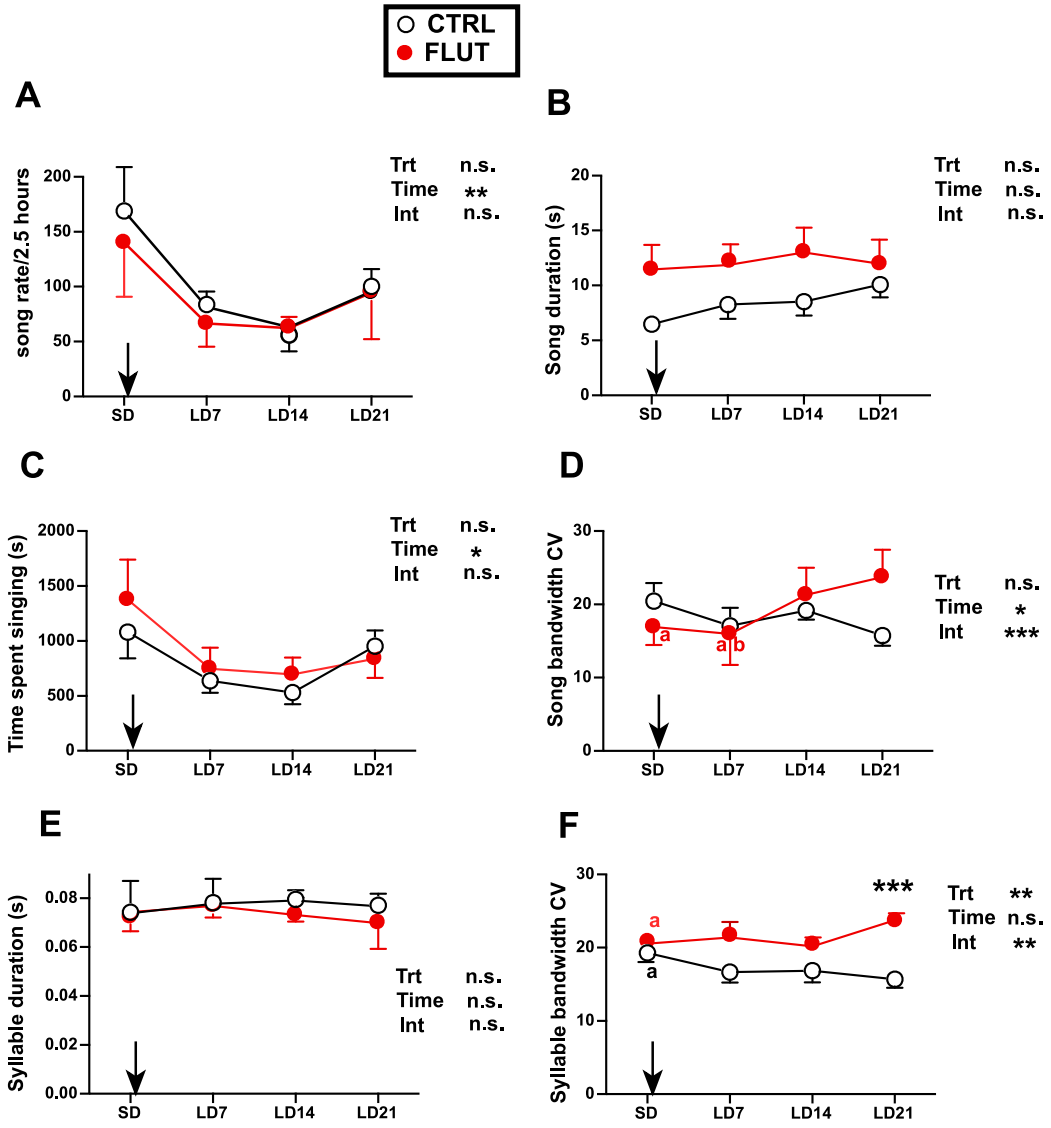

Fig. 4. Six song measurements (A-F) for control (CTRL) and flutamide-treated (FLUT) birds. In each panel, the arrow on the X axis indicates when birds received their testosterone subcutaneous implant and were implanted above LMAN with the cannulae filled with flutamide. Song rate and time spent singing are computed for the entire duration of the daily records i.e. 2.5 h. Each set of data was analyzed by a two-way ANOVA and results are summarized in the inserts (Trt: treatment, Int: interaction). Significant interactions were further analyzed by Sidak post-hoc tests: comparing the two groups at each time point and these results are represented by asterisks (*: $p < 0.05$, ***: $p < 0.001$). Comparisons of the different time points within each group are represented by letters (a: $p < 0.05$ vs. LD21, b: $p < 0.05$ vs. LD14; the letter color indicates the group concerned by the comparison). All data are means ± SEM.

Table 1
Results of the analysis by two-way ANOVA of the 6 song features that were measured. The table shows the F ratio, associated degrees of freedom (df), probability for each main factor and for the interaction and associated effect size (partial eta square). Significant effects are bolded.

Feature	TRT		TIME		INT.	
	F (df)	P (η_p^2)	F (df)	P (η_p^2)	F (df)	P (η_p^2)
Nbr of songs	$F_{(1,9)} = 0.150$	0.707 (0.016)	**$F_{(3,27)} = 7.087$**	**0.002 (0.441)**	$F_{(3,27)} = 0.190$	0.902 (0.021)
Song duration	$F_{(1,9)} = 3.381$	0.099 (0.273)	$F_{(3,27)} = 1.719$	0.187 (0.160)	$F_{(3,27)} = 0.943$	0.434 (0.095)
Time singing	$F_{(1,9)} = 0.269$	0.616 (0.029)	**$F_{(3,27)} = 4.213$**	**0.014 (0.319)**	$F_{(3,27)} = 0.403$	0.752 (0.043)
Song Bdw CV	$F_{(1,9)} = 0.210$	0.657 (0.023)	**$F_{(3,27)} = 3.390$**	**0.032 (0.274)**	**$F_{(3,27)} = 7.761$**	**<0.001 (0.463)**
Syll. duration	$F_{(1,9)} = 0.098$	0.761 (0.011)	$F_{(3,27)} = 2.279$	0.102 (0.202)	$F_{(3,27)} = 2.214$	0.109 (0.197)
Syll. Bdw CV	**$F_{(1,9)} = 10.850$**	**0.009 (0.547)**	$F_{(3,27)} = 1.173$	0.338 (0.115)	**$F_{(3,27)} = 5.829$**	**0.003 (0.393)**

associated with a significant interaction with treatment apparently reflecting the fact that this CV progressively increased with time in the flutamide group. The post-hoc analysis of the time effect and interaction by the Sidak tests comparing the different time points separately for the two groups of birds identified no change over time in the controls but showed that in FLUT males bandwidth CV was higher on LD 21 than in SD and in LD7 ($p = 0.007$ and $p = 0.002$ respectively).

Two examples of the distribution of song bandwidth in a control and a flutamide- treated bird illustrating the larger CV in the latter are shown in Fig. 5.

3.2. Acoustic variability of syllables

Quantification of syllable variability focused on their duration and on the CV of their bandwidth. Syllable duration was not affected by flutamide treatment (Fig. 4E). There was again an interaction between treatments and time for the syllable bandwidth CV (Fig. 4F). In this case there was also an overall effect of treatment. Post-hoc tests indicated that the difference between controls and flutamide-treated birds was significant on LD7 and LD21.

3.3. Syllable usage and syllable sequence variability

We annotated individual syllables and their sequences in sound spectrograms collected while birds were in SD and then on LD7 and 21. The mean repertoire size for the two groups, at the different time points, ranged between 14.8 and 17.6 different syllables and it did not vary as a result of flutamide implantation in LMAN. A two-way ANOVA failed to detect any effect of the treatment on the repertoire size ($F_{1,9} = 0.028$, $p = 0.870$, $\eta_p^2 = 0.003$). Repertoire size decreased slightly over time during the experiment ($F_{2,18} = 3.970$, $p = 0.037$, $\eta_p^2 = 0.306$), but there was no interaction between time and treatment ($F_{2,18} = 0.162$, $p = 0.852$, $\eta_p^2 = 0.018$). The time effect was small and post-hoc Sidak tests failed to detect significant differences between time points (SD vs LD7: $p = 0.072$, SD vs. LD21: $p = 0.075$, LD7 vs. LD21: $p = 0.999$).

We calculated the variability in how often different syllables were sung as the normalized zero-order entropy (see Methods). Flutamide treatment in LMAN did not affect the entropy measurement ($F_{1,9} = 0.877$, $p = 0.373$, $\eta_p^2 = 0.089$; Fig. 6A), which was also stable over time during the study, although a tendency to increase was observed after transfer to LD ($F_{2,18} = 3.483$, $p = 0.052$, $\eta_p^2 = 0.279$). There was no interaction of time with treatment ($F_{2,18} = 0.297$, $p = 0.746$, $\eta_p^2 = 0.032$).

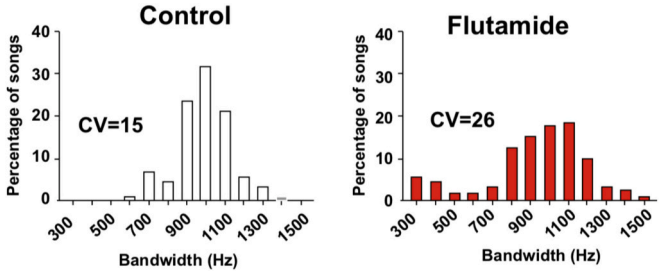

Fig. 5. Histogram illustrating the more variable distribution of bandwidth of whole songs sung by a control bird (left) and a bird that had received a flutamide implant near LMAN.

Besides their differential use in the entire song, syllables can also be used in variable sequences. This aspect of song organization was also quantified by a normalized measure of entropy that was calculated separately for the most frequently used (dominant) syllable and for the 5 most frequently used syllables (Fig. 6B-C). These two aspects of song structure were not affected by the flutamide treatment (Dominant: $F_{1,9} = 0.684$, $p = 0.429$, $\eta_p^2 = 0.071$; Top 5: $F_{1,9} = 0.727$, $p = 0.416$, $\eta_p^2 = 0.075$) nor by the interaction of this treatment with time (Dominant: $F_{2,18} = 0.211$, $p = 0.812$, $\eta_p^2 = 0.023$; Top 5: $F_{2,18} = 0.295$, $p = 0.748$, $\eta_p^2 = 0.032$). There was however a significant increase over time for both measures (Dominant: $F_{2,18} = 8.596$, $p = 0.002$, $\eta_p^2 = 0.489$; Top 5: $F_{2,18} = 56.93$, $p < 0.001$, $\eta_p^2 = 0.863$). These overall time effects were further analyzed by Sidak post-hoc tests that identified significant differences between LD 21 and the two other time points (SD and LD7).

4. Discussion

The present study demonstrates that blocking androgen receptors in LMAN by implantation of an adjacent cannula filled with the anti-androgen flutamide specifically increases acoustic variability of the canary male song over successive renditions without affecting several other features of the song such as number of songs produced, song duration and syllable use. Quite unexpectedly, the number of songs produced and the total time spent singing actually decreased in both control and flutamide birds following transfer to LD and systemic treatment with exogenous testosterone. The reasons for these changes observed in both groups of birds remain unclear. They could relate to the stress associated with the stereotaxic surgery, testosterone implantation and transfer from SD to LD, which would be consistent with the fact that there was apparently some recovery on LD21. Alternatively, we can hypothesize that the higher singing activity in SD reflects the photosensitive but non-photostimulated condition of the subjects. Indeed in a previous study of the annual cycle in singing activity of adult canaries, we observed that during the fall and early winter males sing at higher rate than in the spring (Cornez et al., 2020a). In the fall, under the influence of SD, canaries break the photorefractoriness they had developed during the previous summer (Hurley et al., 2008; Nicholls and Storey, 1977). During this period, canaries also sing a plastic song at a high rate presumably to try matching their production with the template they have stored in memory. During the annual cycle, song rate is thus higher during the fall/winter plastic phase than after crystallization in the spring (Cornez et al., 2020a). At the beginning of the present experiment, birds had been exposed to SD to induce photosensitivity which might explain their high singing rate that decreased following exposure to long days independently of the flutamide treatment. Additional experiments would be needed to discriminate between these possibilities.

It should be noted that while behavioral effects were observed here, no major change in the bird's general health was detected. Although a

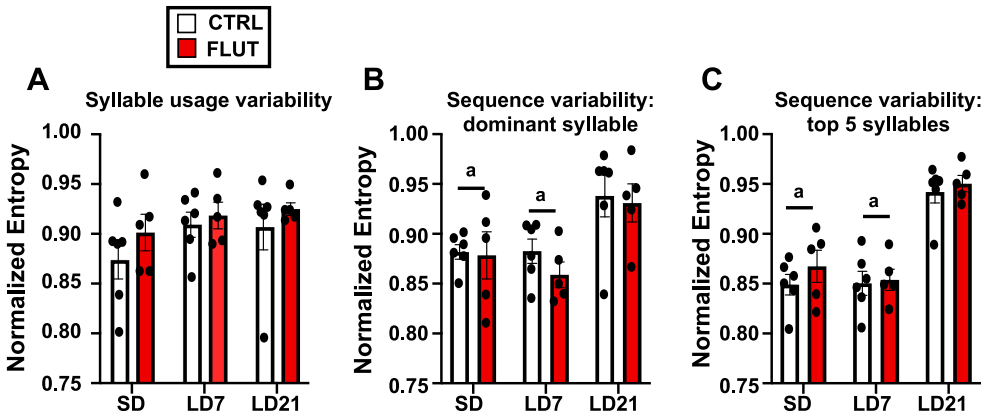

Fig. 6. Effects of flutamide acting on LMAN on (A) syllable usage variability, (B) syllable sequence variability of the dominant syllable and (C) sequence variability of the five most frequently sung syllables in male canaries as compared to control birds. Significant effects of time were further analyzed by Sidak post-hoc tests comparing grouped data of the two groups for each pair of time points. Results of these tests are represented by letters (a: $p < 0.05$ vs. LD21). All data are means ± SEM, individual data points are also plotted.

slight decrease (6 %) in body mass took place in both groups of subjects, all birds apparently remained fully active, eating, drinking and singing at the same rate after flutamide treatment as in controls. The same conclusion concerning the central and limited action of flutamide implants had actually been reached in two separate experiments analyzing the role of androgens on song specifically in HVC and in RA: no general effect on health and no leakage of the drug to the periphery was also observed in these experiments (Alward et al., 2017a).

At the behavioral level this change in song variability across renditions was also quite specific. Measures of song duration and singing rate were not affected by flutamide. Similarly, measures of syllable usage and syllable sequencing variability showed no effect of flutamide treatment. These results agree well with data accumulated over the last 20–30 years based on lesions or electrophysiological studies of LMAN. It has indeed been demonstrated that besides being critically implicated in song learning during development (Aronov et al., 2008; Fee and Goldberg, 2011; Scharff and Nottebohm, 1991), LMAN plays a key role in the maintenance of song stability largely by generating variability in acoustic features of the whole song and of individual syllables across successive song renditions that can then be compared with the learned template (Fee and Scharff, 2010; Kojima et al., 2018; Moorman et al., 2021; Williams and Mehta, 1999).

It is of particular interest that the measures of syllable use and syllable sequence were not affected by blocking androgen receptors in LMAN. Our previous work indeed demonstrated that these aspects of song are controlled by androgen action at the level of HVC: flutamide blockade of androgen receptors in HVC controls song variability by increasing the variability of syllable-type usage and syllable sequences (Alward et al., 2017a). The control mechanisms for the order and use of syllables are thus based on androgen action in HVC and LMAN is not implicated.

We were, however, somewhat surprised by the observation that although flutamide did not affect syllable sequence variability, this measure was markedly increased on LD21 as compared with measures taken while birds were still in SD and had not yet been implanted with Silastic capsules filled with testosterone (see Fig. 6B-C). We wondered whether this effect could potentially be related to lesion by the cannula track of an unidentified brain region that is necessary to reducing stereotypy. Analysis of songs recorded on LD 7 makes this interpretation less likely. Indeed, on LD7 the cannulae had been in place for a week and this increased variability was not yet present. It was also not observed in the control birds. It is therefore likely that the increased variability on LD21 reflects a slowly developing effect of the transfer to long days plus exogenous testosterone treatment in intact birds. This then begs the question of why syllable usage variability would increase in conditions mimicking reproduction and how this compares to previous work. Unfortunately, to our knowledge, this specific aspect of the canary song has never been quantified in short day birds so this comparison cannot be made. In addition, if this effect was due to an LMAN lesion specifically, it would be expected to have the opposite direction of what is observed based on previous work. Indeed multiple studies demonstrate that LMAN promotes endogenous variability that serves to guide the trajectory of vocal motor learning in juvenile males (Kao et al., 2005; Olveczky et al., 2005; Thompson et al., 2011). Even in adulthood, as shown in a previous experiment, testosterone-treated adult female canaries that were given a chemical lesion of LMAN produced a song that was less variable than in control birds: diversity of syllables and phrase types was smaller in lesioned birds as compared to controls (Rouse and Ball, 2016). An interpretation based on lesions is thus unlikely and this finding deserves further study.

The present demonstration that androgen action in LMAN decreases song bandwidth variability of whole songs and individual syllables provides a clear anatomical substrate for the effects of androgen on song variability that had been previously described. It is well established that treatment of juvenile songbirds with testosterone precipitates crystallization of their song, i.e., decreases its variability (Marler et al., 1988). In addition song variability varies across the annual cycle being lower during periods of active reproduction when circulating testosterone concentrations are high (Brenowitz et al., 1998; Cornez et al., 2020a; Smith et al., 1995) (see (Williams et al., 2003) for similar data in zebra fnches). This correlation actually reflects a causal link as demonstrated by the fact that treating adult males with exogenous testosterone also leads to the development of a song with a high degree of stereotypy (Cornez et al., 2020b).

The increased bandwidth variability observed in males treated with flutamide in LMAN could be based on two alternative mechanisms: an increased variability in bandwidth of syllables that were already included in the songs or a change in syllable usage with incorporation in the song of new syllables with larger bandwidth and/or decreased usage of syllables with a low bandwidth. Based on the fact that flutamide in LMAN did not modify the mean repertoire size and also did not change syllable usage variability (how often the different syllables were sung), it seems likely that the first of these options applies even if we cannot completely rule out the other interpretation. It would be interesting in future studies to analyze in greater detail syllable usage after flutamide treatment to further test these possibilities.

It is fairly well established that song plasticity is triggered by LMAN to RA inputs and testosterone silences these inputs thus driving more stereotyped vocalizations (Kojima et al., 2018; Moorman et al., 2021; Woolley and Kao, 2015); see (Rouse and Ball, 2016) for more

discussion). Several mechanisms could mediate this effect including changes in myelination, modulation of catecholaminergic inputs, changes in synaptic properties and remodeling of neuronal architecture. It is however impossible to determine based on the present data what cellular processes are affected by the androgen receptor blockers.

In conclusion the present data indicate that testosterone action in LMAN modulates bandwidth variability of whole songs and individual syllables. This effect is presumably most prominent during the reproductive season when circulating concentrations of testosterone are high, while in the absence of androgens this nucleus generates variability in song bandwidth between renditions. A decrease in song variability and a concomitant increase in song stereotypy is a hallmark of song crystallization that occurs in temperate zone songbird species in the spring of their first year when onset of crystallization is dependent on testosterone (Marler et al., 1988). The findings in our study are consistent with the hypothesis that one key brain site of action of testosterone on song crystallization is LMAN. As previously reviewed LMAN input into RA is a key source of song variability needed during the song learning process and this functional input of variability continues in adulthood. LMAN prominently expresses androgen receptors (Balthazart et al., 1992). Androgen action increases song stereotypy in the context of song crystallization (Marler et al., 1988), in relation to seasonal variation in song (Brenowitz et al., 1998; Smith et al., 1995) and based on exogenous testosterone treatment prior to crystallization (Korsia and Bottjer, 1991; Whaling et al., 1995). Our results as well as the lesion studies indicating that the induction by testosterone of sensorimotor song development in female canaries requires an intact LMAN (Rouse and Ball, 2016) are consistent with the hypothesis that LMAN is the critical site of action of testosterone in the gating of song crystallization associated with a decrease in the variability of acoustic features of syllables and songs across renditions, which, in canaries can occur in a seasonal context and during ontogeny at the onset of sexual maturity (i.e. crystallization).

This work completes a series of studies initiated about 10 years ago to identify the brain sites where androgens control diverse aspects of song. Androgen receptors in canaries are expressed in three nuclei of the vocal control system in addition to the medial preoptic nucleus. Taken together these studies have shown that at each of these brain sites testosterone plays a specific role in the control of song. In HVC, androgens regulate variability in song syntax (Alward et al., 2017a), in RA they regulate variability in phonology (Alward et al., 2017a) and now in LMAN, we show that they control bandwidth stereotypy. This is in addition to the effects of androgens on singing motivation related to their action in the medial preoptic area (Alward et al., 2013; Alward et al., 2016b) and their effects on the muscles of the syrinx that modulate sound production and are known to express androgen (Alward et al., 2016a; Dos Santos et al., 2023) (for review, see Alward et al., 2017b; Ball et al., 2020). Testosterone thus clearly has multiple specific effects in the control of song acting at least at five different locations inside and outside the brain.

CRediT authorship contribution statement

Beau A. Alward: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Jacques Balthazart:** Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Data curation. **Gregory F. Ball:** Writing – review & editing, Project administration, Funding acquisition, Data curation, Conceptualization.

Data availability

Data will be made available on request.

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