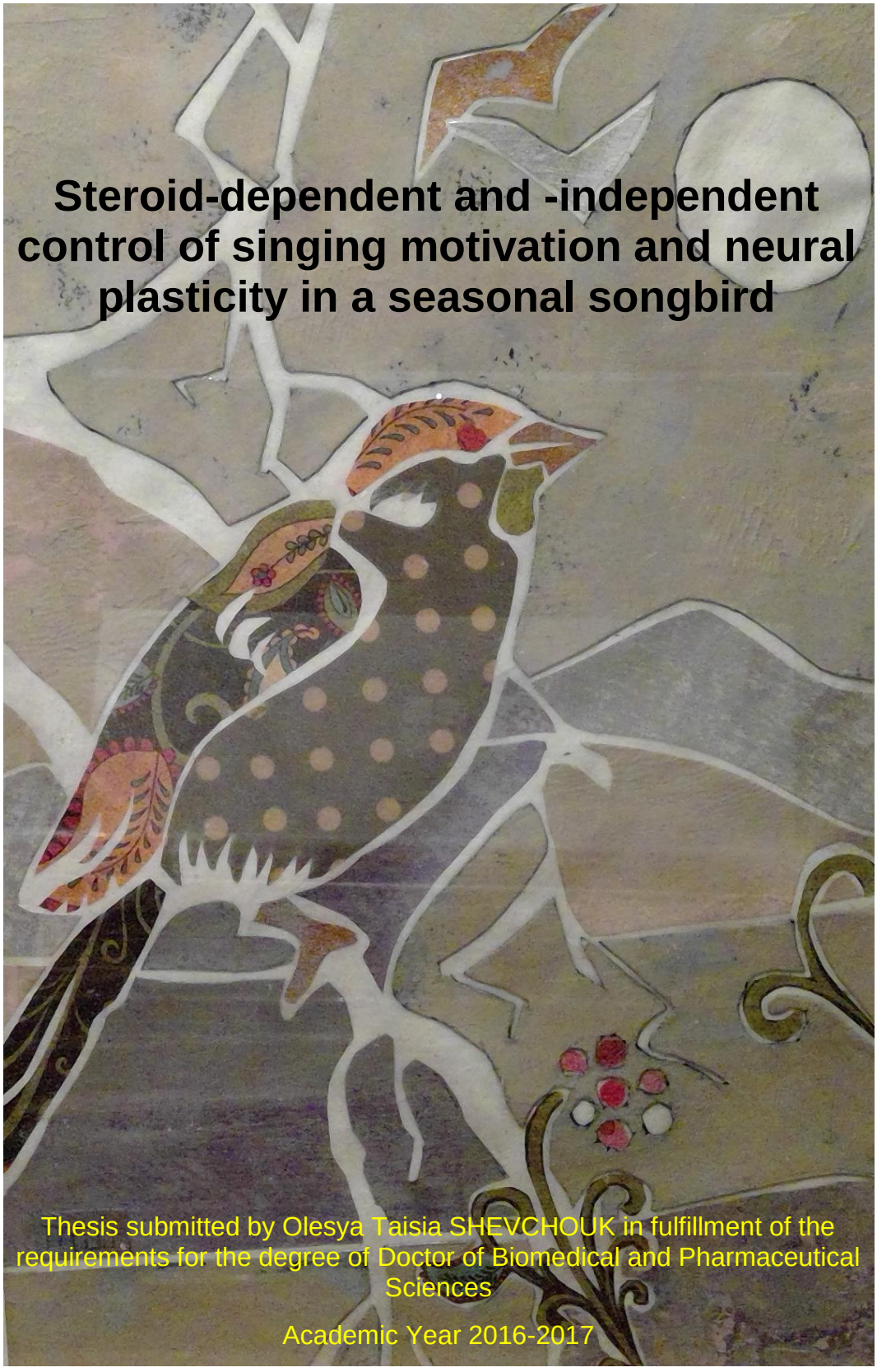
Steroid-dependent and -independent control of singing motivation and neural plasticity in a seasonal songbird



Thesis submitted by Olesya Taisia SHEVCHOUK in fulfillment of the requirements for the degree of Doctor of Biomedical and Pharmaceutical Sciences

Academic Year 2016-2017

Steroid-dependent and -independent control of singing motivation and neural plasticity in a seasonal songbird

Titre du doctorat en français: Contrôle de la motivation à chanter et de la plasticité neurale chez une espèce saisonnière d'oiseau chanteur - Effets hormonaux et non-hormonaux

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Tot candje si tél'mint vite, mès djins, qu' "îr" c'èst dèdja "li bon vî tins" (Walloon expression: Everything changes so fast, my friends, that "yesterday" is already "the good old days").

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Introduction

This thesis is dedicated to the analysis of singing behavior and the neural structures that underlie its learning and production in a seasonal songbird species, the domesticated canary (*Serinus canaria*). We will start by describing the utility of the songbird species in neuroscience research and describing the neural network controlling song. Next, we will outline the mechanisms controlling seasonality in avian species. Sex steroid hormones play a critical role in the seasonal modulation of the song system and therefore the following section will explain the steroid hormone actions involved. The last two sections of this introduction will then characterize in more detail the plasticity of the song control system and the nature and controls of birdsong itself.

Part 1. The songbird model

A model for human vocal development

Vocal learning is a rare trait in the animal kingdom. Among non-human mammals it has been identified only in cetaceans, elephants and a few species of bat. The oscines, also called songbirds, comprise 4500+ species that learn their species-specific vocalizations and use them as a form of communication. A major reason for interest in songbirds as a model comes from the translational perspective – the way songbirds learn to sing is similar in many ways to how human babies learn to speak. The findings generated by songbird research have been applied with some success to studies of human conditions with deficits in vocal learning, such as the autistic spectrum disorder (Panaitof, 2012).

Learning to sing or speak requires a complex interaction of the motor and perceptual systems. Disruption of auditory feedback at any point in the individual's life has a substantial detrimental effect on singing and speaking, although this is particularly damaging when this disruption takes place early in vocal development. Both in humans and songbirds vocal learning starts with listening. During this purely 'sensory' period, songbirds save a template of their tutor's song, which they use in their later efforts of matching their own song to the tutor song. In fact, thanks to the neural template, continued exposure to the tutor is not necessary at the latest stages of vocal development. After the sensory phase and before the production of mature vocalizations, birds start producing sounds and comparing them both to the live tutor

(if still present) and to the saved template; in this way they slowly increase the similarity between their own vocalizations and those of the tutor. In humans, this phase is commonly referred to as babbling, in songbirds it is divided in two phases, first subsong followed by plastic song. Both humans and songbirds start by producing a wide range of sounds, with time narrowing them down to the sounds typical to their native language or species, respectively. Another parallel between human languages and birdsong is the presence of dialects in both. Even the geographical distribution of songbird dialects is not too dissimilar to that of human dialects, at least in some parts of the world (Planqué et al., 2014).

Another important similarity between songbirds and human vocal behavior concerns the timing of ontogenetic development. Correlative studies and case studies have shown that humans are only capable of learning to speak up to a certain age (Friedmann and Rusou, 2015). Having missed the 'critical window' of opportunity, they will not be able to learn to speak if exposed to language only at later phases (Fromkin et al., 1974). In songbirds, it has been experimentally established that a critical window for learning also exists. Zebra finches deprived from a tutor until the age of 100 days post-hatch develop abnormal, simplified songs compared with normal, learned song (Marler, 1970) although they still show some features of species-specific song (Marler and Sherman, 1985), indicating that there are some innate predispositions towards learning and developing species-specific song. Multiple morphological and physiological changes in the brain occur simultaneously with the closure of the critical period and possibly constitute its mechanistic basis, including changes in spine density, axonal arborization, NMDA receptor current decay and perineural nets (reviewed by Brainard and Doupe, 2013). The precise timing of critical period closure depends on the individual's experience and hormonal state – it can be prolonged by social isolation or sensory deprivation, but testosterone speeds up the development of the fully mature stable song also called its crystallization (Templeton et al., 2012; Whaling et al., 1995) while decreasing testosterone concentrations via castration prolongs the critical period for learning (Marler et al., 1988).

Stereotypical behavior and clear anatomical substrate

An important reason for the popularity of the songbird model in research resides in the clear demarcation of the behavioral and neuroanatomical measures that can be collected. It is reasonably unambiguous to define a song, when it starts and finishes,

to break it down into subparts such as phrases, syllables and notes (see figure 1) and to compare different versions of it across time in the same individual or in different individuals within the same species.

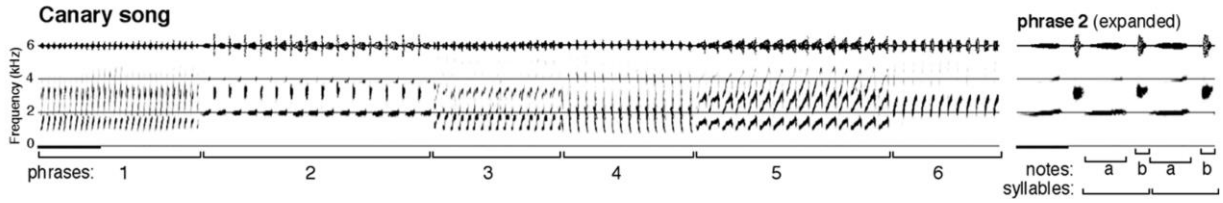

Figure 1. Example of a spectrogram of a recording of canary song with the different phrases constituting it indicated below by numbers (left) and a magnified view of phrase two from the same song showing the notes and syllables in this phrase (right), (Williams, 2004).

At the same time, since the discovery of the song control nuclei in the 1970s, it has also become possible to identify and study the specific brain regions controlling individual features of song. These brain nuclei differ from their neighboring regions by many anatomical, neurochemical and physiological parameters, making them distinct and well-defined. The combination of a well-defined neural substrate associated to a specific behavior provides the opportunity to make relatively straight-forward conclusions about the mechanisms of neural regulation of this behavior. Furthermore, the hierarchical organization, gene expression patterns and morphological properties of the song control system in songbirds is similar in many ways to the equivalent circuits controlling vocal production in the human brain (see figure 2).

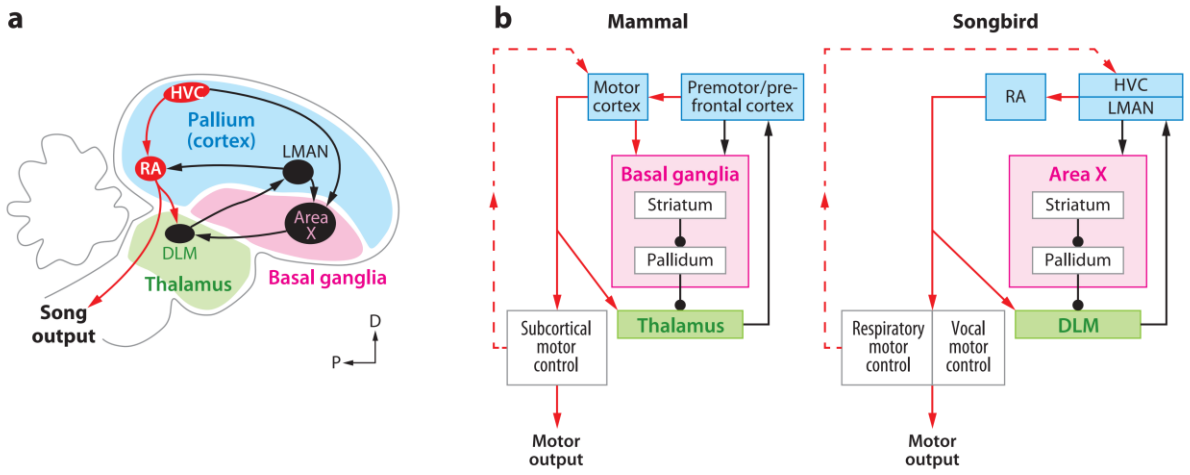

Figure 2. Schematic representation of song control nuclei (a) and diagram showing the homology between songbird and human brain regions controlling vocal production, (Brainard and Doupe, 2013).

The song control system

The songbird brain contains a specialized neural network that controls the learning and production of song, the so-called song control system (Nottebohm et al., 1976). This series of nuclei, discovered through tract-tracing and lesion experiments, is absent in other avian species. The song control system is generally parsed into two interconnected pathways – the motor and learning pathways (see figure 3 – motor pathway in green and learning pathway in yellow) and includes telencephalic, diencephalic, mesencephalic and myelencephalic nuclei (reviewed by Balthazart and Ball, 2016). Both are connected also with auditory areas (see figure 3, blue and red arrows), whose inputs are very important for learning and maintaining song throughout life.

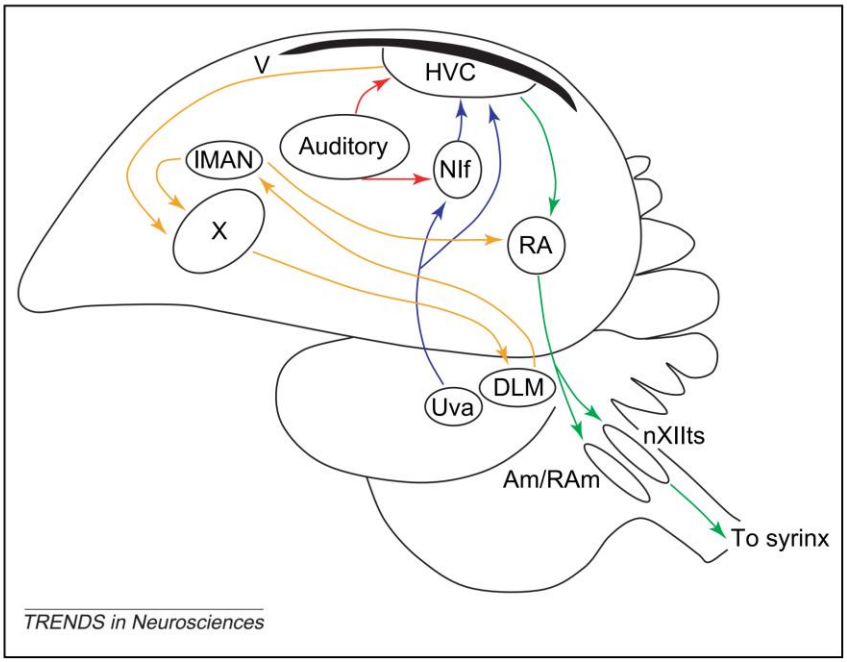

Figure 3. Schematic representation of the song control system. The motor pathway is indicated in green, the anterior forebrain (learning) pathway in yellow, the ascending auditory input in blue and the higher-level auditory inputs in red, (Brenowitz and Beecher, 2005).

Motor pathway

The motor pathway starts with the pre-motor nucleus HVC (high vocal center, used as a proper name, Reiner et al., 2004) where the structure and organization of song is determined (Yu and Margoliash, 1996). Signals generated in HVC are transmitted to the motor nucleus RA (robust nucleus of the arcopallium), which in turn projects to the tracheosyringeal part of the nucleus of the XIIth cranial nerve (nXIIts) that innervates the vocal production organ, the syrinx (Wild, 2004, 1994). Bilateral lesions of nuclei in

this motor pathway eliminate the production of song (Nottebohm et al., 1976; Simpson and Vicario, 1990). Detection of immediate early genes and electrophysiological responses during singing in these nuclei (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997; Yu and Margoliash, 1996) provide further evidence of their role in song production. HVC neurons firing shapes the electrophysiological pattern of activity of RA neurons (Dave et al., 1998; Leonardo and Fee, 2005), which in turn determines the spectro-temporal features of song (Vu et al., 1994). HVC encodes a different level of song structures than does RA (Yu and Margoliash, 1996): microstimulation in HVC causes an interruption of singing, while the same stimulation in RA disrupts only the structure of syllables without altering song patterning (Vu et al., 1994).

RA also projects to several other medullary components of this circuit, including two nuclei that coordinate respiration with song production, the nucleus retroambigualis (RAm) and the nucleus parambigualis (PAm) (Schmidt and Wild, 2014). Additionally, RA has an indirect projection to these same medullary nuclei via the dorsomedial portion of (DM) of the nucleus intercollicularis (ICo). DM is equivalent to parts of the periaqueductal gray (PAG) of mammals (Holstege, 1989; Wild and Balthazart, 2013) and when electrically stimulated will drive vocalizations accompanied by appropriate respiratory patterning in both songbirds and non-songbirds (Wild, 1997; Wild and Balthazart, 2013). Finally, the respiratory centers send recurrent information back to HVC via the thalamic nucleus uvaeformis (Uva) and the auditory interfacial nucleus (Nif) indicating the importance of bidirectional coordination between telencephalic and brain stem structures in vocal control (Schmidt et al., 2012). These bilateral projections are likely also involved in mediating the hemispheric coordination observed during song production (Ashmore et al., 2007; Schmidt, 2003; Wild et al., 2000). Uva and Nif are implicated in organizing syllables into larger units of vocalization (Margoliash, 1997).

Learning pathway

In this pathway, not required for song production but critical for song learning and maintenance (Jarvis et al., 1998), HVC also projects to RA but indirectly via an anterior forebrain loop. Anatomical, physiological and behavioral evidence supports the identification of this pathway as a specialized basal ganglia-thalamo-cortical loop, homologous to the cortico-basal ganglia reward circuit in mammals (Fee and Scharff, 2010). HVC projects to Area X, a homologue of parts of the mammalian striatum and

pallidum. The pallidal segment of Area X sends inhibitory projections to the medial nucleus of the dorsolateral thalamus (DLM). DLM projects to the lateral magnocellular nucleus of the anterior neostriatum (now nidopallium, LMAN), a frontal cortex-like nucleus and from here the circuit is closed with a projection to RA. This excitatory projection constitutes the integrated output of the anterior forebrain pathway processing (Brainard and Doupe, 2013). LMAN additionally projects to Area X, both of which are targets of strong midbrain dopamine projections (Bottjer and Johnson, 1997).

LMAN input into RA injects variability into song which is essential for sensorimotor song learning (Nottebohm, 2005). LMAN appears to facilitate motor variability via its glutamatergic projections to RA and this results in a functional remodeling of RA circuits that in turn modifies behavioral output (reviewed in Nottebohm, 2005). Similar to what is observed in the motor pathway, singing activity leads to induction of immediate early genes in this pathway (Hessler and Doupe, 1999; Jarvis and Nottebohm, 1997; Jin and Clayton, 1997). One theory of how the anterior forebrain pathway maintains song structure is via an error correction process (Benton et al., 1998; Brainard, 2004; Brainard and Doupe, 2000).

Lesions of the nuclei in this circuit during song learning prevent birds from developing normal adult songs (Bottjer et al., 1984; Sohrabji et al., 1989a), consistent with a function in sensory or sensorimotor learning. More specifically, lesions of the output nucleus, LMAN, during development causes a strong reduction in song variability and an inability of developing song to fully match the tutor song (Bottjer et al., 1984; Scharff and Nottebohm, 1991). Lesions of Area X similarly disrupt song learning but do not affect song variability (Scharff and Nottebohm, 1991). On the other hand, lesions to nuclei in this pathway in adult birds do not immediately alter song production (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Sohrabji et al., 1990), however there is a subtle decrease in song variability and in some forms of adult song plasticity (Brainard and Doupe, 2000; Kao et al., 2005; Thompson et al., 2007). DLM seems to have a role in song initiation, a lesion of this nucleus in combination with an HVC lesion has an overall effect on reducing the song output, compared to HVC lesion alone (Chen et al., 2014).

Auditory inputs

The primary auditory cortex homologue in songbirds is Field L, which projects to higher level processing regions, the caudo-medial nidopallium (NCM) and caudo-medial mesopallium (CMM). Both regions display immediate early gene expression and neurophysiological activity in response to hearing song (Knudsen and Gentner, 2010; Moorman et al., 2011). Auditory information from the cochlea reaches field L via the thalamic nucleus ovoidalis (Ov) and the mesencephalic dorsal part of the lateral mesencephalic nucleus (MLd, reviewed in Hahnloser and Kotowicz, 2010). A discrete sub-region of field L, the interfacial nucleus (Nif) projects to HVC, constituting the primary auditory input into the song control system (Fortune and Margoliash, 1995). During the initial song memorization period of song learning, Nif is required for successful imitation of the tutor song, lesions lead to impaired song imitation (Roberts et al., 2012). In adult songbirds, lesions of Nif do not significantly affect singing except during the first two days following the surgery (Otchy et al., 2015), indicating that Nif is not necessary for maintenance of crystallized song. However, activity in Nif increases shortly before the onset of vocalizations, suggesting a premotor role for Nif in song production (McCasland, 1987). A subregion of the CMM, the avalanche nucleus (Av) also has bidirectional connections both with HVC and Nif, Av additionally receives projections from the thalamic nucleus Uva (Akutagawa and Konishi, 2010).

Different learning trajectories

The study of singing in oscine species has been particularly informative due to the diversity of species in this clade. The different learning trajectories and neurochemical adaptations across species provide an opportunity to learn about the control of vocalization through a comparative approach (see figure 4). The zebra finch, the songbird species most commonly studied (Williams, 2004), is an example of a closed-ended learner: song development entirely happens during the first 90 days after hatching and is completed around the time of sexual maturity (Bohner et al., 1990; Eales, 1985). The sensitive period for song memorization covers the first 25–60 days post-hatch, while the sensorimotor phase begins around 35 days post-hatch. Song is gradually improving in structure until it is crystallized in its adult form around 120 days post-hatch (Nordeen and Nordeen, 2004). Each zebra finch develops its own ‘song motif’ which is made up of elements usually repeated in the same order (Williams, 2004). Although small variations in note number and sequence persist beyond

puberty, the acoustic structure of individual notes remains fixed (Brainard and Doupe, 2001; Nordeen and Nordeen, 1993, 1992).

In contrast, canaries and starlings are open-ended learners, they are able to modify their songs or syllables in adulthood and continue to add new songs to their repertoires every year (Chaiken et al., 1994; Nottebohm and Nottebohm, 1978). Song sparrows are closed-ended learners but the stereotypy and rate of their song production varies seasonally even if syllables that are sung do not vary after birds reach adulthood (Marler and Peters, 1987; Smith et al., 1997). Other species, like brown-headed cowbirds are not able to learn new songs throughout life, but they delay the rehearsal and production of some of the phrases learned in early ontogeny to their second and/or third year of life (O'Loghlen and Rothstein, 2002).

Although research on zebra finches has been very useful in elucidating the mechanisms of song learning and production, this species is unusual compared to other songbird species in multiple ways. As already discussed, the sensitive period for song learning is taking place very early and is short compared to other species, and the song repertoire is a single song, while other species have up to tens or hundreds of songs in their repertoire. Additionally, zebra finches imitate their tutor closely and only if it is a zebra finch song, while many other species can also imitate songs from species other than their own or copy the tutor song only partially, modifying song elements to create novel songs. Lastly, in some species song development is possible even when subjects are raised in isolation: this has for example been shown in grey catbirds and sedge warblers (Brenowitz and Beecher, 2005). The diversity of song learning strategies along all these parameters allows for a very informative comparative approach for determining the mechanisms of song learning, choosing the species most appropriate for answering specific questions or directly comparing across species. Examples of the former approach include studying the correlation between neurogenesis and song memorization versus song rehearsal in swamp sparrows where these two processes take place at different times during development (Nordeen et al., 1989), as opposed to zebra finches where they overlap. An example of a useful species comparison concerns the study of electrophysiological responses of different types of HVC neurons to the birds own song using different song types in swamp sparrows who have a repertoire size of 2-5 song types (Mooney et al., 2001) rather than just one song type in zebra finches. The comparative approach has also

been used to show that adult HVC neurogenesis is not necessarily linked to adult song plasticity. This had been thought to be the case based on studies in canaries who have higher rates of neurogenesis during the seasons when they are adding new song syllables (reviewed in Nottebohm, 2004), but song sparrows do not demonstrate song learning in adulthood while they show seasonal peaks in HVC neurogenesis (Tramontin and Brenowitz, 1999).

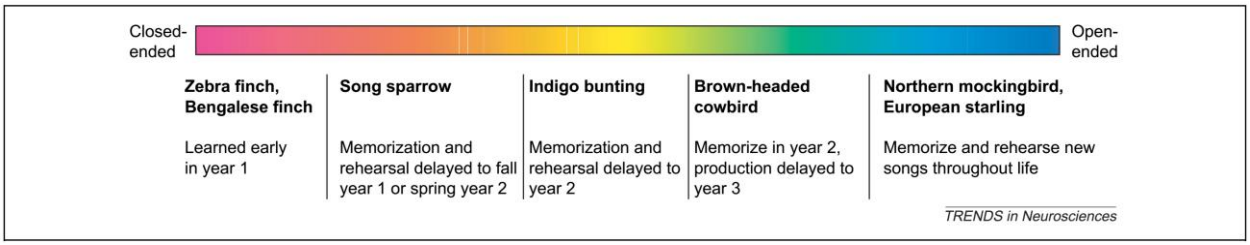

Figure 4. Continuum of song learning strategies in different species of songbirds, (Brenowitz and Beecher, 2005).

Sex dimorphism

In many songbird species, particularly the species that live in temperate climates, singing is a sexually differentiated behavior and examining the details of sex differences in singing and in the song control system provides further insight into brain-behavior relationships. Males sing to attract females during the breeding season, while females respond to high quality male songs by breeding with them. In many species, females will develop a male-like song (Nottebohm, 1980a) and at the same time male-like neural attributes in the song system (DeVoogd and Nottebohm, 1981a; Madison et al., 2015) after being treated with testosterone. In some songbird species that live in tropical climates females sing to the same extent as males and often engage in duets with them (Fortune et al., 2011).

A comparative approach in examining sex differences is also useful. Songbird species can be categorized along a continuum ranging from those species in which only males sing to those where males and females engage in complex, interactive duets (Brenowitz and Arnold, 1986). Comparing across these different species it can be noted that there is in general a direct relationship between the degree of sex differences in singing behavior and of the volumes of song control nuclei, suggesting that the capacity to sing may be constrained by the size and number of neurons in the song control system (Brenowitz and Arnold, 1986; Kirn, 2010; MacDougall-Shackleton and Ball, 1999), even if there are some exceptions to this rule. In the case of zebra finches where females do not sing at all, even after treatment with exogenous

testosterone, the major motor projection connecting HVC to RA, is very diminished and the projection from HVC to Area X is even completely absent (Konishi and Akutagawa, 1985).

Part 2. Seasonality

Avian photoperiodism

Seasonal songbirds use day-length as a predictive cue to anticipate the onset of the forthcoming breeding season and adjust their physiology accordingly. This is particularly important for species living in the temperate zone. In spring increasing daylengths stimulate the secretion of gonadotropin-releasing hormone (GnRH) leading to the gonadal maturation necessary for breeding (Dawson et al., 2002). After a certain duration, the same long photoperiod that stimulated the growth of the gonads also causes their regression, a state called photorefractoriness. During this phase, even 24-hours of light cannot stimulate growth of the gonads (Hamner, 1968). The mechanism of onset of photorefractoriness under the same photoperiod that was previously stimulating is yet unknown. A short-day photoperiod dissipates photorefractoriness, reinstating the sensitivity to light (Lofts and Coombs, 1965; Steel et al., 1975). However, some seasonally breeding avian species, such as Japanese quail, do not enter a photorefractory state during the same long-day photoperiod that was previously photostimulating. Instead a decrease in daylength is needed in order to start gonadal regression (Robinson and Follett, 1982). In these species, the daylength that will initiate regression is however longer than the photoperiod that is initiating gonadal growth, therefore there is still an asymmetry in the breeding season; these birds are considered to show relative photorefractoriness, as opposed to absolute photorefractoriness.

Male and female canaries of the Border strain have small gonads when maintained on a photoperiod of 8 hours of light and 16 hour of dark (8L:16D) but exhibit a robust gonadal growth when transferred to 16L:8D (Storey and Nicholls, 1978). Furthermore, after 6 weeks of exposure to a long-day photoperiod they show a spontaneous gonadal regression (Follett et al., 1973; Hurley et al., 2008; Storey and Nicholls, 1978, 1976), indicating that they have reached absolute photorefractoriness. Around this period molting begins, which is necessary because feathers tend to get

worn out over the year and good quality feathers are critical for both flying and insulation. Breeding and molting both have high metabolic costs and therefore are often timed to proceed sequentially.

Species that show absolute refractoriness exhibit a marked plasticity of GnRH immunoreactivity in the preoptic area and accordingly in Border canaries GnRH immunoreactivity is high in during photostimulation, low in photorefactory individuals and intermediate in photosensitive birds (Hurley et al., 2008). Due to this intermediate expression during photosensitivity, canaries are in a state of readiness during late winter and indeed, studies of wild canaries have shown that gonadal development can start as early as 6 weeks prior to the day when daylength reaches 12L:12D (Leitner et al., 2003), suggesting that cues other than photoperiod also can trigger gonadal development (to be further discussed later in this section).

Circadian and circannual cycles are critical in regulating avian reproduction. The daylength determines the photoperiodic state of the individual, however, the critical variable is not the number of hours of light, but the time relative to dawn during which the light is received. Short light pulses (e.g. 14D:0.5L:9.5D) applied during the 12–16 hours after subjective dawn place the bird in a subjective long day and lead to the release of gonadotropins from the pituitary gland and gonadal recrudescence, a phenomenon called photoinduction. If light is detected during the photoinducible period, thyroid-stimulating hormone beta-subunit (TSH-β) expression in the pars tuberalis segment of the pituitary increases (Nakao et al., 2008). This leads to an increased expression of type II deiodinase and a decreased expression of type III deiodinase, both of which result in an increased production and concentration of the bioactive thyroid hormone - triiodothyronine (T3). T3 acts on the median eminence leading to a retraction of glial endfeet (Yamamura et al., 2004). Under a short-day photoperiod, the glial endfeet are surrounding GnRH-I nerve terminals. Their retraction leads to a greater contact of GnRH-I nerve terminals with the basal lamina of the median eminence, allowing the release of gonadotropins and consequently gonadal growth (Yamamura et al., 2004).

Perception of light

Birds perceive light not only through their eyes, but also through light-sensitive receptors in the medial basal hypothalamus. In fact, as far back as 1935 Benoit showed that photoperiodic gonadal growth in ducks does not require the bird to have

eyes (Benoit, 1935). In fact, neither the eyes nor the pineal gland are necessary for either photostimulation of gonadal growth by long-day photoperiods, development of photorefractoriness after a period of long-day photoperiod or dissipation of photorefractoriness by short-day photoperiod (Wilson, 1991). However, the precise nature of the receptors mediating this response and the mechanism involved remained elusive until more recently. The wavelength capable of inducing these responses was identified as 492 nm, similar to the wavelength affecting rhodopsin (Foster et al., 1985) and a variety of opsins have been suggested as the photopigment involved (Davies et al., 2012; Nakane et al., 2010; Wang and Wingfield, 2011). Three main candidates have been considered. Melanopsin (OPN4) has been suggested based only on anatomical and correlational studies (Kosonsiriluk et al., 2013). Neuropsin-5 was first discovered in the mammalian brain (Tarttelin et al., 2003) and, like the other opsins, has a spectral sensitivity close to what the avian hypothalamic light-sensitive region could respond to (Nakane et al., 2010). In quail it is expressed in the paraventricular organ, a circumventricular organ within the medial basal hypothalamus (Nakane et al., 2010). The knockdown of neuropsin-5 via small interfering RNA antisense increases TSHβ expression (Stevenson and Ball, 2012), providing a potential mechanism of photoregulation of avian reproduction. Unlike the others, the vertebrate ancient opsin (VA) is expressed in the GnRH neurons themselves, where it could have a much more direct action on the HPG axis (García-Fernández et al., 2015).

Social and other supplementary cues

Notwithstanding the importance of the photoperiod, the optimal conditions to breed are not dictated solely by the daylength. Therefore, avian species have adapted to use additional information from the environment to fine-tune the exact timing of gonadal development and breeding. Supplementary cues such as temperature, rainfall, or food availability can influence the timing of breeding (Hahn et al., 1997; Voigt et al., 2011; Wingfield et al., 1999). For example, song sparrows that live at similar latitudes but different altitudes develop their gonads at different times, with up to 2 months of difference. These timings are correlated to differences in the daily temperature range, appearance of new green shoots and flowers (Perfito et al., 2004).

Another important cue for controlling when to start breeding is the social environment. Social cues can modify hormone-dependent behaviors by acting on peripheral reproductive physiology including circulating hormone levels (Hinde, 1965;

Hinde and Steel, 1976) or central physiology including gonadotropin-releasing hormone (GnRH) expression (Stevenson et al., 2008). Supplementary cues, especially social signals, seem to be particularly important for female songbirds. For example, in a laboratory setting, while males respond to photostimulation alone with a full activation of the reproductive axis, including complete growth of the testes, maturation of gametes, and stimulation of sexual behaviors including song (Farner and Wilson, 1957), in females, photostimulation does activate the hypothalamus and pituitary, but ovarian follicles often only show partial development (Farner et al., 1966; Wingfield et al., 2003). Exposure to males stimulates follicle development and egg-laying (Perfito et al., 2015; Silverin and Westin, 1995; Stevenson et al., 2008). Even presentation of male song alone is sometimes enough to stimulate enhanced follicular development and females exposed to male song lay eggs earlier and at a greater frequency (Bentley et al., 2000). In female canaries, male song-playback, and in particular specific types of song elements called “sexy syllables”, also elicit a sexual behavior pattern called copulation solicitation display, a posture that facilitates mounting and signals to the male the female’s sexual receptivity (Kreutzer et al., 1994; Leboucher et al., 1994; Vallet and Kreutzer, 1995). During this display, the female raises her tail and head while rapidly fluttering her wings and producing a characteristic vocalization.

In males, singing behavior and the underlying neuroplasticity are strongly modulated by the social environment. In many species males greatly decrease their singing activity when exposed to a female (Alward et al., 2014; Boseret et al., 2006; Catchpole, 1973; Cuthill and Hindmarsh, 1985; Krebs et al., 1981; Shevchouk et al., 2017a). The social group composition can also influence song control system neuroplasticity (Adar et al., 2008; Alward et al., 2014; Boseret et al., 2006; Lipkind et al., 2002; Shevchouk et al., 2017a; Voigt et al., 2007; Voigt and Gahr, 2011; Walton et al., 2012).

Part 3. Sex hormones

Hypothalamic-pituitary-gonadal axis

We have seen in the section on seasonality that the photoperiod regulates reproduction by acting on gonadotropin-releasing hormone (GnRH) neurons whose

activity leads to an increase of sex hormone production. Now we will review this process in more detail. GnRH neurons, sometimes called the master-regulators of reproduction, are present in different nuclei of the hypothalamus (Blähser et al., 1986; Hahn and Ball, 1995; Saab et al., 2010; Saldanha et al., 1994) however, the GnRH population that is crucial for fertility resides in the preoptic area (POA). A second form of GnRH, GnRH-II is expressed in the brainstem and is involved in the regulation of sexual behavior but not in fertility (Maney et al., 1997), so it will not be considered here. GnRH neurons release the GnRH peptide in a pulsatile manner into the median eminence, a region devoid of the blood-brain barrier and connected to the hypophyseal portal system – a local blood circulation carrying molecules from the hypothalamus to the pituitary gland (Knobil and Neill, 1988). GnRH neurons are relatively few in number, but they can have an impact on their targets in the pituitary by synchronizing GnRH release into the median eminence. The mechanism of synchronization has not been fully elucidated yet but there is a possible involvement of gap junctions between GnRH fibers and cells in the preoptic area and median eminence (Pinet-Charvet et al., 2015). The portal system transports the GnRH peptide to gonadotrophs in the anterior pituitary, which respond by releasing two hormones into the general blood circulation. Pituitary beta cells produce follicle-stimulating hormone (FSH) and gamma cells produce luteinizing hormone (LH, Tixier-Vidal and Follett, 1973). LH and FSH work together to act upon the gonads, supporting the production of gametes as well as stimulating secretion of gonadal steroids. In turn, the gonadal hormones provide feedback signals to the brain and the pituitary gland.

Testosterone and estrogens production

In males FSH induces proliferation and development of spermatogonia and Sertoli cells, with little effect on the Leydig cells, while LH primarily induces differentiation of the testicular interstitium (Brown et al., 1975) and stimulates androgen secretion (Maung and Follett, 1977) from Leydig cells (Idler, 2012; Nakamura and Tanabe, 1972). In seasonally breeding avian species the testis size changes on a scale of 100-fold between the breeding and nonbreeding seasons. These changes are much more pronounced than in seasonally breeding mammals, where changes tend to be in the range of three- to five-fold in magnitude (Dawson et al., 2001). This difference in magnitude is associated with differences in the underlying cellular mechanism. In European starlings, seasonal regression of the testis involves apoptosis of Sertoli

cells, a cell type not known to undergo cell death in mammalian species (Young et al., 2001). In females, LH increases levels of progesterone and testosterone in the plasma and thecal cells (Shahabi et al., 1975), whereas LH and FSH together increase plasma levels of progesterone (Camper and Burke, 1977). During ovarian follicle development progesterone is enzymatically converted into testosterone and then aromatized into estradiol in the granulosa and theca layers (Bahr et al., 1983; Johnson, 1986).

Transport, metabolism and neurosteroids

Due to their lipophilic properties, sex steroid hormones can freely cross cell membranes, as well as the blood-brain barrier. On the other hand, steroids do not dissolve well in water and therefore in order to be efficiently carried through the blood circulation system, they bind to water-soluble carrier proteins. Both testosterone and estrogens bind and are transported by sex hormone binding globulins (SHBG, Hammond, 1995).

Testosterone is a substrate for three main enzymes – aromatase, 5α-reductase and 5β-reductase. The products of these thermodynamically irreversible enzymatic conversions are estrogens (estradiol or estrone), 5α-dihydrotestosterone (DHT) and 5β-DHT. 5α-DHT is an androgenic steroid with an affinity for the androgen receptor 2-3 times stronger than testosterone (Bruchovsky and Wilson, 1968). 5β-DHT has a very weak affinity for the receptor and does not activate androgen-dependent behaviors. All these enzymatic reactions can take place in the brain as well as some peripheral organs such as the adrenal glands, which also produce dehydroepiandrosterone (DHEA) that can be converted within the brain to androstenedione and further into androgens and estrogens (Labrie et al., 2001). Additionally, the brain, including the song control system, can produce sex steroid hormones *de novo* starting from cholesterol or from any steroid intermediates in the steroidogenesis pathway (London et al., 2009). Therefore, there is a gonadally-independent and very specific regulation of sex hormone action in brain regions relevant to singing behavior.

Modes of action

Androgen and estrogen receptors can be nuclear or located at the cell membrane. The nuclear action of androgens and estrogens, often called the “classical pathway”, modulates gene transcription and affects physiological or behavioral responses after hours to days. The androgen receptor (AR) and two of the estrogen receptors (ER), estrogen receptor α (ERα) and estrogen receptor β (ERβ), exert a large part of their

effects via this nuclear mode of action (Charlier et al., 2010; O'Malley and Tsai, 1992). After binding to their ligand, the receptors dimerize and enter the nucleus where ERs bind to estrogen response elements (EREs) and ARs to androgen response elements (AREs). These are short sequences of DNA within the promoter region of a gene that are able to bind a specific hormone receptor complex. For example the promoter of the gene coding for the neurotrophin called brain-derived neurotrophic factor (BDNF) contains an ERE (Sohrabji et al., 1995). Additionally, coregulators form a complex with the liganded receptor enhancing (coactivators) or repressing (corepressors) transcription at specific sites. These mechanisms thus increase the spatial and temporal precision of regulation of transcription by steroids and their receptors (Duncan and Carruth, 2011). For example, the coactivator SRC-1 is expressed in a sexually differentiated way specifically in many brain regions relevant to song control (Charlier et al., 2003).

In addition, to their role as nuclear receptors, ERα and ERβ can translocate to the cell membrane where their binding to estrogens will activate multiple intracellular signaling cascades and thus biological responses that do not depend on the synthesis of new proteins (Cornil et al., 2012). Three additional membrane ERs have been identified or at least suggested which do not seem to have nuclear actions – ER-X, GPR30 and Gq-mER (Filardo and Thomas, 2005; Qiu et al., 2006; Toran-Allerand et al., 2002). Although androgen receptors have been detected in the membrane of some cell types (Berg et al., 2014), little is known about their function and it is not clear how many neurons have membrane AR and where these are located. Membrane-associated ERs act by interacting with G-protein coupled receptors, growth factor receptors such as the epidermal growth factor (EGF) receptor and insulin-like growth factor (IGF) receptor. They can trigger the phosphorylation of intracellular messengers (reviewed by Levin, 2009) and interact with metabotropic glutamate receptors (mGluRs) whose trafficking to the membrane is influenced by estrogens (Bondar et al., 2009; Micevych and Dominguez, 2009). These intracellular events can take place in a few seconds to minutes (reviewed in Cornil et al., 2006), with their behavioral effects following slightly later, after a few minutes (Cornil et al., 2012).

Part 4: Plasticity in the song control system

Sex steroid hormone metabolism and action in SCS

Sex steroid hormones exert a powerful influence on the song control system, regulating many aspects of its neuroplasticity. These effects are mediated by receptors for sex steroid hormones located inside the song control system. The main song control nuclei express androgen receptors, including HVC, RA, Area X, LMAN, DLM, nXIIts as well as the “auditory” nucleus Nif (see Fig. 5, Balthazart et al., 1992a; Kim et al., 2004; Metzdorf et al., 1999; Nastiuk and Clayton, 1995; Sohrabji et al., 1989b). In HVC, RA-projecting neurons but also to a smaller extent Area X-projecting neurons have androgen receptors, while estrogen receptors are only expressed in the Area X-projecting neurons, their density is particularly high in the region medial of HVC, sometimes called the paraHVC (Johnson and Bottjer, 1995, 1993; Sohrabji et al., 1989b). In fact, HVC is the only song control nucleus that expresses ERα (Bernard et al., 1999; Fusani et al., 2000; Gahr et al., 1987; Nordeen et al., 1987). ERβ is not expressed in any song control nucleus but is densely expressed in the medial preoptic nucleus (POM) which is implicated in the control of singing motivation as well as in the auditory region NCM (Bernard et al., 1999). The newly discovered membrane estrogen receptors have not been systematically studied in the song control system, however one study has demonstrated the presence of a sexually dimorphic expression of the membrane ER GPR30 in HVC, RA and to a lesser extent in Area X and LMAN (Acharya and Veney, 2012).

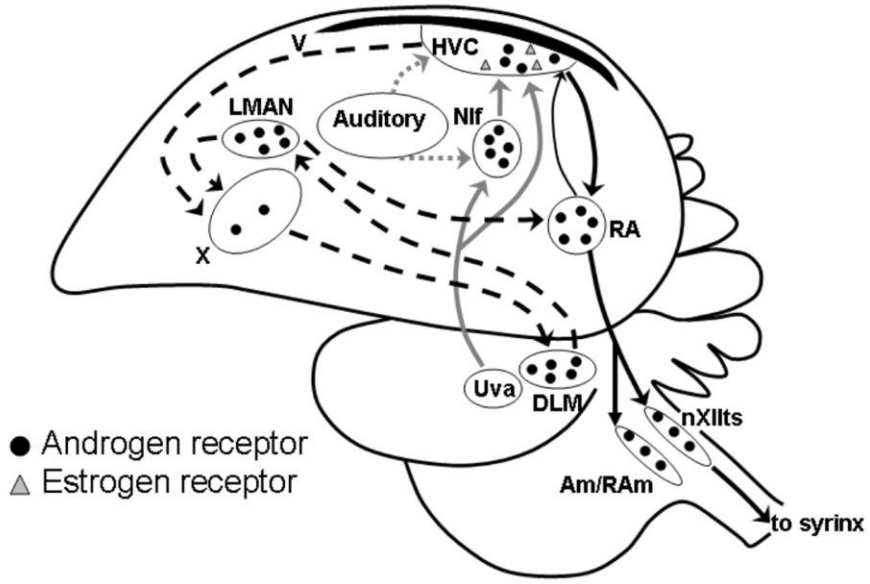

Figure 5. Expression of androgen and estrogen receptors in the song control nuclei, (London et al., 2009).

Aromatase is not expressed inside any song control nucleus but is highly expressed in the auditory region NCM as well as in regions of the caudal nidopallium and hippocampus, close to nucleus HVC and in the POM (Pinaud et al., 2006; Saldanha and Coomaralingam, 2005). In zebra finches, the sex difference in aromatase concentration is most pronounced in synaptosomes purified from the auditory telencephalon. Synaptic aromatase in NCM is selectively upregulated in singing birds compared to non-singing birds, although it has not yet been formally demonstrated that the increased synaptic aromatase was due to the singing activity *per se* and was not pre-existing before the experiment (Remage-Healey et al., 2009). Synaptic aromatase activity is most likely regulated by acute interactions between steroids and modulatory neurotransmitters. Aromatase is colocalized with NMDA receptors in the zebra finch brain (Saldanha et al., 2004) and both aromatase enzyme activity in the quail preoptic area (Balthazart et al., 2006) and forebrain estradiol levels in zebra finches are acutely regulated by glutamate (Remage-Healey et al., 2008). Finally, many other steroidogenic enzymes are present in the song control nuclei (see figure 6), suggesting that there is a very specific local sex hormone concentration regulation. For example, in HVC there is an expression of all enzymes necessary to biosynthesize every steroid in the pathway from cholesterol to testosterone (London et al., 2006).

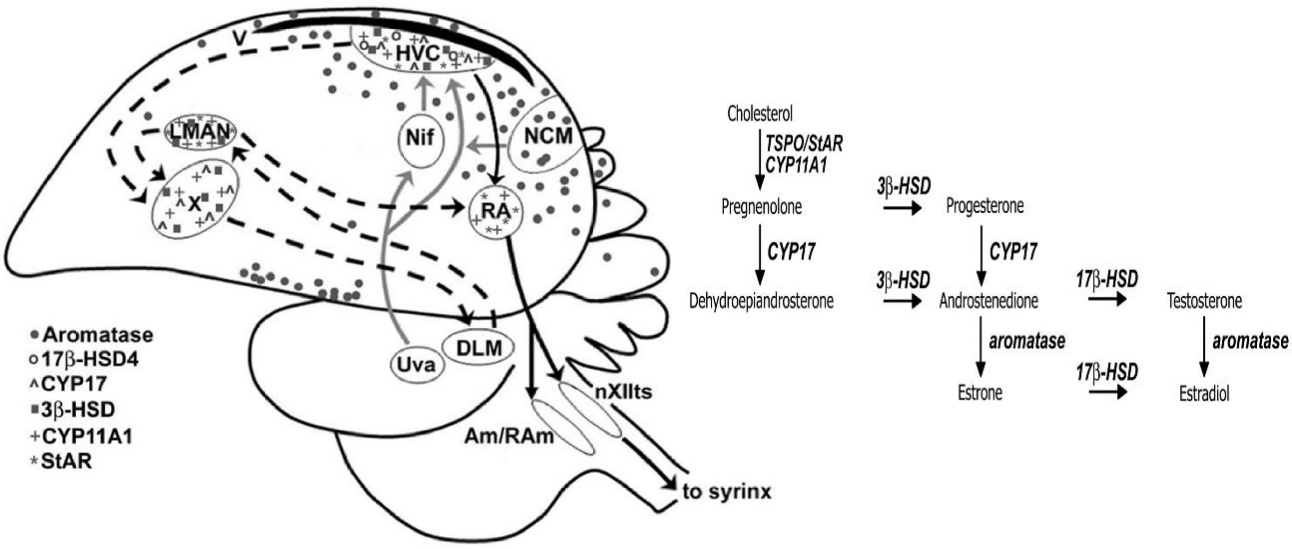

Figure 6. Expression of genes coding for steroidogenic enzymes within the song control nuclei (left) and the steroidogenic pathway (right), (London et al., 2009).

Mechanisms of plasticity

Nuclei volumes

In species living in the temperate zone, the song control system undergoes profound changes across seasons and in response to other environmental stimuli. Now, we will look more closely at the nature of this neuroplasticity. The most simple and common method to evaluate whether a particular stimulus has impacted the song control system is to measure the volume of the song control nuclei. In seasonal songbird species the volumes of HVC, RA, area X (see figure 7) and nXIIts are increased by the rise in testosterone during spring (DeVoogd et al., 1991; Nottebohm, 1980a; Riters et al., 2002). HVC and RA show the most dramatic changes in size, reaching during the breeding season values up to 3 times larger than the nonreproductive baseline (Tramontin and Brenowitz, 2000). In female canaries androgenic and estrogenic metabolites act synergistically to increase HVC and RA volume, while in Area X DHT alone partially increases the volume above the level of controls (Yamamura et al., 2011). However, not all song nuclei exhibit seasonal volumetric changes. For example, although multiple studies have evaluated the volume of LMAN in different seasons, no one has reported a change in this measure (Brenowitz et al., 1998; Smith et al., 1997; Tramontin et al., 1999). The vernal growth of song control nuclei depends on different cellular mechanisms depending on the nucleus. In HVC there is an increase of neuron number due to sex hormone-modulated neurogenesis during breeding season (see following section) and a rapid cell death when testosterone concentrations fall at the end of the breeding season (recently reviewed by Balthazart and Ball, 2016). Increases in the volume of RA and Area X are due to changes in the size of the neuronal soma and the dendritic tree (see next section). Testosterone increases the volume of HVC by acting directly on the estrogen and androgen receptors in this nucleus, while the increase of RA and Area X volumes are dependent, at least in part, on a trans-synaptic effect of testosterone action in HVC (Brenowitz and Lent, 2002, 2001).

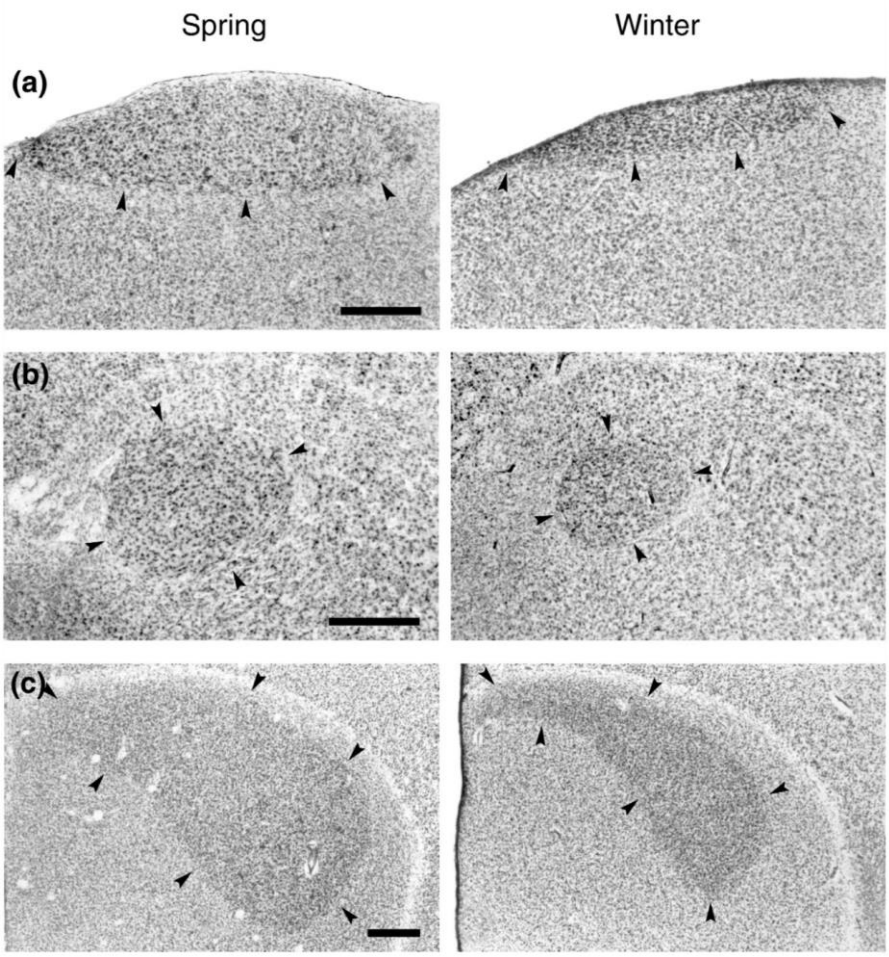

Figure 7. Nissl-stained sections showing (a) HVC, (b) RA and (c) Area X cross-sections during spring (left) and winter (right) in wild male spotted towhees, (Tramontin and Brenowitz, 2000).

The volume of the song control nuclei is sexually dimorphic in all temperate zone songbird species studied (MacDougall-Shackleton and Ball, 1999; Nottebohm and Arnold, 1976) and these differences are established during development primarily due to differential concentrations of sex hormones in the brain (Holloway and Clayton, 2001), although there is also a contribution of the genetic sex (Agate et al., 2003). The caudal portion of the hypoglossal nucleus (tracheosyringeal, nXIIts) which contains the motor neurons that innervate the syrinx is 83% larger in male than in female canaries (DeVoogd et al., 1991). This is caused primarily by a sex difference in neuropil volume as there is no significant sex difference in the number of neurons. nXIIts grows by 34% in females given testosterone as adults. It is 8% larger on the left than on the right side, likely reflecting the left syrinx dominance during song production in canaries and some other songbird species (Hartley et al., 1997; Nottebohm and Nottebohm, 1976). Besides sex steroid hormones, many other factors have been

shown to modulate the volume of the song control nuclei including photoperiod (Bernard and Ball, 1997), melatonin (Bentley et al., 1999), social context (reviewed in Balthazart and Ball, 2016 and in chapter 1 of this thesis) and stress (Buchanan et al., 2004).

Neuronal size and spacing

It has been demonstrated that neuronal spacing is modulated by either season or testosterone in HVC, RA and Area X (Rasika et al., 1994; Thompson and Brenowitz, 2005; Tramontin et al., 2000). Testosterone also increases neuronal soma size in these three song control nuclei (Rasika et al., 1994; Thompson and Brenowitz, 2005; Tramontin et al., 2000; Tramontin and Brenowitz, 1999). In fact, in RA and Area X the increases in neuronal spacing and soma size are the primary cellular changes that account for the seasonal change in nucleus volume (Smith et al., 1997; Thompson and Brenowitz, 2005). The increase of neuronal soma size in RA during long-day photoperiods depends on a synergistic effect of estrogen and androgen receptors in HVC and can be replicated under short-day photoperiod conditions with stereotaxic infusions of DHT and E2 near HVC but not near RA (Meitzen et al., 2007). Androgen receptor activation in RA is permissive but not sufficient for an increase in RA soma area. Increased spacing between neurons can be an indicator of a larger dendritic tree and indeed in RA it has been shown in female canaries that testosterone increases the size of the dendritic tree through a synergistic effect of its estrogenic and androgenic metabolites (DeVoogd and Nottebohm, 1981a). The difference in testosterone concentrations explains the sex difference in dendritic length and distribution (DeVoogd and Nottebohm, 1981b).

Electrophysiology and synaptic plasticity

In RA, spring-like conditions increase the spontaneous firing rate of neurons through a synergistic effect of androgenic and estrogenic metabolites (Meitzen et al., 2007; Park et al., 2005). Since there are no estrogen receptors in RA, the estrogenic component of the effect must be trans-synaptic, akin to the trans-synaptic effect of testosterone action in HVC mediating the increase in RA volume (Brenowitz and Lent, 2001). Indeed, long-day photoperiods increase the firing rate in RA, but systemic aromatase inhibition, HVC lesions or androgen and estrogen receptor antagonism in HVC, prevent this effect, while DHT and E2 implants near HVC are sufficient to increase the firing rate in RA even under short-day conditions (Meitzen et al., 2007).

Testosterone and spring-like conditions both increase the number of synapses onto RA neurons by 51% in female canaries, as well as the number of synaptic vesicles per synapse and the size of the pre- and post-synaptic process (DeVoogd et al., 1985; Hill and DeVoogd, 1991). Testosterone also increases the number of synapses and the synaptic efficacy of neurons in nXIIts, however this effect is much smaller than the massive increase in synaptogenesis seen in RA following treatment with testosterone (DeVoogd et al., 1991). In HVC, males in breeding conditions have a higher proportion of neurons firing action potentials of a shorter duration and higher frequency than either non-breeding season males or females recorded in either season (Del Negro and Edeline, 2002). Seasonal changes in electrophysiological properties have also been reported in the auditory regions. In females, spring-like conditions increase the proportion of cells in the auditory forebrain that use spike timing information rather than firing rate information and increase the temporal resolution required for optimal intensity encoding, while no effects of season were found in males (Caras et al., 2015).

Connectivity

In addition to the changes seen across seasons within the song control nuclei, the connections between them also change. Using diffusion tensor imaging, which detects the directionality of water molecule movements in brain tissue, De Groof and colleagues (2008) showed that in breeding condition male European starlings have an increased connectivity between HVC and RA, more fibers around RA and an increased myelination of the fibers from RA to the mesencephalic dorsomedial nucleus of the intercollicular complex (DM). In canaries, testosterone increases myelination of both HVC and RA (Stocker et al., 1994). Perineural nets are aggregates of proteoglycans that surround neurons, primarily interneurons, limiting their capacity to make new synaptic contacts and therefore modulating their plasticity (reviewed in Cornez et al., 2017). Although perineural nets do not seem to be modulated seasonally in European starlings (Cornez et al., 2017), testosterone increases the number and/or density of perineural nets in HVC, RA and Area X in female canaries (Cornez et al., 2016).

HVC neurogenesis and cell death

HVC is one of the neurogenic regions of the songbird telencephalon and neurogenesis largely accounts for the increased volume of HVC during the breeding season (Meitzen and Thompson, 2008). Testosterone and its metabolites modulate

neurogenesis via several mechanisms (Balthazart and Ball, 2016), which will be outlined after a brief general overview of HVC neurogenesis.

Birth

Adult neurogenesis occurs almost exclusively in the ventricular zone (VZ) lining the lateral ventricle walls (Goldman and Nottebohm, 1983), although the density of neural progenitor cells along the VZ is not the same everywhere. Regions of the VZ concentrating the proliferating cells are located in the ventrolateral and dorsomedial parts of the ventricle. These regions also have a higher number of radial glial cells (Alvarez-Buylla et al., 1990), along which the newborn neurons migrate, at least the first part of the route through the parenchyma, to their final destination (Alvarez-Buylla and Nottebohm, 1988; Scott et al., 2012). Based on mathematical modeling (Vellema et al., 2010), on developmental neurochemical studies of VZ specialization (Scott and Lois, 2007), on a small-scale live-imaging study following the movements of GFP-labeled neuroblasts in transgenic zebra finches (Scott et al., 2012) and on a lesion-study (Chen et al., 2014), the region of the VZ directly dorsal to HVC has been assumed to be the origin of (most) adult-born HVC projection-neurons, although conclusive evidence for this notion is lacking so far. Expression of the neuronal marker Hu starts as early as four hours after the final division of the progenitor cells. By twenty-four hours after progenitor division one third of cells express the marker, reaching 100% in the following 4 days (Barami et al., 1995).

Migration and differentiation

During the same time-frame (1-4 days) the newborn neurons down-regulate N-cadherin and migrate into the parenchyma (Barami et al., 1994), reaching HVC within about one week (Kirn et al., 1999). One month after their final division, a large proportion of the newborn neurons have extended axons to target RA neurons (Kirn et al., 1999) but adult-born HVC neurons do not project to Area X (Alvarez-Buylla et al., 1988; Kirn et al., 1999). Selective laser photo-ablation of RA-projecting HCV neurons increases the incorporation of new RA-projecting neurons, but the equivalent manipulation of Area X neurons does not induce any incorporation of Area X-projecting HVC neurons (Scharff et al., 2000). HVC interneurons are most likely not replaced in adult songbirds as birth-labeled neurons do not co-express GABA, parvalbumin, calretinin or calbindin (Scotto-Lomassese et al., 2007; Walton et al., 2012), the most common markers of inhibitory interneurons (but see Scott and Lois, 2007). The

neurons that project to the two different target nuclei and interneurons constitute three distinct neuronal populations with different morphological and physiological properties (Dutar et al., 1998). HVC neurons are often found in clusters with soma-soma contacts (Burd and Nottebohm, 1985) and newborn neurons preferentially arrest their migration in close contact to a mature Area X-projection neuron (Kirn et al., 1999; Scott et al., 2012). It is possible that the newborn neurons entrain the electrophysiological signals of their neighboring mature Area X neurons through gap junctions, which have been shown to exist between HVC neurons by freeze fracture (Gahr and Garcia-Segura, 1996) and by demonstration of passage of small molecular dyes between HVC RA-projecting neurons (Dutar et al., 1998). This speculation could explain how the circuit maintains the information necessary for a relative stability of song repertoire despite an annual replacement of about 50% of RA-projecting HVC neurons (Kirn and Nottebohm, 1993).

Survival

As newborn neurons mature, an increasing proportion of them is activated by singing activity (Tokarev et al., 2016). Post-synaptic activity increases the chance of the newborn neurons survival: infusion of a GABA-receptor agonist in RA decreases its spontaneous activity and HVC newborn neuron incorporation, while an infusion of KCl in RA increases the spontaneous activity in RA and the survival of newborn neurons in HVC (Brenowitz and Larson, 2015; Larson et al., 2013). On average about half of the newborn neurons that enter HVC one week after their final division die within their first three weeks of life (Kirn et al., 1999). The exact proportion of newborn neurons that dies depends on the season during which they were born (Nottebohm et al., 1994) as well as the hormonal environment, as will be seen in the next section.

Functional modulation

Adult neurogenesis decreases with age across songbird species (Absil et al., 2003; Larson et al., 2014; Walton et al., 2012; Wang et al., 2002; Wilbrecht and Nottebohm, 2004) but seems to be increased by singing activity (Alvarez-Borda and Nottebohm, 2002; Alward et al., 2016a; Alward et al., 2013; Li et al., 2000). The positive trophic feedback of singing activity on neurogenesis in HVC is also suggested indirectly by the study of Tang and Wade (2014) in which a tracheosyringeal nerve transection (a surgery which results in the bird's muteness) decreased BDNF in HVC; BDNF is required for the survival of newborn neurons (Rasika et al., 1999).

Hearing song and the social environment also have complex effects on adult neurogenesis in songbirds. Male canaries housed with a female have a higher density of newborn neurons in HVC despite having a lower song rate than males housed in isolation (Alward et al., 2014; Balthazart et al., 2008), although this effect might depend on the photoperiod (Shevchouk et al., 2017a, see chapter 1 of results). In zebra finches, a highly gregarious species, there is no difference in newborn neuron incorporation between isolated and paired individuals, however housing in a large group increases the survival of newborn neurons compared to either isolation or paired housing (Lipkind et al., 2002). In the zebra finch NCM, younger newborn neurons (40 days) survive more after a larger change in the social environment, while slightly older neurons (60 days) have a higher survival following a mild social change (Barnea et al., 2006). Together these data suggest that in songbirds, just as in mammals (Belnoue et al., 2011), newborn neurons have critical periods during which their survival is sensitive to different environmental stimuli. In zebra finches both deafening (Wang et al., 1999) and paralysis of the vocal muscles (Pytte et al., 2011) decrease the incorporation of newborn neurons into HVC, suggesting that newborn neuron survival is increased not only by activity in RA but also by trophic inputs from auditory regions. However, other studies have found no difference (Pytte et al., 2012) or the inverse relationship between deafening and HVC neurogenesis (Hurley et al., 2008)

Sex differences and sex hormones

I. During development

The sex difference in HVC volume seems to be established, at least in part, during development by an increased concentration of centrally produced estradiol in males (Holloway and Clayton, 2001). In zebra finches HVC is first visible at 4 days post-hatch (dph), but its volume is equal across sexes until at least 11 dph (Kim and Arnold, 2005). In Bengalese finches at 15 dph proliferation rates in the VZ dorsal to HVC (Zeng et al., 2007) are higher in males. In zebra finches by 20 and 30 dph there is no sex difference in proliferation in this VZ region (DeWulf and Bottjer, 2002). Nevertheless, male zebra finches at all post-hatching stages (between 2 days and 30 days post-hatch) have approximately 6 times more newborn neurons in HVC than females (Kirn and DeVoogd, 1989), suggesting a role for neuronal death in establishing the sex difference. At 15 dph there is a sharp increase in apoptosis in the female HVC, with up to 25,000 neurons being lost in 5 days (Burek et al., 1997; Kirn and DeVoogd, 1989). The HVC of an adult female has only 15% of the neurons of a 15 dph female

(Kirn and DeVoogd, 1989). Implanting a zebra finch with estradiol in the first days posthatch will masculinize the proliferation rates in the VZ dorsal of HVC (Zeng et al., 2007) and apoptosis in HVC (Burek et al., 1997).

II. Proliferation

In adults, the rate of proliferation of progenitors giving rise to HVC neurons is not markedly different between the sexes. Mirzatoni and colleagues (2010) cultured male and female zebra finch VZ explants in a medium containing BrdU for 2 hours and found no sex difference in proliferation. However, the number of labeled cells after 18 hours compared to 2 hours was reduced in female but not male explants, suggesting more cell death early after mitosis in females. However, Barker and colleagues (Barker et al., 2014) investigated the proliferation in male and female adult canaries throughout the VZ and found that females had higher proliferation rates in the VZ close to Area X, but not dorsal to HVC while testosterone increased the proliferation rate at the level of the septum and of HVC but only in the ventral VZ. Other studies investigating proliferation rates only in the VZ dorsal to HVC have not found any effect of either testosterone or estrogen on the mitotic rates (Brown, 1993; Hidalgo et al., 1995; Rasika et al., 1994).

III. Migration

Neural progenitor cells express N-cadherin whose down-regulation allows the newborn neuron to migrate away from the VZ (Barami et al., 1994). In rodents N-cadherin is regulated by both testosterone and estradiol (Monks et al., 2001a, 2001b). Additionally, estrogens upregulate IGF-1 (Norstedt et al., 1989; Sahlin et al., 1994) which in turn also downregulates N-cadherin in rodents (Roark et al., 1992). Furthermore, in songbirds IGF-1 increases the number of neurons emigrating from VZ explants (Jiang et al., 1998), although this effect happens on a slower time scale, starting after 6 days of treatment. Finally, estradiol facilitates newborn neuron migration via developmentally-restricted NgCAM-dependent calcium signaling (Barami et al., 1994; Williams et al., 1999). The timing of this calcium response to NgCAM corresponds to when the newborn neurons transverse a subventricular layer of estrogen-receptive neurons, 3-4 cells deep from the VZ (Hidalgo et al., 1995; Jiang et al., 1998; Williams et al., 1999).

IV. Recruitment, survival and integration in HVC

In contrast to proliferation, the recruitment and/or survival of newborn neurons in HVC is clearly increased by testosterone and estradiol both during development (Chen et al., 2014) and adulthood (Balthazart et al., 2008; Goldman and Nottebohm, 1983; Hidalgo et al., 1995; Rasika et al., 1994). Administering testosterone 20 days after the final division of newborn neurons still increases their survival, indicating that a substantial part of the effect of testosterone on neurogenesis is taking place when the neurons are already in HVC and are starting to integrate within the song control circuit. Doublecortin (DCX), a microtubule-associated protein, labels migrating bipolar (fusiform DCX+) neurons and larger, multipolar (round DCX+) neurons, that are either post-migratory or in the 'wandering' phase of migration (Scott et al., 2012). Testosterone, E2 or DHT increase the density of fusiform but not round DCX neurons in the HVC of female canaries (Yamamura et al., 2011) and both fusiform and round DCX neurons in male canaries (Balthazart et al., 2008). Moreover, testosterone increases the number of new endothelial cells and the diameter, perimeter and area of capillaries in HVC via VEGF-signaling. HVC endothelial cells are a local source of brain-derived neurotrophic factor (BDNF, Louissaint et al., 2002) and therefore with greater vascularization there is an increased production of BDNF. BDNF is critical for newborn neuron survival; blocking BDNF signaling in testosterone-treated female canaries decreases the newborn neuron density to the level of subjects not treated with testosterone (Rasika et al., 1999). BDNF is up regulated in response to testosterone but only after 2 weeks (Louissaint et al., 2002; Wissman and Brenowitz, 2009) and newborn neurons are maximally responsive to BDNF in the period 14-20 days after their birth (Alvarez-Borda et al., 2004). BDNF administered when newborn neurons are either 4–10 or 24–30 days after birth, does not enhance their survival compared to untreated subjects (Alvarez-Borda et al., 2004). Additionally, the increased VEGF induces matrix metalloproteinase release (Kim et al., 2008), promoting the breakdown of the HVC interstitial matrix. This facilitates the migration of new neurons, the remodeling of the HVC neuropil and liberates sequestered growth factors, including VEGF, from bound matrix stores (Chen et al., 2013). Testosterone also increases gap junctions between HVC neurons (Gahr and Garcia-Segura, 1996), facilitating the incorporation of newborn neurons into existing networks.

V. Neuronal death

At the end of the breeding season testosterone concentrations fall causing the song control nuclei to regress. Withdrawal of testosterone causes HVC to collapse within 12 hours after changing from a long-day (LD+T) to a short-day photoperiod (SD-noT). This shrinkage is initially due to a decrease of the inter-neuronal space (Thompson et al., 2007). However, a decrease in neuron numbers via cell death follows soon thereafter. One day after the change from LD+T to SD the number of neurons in HVC is intermediate between LD+T and SD, while the number of cells positive for activated caspase-3 (a marker of programmed cell death) is significantly increased by two days after the switch and intermediate between these two values on day 1 (Larson et al., 2014). Interestingly, blocking neuronal death by a mixture of caspase inhibitors decreases the rate of proliferation in the VZ (Larson et al., 2014) and the incorporation of newborn neurons in HVC (Thompson and Brenowitz, 2009) providing support for the hypothesis that newborn neurons migrate to HVC to fill in the “vacancies” created by apoptotic neurons (Nottebohm, 2004; Scharff et al., 2000).

Part 5. Singing behavior

Function

Birdsong is a form of communication – a series of sounds which serve to pass on information to other members of the same species, enabling the receiver to better predict the attributes of the sender, for example their species, sex, size, state of health, motivation, etc. One way we know that birdsong is a form of communication is because it modifies the behavior of the receiving individual (Slater, 1983), however this definition excludes passive signal detection. Singing has evolved to increase the individual’s reproductive success - in males by attracting females and warding off other males from their territory, while in females the detection of certain features of the males’ song enables her to discriminate ‘high quality’ from ‘low quality’ males for mating. Some species also use singing as a signal to warn about predators or to ensure flock cohesion.

In most species, the peak of singing activity is associated with breeding when males often sing for hours without obviously interacting with other birds, and it is difficult to know whether females, other males, or both are the intended audience. Evidence that song deters competitor males comes from experiments where male songbirds have been muted by syringeal denervation or interclavicular air sac

puncture. These individuals progressively lose all or part of their territory (McDonald, 1989; Peek, 1972; Smith, 1979). On the other hand, removing a male from his territory and periodically broadcasting his species-specific song from that territory, will delay re-occupation of the territory by a new male compared to the re-occupation of territory without the song broadcasting (Falls, 1988; Göransson et al., 1974; Krebs, 1977; Nowicki et al., 1998b; Yasukawa, 1981).

The role of singing in mate attraction is supported by studies showing that song rate increases in males upon removal of their mate and decreases again when the mate is reinstated (Catchpole, 1973; Cuthill and Hindmarsh, 1985; Krebs et al., 1981; Otter and Ratcliffe, 1993). Male canaries in the laboratory also show lower song rates when housed with a female than when housed alone or with another male (Alward et al., 2014; Boseret et al., 2006; Shevchouk et al., 2017a). These studies are thus consistent with the role of song in mate attraction: song is no longer necessary when the female is continuously present. Females are more likely to approach and enter a nest-box that contains a male decoy and is broadcasting species-specific song than a nest-box just containing a male decoy (Eriksson and Wallin, 1986; Johnson and Searcy, 1996; Mountjoy and Lemon, 1995). In addition, male song stimulates female reproductive behavior and physiology on many levels. Male song will often induce a female to display a sexual receptivity behavior - the female raises her tail and head while rapidly fluttering her wings and producing a characteristic vocalization (Maney et al., 1997; Searcy, 1992). Male song can also induce a higher rate of nest building, egg-laying and ovarian growth (Bentley et al., 2000). Specific song features determine how much a female will be attracted by a male: these features include song rate (Eens et al., 1991; Kempenaers et al., 1997; Wasserman and Cigliano, 1991), song complexity (Buchanan and Catchpole, 1997; Hasselquist et al., 1996; Searcy, 1984; Yasukawa et al., 1980), local song structure (Searcy, 1992) and vocal performance (Ballentine et al., 2004; Vallet and Kreutzer, 1995). The “nutritional stress hypothesis” suggests that the link between song and male quality depends on the fact that brain structures underlying song learning largely develop during the first few months post-hatching and that during this period birds are likely to be susceptible to developmental stress, largely due to undernutrition, that will handicap the attainment of their full potential (Nowicki et al., 1998a). Therefore, males who are able to produce a high quality song are more likely to be more healthy and robust in general.

Seasonal modulation

Although the most active singing happens during the breeding season, many songbird species continue to sing year-round. Non-breeding season song often has a different structure and function than breeding season song (Leitner et al., 2001a; Rost, 1990; Smith et al., 1997) and its mechanistic basis has been less studied. For example, European starlings sing throughout the year, but their songs are shorter in autumn than in spring (Riters et al., 2000). In this species, some neurotransmitter systems are modulated by singing in a season-specific manner (DeVries et al., 2015; Heimovics et al., 2009; Heimovics and Riters, 2008; Kelm-Nelson and Riters, 2013).

The mechanistic basis of non-breeding song has been studied to some extent in male song sparrows, who continue to sing in autumn but with greater variability compared to spring (Baker et al., 1984; Smith et al., 1997). During the non-breeding season the plasma testosterone levels are very low and castration does not decrease non-breeding season song, however song rate is decreased by a combination of aromatase inhibition and androgen receptor blocking (Soma et al., 1999) and also by treatment with an aromatase inhibitor alone (Soma et al., 2000). Dehydroepiandrosterone (DHEA), an androgen produced by the adrenals is, in contrast to testosterone and estrogen, elevated during the non-breeding season in song sparrows (Soma and Wingfield, 2001). It is this steroid that is likely activating the non-breeding season singing, via its neural metabolism to androgens and estrogens, but possibly via other mechanisms too (for review see Soma et al., 2014).

Although wild canaries living in the Canary Islands and in Madeira, the ancestors of domesticated canaries, sing highly stereotyped syllable types throughout the seasons and the size of the repertoire does not change (Gahr et al., 2001), 25% of their songs in the repertoire do change seasonally (Leitner et al., 2001b). Additionally, changes in singing behavior across seasons have become more pronounced in domesticated canaries that have been living for centuries in the temperate zone, where daylength changes across seasons are more important, such as Europe or North America. These changes could be partially due to selective breeding that was implemented to reinforce specific aspects of song. The common domesticated canaries studied in Seewiesen, Germany, do not change their repertoire size across seasons, however the song duration, number of sexually attractive syllables and proportion of repeated syllable types are all higher in breeding season

compared to non-breeding season (Voigt and Leitner, 2008). Waterschlager canaries, additionally have a period of “unstable song” during non-breeding season when new syllable types are added to the song repertoire, resulting in an overall increase in repertoire size with age (Nottebohm et al., 1987, 1986; Nottebohm and Nottebohm, 1978).

Song quality versus motivation

There is a wide variety of ways in which the songs of different songbird species are organized. Zebra finch songs, which are among the least complex and variable, consist of relatively few notes that are delivered in a fixed sequence. Yet even these songs have a limited form of variability: sequences of song notes can be omitted, with the song stopping short or skipping the initial syllables (Williams, 2004). Canaries, on the other hand, usually have a repertoire of dozens of notes grouped into syllables that are repeated rapidly to form trills. Together the string of different trills constitutes a song and can continue for well over a minute. Although transitions between the different trills are highly predictable, they are nevertheless somewhat variable and the exact sequence within a given song is very rarely an exact replicate of the previous song (Williams, 2004). Overall, these features make the canary adult song highly stereotypical, both in the acoustic structure of the song syllables and in the sequence in which syllables are sung. Song stereotypy is particularly high during the breeding season, when testosterone levels are high (Alward et al., 2013; Madison et al., 2015) and stereotyped song is indeed more effective in attracting a mate (Botero et al., 2009; Byers et al., 2010; Sakata and Vehrencamp, 2012). To measure the stereotypy of a bird’s singing during a certain period, the different renditions of singing are compared for similarity by calculating the coefficient of variation of some of their parameters, for example the song duration, bandwidth or entropy.

As will be discussed in the following section, the stereotypy of song is under control of testosterone, but it takes weeks of exposure to high levels of testosterone to increase the stereotypy of singing to breeding season level (Tramontin et al., 2000). On the other hand, song rate is increased by testosterone in a faster manner, reaching breeding season levels within days. Song rate and song duration are considered to be variables that reflect the bird’s motivation to sing and are regulated independently of song quality variables such as stereotypy and complexity (Alward et al., 2017).

Control by testosterone and its metabolites

Field studies have revealed a remarkable coincidence between the timing of seasonal peaks in plasma testosterone concentrations and in singing activity (Rost, 1992), providing correlative evidence for the role of testosterone in the control of song rate. Testosterone administered to juveniles who are still developing their song leads to premature crystallization (Korsia and Bottjer, 1991; Templeton et al., 2012; Whaling et al., 1995), in some cases producing an abnormal song (Whaling et al., 1995). In adult songbirds, castration causes a decrease in singing activity during the breeding season (Arnold, 1975b; Nottebohm, 1980a), although not if the surgery is performed during the non-breeding season (Alvarez-Borda and Nottebohm, 2002; Pinxten et al., 2002) when singing rates are already low in many species and song likely serves a different purpose (Riters, 2012). Conversely, implanting testosterone increases the frequency and duration of singing in castrated males (Arnold, 1975a; Nottebohm, 1980a; Pröve, 1974) and females (Leonard, 1939; Madison et al., 2015; Nottebohm, 1980a).

While systemic implants of testosterone induce an increase in singing, implanting the steroid stereotaxically either near HVC or near RA does not induce any singing (Alward et al., 2016c; Brenowitz and Lent, 2002). These studies suggest that testosterone-action in the SCS alone is not sufficient to activate singing behavior. An alternative strategy to examine the role of testosterone-action in the song control system is to inhibit androgen and estrogen action in this region while keeping the birds intact and on a stimulating photoperiod. Using this approach Meitzen and colleagues observed a decrease in song stereotypy, but no change in the rate of singing (Meitzen et al., 2007). This suggests that testosterone action in the song control system plays a role in regulating song quality but not song motivation.

The actions of testosterone on singing are mediated at least partially through its conversion to an estrogen (Fusani et al., 2003; Fusani and Gahr, 2006; Harding, 2004; Harding et al., 1988, 1983; Sartor et al., 2005). The action of estrogens on song is, at least in part, rapid as confirmed by the observation that inhibiting aromatase activity in male canaries decreases the song rate within hours of treatment (Alward et al., 2016b). Blocking aromatization in T-treated female canaries increases song duration by increasing the number of tours (a series of repetitions of the same syllable) per song relative to females treated with testosterone alone (Fusani et al., 2003). The organization of songs in stable sequences of different tours marks the transition from

plastic to crystallized song (Güttinger, 1979), therefore the study of Fusani and colleagues suggests that the estrogenic component of testosterone signaling is necessary for the full development of song structure. Another feature of the songs of the aromatase-inhibited females was a decreased number of tours with a rapid syllable repetition rate compared to the females treated with testosterone only. Females show more sexually-receptive behaviors in response to songs with a high repetition rate of syllables (Vallet and Kreutzer, 1995), even if these songs are recorded from testosterone-treated females (Kreutzer et al., 1996). In conclusion, syllable repetition rate, a song variable which strongly affects the reproductive success of a male songbird seems to depend on estrogens.

Although testosterone can induce singing under both long (Madison et al., 2015) and short-day photoperiod (Sartor et al., 2005), the photoperiodic condition of birds modulates their response to the steroid, with a higher song rate elicited by testosterone in photosensitive than in photorefractory male European starlings (Rouse et al., 2015). In photosensitive male canaries, androgenic and estrogenic metabolites act synergistically to increase the song rate, while either metabolite by itself is not able to increase the song rate above the level of castrated controls (Sartor et al., 2005).

Motivation to sing

SCS-independent

Testosterone circulating in the songbird's periphery is highly correlated with song rate, however manipulating testosterone action in HVC has no effect on the rate of singing (Meitzen et al., 2007), suggesting that song motivation is regulated outside of the SCS. This is also supported by a case study where a male canary with bilateral lesions of HVC displayed no audible components of singing, however continued to display the posture and movements associated with singing (Nottebohm et al., 1976). In photostimulated male canaries, a blockade of androgen receptors in HVC or RA decreased the variability of syllable usage and sequencing and syllable and trill acoustic variability, respectively (Alward et al., 2016a). Other regions in the SCS are only involved in the learning aspects of singing and therefore are not likely to be important for song motivation. Three areas outside of the song control system have been suggested to modulate the motivation to sing (reviewed in Ball and Balthazart, 2010).

Catecholaminergic nuclei of the mesencephalon

The catecholaminergic nuclei of the mesencephalon and pons express AR and ER (Maney et al., 2001) and project to the SCS (Appeltants et al., 2002, 2000). Testosterone increases the area covered by tyrosine hydroxylase-immunoreactive structures (fibers and varicosities) in the song control nuclei HVC and RA (Appeltants et al., 2003). Song rate is correlated with expression of immediate early genes in the VTA and PAG (Lynch et al., 2008; Maney and Ball, 2003), however there is so far, no direct evidence for a regulation of singing motivation by the catecholaminergic system.

Vasotocinergic cells of the lateral septum

The lateral septum expresses vasotocin in a sexually dimorphic manner. This expression is decreased by castration and increased by implanting with testosterone (Aste et al., 1997; Voorhuis et al., 1988). Bilateral lesions of the lateral septum decrease song rate, while vasotocin infusions into the lateral septum increase the rate of singing behavior (Goodson, 1998; Goodson et al., 1999).

Medial preoptic nucleus (POM)

The medial preoptic nucleus (POM) plays a critical role in the appetitive aspects of sexual behavior (Balthazart et al., 1998; Balthazart and Ball, 2007; Panzica et al., 1996). Breeding season song is a sexually-motivated behavior and indeed bilateral lesions of the POM decrease song rate in male starlings during the breeding season without affecting neither the functioning of the HPG axis nor motor behavior in general (Alger and Riters, 2006; Riters and Ball, 1999). During the non-breeding season POM lesions increase singing rate, suggesting it is inhibitory for non-sexually motivated singing behavior (Alger and Riters, 2006). The POM expresses AR, ERα and ERβ (Bernard et al., 1999) as well as high levels of aromatase during the season (Riters et al., 2000). Male starlings in breeding condition that have obtained a nest-box (necessary to attract a female for breeding) sing more and have a larger POM volume than breeding condition males without a nest-box or males in non-breeding condition (Riters et al., 2000). Both dopamine and opioid signaling in the POM have been related to singing behavior in a context-dependent manner. Expression of tyrosine hydroxylase and D1 but not D2 receptors in the POM is negatively correlated to time spent singing sexually-motivated song, but only in breeding season (DeVries et al., 2015; Heimovics et al., 2009; Heimovics and Riters, 2005). On the other hand, non-breeding season singing is correlated with the expression of mu-opioid receptors (Kelm-Nelson and Riters, 2013), which also correlate to the extent to which the bird

find singing rewarding (as measured by a conditional place preference assay, Riters et al., 2014).

Recent studies have shown that testosterone action in the POM is sufficient to increase the rate of singing in castrated male canaries to the same level as systemic testosterone (Alward et al., 2016c; Alward et al., 2013). On the other hand, the quality of the song in these birds remains inferior compared to those treated with testosterone either systemically (Alward et al., 2013) or in both POM and HVC (Alward et al., 2016c). These studies provide further evidence for the important role of POM in regulating the motivation to sing.

Goals of this research

The aim of this PhD project was to study the seasonal plasticity of brain regions involved in regulating singing behavior, especially the song control nucleus HVC (formerly high vocal center, now used as proper name) and the medial preoptic nucleus (POM), which controls song motivation in canaries. Furthermore, to address the question of sex differences we endeavored to include both male and female canaries in the experiments whenever possible. Seasonal transitions involve changes in photoperiod, sex hormones, social group composition/relevance as well as other environmental changes concerning for example temperature and vegetation. We were particularly interested in the former three variables and in the time course of neuroplastic changes induced by these variables. Thus, the first experiment was designed to evaluate the impact of different social contexts on HVC neurogenesis in males and females (**chapter 1**). Since the effect of stimuli on newborn neurons can vary according to their age, we developed methods to simultaneously quantify neurons born at different times relative to the experimental manipulation by combining two exogenous and one endogenous marker of neurogenesis. Additionally, we addressed the question of mechanisms by testing the hypothesis that differences could be driven by differential levels of stress. Since the first experiment indicated that the social composition modulated song rate, we decided to explore in more detail how the bird's perception of their cagemate affects their singing behavior. Therefore, in **chapter 2**, we explored the behavioral effects of introducing a mirror in the homecage.

The next question we intended to address was the time course of changes, following transition from a short-day photoperiod and low levels of testosterone to a long-day photoperiod with or without treatment with exogenous testosterone, in song control nuclei volumes, HVC neurogenesis, POM volume and POM aromatase expression in female and male canaries. Due to an unexpected activation of singing behavior in the majority of males while they were castrated and exposed to short days, the time course study was only performed in females (**chapter 3**). The experiment in males was thus, performed only with those few castrated birds that did not sing and therefore was limited to an attempt to replicate one of the main findings of the female time course in males namely the very rapid increase in POM volume and aromatase

expression in this nucleus after treatment with testosterone (**chapter 4**). Implants of testosterone in POM increase not only singing rate, but also the volume of song control nuclei. In **chapter 5** we attempted to explain the mechanism of this increase in song control nuclei volume. The experiment was designed to distinguish between an increase mediated by singing-activity feedback and an increase mediated by trophic signals being transported from POM to song control nuclei via poly-synaptic connections by collecting brains at different time points.

The castrated males who had initiated singing during exposure to short days offered an opportunity to study the mechanism of song activation in non-breeding condition. Therefore, we modified the aim of this study and tested instead whether the singing of males in this condition and the volume of their song control nuclei, was dependent on sex steroid hormone action by pharmacologically blocking androgen receptors and inhibiting aromatase in half of the subjects (**chapter 6**). Finally, in view of some surprising results obtained in some of the experiments, the nature of the photoperiodic responses in the strain of canaries that we used could be questioned. Therefore, we designed a final experiment to characterize the changes in males and females of this strain of canaries in response to changes in photoperiod (**chapter 7**), in order to validate them as a model of seasonal plasticity in the song system.

Results

Chapter 1. Studies of HVC Plasticity in Adult Canaries Reveal Social Effects and Sex Differences as Well as Limitations of Multiple Markers Available to Assess Adult Neurogenesis

PLOS ONE

RESEARCH ARTICLE

Studies of HVC Plasticity in Adult Canaries Reveal Social Effects and Sex Differences as Well as Limitations of Multiple Markers Available to Assess Adult Neurogenesis

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Abstract

In songbirds, neurogenesis in the song control nucleus HVC is sensitive to the hormonal and social environment but the dynamics of this process is difficult to assess with a single exogenous marker of new neurons. We simultaneously used three independent markers to investigate HVC neurogenesis in male and female canaries. Males were castrated, implanted with testosterone and housed either alone (M), with a female (M-F) or with another male (M-M) while females were implanted with 17β-estradiol and housed with a male (F-M). All subjects received injections of the two thymidine analogues, BrdU and of EdU, respectively 21 and 10 days before brain collection. Cells containing BrdU or EdU or expressing doublecortin (DCX), which labels newborn neurons, were quantified. Social context and sex differentially affected total $BrdU^+$, EdU^+, $BrdU^+EdU^-$ and DCX^+ populations. M-M males had a higher density of $BrdU^+$ cells in the ventricular zone adjacent to HVC and of EdU^+ in HVC than M-F males. M birds had a higher ratio of $BrdU^+EdU^-$ to EdU^+ cells than M-F subjects suggesting higher survival of newer neurons in the former group. Total number of HVC DCX^+ cells was lower in M-F than in M-M males. Sex differences were also dependent of the type of marker used. Several technical limitations associated with the use of these multiple markers were also identified. These results indicate that proliferation, recruitment and survival of new neurons can be independently affected by environmental conditions and effects can only be fully discerned through the use of multiple neurogenesis markers.

Highlights

- Endogenous and exogenous markers of new neurons differentially identify neurogenesis
- Thymidine analogues label neuronal populations born at specified moments
- Doublecortin gives an integrated view of neurogenesis changes over extended periods
- BrdU antibodies detect EdU-positive cells to a variable extent depending on their age
- Young and slightly older HVC neurons are differentially affected by social conditions

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Introduction

Adult neurogenesis was first discovered in the rat hippocampus [1], however, it was a series of experiments in songbirds that conclusively demonstrated the production, functional integration and electrophysiological activity of newborn neurons in the adult brain [2], triggering a new wave of interest in the phenomenon. Songbirds continue to be a useful model for the study of adult neurogenesis due to some unique features of the phenomenon in this taxon such as widespread migration of newborn neurons throughout the telencephalon, higher rates of proliferation than in mammals and the establishment of long-distance projections made by the newborn neurons in certain cases [3,4]. One specific neurogenic region, the song control nucleus HVC (used as a proper name), is of particular interest due to its important and specific role in the regulation of song behavior. By investigating the regulation of HVC neurogenesis, we can not only gain insight into the molecular and cellular aspects of this process, but also probe for the function of adult neurogenesis.

HVC is at the crossroad of three pathways involved in the learning, maintenance and production of song–the caudal motor pathway, the anterior forebrain 'feedback' pathway and the auditory pathway related to the perception of species-typical auditory signals. HVC is highly plastic and sensitive to a range of modulating factors including hormones and a variety of environmental stimuli. In seasonally breeding songbirds the volume of HVC during the breeding season is 1.3 to 3 times larger than during the non-breeding season (reviewed in [5]). Neurogenesis contributes importantly to this growth, although soma size also changes across season [6]. Neuronal proliferation takes place in the lateral ventricle [7]. The neuronal progeny then migrate along radial glia into the parenchyma [8] reaching HVC within 1–2 weeks. During this same period only about 50% of these newborn neurons will survive [9] and this survival rate is enhanced by testosterone [10] and estradiol [11]. Post-synaptic activity also enhances the new neurons survival during their first month of life once they have extended their axon to the nucleus robustus arcopallialis [12].

Traditionally neurogenesis is studied with the use of one of two thymidine analogues, [^{3}H]-thymidine or 5-bromo-2'-deoxyuridine (BrdU) and there are a few cases when these two markers have been combined in the same study (see [13] for an example in birds). More recently endogenous markers that label specific phases in proliferation and neuroblast development have also been used. Both exogenous and endogenous markers of neurogenesis have advantages but also pitfalls (for a comprehensive review see [14]). For example, BrdU, considered the gold standard for measuring neurogenesis by many, only stays in the circulation for a limited period after injection (less than an hour for songbirds: [15]) and therefore labels only a limited population of newborn neurons that replicated their DNA during a short time window. Given that newborn neurons have critical periods to environmental influences [16], analyzing only one specific cohort of neuroblasts born at a specific time potentially leads to overlooking experimental effects on neuroblasts born at a different time. In addition, BrdU incorporates into all proliferating cell types and does not discriminate between newborn neurons, glia and endothelial cells unless an additional cell-type marker is used in combination, which is technically more challenging and can decrease the detection sensitivity. Increasing the dose or number of injections can also lead to DNA damage followed by DNA repair during which BrdU is incorporated [17], so that cells with newly repaired DNA can be falsely counted as newly born cells. This type of labeling is however rare with the doses and injection schedules that are generally used by most investigators and the vast majority of BrdU-labeled cells after a substantial survival time are in fact new neurons as attested by the fact that they co-express in high proportion markers of young neurons such as doublecortin [18,19].

There are equally important problems associated with the use of endogenous markers of neurogenesis such as calretinin, Ki67, PCNA, pHH3, PSA-NCAM and doublecortin. Although

several of these endogenous markers have still not been used in birds due to the lack of antibodies that cross-react with the avian antigens, doublecortin (DCX) has been validated as a marker of neuroblasts in neurogenic regions of the songbird brain [20]. DCX labels newborn neurons that present two distinct types of morphology–fusiform DCX cells that are presumably young migratory neurons and round DCX cells that likely represent neurons in the early differentiation stage [20,21]. At the sub-cellular level, DCX plays a role in controlling the polymerization of microtubules and stabilization of the cytoskeleton [22–24] both of which are important for the migration of young neurons [21,25]. However these mechanisms are also involved in reorganization of the dendritic arbor, neurite outgrowth and synaptogenesis [26–28]. Thus neurons undergoing these processes could also express DCX, but it has been demonstrated that DCX-positive cells found in neurogenic regions are in the vast majority of cases newborn neurons. For example, in rats that have been injected with BrdU for 12 days, 90% of DCX-immunoreactive cells in the dentate gyrus are strongly positive for BrdU and therefore are very likely to be newly born neurons [29]. In canaries injected with BrdU twice a day for 5 days, over 70% of doublecortin-positive neurons in HVC co-label for BrdU 10 days after the first injection [18,19]. Between 10 and 30 days post-BrdU injection the proportion of BrdU-positive DCX-immunoreactive neurons displaying a fusiform morphology decreases while the proportion of round DCX neurons increases, suggesting that indeed the fusiform phenotype is the more immature form that later develops into round DCX neurons.

The social environment profoundly influences the behavior and physiology of songbirds [30–32]. For example, in several species it has been shown that the presence of a female greatly reduces a male's song output [33–35], while removal of the female reinstates high levels of singing [36]. Although there is evidence that singing activity per se has a positive feedback effect on HVC neurogenesis [37,38], one study from our laboratory indicated that males housed with a female have a larger volume of HVC than their more actively singing counterparts housed with another male [39], suggesting either a stimulatory effect of the female or an inhibitory effect of the male presence. Additionally, males housed with a female sing less than males housed in isolation, yet their density of newborn neurons in HVC is higher [35]. In zebra finches a rich social environment increases newborn neuron survival in HVC, Area X and the caudal nidopallium [40]. In the latter region younger newborn neurons (40 days) survive more after a larger change in the social environment, while slightly older neurons (60 days) have a higher survival following a mild social change [41,42]. Together these data suggest that in songbirds, just as in mammals [16,43], newborn neurons have critical periods during which their survival is sensitive to different environmental stimuli.

On the other hand, in many temperate zone songbird species, including canaries, females rarely sing and have smaller song control nuclei, including HVC, than males [44] even though the presence of song in females seems to be an ancestral feature [45]. Although female canaries are frequently used as a model to study the activational effects of testosterone on song behavior and growth of song control nuclei in adults in response to testosterone [46] including HVC neurogenesis [10,47], few studies have directly compared the male and female HVC volumes and neurogenesis in the same study. A sex difference in HVC volume persists in canaries when both sexes are treated with the same amount of exogenous testosterone [48]. Similarly, male starlings in the same hormonal condition as females continue to have a larger HVC volume but a lower density of newborn neurons [49]. Other studies have compared the neurogenesis in males and females in different endocrine conditions. Male canaries had a higher density of fusiform DCX-positive (DCX^+) neurons than females in all photoperiodic conditions that they experience during an annual cycle, i.e., irrespective of whether they are photosensitive, photostimulated or photorefractory [18]. Male brown-headed cowbirds and red-winged

blackbirds in breeding condition had larger HVCs and a lower density of DCX^+ neurons than the females of their species [50].

To investigate the mechanism mediating the sex differences and the social effects on HVC volume and neurogenesis, we compared here males housed in three conditions–alone, with a female or with a male. The females housed with a male were treated as experimental subjects as well as stimuli in order to simultaneously investigate sex differences in neurogenesis. Sex steroid concentrations were clamped at levels representative of breeding condition for each sex via subcutaneous Silastic™ implants filled with testosterone in males and with estradiol in females. This was done to distinguish between direct effects of the social conditions on HVC (female presence, male-related stress, . . .) and effects mediated by the possible activation by the female of the hypothalamo-pituitary-gonadal axis [51,52]. Given the limitations associated with the use of only one type of marker for labeling newborn neurons, we decided to use a combination of markers of neurogenesis to test whether a better understanding of the regulation of HVC neurogenesis by the social environment and sex of the bird could be gained through this approach. All birds were thus injected with two analogues of thymidine at different times points to label neuronal populations born at two different times and evaluate whether neurons of different ages are differentially sensitive to the social environment. We complemented this approach with a quantification of the endogenous marker of newborn neurons, DCX. Previous studies in mammals and birds have combined the use of two markers for labeling new neurons (e.g., [13,53,54], see [14] for discussion) but this is to our knowledge the first time that two exogenous and one endogenous marker are used simultaneously, in particular for analyzing neurogenesis in the songbird HVC, a model system characterized by a very intense neurogenesis (much more active than in the mammalian hippocampus) that provides more sensitive measures of changes in the neurogenesis process.

Methods

Animals

A total of 18 canaries of the Fife fancy breed were used in this study. All birds were in their second year; they were born and had gone through a full breeding cycle in the colony maintained at the University of Antwerp, Belgium during which they had been exposed to a minor immune challenge or its control manipulation and their body temperature had been recorded between 9 and 12 months of age (see [55] for details on the procedure and its effects). This manipulation was balanced across the groups formed for the current study so that it could not affect the group differences to be observed here. All experimental procedures complied with Belgian laws concerning the Protection and Welfare of Animals and the Protection of Experimental Animals, and this experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC) called the Ethics Committee for the Use of Animals at the University of Liege (Protocol number 926). The canaries were exposed to a natural photoperiod for the months preceding their arrival in our lab at the University of Liege in September and were then housed in single sex groups of 9–10 subjects on short days (8L:16D) for 2 months before the start of the experiment to induce photosensitivity. One month after arrival (at the time of castration), it was confirmed that the males had regressed testes and were in a nonbreeding condition. Throughout their stay in the laboratory, all birds had *ad libitum* access to food (a mix of various seeds designed for canaries), grit, cuttlebone and water for drinking and bathing. A small amount of egg yolk food was additionally added approximately once a week. Their health status and food/water provision was checked daily including during weekends as required by the Belgian law on the use of experimental animals.

Experimental procedures

Castration of all males was performed under general isoflurane anesthesia (3% for induction followed by 2–2.5% for maintenance) as described previously by Sartor and colleagues [56]. Each testis was removed through an ipsilateral incision during two surgeries separated by one week of recovery. Birds were then maintained under a warm lamp under visual inspection until they fully recovered from the anesthesia (a process that took only a few minutes) after which they were returned to their home cage. They were then checked several times during the next 24 hours to detect any possible problem. All subjects were observed to perch and feed within one hour. After both testes had been removed, males were allowed to recover for a minimum of two weeks. One day before being transferred to the experimental conditions, all subjects received a Silastic™ implant (Degania Silicone; internal diameter 0.76 mm, external diameter 1.65 mm, length 10 mm) which had been pre-incubated in 0.9% saline at 37˚C overnight. For males, the Silastic™ implant was filled with crystalline testosterone, for females with 17β-estradiol (both Fluka Analytical, Sigma-Aldrich), in order to clamp the concentrations of these sex steroid hormones in both sexes to high values typical of the reproductive season. These experimental conditions were selected to allow us to separate direct effects of social conditions on neurogenesis from indirect effects that would result from a change in circulating concentrations of testosterone induced by the different social conditions. Females were treated with estradiol to ensure they would be receptive and thus provide optimal stimuli for the males. These implants have been shown to establish in the canary blood stable concentrations of testosterone or estradiol and activate morphological and behavioral responses that are typical of what is observed during reproduction for periods longer than 3 weeks (e.g., [46,48,57–60]).

One day after implantation of the Silastic™ capsules, subjects were moved from their group housing cages to their respective experimental social context and the photoperiod was changed from 8L:16D to 11L:13D. During that day subjects were also injected intraperitoneally with bromodeoxyuridine (BrdU, Fluka [Sigma Aldrich], ref no. 16880; 10mg/mL in 0.01M Phosphate Buffer Saline, PBS) 5 times with 2 hours between each injection, at a dose of 50mg/kg per injection. The first of these injections was given at the same time when birds were moved from their group housing to the experimental social conditions and other injections followed 2 hours apart. On day 12 of the experiment all subjects were injected 5 times with 5-Ethynyl-2 ´-deoxyuridine (EdU, Invitrogen, ref no. E10187) at a dose equimolar to the dose of BrdU, i.e. 41.07 mg/kg EdU in 0.01M PBS, following exactly the same injection schedule as for BrdU.

Social context manipulations and behavioral observations

The social context manipulations consisted of housing birds in one of the three following conditions: testosterone-treated male housed alone (M; n = 3), testosterone-treated male housed with another male in the same endocrine condition, i.e. treated with testosterone (M-M; n = 6), testosterone-treated male housed with an estradiol treated female (M-F). In the M-F condition both birds served as experimental subjects; results of the male in the pair will be labeled M-F (n = 5) while results of female in the pair will be labeled F-M (n = 4 due to the loss on one brain). Care was taken to distribute birds from the same pre-experimental cage across different treatment groups and to ensure that birds housed together for the experiment were neither siblings nor members of a previously breeding couple. Due to time constraints, the experiment was run in 4 replicates that were started 2 days apart. Each replicate contained roughly equal numbers of birds in each of the 4 treatment groups. The subjects from one replicate were placed in adjacent cages to facilitate simultaneous behavioral observations. Subjects in one social condition were distributed randomly in the room. All subjects were in the same

room in visual but not acoustic isolation from other cages. Final numbers of subjects in each group and for each dependent variable are indicated in all figures.

Every 2nd day of the experiment starting from day 3, song rate of male subjects was quantified during a total of 10 min per day (total of 10 observation days). Each male subject was monitored for number of songs produced for 5 minutes in the morning and 5 minutes in the afternoon. During these 5 minutes, the observer sat quietly in front of the cages and noted the number of songs produced by each male. The different birds were observed each time in a different randomized order. We operationally defined song as a vocalization longer than approximately one second in duration after at least a 500 msec period of silence.

EdU-BrdU cross-reactivity validation

The BrdU antibody we used (ABD Serotec, OBT0030, clone BU1/75) to quantify $BrdU^+$ cells is known to cross-react with EdU [61] which could confound the detection of cells that incorporated only BrdU. To quantify the extent of cross-reactivity, we injected an additional four male canaries (obtained from a local breeder in Belgium) with EdU only at the same dose and following the same protocol as for other subjects in this experiment. Either 4 hours (n = 2) or 24 hours (n = 2) after the 5th injection birds were killed by transcardial perfusion. The perfusion, brain collection, cryoprotection and processing followed the same protocol as for the other subjects in this study. One series of brain sections obtained from these subjects were labeled for BrdU and EdU.

Blood collection and hormone measurements

Blood samples were taken from all subjects 4 to 7 days before the experiment (baseline), 4 days after the onset of social context manipulations, and during brain collection. 30–100 μL of blood was drawn from the brachial vein, in most cases within 3 minutes of catching. Samples collected between 3 and 4 minutes did not show increased corticosterone concentrations above baseline, therefore only a few samples collected more than 4 minutes after the initial bird capture were excluded from analyses of corticosterone. Blood samples were collected into heparinized micropipettes (Brand, Wertheim, Germany), transferred into Eppendorf™ microtubes and stored on crushed ice. Samples were centrifuged at 9000 g for 9 minutes, the supernatant plasma was collected and stored at -80˚C until further use.

Before hormone enzyme immunoassays (EIA), steroids were extracted from the samples to remove potentially interfering compounds using liquid phase extraction. Spiked samples were always processed in parallel with the experimental samples to assess extraction recovery. Spiked samples consisted of the same type, amount and dilution of plasma as experimental samples but contained in addition 30,000 counts per minute (CPM) of the tritiated hormone of interest. Recovery rates (ratio of CPM recovered to CPM added) were used to correct all assay results.

10 μL of plasma was diluted in 150 μL of deionized water (MilliQ) in glass test tubes, samples were kept at +4˚C for 30 minutes, and 2 mL of the non-polar organic solvent dichloromethane was added. Samples were vortexed and then left immobile for 1–2 hours to allow separation of the organic and aqueous phases. The organic phase was moved to a new test tube and dried under nitrogen gas at 40˚C. The dichloromethane extraction was repeated a second time and pooled extracts were kept at -20˚C until the EIA assay. Average recovery rates were 80% for testosterone and 84% for corticosterone. Testosterone and corticosterone concentrations were measured using EIA kits (Cayman Chemicals). On the day of the assay, samples were re-suspended in 400 μL EIA buffer from the kit (including 10% ethanol in the case of testosterone) and placed on a shaker set to 1350 rpm for 1 hour. The assay was performed

immediately after following instructions provided with the kit. These assays have been previously validated for measuring these hormones in avian plasma [62–67].

Brain collection and processing

After 21 days of exposure to the experimental conditions, all birds were deeply anaesthetized by an injection of 0.03–0.04 mL Nembutal™ (Sodium Pentobartbital 60mg/ml) and brains were collected from all subjects after transcardial perfusion. Blood was cleared from the brain by perfusion with 200 mL PBS 0.01M, followed by 200 mL 4% paraformaldehyde (PFA; 4.3 g/L NaOH, 40 g/L paraformaldehyde, 18.8 g/L $NaH_2PO_4.H_20$). The brain was extracted from the skull immediately after perfusion and post-fixed overnight in 15 mL PFA. The following day brains were cryoprotected in 30% sucrose (15.6 g/L Na_2HPO_4, 1.5 g/L KH_2PO_4, 300g/L sucrose) until they sunk to the bottom of their vial. Brains were then frozen on dry ice and stored at -80˚C until further use. Brains were cut coronally into 4 series of 30 μm thick sections on a Leica CM 3050S cryostat and stored in anti-freeze (0.01M PBS with 10 g/L polyvinylpyrrolidone, 300 g/L sucrose, and 300 mL/L ethylene glycol) at -20˚C.

Nissl and immunohistochemical staining

Nissl staining and volume reconstruction. One series of sections was mounted on Superfrost slides, dried at least overnight, and Nissl-stained with toluidine blue. After differentiation in Walpole buffer and molybdate, they were dehydrated in a series of increasing isopropanol concentrations, in 99% ethanol and finally in xylene and coverslipped using Eukitt as a mounting medium. To reconstruct HVC volumes, photomicrographs were taken of each section in the series containing the nucleus, in both left and right hemispheres with a camera connected to an Olympus BH-2 light microscope at 4x magnification. An outline was drawn around the perimeter of each cross-section of the nuclei using ImageJ v1.47v (National Institutes of Health) and the delimited area was measured. When a section was missing, the area was estimated by taking the average of the two sections immediately rostral and caudal to it. The volumes of nuclei were calculated by summing the areas and multiplying by 120 μm, the distance between two successive sections in the series. The volume of the nuclei in each hemisphere was calculated separately and the average of the two measures was used for statistical analyses.

Doublecortin (DCX) and BrdU immunohistochemistry. A second series of sections was double-labeled by immunocytochemistry for BrdU and doublecortin (DCX). Sections were washed 3 times in Tris-Buffer Saline (TBS) 0.05M at the start and between each subsequent step except prior to primary antibody incubation. All sera and antibodies were diluted in TBST (TBS + 0.1% Triton-X100), while H_2O_2, avidin, biotin and diaminobenzidine (DAB) were diluted in TBS. Endogenous peroxidases were blocked using 0.6% H_2O_2 for 20 minutes, DNA was denatured to reveal the BrdU epitope in the chromatin by incubating the tissue in 2N HCl at 37˚C for 20 minutes. The pH of the tissue was then neutralized during a 10-minute incubation in 0.1M sodium borate buffer. The non-specific binding of the secondary antibody was blocked by incubation in 10% donkey serum for 30 minutes and BrdU was labeled overnight with a primary rat anti-BrdU antibody (ABD Serotec, OBT0030) at a concentration of 1:2000. On the next day, sections were incubated for 2 hours with donkey anti-rat biotinylated antibody (Jackson, 1:2000), the signal was amplified by incubation for 90 minutes in ABC kit (Vectastain Elite PK-6100, Vector Laboratories), both components A and B being used at a concentration of 1:400. The BrdU antibody binding sites were revealed using 0.04% DAB in 0.012% H_2O_2 for 10 minutes, which produced a brown precipitate (Fig 1A and 1C).

For the second immunostaining sequence, sections were again incubated for 20 minutes in 0.6% H_2O_2, blocked for non-specific binding of the second biotinylated antibody by incubation

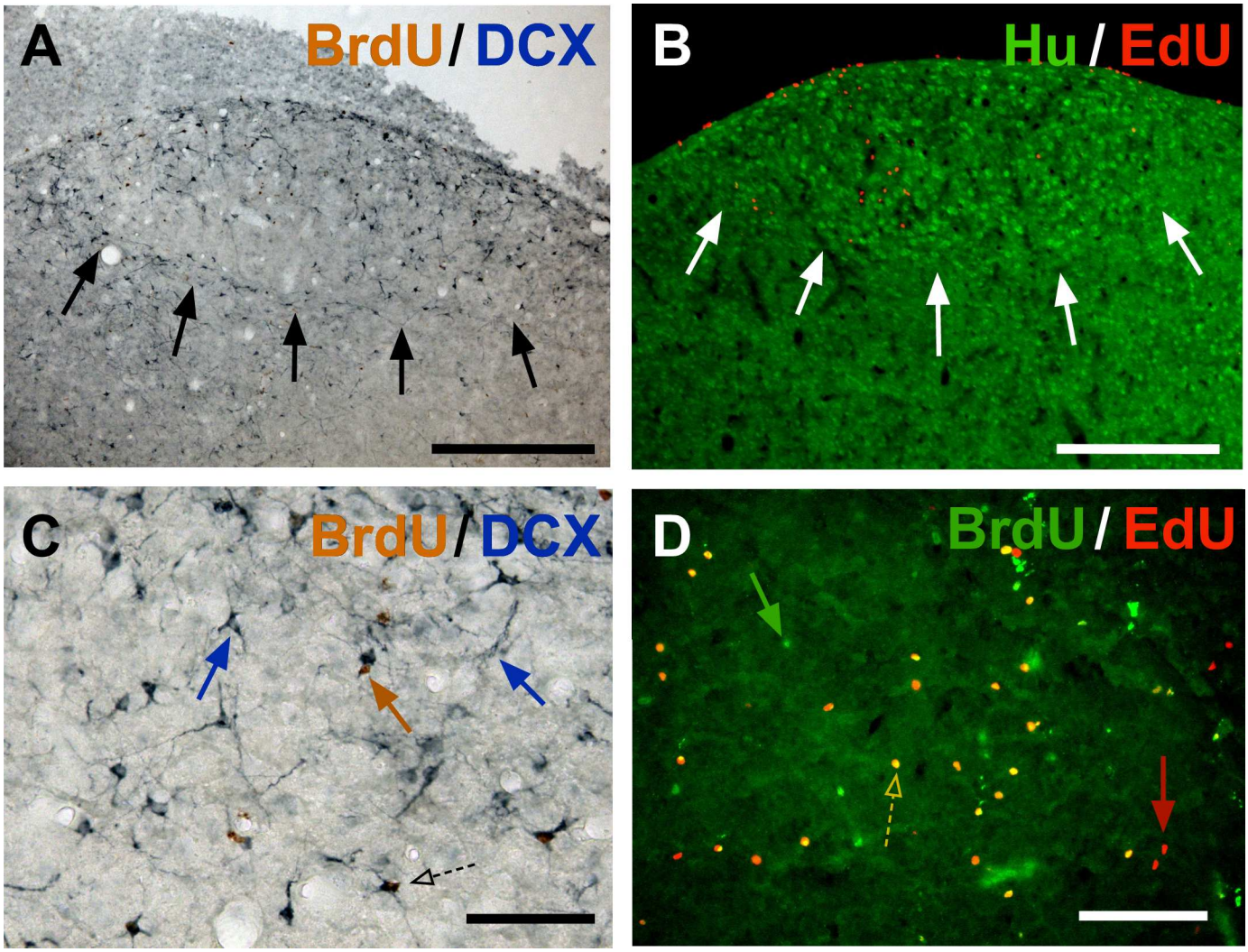

Fig 1. Photomicrographs illustrating the different labels used to identify new neurons in HVC at low (A-B) and high (C-D) magnifications. The first two panels show sections through the entire HVC that were stained for BrdU (brown) and DCX (blue; A) or with Hu (green) and EdU (red; B) illustrating the fact that these markers highlights the boundaries of HVC (arrows). Panel C shows at higher magnification a cell labeled for BrdU only (brown arrow), two DCX-positive neurons indicated by blue arrows, one round (left) and one fusiform (right) in shape) and one DCX-positive neuron also containing BrdU in its nucleus (dotted arrow). Panel D illustrates the double label for BrdU and EdU with arrows pointing to cells labeled for BrdU only (green), EdU only (red) and for both thymidine analogs (dotted arrow). Magnification bars are 200 μm in A-B and 50 μm in C-D.

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for 15 minutes each, first in avidin then biotin solution, both at a concentration of 1:5. Sections were blocked in 10% rabbit serum and incubated for 2 hours at room temperature and then for two days at 4˚C in goat anti-DCX (Santa Cruz, sc-8066) at a concentration of 1:200. Next, sections were incubated with 1:100 biotinylated rabbit anti-goat antibody for 2 hours, for 90 minutes in ABC kit as previously, and finally revealed for the DCX antibody binding sites with Vector SG Substrate kit (Vector laboratories, 15 μL chromogen and 24 μL H_2O_2 per mL of TBS) for 6 minutes, which produced a blue-gray precipitate (Fig 1A and 1C). Sections were mounted on glass slides and coverslipped using Eukitt (Sigma-Aldrich) as a mounting medium.

Hu immunohistochemistry combined with EdU Click-IT reaction. The Click-IT kit (Invitrogen, Catalog number C10338, using Alexa Fluor®555 azide, 555/565 nm excitation/emission, as fluorochrome) was used to label cells that incorporated EdU. In order to delineate

the HVC area, this was combined with immunohistochemistry for Hu, a neuronal marker that has been shown in songbirds to be expressed specifically by neurons starting soon after their birth [68]. Many neurons in HVC are larger and denser than in the surrounding nidopallium and therefore the Hu-staining can be used to visualize the borders of HVC (Fig 1B). EdU-labeling was performed according to the instructions of the kit and Hu immunohistochemistry was started immediately after. PBS 0.01M and PBST 0.1% were used and 3 washes followed each step. The epitope was unmasked by incubating sections in citrate buffer (2.1g/L citric acid, adjusted to pH 6 using 1N NaOH, 0.5 mL/L Tween 20) for 2 hours at 37˚C. The primary antibody incubation (1:100 mouse anti-Hu, Invitrogen, A-21271) was combined with 10% normal goat serum blocking and was performed for 2 hours at room temperature followed by overnight incubation at 4˚C. Sections were then incubated with biotinylated goat anti-mouse secondary antibody at a concentration of 1:100 for 2 hours at room temperature. Finally, sections were incubated with streptavidin conjugated to Alexa Fluor 488 at a concentration of 1:100 for 90 min at room temperature. After the final wash, the sections were mounted on glass slides and coverslipped using Vectashield with DAPI as a mounting medium.

BrdU immunohistochemistry combined with EdU Click-IT reaction. EdU-labeling was performed according to the instructions provided by the manufacturer and BrdU immunohistochemistry was started immediately after. PBS 0.01M and PBST 0.1% were used for washes and antibody/serum dilutions. The sections were washed 3 times at the start to remove the antifreeze and following each incubation. DNA was denatured to reveal the BrdU epitope in the chromatin by incubating the tissue in 2N HCl at 37˚C for 20 minutes. The pH of the tissue was then neutralized during a 10-minute incubation in 0.1M sodium borate buffer. The non-specific binding of the secondary antibody was blocked by incubation in 5% goat serum with 1% bovine serum albumin for 60 minutes and BrdU was labeled overnight at 4˚C with a primary rat anti-BrdU antibody (ABD Serotec, OBT0030) at a concentration of 1:500. Sections were then incubated with biotinylated goat anti-rat secondary Alexa Fluor-488 antibody at a concentration of 1:500 for 2 hours at room temperature (Fig 1D). After the final wash, the sections were mounted on glass slides and coverslipped using Vectashield with DAPI as a mounting medium.

Microscopy

BrdU and/or DCX-positive cells in HVC. A representation of the HVC borders was drawn on paper with the help of a camera lucida and a symbol was added on the drawing for each labeled cell, categorized as following: $BrdU^+DCX^-$, $BrdU^-$Fusiform-DCX^+, $BrdU^-$Round-DCX^+, $BrdU^+$Fusiform-DCX^+ and $BrdU^+$Round-DCX^+. The numbers of the different cell types were summed up for each HVC and the procedure was repeated on both sides of the brain and for 3–4 sections containing HVC per subject (except for 1 female where only 1 section with HVC could be counted). The total from both hemispheres was then averaged for all sections of a given subject and these mean values per section were used for analysis (see Statistical analysis). In addition, the $BrdU^+$ cells in the ventricular zone (VZ) dorsal to each of these HVCs were quantified separately, as well as the length of this segment of VZ. For these counts, we only considered cells that were entirely included in the thickness of the VZ and ignored labeled cells as soon as their perikaryon had migrated out of this periventricular cellular layer.

EdU-Hu. EdU^+ cells were quantified with a Leica fluorescence microscope (Leica DMRB FL.100; excitation filter BP545/30, dichromatic filter 565, suppression filter BP10/75) connected to a digital camera (Leica DFC 3000G). A photomicrograph was taken of each HVC, as detected by the dense group of Hu^+ cells (observed with excitation filter BP480/40, dichromatic filter 505, suppression filter BP 527/30), in one series at 5x magnification and was used

to quantify both the area of the nucleus and the number of EdU^+ cells. The area was delineated based on the limit of the brighter, larger somas of neurons in HVC compared to surrounding nidopallium. EdU^+ cells were counted manually in the entire cross section of HVC on these photographs in 3 to 6 sections per subject (except two females and one male with 1–2 sections of HVC counted). The EdU^+ cells in the VZ dorsal to each of these HVCs were quantified separately, as well as the length of this segment of VZ, as described for BrdU and DCX cells.

EdU-BrdU. To quantify EdU^+ and $BrdU^+$ cells, the brain sections were visualized with a Leica fluorescence microscope (Leica DMRB FL.100; for EdU see filter specifications in 1.8.2; for BrdU: excitation filter BP480/40, dichromatic filter 505, suppression filter BP 527/30) and photomicrographs were taken with a digital camera (for cross-reactivity validation study—Leica DFC 480, for social context experiment—Leica DFC 3000G). In the cross-reactivity validation the EdU^+ and $BrdU^+$ cells in the VZ were counted on photomicrographs taken at 20x magnification on 4–6 sections from each brain. For the social context experiment brains, EdU^+ and $BrdU^+$ cells in one section of HVC were quantified on photomicrographs taken at 10x magnification.

Statistical analyses

Densities of cells labeled by neurogenesis markers (DCX, BrdU, EdU) were obtained by dividing number of positive cells by the cross-sectional area of the HVC they had been counted in (number of cells/mm^2). The density of cells/mm^2 was corrected by the section thickness (30 μm) to obtain a density per mm^3 (multiplied by 1000/30 = 30,3) and this density was then multiplied by the volume of the nucleus in mm^3 to obtain an estimate of the total number in the entire nucleus. HVC volumes for this calculation were obtained from quantifications in Nissl-stained sections. These numbers that are extrapolated to the entire HVC provide a useful index to compare groups within the present experiment but should not be used for comparisons with independent studies since they are not absolute and depend on several parameters of the current study such as the section thickness and microscopic depth of field with the objective that was used, not to mention the staining efficiency.

The number of $BrdU^+$ and EdU^+ cells in the VZ was analyzed both as a number of cells in the entire VZ adjacent to HVC (per section, regardless of length) or as number of cells per mm of VZ adjacent to HVC. Although the VZ length per section was obviously different between males and females and possibly across male groups, since a large number of the new HVC neurons seem to originate in the adjacent VZ ([69] but see [4] for discussion), both analysis provide complementary information: data for the entire VZ relate more or less directly to the numbers of cells calculated for the entire HVC whereas data per mm of VZ relate to the densities within HVC.

A larger group of animals had initially been included in the experiment, however a subset of birds lost their Silastic™ implants during the experiment and thus had to be eliminated from the analysis. Additionally, in 3 males and 1 female the HVC was not complete in the sections that were collected because the caudal end of brain had been lost during processing. These subjects are excluded from the analyses that require the full extent of HVC, such as volume, but not other analyses, such as density of neurons where these markers were evaluated in only a subset of HVC sections. Extrapolation of these cells densities to the total numbers of cells in the entire HVC was obviously also impossible in these cases. The final numbers of data points available for each analysis are indicated in all graphs that also contain a representation of individual data points so that intragroup variability can be accurately appreciated. These small sample sizes obviously call for caution when interpreting the observed group or sex differences but they nevertheless allow us to establish the clear value of using multiple markers of neurogenesis in the

same subjects. This approach should in the future be used more generally to dissect the time course of the production and incorporation of new neurons in HVC.

Analyses of the total numbers of different cell types in HVC were performed after either eliminating the subjects without a full HVC or after substituting the group means for these subjects. These two approaches yielded the same results, therefore only results of the former approach will be reported. In one female no $BrdU^+$ cells were detected in the brain, thus this subject was excluded from all BrdU-analyses. For pre-experimental corticosterone measurements three male subjects were excluded because the volume of plasma collected was too small to be assayed reliably.

All behavioral and morphological measures including measures of neurogenesis were analyzed by non-parametric methods as the majority of data sets were not distributed normally, as determined by the Kolmogorov-Smirnov test. For each variable, the data from the three groups of males and one group of females is displayed side by side on a single graph for each measure, however the statistical analyses were performed separately. A Kruskal-Wallis ANOVA was used to compare the three groups of males and a Mann-Whitney U test to compare the males to the females in the M-F group. When the Kruskal-Wallis ANOVA indicated a significant difference between groups, a Dunn's Multiple Comparisons post-hoc test was performed. All analyses of the three male groups were additionally performed taking the average data per male-male dyad rather than using individual data of the two males in the dyad, in order to test whether social interactions within a dyad had a major impact on the conclusion. The results obtained in these two approaches were very similar and therefore only the latter type of analysis is reported. Corticosterone plasma concentrations were analyzed by a repeated-measures ANOVA with social context/sex and time as factors and the Bonferroni procedure was used as a post-hoc test. Linear regression was performed to correlate the number of EdU^+ and $BrdU^+$ cells in the VZ in the cross-reactivity validation study. Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc.) or STATISTICA (StatSoft). All data are presented by the mean ± standard error of the mean (SEM). Effects were considered statistically different if the p-value (two tailed for comparisons of two groups unless otherwise mentioned) for the analysis was equal to or lower than 0.05.

Results

Singing behavior

Total numbers of songs recorded over the three weeks of observation were compared across the three male social conditions. The highest rate of singing was observed in male subjects housed alone (M) followed by males housed with another male (M-M) and then by males housed with a female, (M-F) (Fig 2A). Statistical analyses confirmed that the differences between groups were significant ($H (df = 2, N = 14) = 8.251$, $p = 0.0162$). Dunn's Multiple Comparison post-hoc test showed that the male-alone group was significantly different from male with a female. Note however that a hypothesis-driven Mann Whitney test directly comparing the singing rates in the M-M and M-F groups suggested the existence of a significant difference also between these groups ($U (N1 = 6, N2 = 5) = 3$, one-tailed $p = 0.015$).

HVC volume

As expected based on previous work [44,48] in the M-F dyads, HVC volume was bigger in males than in females ($U (N1 = 4, N2 = 3) = 0$, one-tailed $p = 0.0286$; Males: 0.257±0.021, Females: 0.122± 0.020, all means ± SEM; Fig 2B). HVC volume was not significantly different in males housed in different social conditions ($H (df = 2, N = 11) = 4.303$ $p = 0.1161$). The

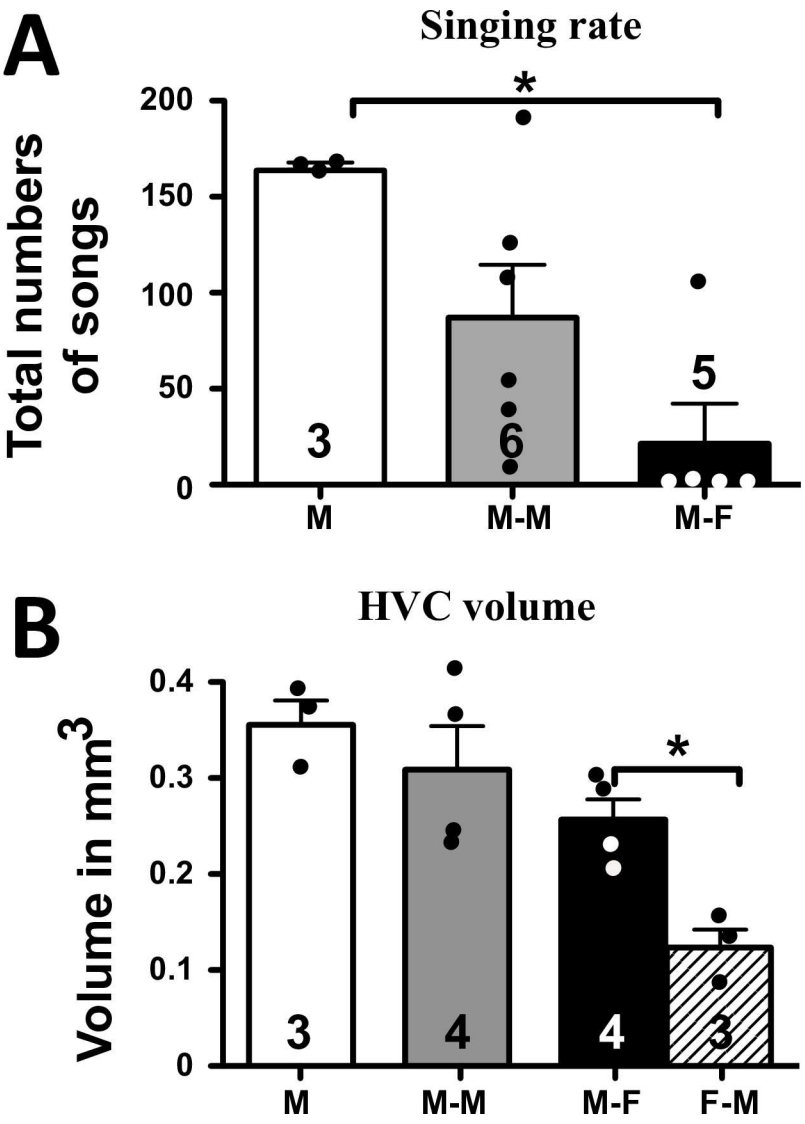

Fig 2. Effect of social conditions on total number of songs measured during all observations (A) and on the volume of HVC measured in Nissl stained section (B). HVC volume in females is also shown. M: male-alone, M-F: male housed with female, M-M: male housed with another male, F-M: female housed with a male. The figures on the bars indicate the numbers of available data. * = p<0.05.

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limited numerical differences between groups followed however the same general pattern as the differences in singing behavior (M > M-M > M-F).

HVC neurogenesis

BrdU$^+$ cells. The overall number of BrdU$^+$ cells in the VZ dorsal to HVC was significantly different between three groups of males subjected to different social conditions during 21 days (H (df = 2, N = 14) = 7.47, p = 0.024; Fig 3A). A post-hoc test showed that males housed with another male had more BrdU$^+$ cells in the VZ than males housed with a female, the latter was not different from males housed alone. When the number of BrdU$^+$ cells was normalized by the length of VZ, the difference in BrdU$^+$ cells between the three groups of males was still significant (H (df = 2, N = 14) = 6.70, p = 0.035), however a post-hoc test did not reveal any pairs

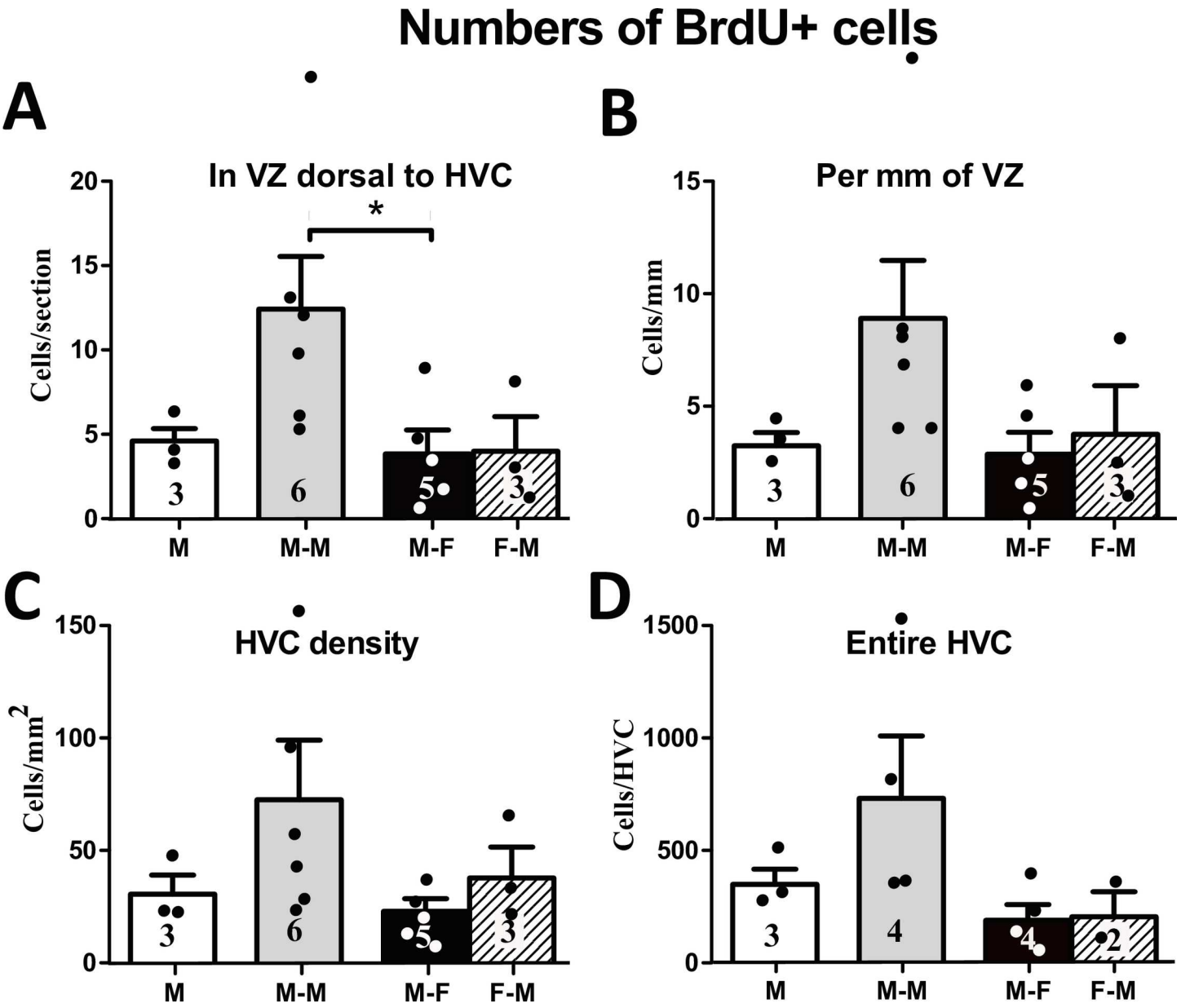

Fig 3. Number of BrdU$^+$ cells in the ventricular zone (VZ) dorsal to HVC as an absolute number per section (A) and normalized by the length of the VZ (B). Density (number per mm^2) of BrdU$^+$ cells in HVC (C) and number of BrdU$^+$ cells estimated for the entire HVC (D). M: male-alone, M-M: male housed with another male, M-F: male housed with female, F-M: female housed with a male. The figures on the bars indicate the numbers of available data points. * = p<0.05.

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of groups that were significantly different from each other Fig 3B). Comparing the BrdU$^+$ cells between males and females in the M-F group revealed no significant difference in terms of cells per section (U (N1 = 5, N2 = 3) = 7, p = 1.000), nor in terms of cells per mm of VZ (U (N1 = 5, N2 = 3) = 7, p = 1.00).

Inside HVC, the density of BrdU$^+$ cells was also higher in male subjects housed with another male than the other two groups of males, but this difference did not reach significance (H (df = 2, N = 14) = 3.89, p = 0.143; Fig 3C). No sex difference in the BrdU$^+$ density in HVC was found (U (N1 = 5, N2 = 3) = 5, p = 0.572). The estimated number of BrdU$^+$ cells in the

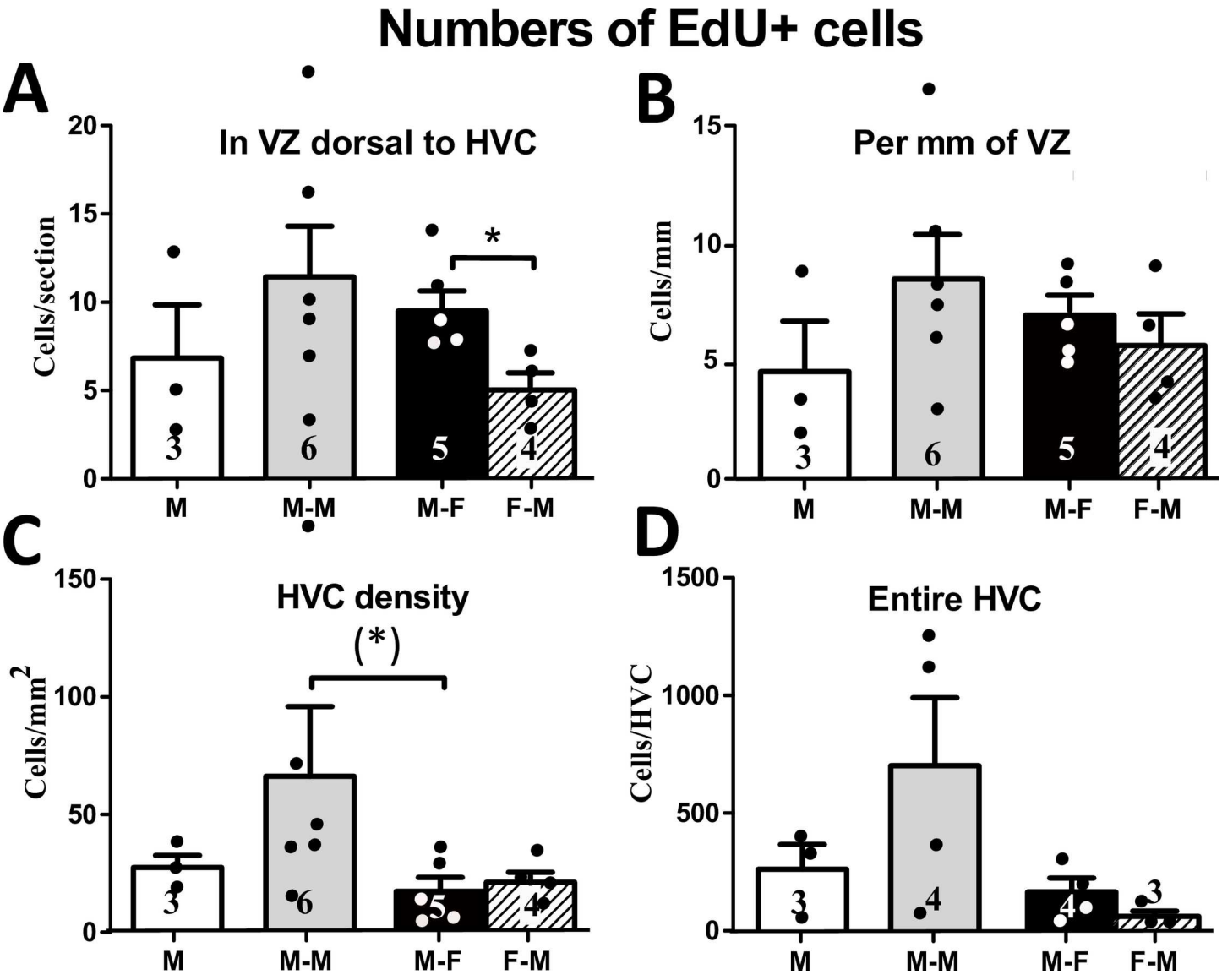

Fig 4. Number of EdU$^+$ cells in the VZ dorsal to HVC as an absolute number per section (A) and normalized by the length of the VZ (B). Density (number per mm^2) of EdU$^+$ cells in HVC (C) and numbers of EdU$^+$ cells estimated for the entire HVC (D). M: male-alone, M-M: male housed with another male, M-F: male housed with female, F-M: female housed with a male. The figures on the bars indicate the numbers of available data points. * = p<0.05; (*) = p<0.05 after an ANOVA showing only a statistical tendency (p = 0.053).

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entire HVC was not significantly different across male subjects in different social conditions (H (df = 2, N = 11) = 4.55, p = 0.103; Fig 3D), neither was it different between sexes of equivalent social conditions (U (N1 = 4, N2 = 2) = 4.00, p = 1.000).

EdU$^+$ cells. No difference between treatment groups was found in the number of EdU$^+$ cells in the VZ dorsal to HVC in males (H (df = 2, N = 14) = 1.37, p = 0.504; Fig 4A). When the number of EdU$^+$ cells in the VZ was normalized by the length of the VZ, no difference was still seen between different social conditions either (H (df = 2, N = 14) = 1.54, p = 0.463; Fig 4B). Comparison of sexes in equivalent social treatments (M-F vs. F-M) showed that females had fewer EdU$^+$ cells per section (U (N1 = 5, N2 = 4) = 0, p = 0.016; Figs 4, 5A and 5B), but this

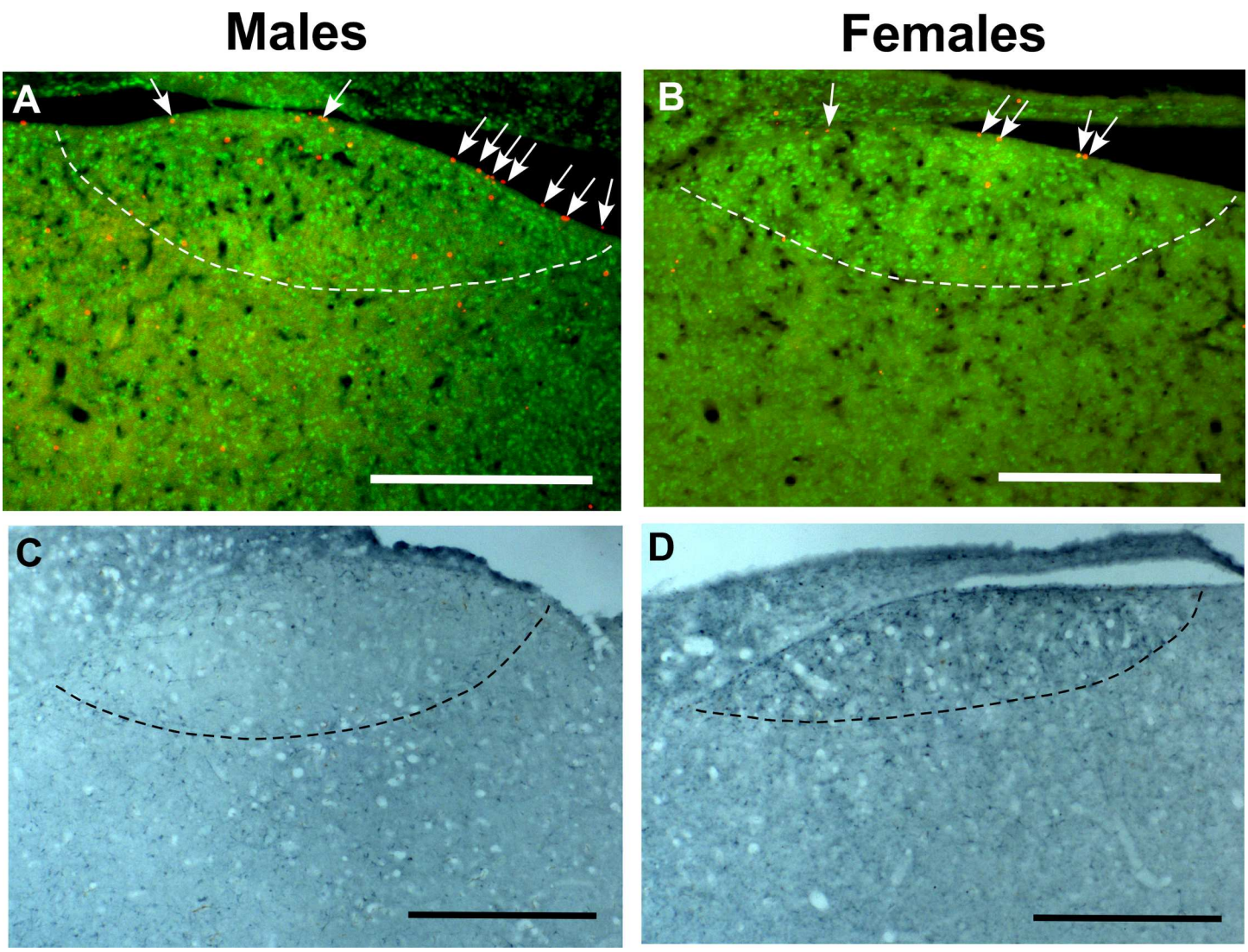

Fig 5. Photomicrographs illustrating the sex difference in numbers of EdU+ cells in the VZ dorsal to HVC (A-B; Males >Females) and in the number of DCX+ neurons in HVC (C-D; Females>Males). In panels A-B, EdU+ cells (white arrows) were labeled in red by the Click-IT reaction while the limits of HVC were identified by the higher density of Hu+ cells labeled in green. In all panels, the ventral edge of HVC is indicated by a dotted line. The magnification bars are set at 500 μM.

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difference disappeared when counts were normalized by the VZ length (U (N1 = 5, N2 = 4) = 0, p = 0.016; Fig 5B).

The density of EdU^+ cells in HVC was marginally different between social condition groups in males (H (df = 2, N = 14) = 5.87, p = 0.053; Fig 4C) largely because males housed with another male tended to have a numerically higher density of EdU^+ in HVC than males housed with a female (even if this difference is not significant). No significant difference was found in this measure between sexes in equivalent social conditions (U (N1 = 5, N2 = 4) = 8.00, p = 0.730). The estimated number of EdU^+ cells in the entire HVC was not different between social conditions (H (df = 2, N = 11) = 2.91, p = 0.233: Fig 4D) neither between sexes in equivalent social conditions (U (N1 = 4, N2 = 3) = 2.00, p = 0.229).

Cross-reactivity validation. In male canaries injected with EdU only whose brains were collected 4 or 24 hours after the last injection there was a high degree of cross-reactivity of

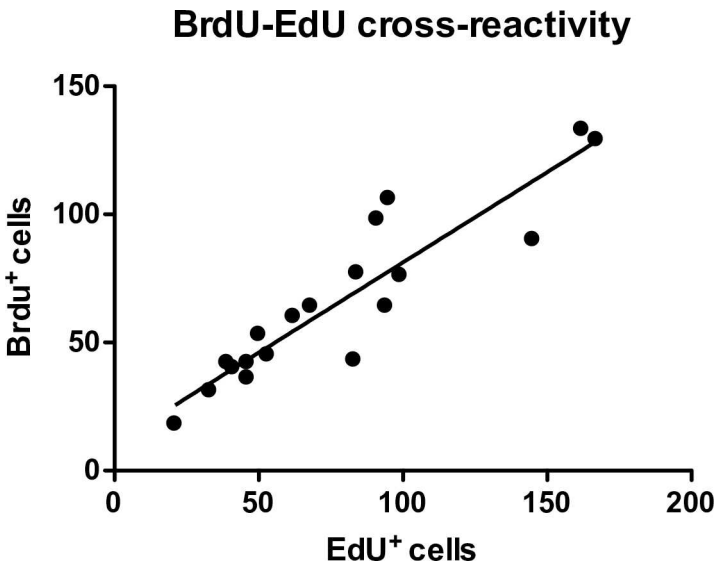

Fig 6. Linear regression of number of EdU-positive cells versus number of BrdU-positive cells in the VZ of the combined sections of birds injected with EdU only 4 and 24 hours before brain collection.

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BrdU antibody (ABD Serotec, OBT0030) with EdU as shown by the correlation of the number of EdU^+ and $BrdU^+$ cells counted in segments of the VZ in the combined sections collected at both intervals after BrdU injections ($r^2 = 0.84$, $y = 0.70x + 10.79$, Fig 6). The average percentage of EdU^+ cells detected by the BrdU antibody was 87.6% (SEM = 3.7%, n = 19 sections). These data thus suggested that it should be possible to quantify both BrdU and EdU in sections from subjects injected with both thymidine analogs by subtracting from the BrdU-positive counts the numbers of EdU-positive cells.

BrdU-EdU co-labeling. Although the cross-reactivity validation showed that the BrdU antibody we used recognizes most EdU^+ cells, the quantification of $BrdU^+$ and EdU^+ cells in the brains of subjects from the main experiments that had been injected with both markers identified some subjects with more EdU^+ cells than $BrdU^+$ cells. There were even social conditions where such a difference (more EdU^+ than $BrdU^+$ cells) was seen in the average counts (e.g. in the VZ of M-F males). This suggested that at least in some cases the cross-reactivity was not as complete as expected. To further investigate this question, we double-labeled for BrdU and EdU one section through HVC from each subject of the main experiment and quantified the single-labeled and double-labeled cells in the VZ dorsal to HVC and inside HVC.

Overall, the pattern of the single-labeled EdU^+ and $BrdU^+$ cells across groups was similar to the quantification of these labels performed on different sets of sections as summarized in Figs 3 and 4 (data not shown). Confirming our hypothesis regarding the lack of complete cross-reactivity in these brains, we observed that, on average, only 43.7 ± 0.06% (n = 18) of EdU^+ cells in the VZ and 42.8% ± 0.07% (n = 18) of EdU^+ cells in HVC were also labeled by BrdU. If we assume that some of these cells were $BrdU^+$ because they had incorporated BrdU as well as EdU, the extent of cross-reactivity would be even lower. This is however unlikely since the BrdU and EdU injections were made 11 days apart.

Putting this methodological limitation aside, these counts of double-labeled cells allowed us to obtain an estimate of how many $BrdU^+$ cells truly contained BrdU only ($BrdU^+EdU^-$) by subtracting from the total $BrdU^+$ cells the number of $BrdU^+EdU^+$ cells. Although this number is possibly an underestimate because some of the EdU^+BrdU^+ cells could have incorporated both compounds (an unlikely event as discussed earlier in this section), this number still

provides a useful estimate of how many of the cells born at the start of the social context manipulation and had incorporated BrdU at that time survived for 21 days. This analysis revealed that males housed with a female tended to have fewer $BrdU^+EdU^-$ cells than the other two groups of males both in the entire VZ ($H = 5.32$ ($df = 2$, $N = 14$), $p = 0.070$) and in HVC ($H = 5.581$ ($df = 2$, $N = 14$), $p = 0.061$; data not shown). This pattern was also present in the analysis of the density (numbers per mm or per mm^2) of $BrdU^+EdU^-$ cells in the VZ ($H = 5.51$ ($df = 2$, $N = 14$), $p = 0.064$, Fig 7A) and in HVC ($H = 6.43$ ($df = 2$, $N = 14$), $p = 0.093$, Fig 7C). Comparing the males and females in the M-F group revealed a numerically larger number (VZ: U ($N1 = 5$, $N2 = 4$) = 3, $p = 0.112$; HVC: U ($N1 = 5$, $N2 = 4$) = 4, $p = 0.190$; not shown) and density (VZ: U ($N1 = 5$, $N2 = 4$) = 3, $p = 0.112$, Fig 7A; HVC: U ($N1 = 5$, $N2 = 4$) = 2, $p = 0.064$, Fig 7C) of $BrdU^+EdU^-$ cells in the brains of the females than in those of the males although these differences did not reach statistical significance.

We also compared across groups the ratio of $BrdU^+EdU^-$ cells over EdU^+ cells as a measure of how many early born compared to late born cells survived. This comparison showed a significant difference between the three groups of males in the VZ ($H = 9.44$ ($df = 2$, $N = 14$), $p = 0.009$, Fig 7B) and a trend in HVC ($H = 5.55$ ($df = 2$, $N = 13$), $p = 0.063$, Fig 7D). Post-hoc tests indicated that males housed alone had a higher ratio of $BrdU^+EdU^-$ over EdU^+ cells in the VZ than males housed with a female. Males housed with a female also had a lower ratio for the VZ than the females they were housed with (U ($N1 = 5$, $N2 = 4$) = 1, $p = 0.032$, Fig 7C) but this difference was not significant in HVC (U ($N1 = 5$, $N2 = 4$) = 3, $p = 0.112$, Fig 7D).

Doublecortin (DCX). The density of fusiform DCX^+ neurons in HVC was not different between social groups (H ($df = 2$, $N = 14$) = 1.07, $p = 0.586$; Fig 8A). The comparison between sexes in the M-F group showed however a significantly higher density of fusiform DCX^+ neurons in females compared to males (U ($N1 = 5$, $N2 = 4$) = 0, $p = 0.016$). A similar pattern was found for round DCX^+ neurons, with no difference between social treatment groups (males: H ($df = 2$, 14) = 0.55, $p = 0.759$ and a significant sex difference with higher densities in females (U ($N1 = 5$, $N2 = 4$) = 0, $p = 0.016$; Fig 8C).

The estimated number of fusiform DCX^+ neurons in the entire HVC was significantly different between the male social condition groups (H ($df = 2$, $N = 11$) = 7.05, $p = 0.029$; Fig 8C). A post-hoc analysis showed that males housed with a female had significantly fewer fusiform DCX^+ neurons/HVC than males housed with another male. Males also tended to have a larger total number of fusiform DCX^+ neurons in HVC than females (U ($N1 = 4$, $N2 = 3$) = 0, $p = 0.056$). There was a significant effect of social condition on the estimated total number of round DCX^+ neurons for HVC in males (H ($df = 2$, $N = 11$) = 7.05, $p = 0.029$; Fig 8B), but the post-hoc analysis did not reveal any pairs of groups that were significantly different from each other. No sex difference was found in the estimated total number of round DCX^+ neurons in HVC (U ($N1 = 4$, $N2 = 3$) = 5, $p = 1.000$).

Hormone measurements

The blood samples collected at the end of the experiment from male subjects were assayed for testosterone concentrations, while samples from males and females collected at all three time points were assayed for corticosterone. Testosterone concentrations in the plasma of male subjects ranged from 1.24 to 6.84 ng/mL (mean = 3.56 ng/mL) and were not different between social conditions (H ($df = 2$, $N = 14$) = 1.07, $p = 0.586$).

A two-way repeated-measures ANOVA of corticosterone concentrations in the three male groups with time and social context as factors did not identify any main effects (time of sampling: $F_{2, 16} = 1.90$, $p = 0.182$, social context: $F_{2, 16} = 0.36$, $p = 0.708$) nor interaction between these factors ($F_{4, 16} = 1.40$, $p = 0.278$; Fig 9). The equivalent ANOVA comparing females and

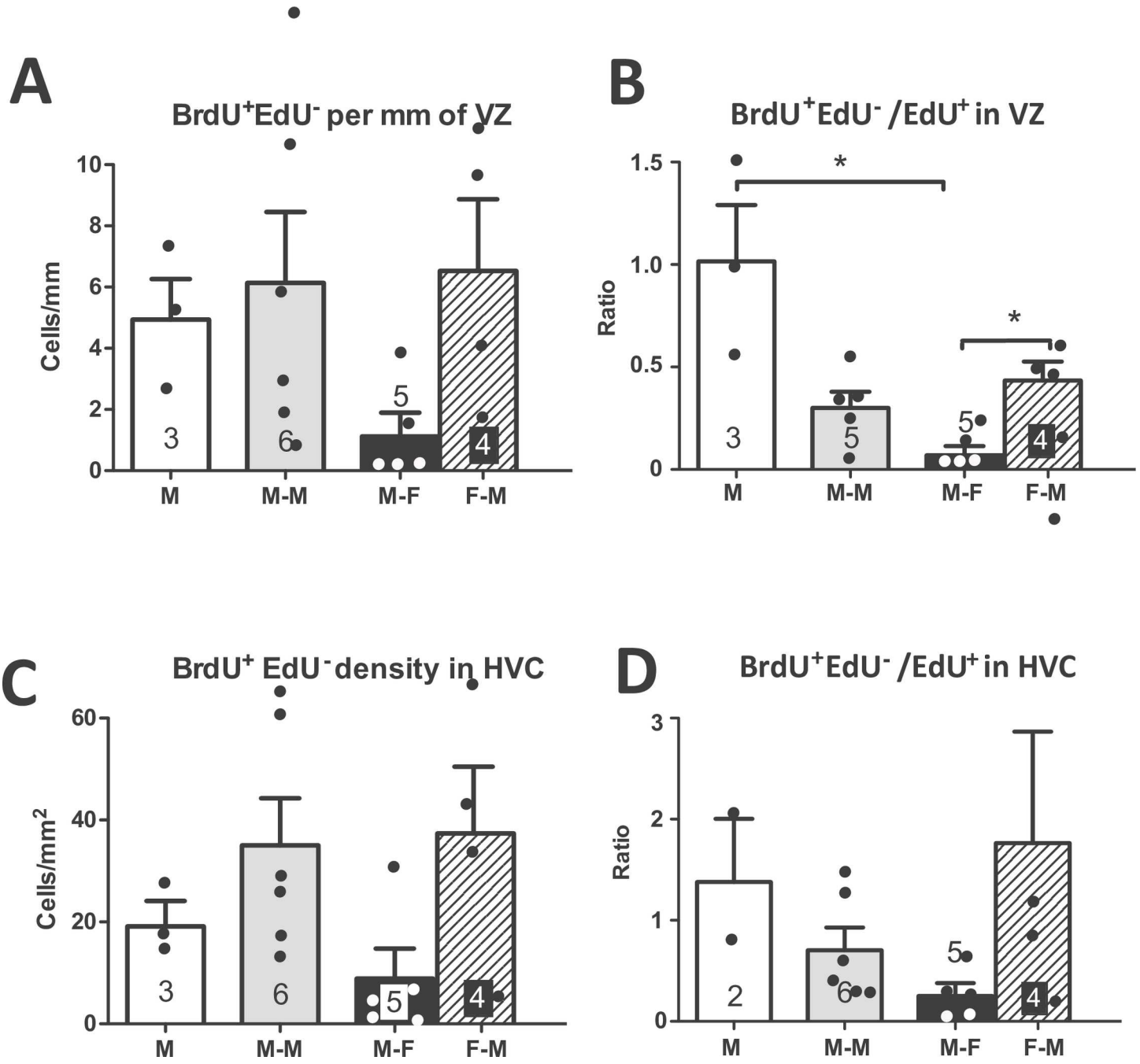

Fig 7. Number of BrdU⁺EdU⁻ cells in the VZ dorsal to HVC per mm (A) and density (number per mm²) of BrdU⁺EdU⁻ cells in HVC (C). Ratio of BrdU⁺EdU⁻ over EdU⁺ cells in VZ (B) and HVC (D). M: male-alone, M-M: male housed with another male, M-F: male housed with female, F-M: female housed with a male. The figures on the bars indicate the numbers of available data points. * = p<0.05.

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males in the M-F group identified no effect of time of sampling ($F_{2, 14} = 2.75$, p = 0.099), no main effect of sex ($F_{1, 14} = 0.65$, p = 0.448) but a significant interaction between the two factors ($F_{1, 14} = 4.22$, p = 0.037). Post-hoc analysis showed that before social context manipulations corticosterone concentrations were higher in males than in females. Interestingly, 4 days after

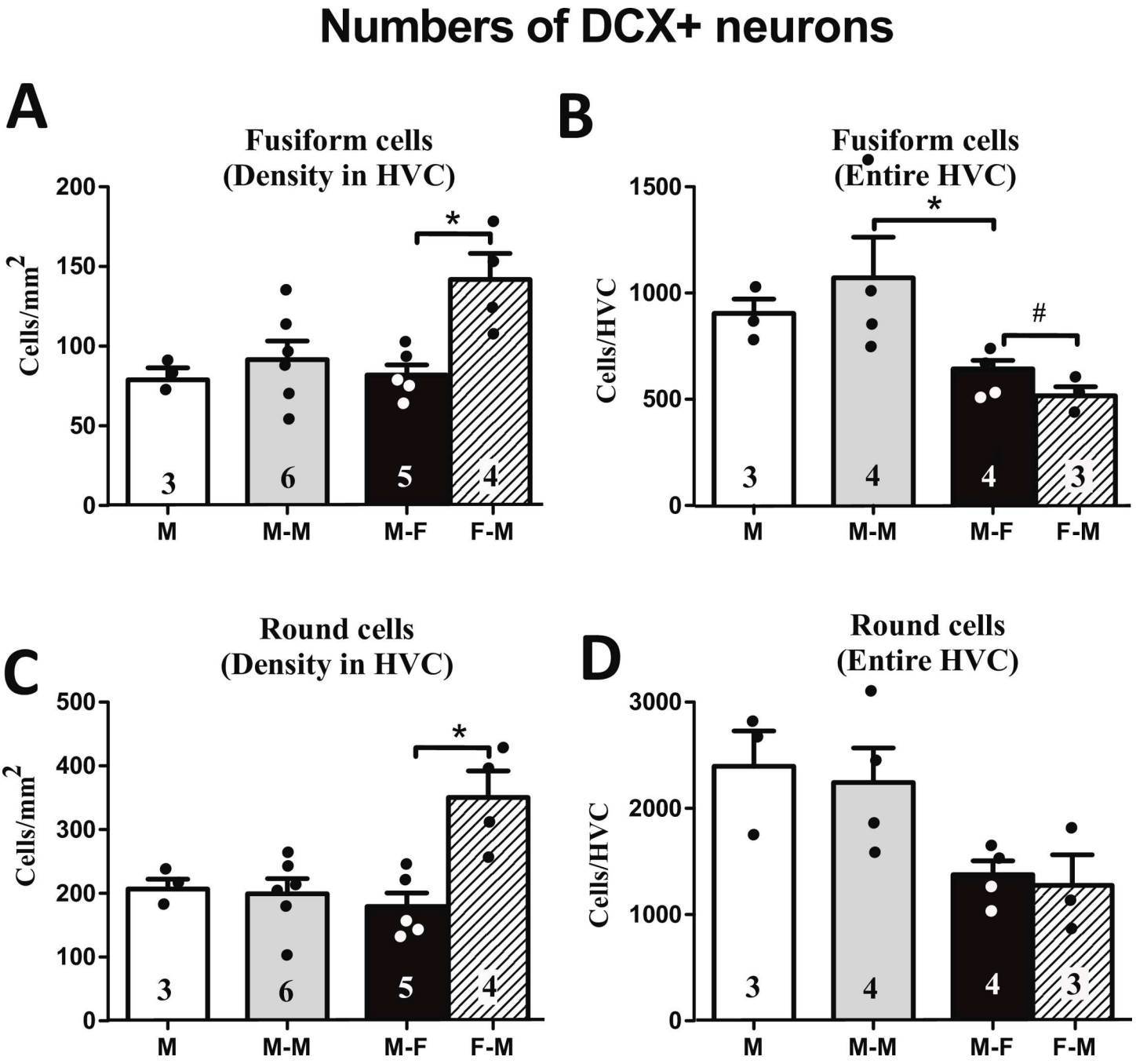

Fig 8. Density of fusiform DCX$^+$ (A) and round DCX$^+$ neurons (C) in HVC. Numbers of fusiform DCX$^+$ neurons (B) or round DCX$^+$ neurons (D) estimated for the entire HVC. The figures on the bars indicate the numbers of available data points. * = p<0.05, # = p<0.06.

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onset of social conditions this pattern was reversed with females having a higher concentration of corticosterone than males, although this difference was not significant.

Discussion

Most studies on adult songbird neurogenesis to have date employed a single proliferation marker. However due to the limitations of each marker, these investigations could be missing

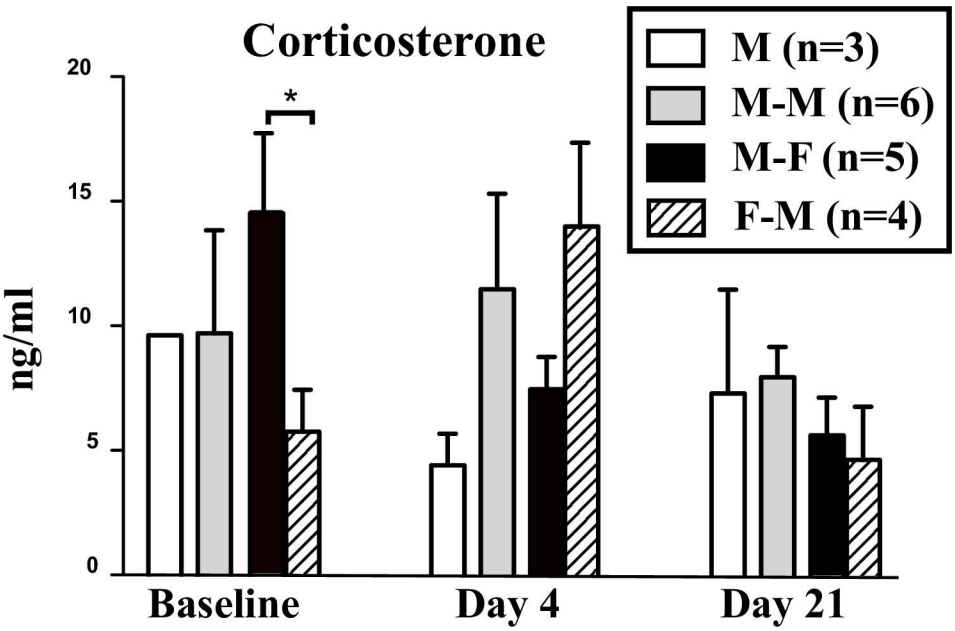

Fig 9. Plasma corticosterone concentrations measured in the 4 experimental groups before the experiment and after 4 and 21 days of exposure to the different social conditions. * = p<0.05.

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valuable information about the dynamics and regulation of neurogenesis. BrdU and other exogenous markers label small populations of cells born at specific times immediately after injections, doublecortin labels a broad population of neurons born over a large period before brain collection, although some disagreement exists concerning how long this labeling will last (see [14,69,70]). Exogenous markers are non-specific as regards the cell type that they label (in the brain they label both new neurons and glial/endothelial cells even if the former are more numerous, >70% of the total than the latter based on the co-localization with DCX; see [70] for discussion concerning the HVC of canaries) but largely label in a specific manner newly born cells. On the other hand doublecortin is neuron-specific but may also label neurons undergoing other types of plasticity. To exploit the advantages of both approaches, we combined here doublecortin and two exogenous markers, EdU and BrdU, to investigate effects of social context on HVC neurogenesis in male and female canaries. Each approach revealed substantially divergent patterns of neurogenesis as a function of the social condition or the sex of the birds.

Methodological issues

EdU was introduced relatively recently and has proven to be very useful for studying cell proliferation (see for example [71–74]). Its detection, using a commercially available kit, is very specific, not labeling any other analogue of thymidine and compared to immunohistochemical staining of BrdU, the labeling is simpler, faster and the signal is stronger. Unfortunately, it is now becoming clear that this tool also has some drawbacks. Since our experiment was performed, a study comparing the survival time of cells labeled with EdU and CldU observed in mouse brain and in primary cultures exposed to EdU (but not to CldU) an increase in pyknotic cells and a decrease in EdU cells starting from 24 hours post-incorporation [53]. We noticed here that on the day after EdU injection, the injected birds were not looking healthy: their feathers were puffed up and some were sleeping with the head turned around in the night sleeping

position. These organismal symptoms disappeared after 1–2 days but the cellular toxicity might have remained. Therefore, this marker should probably be used during *in vivo* studies only when the tissue of interest will be collected within 24 hours after injection.

An additional problem of EdU relates to its dose-dependent cross-reactivity with most anti-BrdU antibodies. Before initiating the main experiment presented here, a cross-reactivity test was performed with canaries that had been injected with EdU only; their brain had been collected 4 or 24 hours later and labeled for both EdU and BrdU. In this test, the cross-reactivity of our BrdU antibody (ABD Serotec OBT0030 rat monoclonal antibody) with EdU was 87.6%. Since this number was close to 100%, we assumed that we would be able to calculate the number of cells containing BrdU in sections of brains injected with both EdU and BrdU by subtracting the number of EdU^+ cells detected from the number of $BrdU^+$ cells detected. However, when the brains from the main study, which had been injected with both thymidine analogs 10 or 21 days before brain collection respectively were labeled for EdU and BrdU, the apparent cross-reactivity of the BrdU antibody with EdU was much lower: on average only 43.7 ± 0.06% (n = 18) of EdU^+ cells in the VZ and 42.8% ± 0.07% (n = 18) of EdU^+ cells in HVC were also labeled by BrdU.

This apparent decrease in cross-reactivity is possibly due to the fact that while at 24 hours most cells that had incorporated EdU had survived, 10 days later the cells that had incorporated a large amount of EdU had died and only those that had incorporated a smaller amount of EdU were still surviving. While the detection of EdU with the EdU kit is very sensitive and powerful, the detection of the low concentration of EdU in these cells by the BrdU antibody might give a weaker signal that was not detectable during quantification. An additional factor potentially decreasing the amount of EdU in cells at 10 days compared to at 24 hours post injection is that some cells had undergone their final division after multiple divisions of a progenitor that had incorporated EdU, each division further diluting the label.

The simultaneous use of these two thymidine analogs is thus bound to result in significant problems that relate to the toxicity of EdU, limiting its use to label cell proliferation just before tissue collection, and to its cross reactivity with most anti-BrdU antibodies [61]. We had also tried in preliminary experiments an anti BrdU antibody that has been claimed not to cross-react with EdU (antibody Exbio mouse monoclonal BrdU antibody, MoBu-1 clone, 11-286-C025: [61]) but this antibody was also very poor at detecting BrdU itself and had to be discarded. Until more specific BrdU antibodies have been developed or identified, we would thus suggest avoiding the simultaneous use of these two markers.

We assessed here for each marker both the density of positive cells as well as their estimated number in the entire HVC calculated based on the volume of this nucleus. In several cases a different pattern of group or sex differences was detected by these two approaches due to the contribution of the differences in HVC volume to the total count. Similarly, Yamamura and colleagues [75] found diverging effects of testosterone and its metabolites, 5α-dihydrotestosterone (DHT) and E2 alone or in combination, on total numbers of DCX neurons in HVC and on DCX^+ neurons density in female canaries. These diverging effects on density and total number of newborn neurons in HVC probably relates to the fact that the growth of HVC is due not only to an increase in neuron number but also to changes in soma size and spacing of neurons. The two types of measures of neurogenesis are thus not necessarily correlated and this raises the question of which measure better relates to functional outcomes such as memory formation or production of specific song features. Although many interesting hypotheses have been proposed about the function of adult neurogenesis such as its role in song learning or song perception, a recent critical review (see [3]) has pointed out that the lack of clear causal links between the occurrence of adult neurogenesis and any behavioral or cognitive outcome suggests that the function of neurogenesis itself largely remains to some extent an open question (see [3]).

Effects of social conditions

Compared to isolated males, the males housed with a female had a reduced rate of singing, a phenomenon that has been described in other songbird species including canaries [35]. In the current study males housed in a same-sex dyad also sang at rates that were intermediate between the M and M-F males. Although the difference of singing rates between M-M and M-F males was not significant in the general non parametric analysis, it became significant in a hypothesis-driven Mann Whitney tests focused on these two groups only, which thus replicates the result of a previously published study [39].

The number of subjects per group was reduced in the present experiment due to technical problems such as the loss of Silastic™ implants and this limited the statistical power of the study. However, the patterns of differences between groups clearly indicated that the way the three populations of newborn neurons labeled by BrdU, EdU and DCX were affected was not always equivalent. In a first step we quantified the BrdU and EdU labels separately on different sets of brain sections. Inside HVC both the EdU and BrdU density/total numbers per HVC tended to be higher in the M-M condition than in the other two groups even if group differences did not reach statistical significance in many cases (see Figs 3 and 4). This similarity probably reflects the fact that many of the BrdU-labeled cells are actually EdU cells detected by the BrdU antibody. Indeed, in the sections double-labeled for BrdU and EdU, almost one half of BrdU cells in HVC were also positive for EdU. This pattern seen in the total BrdU and EdU cells in the separate sets of sections was also observed when BrdU and EdU were co-labeled in the same set of sections (data not shown).

We additionally quantified BrdU and EdU cells in the putative site where many (most?) HVC new neurons are born: the VZ dorsal of HVC [76–78]. Although this measure does not represent the real rate of proliferation since large numbers of cells labeled at this location had plenty of time to migrate away and progenitors remaining in place could have lost a detectable signal over time after multiple divisions, it can serve as an indirect indicator of the proliferation rate at the time of injection or conversely as a negative measure of progenitor migration. Both the cells per section and cells per mm again showed a similar pattern for BrdU and EdU with the M-M group having more labeled cells than the other two groups, although in both measurements the effect was much less pronounced for EdU than for BrdU, thus providing a first suggestion that the cross-reactivity of BrdU with EdU cells does not completely explain the effect seen in BrdU. In the VZ of double-labeled sections approximately half of BrdU cells were also positive for EdU. These neurogenesis measures thus suggest a higher rate of proliferation and survival in males housed with another male, although this group also often displayed high inter-variability that prevented difference with other groups from being significant. This variability could be due to the dominance-hierarchy that was established in some pairs or alternatively reflect simply preexisting differences in birds assigned to the M-M group that potentially had a constitutively more active neurogenesis.

The double-labeled sections (BrdU and EdU) provided a more precise measure of cells that had incorporated BrdU by excluding from the total the BrdU cells that were also positive for EdU. The pattern for these $BrdU^+EdU^-$ cells (Fig 7) was somewhat different from the total $BrdU^+$ or EdU^+ cell counts (Figs 3 and 4). Males housed with a female had here a much lower number and density of these cells both in VZ and HVC than the other groups of males. Furthermore, when we assessed the number of $BrdU^+EdU^-$ cells relative to the number of EdU^+ cells as a measure of relative survival of the older cells, the males housed with a female also had a lower ratio than the other groups, with males housed with another male falling in between the other two groups. These data thus suggest that in presence of a female, newborn cells that had been labeled by BrdU survived less than in other conditions. This effect can relate to

migration, recruitment and/or survival but presumably not to a difference in proliferation since the BrdU labeling occurred when different social conditions had just been established for a few hours. The fact that this difference was not found in the count of EdU^+ cells suggests that either social condition did not have enough time to affect these cells labeled only 10 days before brain collection or that an increase in proliferation compensated the decrease in recruitment/survival.

The density of fusiform or round DCX^+ neurons was not different across social conditions in males, however the estimated number of fusiform and round DCX neurons for the entire HVC was numerically lower in males housed with a female than the other two groups of males, although this difference was significant only for the comparison of fusiform cells between the M-M and M-F groups (Fig 8B). In contrast, previous studies reported that males housed with a female have a higher density of DCX neurons than males housed alone [35] or males housed with another male [18]. Birds in both these studies were however maintained under a long-day photoperiod (16L:8D) while they were here on 11L:13D and in the former study birds were in acoustic isolation and exclusively affected by the social environment inside their cage, whereas the birds in the current and previous [18] study were visually isolated but could hear all other birds present in the same room. They could thus integrate acoustic cues from a large number of other birds and this complex acoustic environment could have partly masked some effects of the partner present in the same cage. These differences in design possibly explain why we saw a different pattern from previously published studies. Photoperiodic gating of the effect of social environment on HVC neurogenesis will be further discussed below.

Singing activity in M-M males was intermediate between activity in the M and M-F groups. Overall the ratio of $BrdU^+EdU^-$ over EdU^+ in HVC and at the VZ level, and HVC volumes followed a similar pattern. The differential levels of singing between the groups could explain differences in some measures of neurogenesis. Singing has been shown to have a positive feedback effect on BDNF expression and neurogenesis in HVC [37]. It is interesting that this pattern is seen in measures of $BrdU^+EdU^-$ cells (presumably older neurons), of HVC volumes and to some extent in the numbers of DCX^+ neurons in the entire HVC but not in the densities or total numbers of EdU^+ cells (presumably younger neurons) nor in measures of total $BrdU^+$ cells that are contaminated by EdU cross-reactivity. This would suggest that singing activity affects the survival of new neurons but only does so after a minimal amount of time that would, based on the current data, definitely need to be longer than 10 days. It has been suggested in mammals [16,43] and in zebra finches [41,42] that newborn neurons have a critical sensitive period when they are responsive to certain environmental cues. For example, work on the social context effect on neurogenesis in the caudal nidopallium of zebra finches indicates that changes in social conditions (transfer from a small group to isolation or to a large group) differentially affects cells labeled by BrdU if brain collection is performed 40, 60 or 150 days after labeling. It is thus possible that the EdU^+ cells that were about 10 days of age at brain collection had not yet reached the sensitive period when the social context can affect their survival. Alternatively, the toxicity of EdU means that a large number of labeled cells must have died before brain collection and it is conceivable that those cells that survived constitute a sub-population that is less or not sensitive to the social environment.

Males housed with a female also had somewhat smaller HVC volumes and reduced levels of singing compared to other males, although this difference was only significant for the songs produced by males alone versus males housed with a female. There was also a lower rate of putative neuronal proliferation and survival in males housed with a female as detected by several measures which contrasts with previous studies that had identified effects in the opposite direction based on measures of HVC volumes or counts of DCX^+ neurons [18,35,39]. Multiple factors could explain this discrepancy.

The effects of the social environment can indeed be complex. Our previous work showed that males housed with a female had a larger HVC than males housed with another male [18,39] but this experiment did not allow us to discriminate between an increase due to the female presence from a decrease due the presence of another male. Other studies have also found that males in acoustic isolation have a smaller HVC than males housed in group although the specific aspect of the social condition playing a key role have not been identified in a definitive manner [79]. The behavior of congeners including their vocalizations could for example be the determining variable here and more work should be done concerning the question of social effects on brain plasticity.

We also wondered whether the multiple manipulations performed in this but not in previous studies (blood sampling on day 4, multiple injections of BrdU on day 0 and of EdU injections on day 12, . . .) had potentially induced a stress preventing the positive effect of female presence. Assays of plasma corticosterone provided however no evidence for this interpretation: concentrations of this steroid were similar in the three male groups and, if anything, decreased in M-F males in the course of the experiment.

Another difference between this study and previous studies concerns the photoperiod the birds were exposed to. We kept birds under an 11L:13D photoperiod that insures they do not become photorefractory during the experiment, while previous studies identifying a positive effects of females on HVC were performed under an 16L:8D photoperiod [35,39,80]. It is possible that males become more sensitive to social cues related to reproduction and/or alternatively that females only start emitting positive signals conducive to enhanced neurogenesis under photoperiods mimicking spring and summer conditions. Moore showed [52] that male sparrows housed with females implanted with estradiol displayed an increased rate of mount attempts and higher testosterone and LH levels than males housed with non-sexually receptive females with empty implants when held on long-days but that under short-days males still mounted more the E2-treated females but no longer showed the increased testosterone and LH concentrations compared to the control males housed with sexually non-receptive females. In addition, the stimulatory effects of female presence on the development of the hypothalamus-pituitary-gonadal axis has been shown in quail to be effective only or more effective in long days [81]. On the female side, effects of estrogens on receptivity and on the response to stimulatory male songs are also influenced by the photoperiod. In ovariectomized canaries for example, exposure to male songs increases the estrogen-induced nest building only in marginally stimulating photoperiods (12L:12D). In short days nest building is not observed at all but in long days, activity is so intense that no further increase can be observed after exposure to male songs [32]. Similarly, in white-crowned sparrows, male song playback increases ovarian growth if females are in 12.5L:11.5D or in 14L:10D but not under 6L:18D or 11L:13D [82,83]. These studies provide evidence that neuroendocrine responses to behavioral cues can be dependent on the photoperiodic condition the bird experiences [83]. It is thus conceivable that under the photoperiodic conditions used here either females did not produce and send the adequate stimulatory stimuli to the males or males were not sensitive to these stimuli.

Sex differences

The comparison of males and females in the M-F group also identified divergent patterns of sex differences as a function of the marker of neurogenesis employed. In HVC, the total numbers and the density of $BrdU^+$ and EdU^+ cells were both similar in the two sexes (Figs 3 and 4) and again, the similarity of these two sets of results probably reflects the cross-reactivity of the BrdU antibody with EdU. In contrast, however, the density of $BrdU^+EdU^-$ cells (Fig 7C), the

ratio of $BrdU^+EdU^-$ to EdU^+ cells (Fig 7D) and the density of both fusiform and round DCX^+ neurons (Fig 7A and 7C) were higher in the female than in the male HVC.

However, when cell numbers were estimated for the entire HVC, the sex difference in round DCX^+ neurons disappeared (Fig 7D) and the difference in fusiform DCX^+ neurons was even reversed (males>females, Fig 7C). Together the data are thus supporting the fact that progenitor proliferation at the VZ and young neuron recruitment by HVC are overall very similar in males and females [84,85] even if small localized differences favoring females do exist in the rostral telencephalon [86] but since HVC volume is smaller in females, the density of these new neurons is larger in females than in males. Why this sex difference in density in favor of females was not detected in the analysis of EdU^+ cells remains unexplained. It could be hypothesized that the cytotoxicity of EdU discussed in the previous sections could block migration of young neuroblasts away from the VZ thus causing a local accumulation and in parallel decreasing the density of EdU^+ cells in HVC so that F-M values are equivalent to values in the M-F group (Fig 4C). Why this would affect females proportionally more than males is however unclear.

It is interesting to note that sex differences in HVC neurogenesis favoring females were detected with markers related to relatively older neurons ($BrdU^+EdU^-$ cells, DCX^+ neurons) but not with EdU that was injected only 10 days before tissue collection and thus labeled comparatively younger cells. This sex difference concerning $BrdU^+EdU^-$ cells and DCX^+ neurons but not EdU^+ cells could be taken as evidence suggesting that female neurons are recruited and/or survive in HVC comparatively longer than male neurons. Unpublished data from our lab indeed suggested that newborn neurons in the HVC of females mature more slowly than in the male HVC, taking longer to down-regulate DCX [87]. This specialized difference related to older neurons could also potentially relate to the lower baseline corticosterone concentration in the females compared to males of the same M-F group. Since this difference was seen in baseline samples, before the establishment of differential social contexts, it has to reflect a pre-existing sex difference that later vanished in samples collected on days 4 and 21. Corticosterone decreases neural proliferation in mammals [88] and in male songbirds [89,90]. Exposure to a higher corticosterone concentration before but not during the experiment might thus explain the lower rate of neurogenesis in males compared to females observed in measures of older but not comparatively younger neurons.

The more active neurogenesis in females compared to males could seem at first sight counter-intuitive given that testosterone is known in males to increase new neurons survival [18,75,84, 91–95] It must be recalled however that females in the present experiment were treated with estradiol and estrogens also increase neurogenesis in the canary HVC [11] while in female starlings, blocking estrogenic action decreases neurogenesis to the same low level as in males [49]. In agreement with the present study, female starlings, red-winged blackbirds and brown-headed cowbirds also had a higher density of DCX neurons in HVC than males [49,50]. This higher density of DCX neurons in females than males contrasts with results of a previous study where males had more fusiform DCX neurons and tended to have more round DCX neurons than females [18]. However, all the canaries in the present study were treated with exogenous hormones—testosterone for the males and 17β-estradiol for females—whereas birds in [80] were only exposed to their endogenous steroids. Although the photostimulated females in this previous study likely had relatively high levels of circulating estrogens, concentrations were not measured and they were possibly not as high as in estradiol-treated females of the present study. The discrepancy of results could also be due to the presence of gonads in males of the study of Balthazart et al [80] but not here, or to the different quantification approaches (counts in the entire cross-section of HVC here vs. counts in a 200 μm x 200 μm square in the center of HVC in [18].

Note also that the pattern of sex difference density per mm of $BrdU^+EdU^-$ cells in the VZ was similar to the pattern observed for the density of these cells in HVC (Fig 7A vs. 7C)

suggesting that even 21 days after injections of the exogenous marker, the density of labeled cells in the VZ still reflects the initial proliferation rate despite the migration away of many labeled progenitors. This was however not true for the density of EdU^+ cells per mm of VZ possibly due to the toxicity of this compound discussed before (Fig 4A vs. 4C). More work should clearly be devoted to understanding how EdU incorporated into cells replicating their DNA affects the subsequent survival of these cells.

Conclusion

These results, despite their limited power related to small final sample sizes, demonstrate that the use of multiple markers is a very useful tool to understand the complexities of environmental influences on HVC neurogenesis. A limited number of endogenous markers have been validated for use in songbirds, including doublecortin that is particularly useful because it is neuron-specific and discriminates two different stages of neuroblast development, especially when combined with different analogues of thymidine which enable us to follow the trajectories of newborn neuron populations born at a specific time relative to the treatments administrated. Technical problems are however associated with the simultaneous use of multiple thymidine analogs including cross-reactivity in their detection and potential toxicity of EdU that should only be used as a marker of proliferation and injected less than 24 hours before brain collection. Even with these limitations, the present data suggest that proliferation, recruitment and survival of new neurons can be independently affected by environmental conditions with DCX providing cumulative information not necessarily reflected in measures of single new populations ($BrdU^+$ or EdU^+).

Supporting Information

S1 Table. Final full data set.
(XLSX)

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Chapter 2. Does a mirror suppress singing in male canaries?

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Key words: songbirds; singing motivation; mirror; social interactions

Abstract

Social context plays a key role in the control of singing rate across songbird species. Male canaries sing at high rates when housed alone but very rarely in the presence of a female. On average a male housed with another male sings at a rate intermediate of an isolated male and a female-paired male, however singing rate in male-male pairs is highly variable likely depending on the specific hierarchical relationship established between the males. Songbirds readily respond to the presence of a mirror, which is often interpreted as showing the presence of a congener even if cases of self recognition seem to exist in oscines. We investigated the effect of a mirror on song rate in male canaries in two small scale studies. The preliminary study showed promising results with a significant suppression of song rate in individuals housed with a mirror. However, the follow-up study failed to support the inhibitory role of a mirror on singing motivation. Early in the experiment, two out of three subjects without a mirror sang at high rates, compared to only one out of five subjects with a mirror. However, later on in the experiment this trend disappeared. The average song rate of the two groups never differed significantly. The reason for the discrepancy between the two studies could be related to the different photoperiods birds were exposed to, different strains of canary used or strong individual differences. Previous studies of budgerigars and European starlings have indeed shown large differences in individual responses to a mirror, which seemed to depend on the subject's social experiences during development.

Introduction

Birdsong is a complex vocal behavior acquired during development in a process similar to the learning of human speech (Brainard and Doupe, 2013). Like human speech, singing is a social behavior—male songbirds often use it in courtship or in territorial defense (Catchpole and Slater, 2008; Collins, 2004). The social context affects singing behavior in many ways. When a male zebra finch sings to a female, variability in syllable sequencing and syllable structure is reduced relative to when males sing alone (Kao et al., 2005; Sossinka and Böhner, 1980). Male white-crowned sparrows and canaries sing at different rates depending on the social housing conditions (Alward et al., 2013; Boseret et al., 2006; Shevchouk et al., 2017a; Tramontin et al., 1999).

A well-established social effect is the suppression of singing in males by the presence of a female which has been shown in European starlings (Cuthill and Hindmarsh, 1985), sedge warblers (Catchpole, 1973), great tits (Krebs et al., 1981) and canaries (Alward et al., 2014; Boseret et al., 2006; Shevchouk et al., 2017a). These studies are thus consistent with the role of song in mate attraction: song is no longer necessary when the female is continuously present. The song rate of males housed with other males is intermediate between female-paired males and isolated males (Shevchouk et al., 2017a). As a territorial behavior, singing rate can reflect the social status of a male songbird. Dominant male canaries sing at higher rates than subordinate males (Boseret et al., 2006; Sartor and Ball, 2005; Wiley et al., 1993), therefore the intermediate singing rate found in male-male pairs in which social hierarchy has not been determined could be a reflection of heterogeneity in the population.

In this study, we tested whether housing male canaries with a mirror would reduce song rate by simulating the effect of the presence of another male. Avian species identify their mirror reflection as a conspecific (Buckley et al., 2017; Diamond and Bond, 1989; Friedman, 1977; Henry et al., 2008; Lott and Brody, 1966) although work with corvids has suggested that some species might be recognizing themselves (Prior et al., 2008; but see Soler et al., 2014). In general the specific behavioral responses of avian species to a mirror reflection are poorly understood and seem to depend on the sex of the bird and its

previous social experience (Buckley et al., 2017; Henry et al., 2008). A mirror can also induce physiological changes. Female ring doves who can hear a dove colony or a single male's vocalizations respond to their own mirror reflection with growth of the reproductive tract, but do not show this response if they have only the auditory stimulation (Friedman, 1977; Lott and Brody, 1966). Cichlid fish exposed to their own reflection aggressively attack it, but they show no change in circulating testosterone concentration, whereas circulating testosterone decreases when males of this species lose a fight and increases when they win a fight (Oliveira et al., 2005). This suggests that although a mirror can evoke territorial behavior, the hierarchy of the "dyad" formed by a subject and its reflection is less clear, making it an interesting situation to examine the social modulation of song rate.

Moreover, a method to reduce song rate in a non-invasive way could be a useful tool for studying the effect of a songbird's own singing on the plasticity of its song control system. Male canaries who were prevented from singing had a lower expression of the neurotrophin BDNF (brain-derived neurotrophic factor) and a lower incorporation of newborn neurons in the song control nucleus HVC, compared to control birds (Li et al., 2000). The method for reducing the song rate in this study, an experimenter waving his hands whenever the birds started to sing, could however be stressful for the birds. Stress induces the release of corticosterone which inhibits neurogenesis both in mammals (reviewed by Mirescu and Gould, 2006) and songbirds (Katz et al., 2008; Newman et al., 2010). Therefore, it is not clear whether the decreased neurogenesis in birds of the study by Li and colleagues (Li et al., 2000) was due to their decreased singing rate or their increased stress. If a mirror could decrease song rate without causing substantial stress, this behavioral setup could be used to confirm the positive effect of the birds' own singing on neurogenesis in HVC, as well as on other forms of neuroplasticity related to singing.

Materials and Methods

Experiment 1

The full methodology of this preliminary experiment is described in chapter 1 of this thesis. Briefly, photosensitive male canaries of the Fife fancy breed were castrated, implanted

with a 10 mm-long Silastic™ implants filled with testosterone and housed individually on a photoperiod of 11L:13D. Being part of a study on HVC neurogenesis all subjects were injected 5 times with 100 µl of a 10 mg/ml Bromodeoxyuridine (BrdU) solution (dissolved in 0.9% saline with 28 mg/L NaOH) one day after receiving the testosterone-filled implants and 5 times with an equimolar dose of 5-ethynyl-2'-deoxyuridine (EdU) in 0.01M PBS eleven days later. Three subjects had a mirror 16 cm wide x 16 cm high attached to one wall of their cage (Mirror group, these subjects were not included in the experiment described in chapter 1 but were treated in the same way as the subjects in that experiment). The other eight subjects (included in chapter 1 experiment) were housed in equivalent conditions but did not have a mirror in their cage – No-mirror group. Singing behavior was recorded by direct observation once every second day for five minutes in the morning and five minutes in the afternoon, for a total of 10 observations.

Experiment 2

Experimental animals

Eight male canaries of an unknown strain used in this study were obtained from a local breeder in Belgium. All subjects had been on natural daylight during the months preceding their arrival in our laboratory at the University of Liege, Belgium, in late September.

Experimental procedures

Upon arrival subjects were housed on medium short day photoperiod (11L:13D) for 7 months and on short day photoperiod (8L:16D) for 5 weeks, in single-sex groups of 5-7. One week after arrival all males were castrated following a procedure similar to that described in Sartor et al., (Sartor et al., 2005). Briefly, under isoflurane anesthesia each testis was removed via an ipsilateral incision posterior of the last rib. Testes were found to be regressed in all subjects. The incision was sutured, the subject was allowed to recover under a heat lamp until perching and then returned to its home cage. One day before the start of the experiment the subjects received a single subcutaneous 10 mm-long Silastic™ implant (Dow Corning reference no. 508-004; inner diameter 0.76 mm, outer diameter 1.65 mm) filled with crystalline testosterone (Fluka Analytical, Sigma-Aldrich) and sealed on both sides with medical silicone (Medical Adhesive Silicone, Dow

Corning). All implants were checked under a stereo-microscope to make sure they were well sealed and were incubated in 0.9% NaCl at 37°C overnight before being inserted subcutaneously. After implanting the Silastic™ the subjects were housed individually in new cages with a mirror on one wall of their cage (exactly the same as in experiment 1) – Mirror group (n=5), or without a mirror (No-mirror group, n = 3). Since these subjects were also part of a study on HVC neurogenesis, the following day they were injected with 100 µl of a 10 mg/ml Bromodeoxyuridine (BrdU) solution (dissolved in 0.9% saline with 28 mg/L NaOH) 5 times with 2 hours in between each injection. The birds weighed on average 20 grams, therefore the BrdU dose per injection was 50 mg/kg. On the same day, the photoperiod was changed to 14L:10D and the behavioral observations commenced (see next paragraph for details). On days 1, 2, 7 and 10 all birds were checked for presence of implants and on day 10 most subjects were found to have implants that started to pierce the skin and fall out. Therefore, on day 14 all subcutaneously implanted Silastic™ capsules were removed and new testosterone-filled Silastic™ implants, prepared in exactly the same way as previously, were inserted under light isoflurane anesthesia (1.5-3%) into the intraperitoneal space via an incision posterior of the last rib. Behavioral observations were resumed 7 days after this surgery, as no subject was found singing during the first 7 days, and continued for another 8 days.

Song observation

The song rate of all subjects was quantified during a total of 10-30 min per day in 10 minute sessions over a total of 13 observation days. During the observations, the observer sat quietly in front of the cages and noted the number of songs produced by each male. Due to the small number of subjects all birds could be observed simultaneously. We operationally defined song as a vocalization longer than approximately one second in duration after at least a 500 msec period of silence. In the first six days the observer was not hidden from the subjects view and they were only observed to produce calls and short simple songs (2-3 seconds long), although longer more complex songs were heard outside of observation periods. This experimental phase will be referred to as the phase 1. For the rest of the experiment a cardboard blind was used to conceal the experimenter during song observation. During these further

observations, long complex songs were observed and therefore calls and short-songs were no longer considered relevant and were not recorded. The observer-hidden part of the experiment was divided into a further 3 phases. The 2nd phase was similar to the first, the only difference being the concealment of the observer. During the 3rd phase in addition to the observer being hidden, a song-playback was used during or directly preceding the song observation. The song-playback was a song recording made from one of the birds in the experiment. The 4th phase had the same conditions as the 2nd phase, however the testosterone-implants were implanted intraperitoneally instead of subcutaneously, as described in the experimental procedures.

All experimental procedures complied with Belgian laws concerning the Protection and Welfare of Animals and the Protection of Experimental Animals, and experimental protocols were approved by the Ethics Committee for the Use of Animals at the University of Liege (Protocol number 926). In all housing situations food, water, baths, cuttlebone and grit were available *ad libitum*.

Statistical analyses

The number of calls, short songs and longs songs in the 4 phases of experiment 2 and total number of songs in experiment 1 were all compared across the two groups (Mirror vs No-mirror) by a t-test. All statistical analyses were performed using GraphPad Prism and differences were considered significant for $p<0.05$. All data are represented here by their mean ± SEM.

Results

Experiment 1

The preliminary study compared the singing rates in males with or without a mirror maintained on intermediately short photoperiod (11L:13D). All No-mirror subjects sang at higher rates than subjects with a mirror (Fig. 1). A t-test confirmed that the difference in singing rate between the groups was significant ($t_9 = 2.40$, $p = 0.040$).

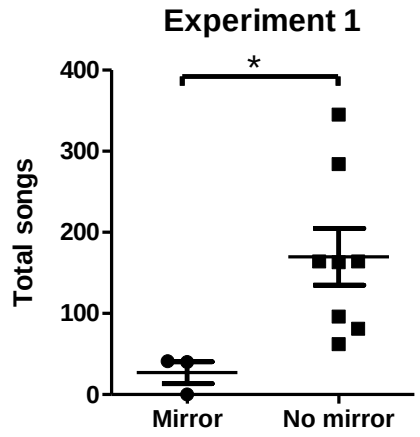

*Figure 1. Total numbers of songs recorded during the 20 observation sessions of experiment 1. *= p<0.05 for individually-housed male canaries with a mirror compared to male canaries housed without a mirror. All data are represented by their mean ± SEM.*

Experiment 2

The interesting result of the preliminary study motivated us to repeat the experiment, this time exposing the birds to a long photoperiod to induce breeding conditions and a maximal singing rate. In **phase 1** the experimenter was visible to the subjects, which seemed to inhibit their singing. Only calls and simple short songs (2-3 seconds) were observed, although complex songs were heard outside of observation periods. Although the average number of calls was higher in the No mirror than in the Mirror group (Fig. 2A), this difference did not reach significance ($t_6 = 1.19$, $p = 0.280$). Most subjects did not sing short songs; there was only one subject in each group who sang at higher rates and no difference was detected between groups (Fig. 2B, $t_6 = 0.34$, $p = 0.744$).

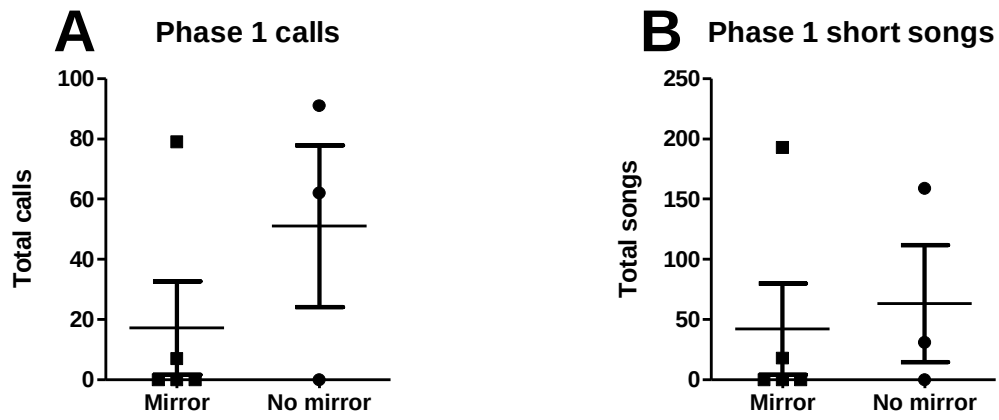

*Figure 2. Total calls (**A**) and short simple songs (2-3 seconds) (**B**) recorded during 6 sessions of phase 1 (experimenter visible to the birds during observation sessions) in male canaries individually-housed with or without a mirror. All data are represented by their mean ± SEM.*

In **phase 2** the experimenter was hidden from the view of the birds and more complex longer songs were produced during the observation sessions, therefore calls and short songs were ignored. Two out of three No-mirror birds sang, while only one out of five males sang in the Mirror group (Fig.3), however this difference in song rate between groups did not reach significance ($t_6 = 1.76$, $p = 0.129$).

Figure 3. Total number of songs recorded during 4 sessions of phase 2 (experimenter hidden from the birds view during observation sessions) in male canaries individually-housed with or without a mirror. All data are represented by their mean ± SEM.

In **phase 3** song-playback occurred during or immediately prior to the song rate observation. This increased the number of birds singing in the Mirror group but not in the No-mirror group (Fig. 4). Overall, song rates were slightly higher than in the previous phase (both phases had 4 observation sessions and so are comparable), but the difference between the two groups was even smaller than previously ($t_6 = 1.26$, $p = 0.256$).

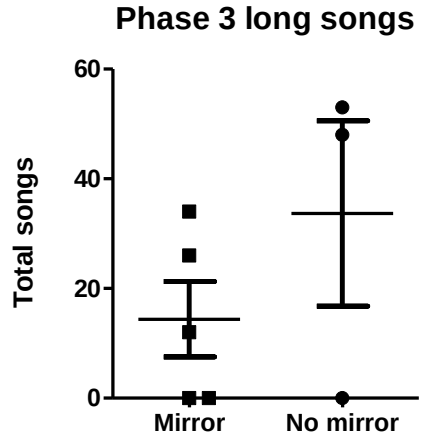

Figure 4. Total number of songs recorded during the 4 sessions of phase 3 (with song-playback during or immediately prior to observation, experimenter hidden from the birds) in male canaries individually-housed with or without a mirror. All data are represented by their mean ± SEM.

During **phase 4**, the birds had already been in the experimental conditions for 3-4 weeks and had been intraperitoneally re-implanted with testosterone-filled Silastic™ implants after having lost the subcutaneously inserted ones. In this phase, 3 out of 5 Mirror subjects were observed to sing and 2 out of 3 No-mirror subjects. Overall, the same birds were singing at high rates in phase 4 as the ones singing in phase 3. There was no significant difference between the song rates of the two groups (Fig. 5, $t_6 = 0.28$, $p = 0.789$).

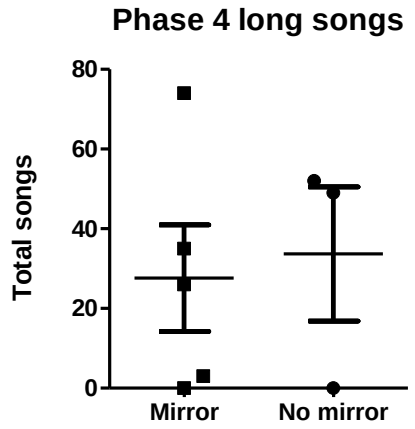

Figure 5. Total number of songs recorded during 10 sessions of phase 4 (experimenter hidden from the birds during observation sessions, 3-4 weeks after onset of experimental conditions, testosterone re-implanted intraperitoneally after subcutaneous implants fell out) in male canaries individually-housed with or without a mirror. All data are represented by their mean ± SEM.

Discussion

In this study the effect of housing with a mirror on singing rate in male canaries was investigated in two small studies. The first study found a significantly suppressed song rate in subjects individually housed with a mirror compared to their counterparts housed without a mirror. Since the only difference between the two groups, which had substantially different song rates, was the mirror, we concluded that the subjects paid attention to the mirror and something about seeing their own reflection there influenced their motivation to sing. This result can be interpreted in a few ways. Since housing with a female also has the effect of suppressing song in male canaries, while housing with another male usually does so to a variable degree, this could mean that the subject regarded his reflection as a female bird, perhaps because that bird did not behave in an aggressive, territorial way. Canaries do not have a sexually dimorphic appearance and it is not yet clear which sensory cues allow canaries to identify the sex of conspecifics. A second interpretation is that the subject perceived the reflection as another male, however since that 'other male' did not attempt to show signs of dominance, he was not seen as a threat and thus, the territorial singing that is usually seen in two males housed together

did not occur. A third possibility is that, because the behavior of the reflected bird in the mirror did not fit into the subject's expectations, this image was stressful for the subject and it was the stress that inhibited the singing activity. In this case, the use of a mirror to study the effect of a birds own singing on HVC neurogenesis would not be better than an experimenter waving his/her hands when the birds start to sing. The interpretation that the subject recognized himself in the reflection is unlikely because that should make them equivalent to subjects housed alone without a mirror and these subjects did not sing at the same high rates as subjects kept alone without a mirror.

The follow-up study showed no consistent inhibition of singing motivation by housing with a mirror. The first phase of the experiment was not conclusive because the sight of the experimenter inhibited all birds from singing during the observation sessions, therefore in all following phases the experimenter hid behind a blind. In phase two, although there was no significant difference in the average song rate between the two groups, there was a suggestion that the mirror might be inhibiting singing in a subset of subjects.

Being a social behavior, singing is produced at higher rates when other individuals around are also singing, at least in zebra finches (Jesse and Riebel, 2012). Since there was a low number of subjects in the experiment and very few of them were singing at all, we hypothesized that the overall low rates of singing in the No-mirror group could be due to the lack of social stimulation. To test this hypothesis, we stimulated the subjects with song-playback and recorded their song rate during and immediately after the song-playback. Although this manipulation did indeed slightly increase the average song rate in the room, the trend towards a difference between groups completely disappeared during this phase of the experiment. In phase four, even though no song-playback was used, the pattern was exactly the same as in phase 3, with exactly the same subjects singing at significant rates and the same subjects producing no songs. The song playback failed to activate singing in one No-mirror and two Mirror subjects, which never were observed to sing in the entire experiment, despite the long-day photoperiod and high circulating testosterone that the implants would presumably have induced.

The first study maintained the male canaries on an intermediate daylength of 11L:13D. Although this photoperiod was longer than the 8L:16D photoperiod they were

previously exposed to and thus was probably partially photostimulating, it is most likely that these subjects were not in fully breeding condition, while the subjects in the follow-up study were housed on 14L:10D and likely were in full breeding condition. This difference might suggest that the mirror can inhibit singing but only under some circumstances, including an adequate photoperiod. It is also conceivable that the effect of a mirror on the subjects' behavior changes over time, with the subject changing its perception of the reflection via "social interactions" with it. A case study of the interaction of a Kea (a parrot, *Nestor notabilis*) with its own reflection shows substantial changes in the nature of the interactions over time (Diamond and Bond, 1989). It is possible that the inhibiting effect of the mirror was strongest early in the experiment and that the suboptimal methods used at the beginning of the experiment prevented us from seeing the inhibiting effect. Another possible difference between the two studies was the strain of canary used. Experiment 1 was conducted with Fife fancy canaries, while the strain of the canaries in experiment 2 was not known (Generic Belgian singers) as they were purchased from a commercial breeder.

Finally, it is possible that the reason why a robust effect of the mirror on singing rate was found in the first but not in the second experiment is the pre-existence of strong individual differences in response to the mirror, which were shaped by the subjects' early social environment. Male starlings that had been raised either in isolation or in a large group responded to a mirror with more "attention focusing" and movement, with more behaviors of the following types: move, rub wings, rub beak, shake and gaze at mirror, while male starlings raised in pairs responded calmly to the mirror, showing a higher frequency of behaviors such as preen, scratch and stretch (Henry et al., 2008). All females in this study also responded calmly to the mirror, possibly because in captivity females tend to form dyads, while males usually form small groups (Hausberger et al., 1995). Budgerigars that had a stronger pair-bond prior to experience with a mirror, spent more time interacting with a mirror than those with a weaker pair-bond (Buckley et al., 2017). Both these studies illustrate the complexity of avian responses to a mirror and their dependence on previous social interactions and possibly also on inborn social dispositions (Buckley et al., 2017). Especially when the subject size of the groups is small, like in the current experiment, there is a chance that the experimental groups are

heterogenous, some birds showing an inhibition of singing by a mirror (such as all subjects in experiment 1 and some in experiment 2) due to their history and inborn preferences, and some birds, with different kinds of early social experiences, not showing this inhibition. This study reminds of the danger in using very small groups to study complex social interactions. In order to use a mirror to suppress song and study the effect of this on HVC neurogenesis, more studies are needed to understand the circumstances in which a mirror exerts an inhibitory effect on song rate, including photoperiodic conditions and the role of past social experience.

Chapter 3. Testosterone-induced neuroendocrine changes in the medial preoptic area precede song activation and plasticity in song control nuclei of female canaries

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Abstract: Testosterone plays a key role in the control of seasonal changes in singing behavior and its underlying neural circuitry. After administration of exogenous testosterone, song quality and song control nuclei volumes change over the course of weeks, but song rate increases within days. The medial preoptic nucleus (POM) controls sexual motivation and testosterone action in POM increases sexually motivated singing. Here, we investigated the time course of testosterone action in the song control nuclei and POM, at the gross anatomical and cellular level. Photosensitive female canaries were injected with BrdU to label newborn neurons. One day later they were transferred to a long day photoperiod and implanted with testosterone-filled or empty implants. Brains and blood were collected 1, 2, 9 or 21 days later. Testosterone increased POM volume within one day, whereas the volume of song control nuclei increased significantly only on day 21 even if a trend was already observed for HVC on day 9. The density of newborn neurons in HVC, labeled by Bromodeoxyuridine (BrdU) and doublecortin, was increased by testosterone on days 9 and 21 although a trend was already detectable on day 2. In POM testosterone increased the number and size of aromatase-immunoreactive neurons already after one day. This rapid action of testosterone in POM supports its proposed role in controlling singing motivation. Although testosterone increased the number of newborn neurons in HVC rapidly (9, possibly 2 days), it is unlikely that these new neurons affect singing behavior before they mature and integrate into functional circuits.

Full text: https://orbi.ulg.ac.be/handle/2268/205891

Chapter 4. Rapid testosterone-induced growth of the medial preoptic nucleus in male canaries

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Key words: Testosterone; medial preoptic nucleus; HVC, singing motivation; songbirds

Abstract

Testosterone acts in the songbird brain to facilitate singing in a myriad of ways. By affecting different forms of neuroplasticity within song control nuclei, testosterone and its metabolites change the quality of singing, however these changes take weeks to develop fully. By contrast, testosterone activates singing within days in castrated males. Lesion and stereotaxic implantation of testosterone have suggested the medial preoptic nucleus (POM) as the site where testosterone mediates the motivation to sing, expressed as differential rates of singing. Confirming this hypothesis, testosterone increases the volume of POM within one day in female canaries, while the increase in volume of song control nuclei takes several weeks. The current study was designed to test for rapid testosterone-induced changes in HVC and POM of male canaries. Four photosensitive castrated male canaries were implanted with testosterone-filled Silastic™ implants and another six with empty implants. Both groups were switched from short- to long-day photoperiod on the same day. Two days later a blood sample and brains were collected from all subjects. Plasma testosterone was elevated in testosterone-treated but not in control subjects. HVC volumes were not different between groups, however there was in the testosterone-treated group a significant increase in POM volume, number and average somal area of aromatase-immunoreactive (ARO-ir) neurons and fractional area covered by ARO-ir material in POM. Comparison with females in an equivalent hormonal and photoperiodic state also suggests the existence of a sex hormone-independent sex difference in POM volume in canaries. These results show that testosterone induces POM growth within two days and support the hypothesis that testosterone increases singing motivation via its action in POM.

Introduction

Male songbirds sing profusely during the breeding season to attract females and defend their territory (reviewed in Catchpole and Slater, 2008; Collins, 2004). Like many courtship behaviors, singing is largely under the control of testosterone (reviewed in Alward et al., 2017; Ball et al., 2003; Schlinger, 1997). Implanting testosterone in adult songbirds increases frequency and duration of singing in a number of species (Madison et al., 2015; Nottebohm, 1980a; Pröve, 1974). Castration causes a decrease in singing activity during the breeding season (Arnold, 1975b; Nottebohm, 1980a; Pröve, 1974), although this is not the case if surgery is performed during the non-breeding season (Alvarez-Borda and Nottebohm, 2002; Pinxten et al., 2002). Testosterone regulates most aspects of song through a synergistic effect of its androgenic and estrogenic metabolites (reviewed in Ball et al., 2003).

The songbird brain contains a specialized neural network, called the song control system (SCS) that controls the learning, maintenance and production of song. Testosterone and its metabolites increase the volumes of nuclei in this network (Nottebohm, 1980a; Yamamura et al., 2011) by altering various cellular properties in a region-specific manner (Ball et al., 2004; Brenowitz and Beecher, 2005). Despite the profound impact that testosterone has on the song control system, testosterone action within these nuclei does not seem to be responsible for the vernal increase in song rate. Meitzen and colleagues (Meitzen et al., 2007) showed that blocking androgen and estrogen receptors in the HVC of white-crowned sparrows induces a decrease in song stereotypy, but no change in song rate. Similarly, blocking androgen receptors in the HVC or RA of male canaries affects different aspects of song structure, but does not decrease song rate (Alward et al., 2016a). Together these studies suggest that testosterone acts in HVC to regulate the quality but not the rate of singing.

The medial preoptic nucleus (POM) is a critical brain site where testosterone acts to activate appetitive aspects of sexual behavior in Japanese quail as well as songbirds (Alger and Riters, 2006; Balthazart and Ball, 2007; Panzica et al., 1996; Riters and Ball, 1999). Bilateral lesions of POM decrease song rate of male starlings during the breeding

season (Alger and Riters, 2006; Riters and Ball, 1999). Conversely implanting testosterone in the POM of castrated male canaries is sufficient to increase the singing rate, but not the song quality, to the same level as systemic treatment with testosterone (Alward et al., 2016c; Alward et al., 2013). Testosterone increases the rate of singing within 3-6 days in female or castrated male canaries (Madison et al., 2015). Therefore if this behavioral effect is mediated by an action in the POM, testosterone-induced changes in POM should be evident after even shorter latencies. Male quail and female canaries implanted with testosterone display an increased POM volume one day after the beginning of the treatment (Charlier et al., 2008; Shevchouk et al., 2017b), while the increase in volume of the song control nuclei HVC, RA and Area X in female canaries takes three weeks (Shevchouk et al., 2017b). The current study investigates whether there is a rapid increase of POM volume following testosterone implantation in male canaries, as previously seen in females.

Materials and Methods

Experimental animals

The 10 male canaries of the Fife fancy breed used in this study were obtained from a colony maintained at the University of Antwerp, Belgium. They were born and had gone through a full breeding cycle in this colony. All subjects had been on natural daylight during the months preceding their arrival in our laboratory at the University of Liege, Belgium, in late March. All experimental procedures complied with Belgian laws concerning the Protection and Welfare of Animals and the Protection of Experimental Animals, and experimental protocols were approved by the Ethics Committee for the Use of Animals at the University of Liege (Protocol number 926).

Experimental procedures

Upon arrival, subjects were housed on long day photoperiod (16L:8D) for five months, on intermediately long day photoperiod (12L:12D) for 2 months and on 8L:16D for five months to transition the subjects first through photoregression and then maintain them in a photosensitive state. During the first two months, the subjects were housed in an aviary containing a large mixed-sex group of canaries. They were subsequently moved to single-

sex housing with 5-6 males per cage in a separate room with no visual contact and minimal acoustic contact with the females. In all housing situations food, water, baths, cuttlebone and grit were available *ad libitum*. Three weeks after the change to short-day photoperiod subjects were castrated following a procedure similar to the procedure described by Sartor and colleagues (Sartor et al., 2005). Briefly, under isoflurane anesthesia each testis was removed via an ipsilateral incision posterior of the last rib. Testes were regressed in all subjects. The incision was sutured, the subject was allowed to recover under a heat lamp until perching and then returned to the same cage with the same male companions as previously. Two months before the onset of the experiment (in February) the subjects were individually housed in sound-attenuated boxes for three days to record their baseline singing behavior. Immediately before the cage change they were weighed, their cloacal protuberance length and width was measured and a blood sample was collected from their wing vein. These recordings showed an absence of singing in all subjects except for one.

Two days before the treatment onset (in May) subjects were transferred again to sound-attenuated boxes for baseline recordings to verify that they were still singing at a minimal rate. Each subject then received a single subcutaneous 10 mm-long Silastic™ implant (Dow Corning reference no. 508-004; inner diameter 0.76 mm, outer diameter 1.65 mm) that was either filled with crystalline testosterone (n= 4, Fluka Analytical, Sigma-Aldrich) or kept empty as control (n = 6). All implants were sealed on both sides with medical silicone (Medical Adhesive Silicone, Dow Corning) and were checked under a stereo-microscope to make sure they were well sealed. They were incubated in 0.9% NaCl at 37°C overnight before being inserted subcutaneously. After implantation of the Silastic™ capsules, the subjects were returned to the same sound-attenuated box as previously and their singing behavior was recorded for two days. On the morning of the 2nd day after implantation a blood sample was collected from the wing vein, the subjects were deeply anaesthetized and perfused to fix their brain (see below for details).

Song recording and analysis

Singing was recorded inside 16 individual custom-built sound-attenuated boxes for two hours each day immediately following lights-on (0900h). Sound was acquired from all 16

channels simultaneously via dedicated microphones (microphone from Projects Unlimited/Audio Products Division, amplifier from Maxim Integrated) and an Allen & Heath ICE-16 multichannel recorder. The sound file was acquired and saved as a .wav file by Raven v1.4 software (Bioacoustics Research Program. (2011). Raven Pro: Interactive Sound Analysis Software (Version 1.4). Ithaca, NY: The Cornell Lab of Ornithology) at a frequency of 44,100 Hertz. The spectrograms were analyzed for song rate manually for post-treatment recordings and with a custom-built program for pre-treatment recordings (for details see methods of chapter 6).

Blood collection and hormone analysis

Blood samples of 150 µl were collected from the wing vein of all subjects during the transfer to sound-attenuated boxes for baseline recordings, just before the subcutaneous implants and on the day of brain collection. Blood collection was always performed within 3 minutes of catching the birds in their cage and within 90 minutes after lights on. The blood was collected into Na-heparinized micropipettes (Brand, Wertheim, Germany) and any further blood flow was stopped by pressing cotton on the vein puncture. Blood was centrifuged at 9,000 g for 9 minutes and the supernatant plasma was collected and stored at -80° C until further use.

Testosterone Enzyme Immunoassay

10 µl of plasma from each sample was diluted in 150 µl of ultra-pure water. Recovery samples were spiked with 20,000 CPM of tritiated-testosterone (Perkin-Elmer). All samples were extracted twice with 2 ml of dichloromethane. The organic phase was eluted into clean tubes, dried with nitrogen gas and stored at -20°C until further use. Average recovery rate was 76.3%.

Extracted samples were re-suspended in 400 µl Enzyme Immunoassay (EIA) buffer by vortexing for 30 seconds and shaking for 90 min at 1350 rpm. Re-suspended samples were assayed for testosterone concentration in a single assay using a Cayman Chemicals testosterone EIA kit (ref. 582701) following manufacturer's instructions. The minimum and maximum detection limit of the EIA measuring testosterone in the samples collected at the end of the experiment, as determined by the lowest and highest

concentration detected, were 10.79 pg/ml and 169.73 pg/ml respectively. The average intra-assay variation for the assay was 11.1%. For the pre-experimental samples the minimum and maximum detection limit of the EIA was 4.66 and 69.44 pg/ml respectively, and the average intra-assay variation was 15.5%.

Brain collection and processing

Two days after the introduction of implants, a blood sample was taken from the wing vein of each subject, they were weighed, their cloacal protuberance was measured and then birds were anaesthetized with ~0.03ml of Nembutal (Ceva, 60 mg/ml). Once reflexes had stopped the birds were perfused intracardially with phosphate-buffered saline (PBS, 1.43 g/L Na_2HPO_4, 0.48 g/L KH_2PO_4, 7.2 g/L NaCl) to remove blood and immediately after with 4% paraformaldehyde (PFA, 4.3 g/L NaOH, 40 g/L paraformaldehyde, 18.8 g/L $NaH_2PO_4.H_20$) to fix the brain. After perfusion, the brain was immediately extracted from the skull and post-fixed overnight in 15 ml PFA.

The syrinx was extracted and weighed, the presence of implants and, when relevant, presence of testosterone inside the implants was confirmed. On the following day, brains were transferred to 15 ml of 30% sucrose solution (15.6 g/L Na_2HPO_4, 1.5 g/L KH_2PO_4, 300 g/L sucrose). Once the brains had sunk to the bottom of the vial they were frozen on dry ice and stored at -80° C until used. Brains were cut on a cryostat into 30 µm thick coronal sections. The sections containing the medial preoptic nucleus were collected separately into one series – these included all sections between the rostral end of tractus septopallio-mesencephalicus (TSM) and 240 µm caudal to the anterior commissure (AC), while the rest of the brain was cut into four series. Sections were stored in anti-freeze (0.01M PBS with 10 g/L polyvinylpyrrolidone, 300 g/L sucrose, and 300 ml/L ethylene glycol) at -20°C until further use.

Nissl staining

One series of sections was mounted on Superfrost slides, dried at least overnight, and Nissl-stained with toluidine blue. After differentiation in Walpole buffer and molybdate, they were dehydrated in a series of increasing isopropanol concentrations, in 99% ethanol and finally in xylene and coverslipped using Eukitt as a mounting medium.

Aromatase immunohistochemistry

A second series of brain sections was stained by immunohistochemistry for aromatase. Washes were performed with Tris-buffered saline (TBS) or TBST (TBS with 0.2% Triton X-100). The blocking sera and antibodies were diluted in TBST with 5% normal goat serum (NGS) and 1% bovine serum albumin (BSA). Sections were washed three times for five minutes to remove antifreeze and after all other steps except for the blocking serum. Endogenous peroxidases were inhibited by incubating the sections in 3% hydrogen peroxide in a solution of 50% methanol for 20 minutes. Sections were blocked in TBST-NGS-BSA solution for 60 minutes and incubated in primary antibody for one hour at room temperature and overnight at 4°C (1:10,000 rabbit anti-quail aromatase antibody, a kind gift from Prof. Harada, Fujita Health University, Toyoake, Japan). This antibody has been especially developed and validated for quail (Foidart et al., 1995a) and also validated in songbirds (Balthazart et al., 1996a). On the following day, sections were blocked again in TBST-NGS-BSA solution for one hour, and incubated with the secondary antibody for two hours at room temperature (1:200 goat anti-rabbit biotinylated, DAKO, ref. E0432). The binding was amplified by incubating sections in ABC kit solution (both solution A and B at 1:400, Vectastain Elite PK-6100 2001). The binding sites were revealed by incubating for 10 minutes in 0.04% diaminobenzidine (DAB) with 0.012% H_2O_2 diluted in TBS. Sections were mounted from TBS with gelatin onto glass slides, dried overnight, immersed in xylene for 10 minutes and coverslipped with Eukitt mounting medium.

Microscopy and image analysis

HVC volume reconstruction in Nissl-stained sections

To reconstruct HVC volumes, photomicrographs were taken of each section in the series containing the nucleus, in both the left and right hemispheres, with a Leica DMRB FL.100 microscope connected to a Leica DFC 480 color camera at a magnification of 5x using the same light settings for all pictures. An outline was drawn around the perimeter of each cross-section of the nuclei using ImageJ v1.47v (National Institutes of Health) and the delimited area was measured. In the few cases that a section was missing, the area was

estimated by taking the average of the two sections immediately rostral and caudal to it. The volumes of nuclei were calculated by summing the areas and multiplying by 120 µm, the distance between two successive sections in the series. The volume of the nucleus in each hemisphere was calculated separately and the average of the two measures was used for statistical analyses.

Aromatase

Aromatase staining was analyzed on a Leica DMRB FL.100 microscope connected to a Leica DFC 480 color camera. Photomicrographs of each POM in the series were taken at 10x magnification using the same light settings for all pictures, starting from the most rostral section containing aromatase-immunoreactive (ARO-ir) cells ventral of the TSM and finishing at the section containing the full extent of the AC. However, since some brains had damage in at least one of the three first sections in this series, the first three sections were excluded from the quantification of POM volume for all brains. The cluster of ARO-ir cells that correspond to the POM was delineated and its surface measured with ImageJ FIJI (National Institute of Health). The volume of the nucleus was calculated by adding these areas in all sections and multiplying by 60 µm, the distance between two consecutive sections.

The photomicrograph representing the middle section of the POM in the rostro-caudal axis in each bird was additionally analyzed for cellular level changes. Within an 852 x 852 µm square surrounding the POM (this square fully included the largest POM cross-section from all subjects) the number of aromatase-expressing neurons, the percentage of area covered by aromatase staining and the mean somal area of the ARO-ir neurons were quantified. Briefly, images were converted to 8-bit, a grey-value threshold was set to include all clearly visible aromatase-expressing neurons, but exclude all background. All particles over 30 µm in area and over 0.15% circularity (circularity = 4π x area/$(\text{perimeter})^2$, with a value of 1.0 indicating a perfect circle) were counted and measured. Additionally, the ARO-ir neurons in the same square were manually counted on the raw, non-thresholded image as a control. A linear regression showed that the manually and automatically counted numbers of neurons were significantly correlated ($r_{17} = 0.92$, $p < 0.001$).

Statistical analyses

A repeated-measures ANOVA was used to analyze the effect of treatment and time on body mass, cloacal protuberance area (length x width measured) and plasma testosterone as measured two months before treatment (February) and on the day of brain collection (May). When a significant interaction was found Tukey's post-hoc test was used to identify the origin of the effect. A t-test was used to investigate whether there is a difference between the control and testosterone-treated group in syrinx mass, HVC and POM volumes, the number of ARO-ir neurons, their average somal area and the % area covered by ARO-ir material in the rostro-caudally middle section of the POM. A linear regression was performed to compare the number of ARO-ir neurons counted manually versus automatically with a grey-value threshold analysis. A one-way ANOVA was used to analyze the POM volume of the two groups in the current study and subjects singing at a high rate from the same batch that were used in a separate experiment (See Chapter 6 of this thesis). A two-way ANOVA was used to compare the POM volumes and plasma testosterone concentrations in the males of the current study and female canaries that had been transferred to long days and implanted with testosterone-filled or empty Silastic™ capsules (See Chapter 3 of this thesis). The brains of the females were collected after two days of treatment. A paired t-test was used to test whether volumes of HVC and POM, the number and area of ARO-ir cells and the fractional area covered by these cells in POM were lateralized. These tests show that none of these measures were lateralized. All statistical analyses were performed using STATISTICA and differences were considered significant for $p<0.05$. All data are represented here by their mean ± SEM.

Results

Morphological measures and testosterone

The subjects were weighed and their cloacal protuberance was measured two months prior to the experiment (February) as a baseline and these measures were compared with those obtained on day of brain collection (May) in a repeated-measures ANOVA. There was no effect of treatment on body mass (Fig. 1A, $F_{1,8} = 1.12$, $p = 0.322$), however over

time both groups showed a slight decrease in body mass ($F_{1,8} = 8.67$, $p = 0.019$), with no interaction between the factors ($F_{1,8} = 0.36$, $p = 0.564$). The cloacal protuberance area was not affected by either time (although a trend toward an increase was present; Fig. 1B, $F_{1,8} = 3.84$, $p = 0.086$) or treatment ($F_{1,8} = 1.63$, $p = 0.238$), and the factors did not interact ($F_{1,8} = 0.14$, $p = 0.719$). Similarly, the treatment did not affect the mass of the syrinx as measured on the day of brain collection (Fig.1C, $t_8 = 0.25$, $p = 0.811$).

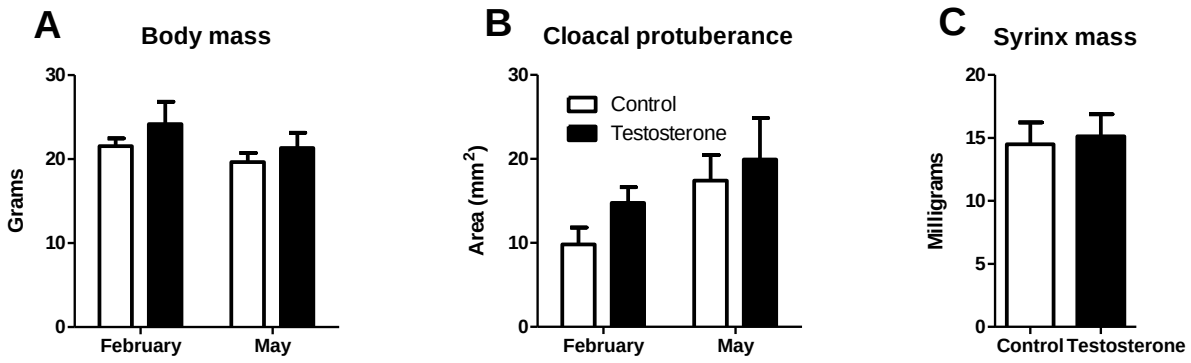

*Figure. 1. Body mass (**A**), area of the cloacal protuberance (length x width, **B**) two months before treatment (February) and at the time of brain collection (May) and mass of the syrinx at time of brain collection (**C**). All white bars represent averages for subjects implanted with empty Silastic™ capsules (n=6), all black bars represent averages of subjects implanted with Silastic™ capsules filled with testosterone (n=4). Brain collection occurred two days after transfer to long-day photoperiod and implantation of Silastic™ capsules. All data are represented here by their mean ± SEM.*

The two-way ANOVA of plasma testosterone concentration revealed a main effect of time ($F_{1,8} = 25.70$, $p < 0.001$), treatment ($F_{1,8} = 28.64$, $p < 0.001$) and an interaction of these factors ($F_{1,8} = 27.65$, $p < 0.001$). A post-hoc test showed that while there was no difference between the control and testosterone-treated birds at baseline, the testosterone concentrations were significantly elevated in the treated group following treatment onset (Fig. 2).

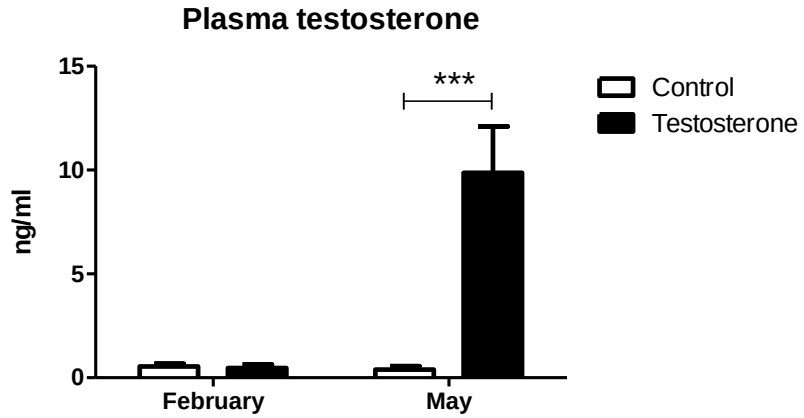

*Figure 2. Circulating testosterone concentration in control and testosterone-treated subjects as measured two months before treatment (February) and on the day of brain collection (May). ***= p<0.001 for testosterone-treated compared to control birds on the same day. All data are represented here by their mean ± SEM.*

HVC volumes

The volume of HVC was not different between the control and testosterone-treated group (Fig. 3) as confirmed by a t-test ($t_8 = 1.38$, $p = 0.205$).

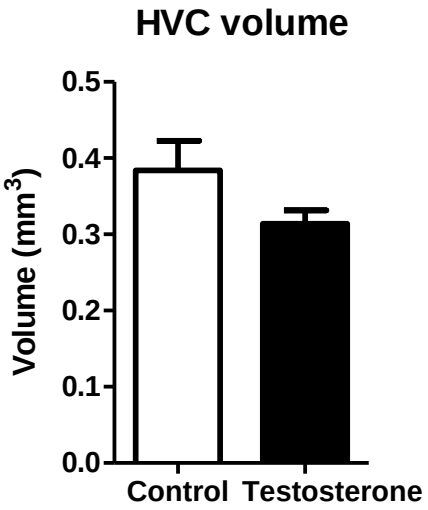

Figure 3. HVC volume in control and testosterone-treated subjects measured 2 days after the transfer to long days and implantation of Silastic™ capsules filled with testosterone or left empty. All data are represented here by their mean ± SEM.

POM volume and ARO-ir properties

The volume of POM was significantly larger in the testosterone-treated group than in the control group (Fig.4 first two bars, $t_8 = 5.13$, $p < 0.001$). A one-way ANOVA was used to test for differences between the two groups of the current study and subjects from the same batch that were singing with a high rate during baseline recordings (N=15, see Chapter 6). Some of these latter subjects had been treated with drugs that inhibit aromatase and block androgen receptors, but this treatment did not affect the volume of their POM, therefore they were pooled for the current analysis. The one-way ANOVA showed a main effect of group ($F_{2,22} = 13.61$, $p < 0.001$). A post-hoc test showed that while the control group was significantly different from both testosterone-treated group and the high-singers with no testosterone, there was no significant difference between the testosterone-treated group and the high-singers with no testosterone (Fig. 4).

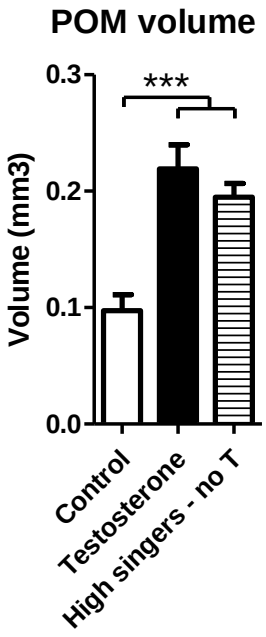

*Figure 4. POM volume measured in subjects from the current study (control and testosterone-treated subjects measured 2 days after the transfer to long days and implantation of Silastic™ capsules filled with testosterone or left empty) and from a separate study of castrated male canaries which had low concentrations of testosterone but were singing at high rates. ***= p<0.001. All data are represented here by their mean ± SEM.*

A previous study also compared the POM volume of female canaries treated for two days with testosterone at an equivalent dose to the current study and controls with no testosterone (see Chapter 3). A two-way ANOVA comparing the males in the current study with the aforementioned female canaries shows a main effect of hormonal treatment ($F_{1,20} = 54.92$, $p < 0.001$), a main effect of sex ($F_{1,20} = 23.37$, $p < 0.001$) and an interaction between these factors ($F_{1,20} = 10.19$, $p = 0.005$). A post-hoc analysis shows that testosterone increases POM volume both in males ($p < 0.001$) and females ($p = 0.018$), however the effect is much stronger in males (Fig. 5A). The post-hoc test also indicates that there is no significant difference between control males and females ($p = 0.636$), but there is a significant difference between testosterone-treated males and females ($p < 0.001$). Comparing the same subjects in an equivalent analysis for circulating testosterone concentrations shows that there is a main effect of hormonal treatment ($F_{1,20} = 23.04$, $p < 0.001$), however no main effect of sex ($F_{1,20} = 2.46$, $p = 0.132$) and no interaction effect ($F_{1,20} = 0.17$, $p = 0.688$), indicating that the males and females were equivalent in terms of circulating testosterone concentrations (Fig. 5B).

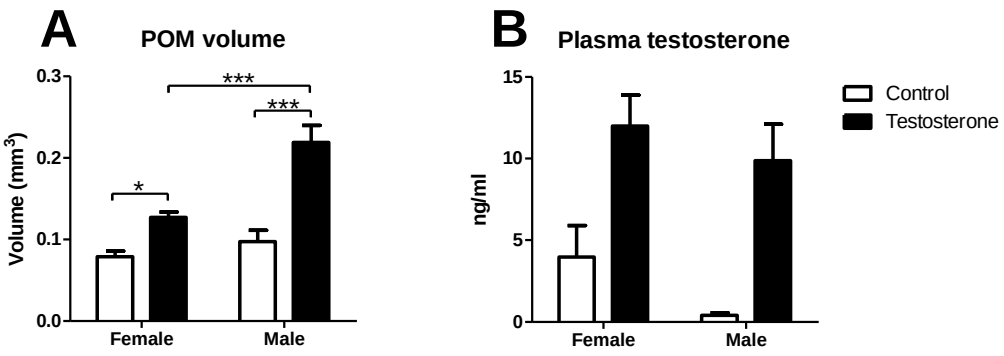

*Figure 5. POM volume (**A**) and plasma testosterone concentration (**B**) in female and male canaries measured 2 days after the transfer to long days and implantation of Silastic™ capsules filled with testosterone or left empty (control). *= p<0.05; ***= p<0.001 for testosterone-treated compared to control birds within the same sex. All data are represented here by their mean ± SEM.*

The testosterone treatment also increased the number of ARO-ir neurons in the rostro-caudally middle section of POM (Fig. 6A, $t_8 = 4.92$, $p = 0.001$), the somal area of

those neurons (Fig. 6B, $t_8 = 6.66$, $p < 0.001$) and the % area covered by ARO-ir material in that section (Fig. 6C, $t_8 = 6.50$, $p < 0.001$).

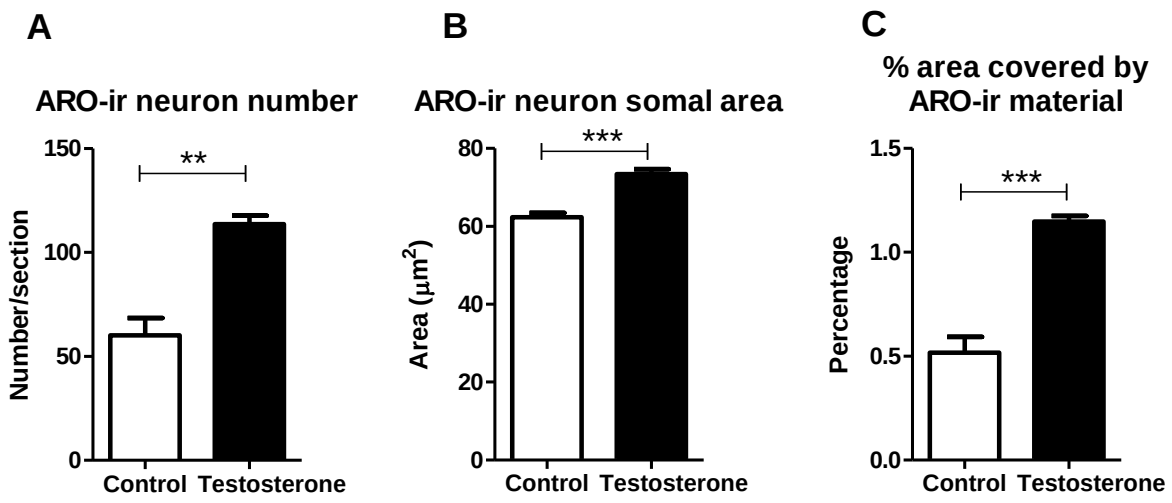

*Figure 6. Number of ARO-ir neurons (**A**), mean somal area of ARO-ir neurons (**B**) and fractional area covered by ARO-ir material (**C**) quantified in a section located in the middle of the POM in the rostro-caudal axis in the brains of control and testosterone-treated subjects collected after 2 days of exposure to long-day photoperiod associated or not with testosterone. **= p<0.01; ***= p<0.001 for testosterone-treated compared to control birds. All data are represented here by their mean ± SEM.*

Singing behaviour

One bird from the control group sang at high rates both before and after treatment. This bird had a POM volume that was somewhat higher than the rest of the control group (data point represented by a diamond in the graph, Fig. 7). Two other birds were not singing during the February recordings, but did start to sing during the May pre-recordings, although at relatively low rates. One of them, which was in the testosterone-treated group, did continue to sing also after treatment at approximately the same rate as before the treatment, the other was in the control group and did not sing after treatment onset. All other birds did not sing either before or after treatment.

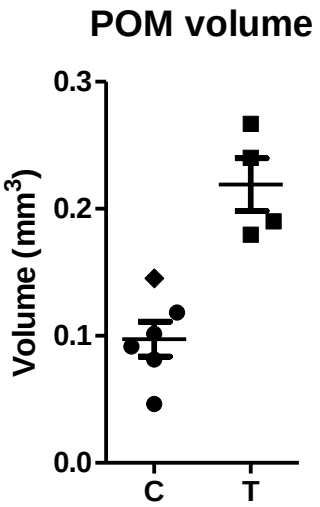

Figure 7. POM volume in low-singer control (circles), high-singer control (diamond) and low-singer testosterone-treated (square) subjects collected after 2 days of exposure to long-day photoperiod associated or not with testosterone. Statistical significance not indicated.

Discussion

Photosensitive male canaries of the Fife fancy strain were treated with testosterone or control implants for two days before harvesting of brains and blood, to investigate whether there is a rapid effect of testosterone-treatment on POM volume, similar to what has been seen in female canaries (Shevchouk et al., 2017b). The testosterone-treatment was effective, as demonstrated by the significantly elevated circulating testosterone concentrations in the treated subjects compared to control subjects. The magnitude of this increase was similar to what was seen in female canaries implanted with the same dose of testosterone for two days. However, unlike what was seen in gonadally intact females, no indication of an increase in testosterone in the castrated male controls of the present experiment following transition from short-day to long-day photoperiod. Neither the area of the cloacal protuberance nor the mass of the syrinx were significantly increased in treated subjects. These two organs increase in size in an androgen-dependent manner across a variety of songbird species (Appeltants et al., 2003; Hall and MacDougall-Shackleton, 2012; Luine et al., 1980; Tramontin et al., 2003, 2000), however it is likely that a duration longer than two days is required for this effect to take place. In

female canaries testosterone increased both cloacal protuberance length and syrinx mass after nine days, but had no effect on the size of these organs after only two days of treatment (Shevchouk et al., 2017b).

The volume of HVC was not increased in testosterone-treated subjects compared to controls. An increase in HVC volume following testosterone treatment has been demonstrated in both male and female canaries but after longer treatment durations (Madison et al., 2015; Nottebohm, 1980a; Rasika et al., 1999; Tramontin et al., 2000; Yamamura et al., 2011). It should however be noted that few studies to date have investigated short-term changes in HVC volume following testosterone treatment in male canaries. The current study suggests that a period longer than two days is necessary for testosterone to increase the volume of HVC. Testosterone-treatment in female canaries increases HVC volume after 21 days, with marginal increases being already detected after 9 days, but no effect at all after 1 or 2 days of treatment. The relatively slow changes seen in HVC following testosterone treatment are compatible with the role of testosterone action in HVC in modulating the quality of song (Meitzen et al., 2007), which takes place over the course of weeks rather than days (Tramontin et al., 2000). We did not measure here the volume of RA and Area X, the other two major song control nuclei, because it has been repeatedly shown that these nuclei grow with a slower time course than HVC (Shevchouk et al., 2017b; Smith et al., 1997; Tramontin et al., 2000).

In contrast, the volume of the POM was significantly larger in the testosterone-treated group than in control birds after only two days. Although in this study POM volume was evaluated based on the ARO-ir cell cluster, previous studies have shown covariation in POM volume as measured by aromatase immunohistochemistry and a Nissl stain (Balthazart et al., 1996b, 1992b; Charlier et al., 2008; Foidart et al., 1994). An increase of POM volume within two days of exposure to testosterone is compatible with the role of this nucleus in the control of singing motivation as song activation in castrated males occurs three to five days following the introduction of a testosterone implant (Alward et al., 2013; Madison et al., 2015; Meitzen et al., 2009).

Comparing the male canaries in current study with female canaries treated with testosterone from a previous study (Shevchouk et al., 2017b) indicates that although

testosterone-treated females had a significantly larger POM volume than controls, this volume remained about twice smaller than in testosterone-treated males. This sex difference was maintained despite the fact that the Silastic™ implants induce similar concentrations of circulating testosterone in males and females. In quail the volume of the POM is larger in sexually mature males than in sexually mature females, it is increased by testosterone in gonadectomized birds but there is no sex difference in its volume when males and females are exposed to the same concentration of testosterone (Panzica et al., 2001, 1996, 1987). Although the sex difference observed here should be confirmed in a future study that would include both testosterone-treated males and females implanted within the same experiment, our results suggest that in canaries there is possibly a sex difference in POM volume that is not eliminated by equalizing the circulating testosterone concentrations.

Although POM volume was clearly increased by testosterone in the present study, a comparison of the POM volumes in subjects of the current study (singing at low rates and treated with T) with subjects from the same batch that were singing at high rates, but had very low levels of circulating testosterone (see Chapter 6), shows that the POM volume of high-singers was equivalent to the POM of the present subjects. This comparison suggests that high concentrations of testosterone are not necessary for the growth of this nucleus, although the causality of the correlation between high rates of singing and large POM volumes is not clear. This observation provides extra support for the involvement of POM in song motivation.

The effects of testosterone on singing behaviour are partially mediated via its estrogenic metabolites (Fusani et al., 2003; Fusani and Gahr, 2006; Harding, 2004; Harding et al., 1988, 1983) and inhibiting aromatase decreases song rate in a rapid manner in canaries (Alward et al., 2016b). Therefore, it was relevant to examine here the changes in the expression of aromatase in POM. Not only was the overall volume of POM as defined by the ARO-ir cell cluster larger in testosterone-treated subjects, but the POM in these subjects also contained a higher number of ARO-ir neurons and a bigger proportion of it was covered by aromatase-stained material. This increase in aromatase expression could be what underlies the increase in POM volume, although other cellular

changes are also probably involved. The average ARO-ir somal area was also significantly larger in testosterone-treated subjects than in the control group. Although it is not possible to compare directly between two separate studies, it can be noted that the area of ARO-ir neurons increased more slowly in female canaries treated with testosterone than in the males used here, with the first significant difference being observed only on day 9 after the initiation of the testosterone treatment and no difference on day 2, contrary to what was seen in males in the current study. This comparison suggests that this parameter changes more rapidly in male than female canaries, although this would need to be confirmed in a study including both sexes in a single experiment.

Alward and colleagues (Alward et al., 2013) showed that implanting testosterone stereotaxically in POM increases within a week the song rate in castrated male canaries to the same level as in subjects peripherally implanted with testosterone. The peripherally-implanted birds were however already singing at a higher rate than castrated control subjects by day 3 following surgery whereas birds with T in POM were at that time still behaviorally inactive and their singing rate reached the level seen in birds treated systemically with testosterone only by day 7. Thus, there was a four-day delay in the activation of singing behavior by testosterone depending on whether it was applied to POM only or to the entire brain as well as to peripheral organs such as the syrinx in systemically treated subjects. The current study demonstrates the presence gross morphological changes in the POM after two days of testosterone-treatment as well as an increase in the number and size of aromatase-expressing neurons in the POM. The reasons for the four-day delay are unclear, but could indicate the involvement of downstream brain regions or suggest that other molecular changes besides the increase of aromatase-expression within POM are necessary for increasing singing motivation. Further studies will be necessary to answer this question, including investigations of the activation of POM neurons that possess anterograde projections to areas potentially mediating song activation and the identification of estrogen-responsive genes that are up-regulated in POM following the increase of aromatase expression in this nucleus.

Chapter 5. Is the action of testosterone implanted in the medial preoptic nucleus on HVC growth activity-dependent?

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Key words: song control system; HVC, POM; testosterone; singing motivation; songbirds

Abstract

Testosterone (T) acts in the songbird brain to regulate the quality of singing via song control nuclei (SCN) and simultaneously controls the motivation to sing via the medial preoptic nucleus (POM). T stereotaxically implanted in the POM increases song rates to the same level as systemic T, but also increases the volume of SCN, a form of neuroplasticity that accompanies seasonal changes in song. Singing activity has a positive feedback on neurogenesis in HVC, therefore the increases of SCN volumes produced by T implanted in POM could be an indirect consequence of the increased rates of singing. Alternatively, the increase could be due to trophic factors that reach SCN from POM via poly-synaptic connections. The current experiment aimed to discriminate between these two mechanisms by comparing the HVC volume of birds with T implanted in POM for 9 days, which is the start of song activation, and the HVC volume of birds that had T in POM for 25 days, 16 days after song activation. A difference between these two conditions would suggest that singing feedback is driving HVC plasticity, while no difference would indicate that poly-synaptic neural connections are primarily driving the HVC volume growth. Circulating T concentrations were elevated in peripherally implanted subjects and low in no T subjects and all subjects with central T implants. However, imprecision of stereotaxic coordinates prevented from having a sufficient number of

subjects per group to run valid statistical tests to test for differences in HVC volume and make a conclusion about the hypothesis proposed.

Introduction

Three families of avian species share with humans the unique ability to communicate via learned vocalizations – oscines (songbirds), hummingbirds and parrots. The study of singing in oscine species has been particularly informative due to the diversity of species in this clade. Songbirds possess a network of nuclei – the song control system - specialized in processing information pertaining to the learning and production of song (Nottebohm, 1980b). HVC and RA are involved in controlling the production of song, the former is also part of the learning and maintenance, along with the nuclei Area X, LMAN and DLM (recently reviewed in Ziegler and Marler, 2008). A particular interest in studying some songbird species is the marked neuroplasticity that is seen in the song control system in adulthood across seasons. Seasonal songbird species, such as canaries, respond to increasing daylengths with an activation of the hypothalamic-pituitary system leading to gonadal growth and ultimately to an increase in the concentration of circulating sex hormones (Wingfield et al., 1980). These changes prepare the songbird for reproduction, including motivational aspects of reproduction such as courtship behavior. Implanting exogenous testosterone in male or female songbirds increases frequency and duration of singing in a number of species (Arnold, 1975a; Madison et al., 2015; Nottebohm, 1980a; Pröve, 1974).

In parallel to changes in singing behavior, the song control nuclei in seasonal songbird species increase in size 1.3-3 times between autumn and spring (Tramontin and Brenowitz, 2000). Subcutaneous testosterone implants increase the volume of song control nuclei in castrated males (Madison et al., 2015; Nottebohm, 1980a). Given that in spring testosterone, singing rate, song quality and song control nuclei volumes all increase, it was long assumed that testosterone acts directly on the song control system to regulate song motivation and song structure via the morphological changes that take place in these nuclei; however some more recent studies have challenged this view. For example, in Corsican blue tits the recrudescence of the song control system has been

shown to occur earlier in the season than the rise in testosterone and in song rates (Caro et al., 2005), suggesting that testosterone is not necessary for growth of the song control nuclei. Furthermore, song control system growth can occur in the complete absence of sex steroid action: in photostimulated white-throated sparrows, castration and treatment with an aromatase inhibitor, ATD, and an androgen receptor blocker, flutamide does not decrease the volume of HVC below the level of photostimulated untreated controls (Robertson et al., 2014).

These two studies show that testosterone action is not always required for song control system plasticity, yet both androgen and estrogen receptors are expressed in the song control system (Bernard et al., 1999). What is then the role of sex hormone signaling in the song control nuclei in singing behavior? In white-crowned sparrows blocking androgen and estrogen receptors in HVC by infusing the anti-androgen flutamide and the ER antagonist faslodex increases the coefficient of variation of multiple song attributes, suggesting these receptors are involved in increasing the stereotypy of song (Meitzen et al., 2007). This pharmacological intervention had however no effect on the rate of singing, suggesting that testosterone action in HVC is involved in regulating the qualitative but not quantitative aspects of singing behavior. In photostimulated male canaries, a blockade of androgen receptors in HVC or RA decreased the variability of syllable usage and sequencing and syllable and trill acoustic variability, respectively (Alward et al., 2016a). Neither manipulation affected the song rate, again suggesting that sex hormone action in the song control system regulates the quality of singing but not the quantity.

The time spent singing can be considered to reflect the motivation of the bird to sing. Being a courtship behavior, frequency of singing is therefore indicative of the bird's sexual motivation (Ball et al., 2002). It may therefore be useful to consider which brain regions are known to regulate sexual motivation in general. Testosterone action in the medial preoptic nucleus (POM) is very important in controlling sexually motivated behaviors, such as copulation (Balthazart and Surlemont, 1990). Lesions in the POM in European starlings decrease singing rate during the breeding, but not during the non-breeding season, consistent with a role of this nucleus in sexually-motivated song (Alger and Riters, 2006; Riters and Ball, 1999). Male canaries implanted stereotaxically with

testosterone in POM sing after one week at rates equivalent to birds implanted with testosterone systemically (Alward et al., 2016c; Alward et al., 2013) and both sing more than controls which have testosterone implanted outside POM or an empty cannula inside POM.

Is this effect indirectly mediated via the song control system? POM does not have any mono-synaptic projection to the song control nuclei, however there are at least four important di-synaptic connections to the song control system via the dorsomedial nucleus intercollicularis (DM), mesencephalic central gray (Gct), area ventralis of Tsai (AVT) and locus ceruleus (LoC) (Riters and Alger, 2004). In fact, HVC, RA and Area X volumes in birds stereotaxically implanted with testosterone in POM are larger than in controls with no testosterone in POM and not different from birds with systemic testosterone implants (Alward et al., 2016c; Alward et al., 2013). It is not yet clear how testosterone implanted in the POM can affect the growth of song control nuclei. One hypothesis is that POM stimulates growth of song control nuclei by inducing higher rates of singing activity, which in turn has a positive feedback effect on song control system plasticity. HVC volumes and singing rates are correlated in canaries (Nottebohm, 1981; Nottebohm et al., 1987). Furthermore, male canaries that naturally sing at lower rates incorporate fewer newborn neurons into HVC than actively singing canaries (Li et al., 2000) and HVC neurogenesis is a major contributor to HVC growth. Another study showed that there is a strong correlation between song rate and number of newborn neurons in the HVC in castrated but not in T-implanted males canaries (Alvarez-Borda and Nottebohm, 2002). The growth of song control nuclei after implantation of T in the POM could thus be activity-dependent. A second hypothesis proposes that the growth of song control nuclei in birds implanted with T in POM could be independent of singing activity, but mediated by trophic signaling originating from POM and reaching the song control system via an intermediate locus such as DM, Gct, AVT or LoC (Riters and Alger, 2004). In order to discriminate between these two hypotheses, photosensitive male canaries were transferred to a long photoperiod and implanted with testosterone in POM. Their brains were then collected and HVC volume was measured either after 9 days, when singing activity is just beginning to be established) or after 25 days when birds have been able to sing for an extensive period of time.

Materials and Methods

Experimental animals

Twenty 3-year old male canaries of the Fife fancy breed raised in the breeding colony maintained at the University of Antwerp, Belgium were used for this study. All subjects had been part of previous experiments but these involved only behavioral observation. Some subjects had bred and raised chicks previously, others had not and this was balanced across groups for the experiment.

All males were bilaterally castrated under isoflurane anesthesia, with one week of recovery between the removal of each testis, following the procedure described in Sartor et al. (2005). After both testes had been removed, males were allowed to recover for two months to ensure that physiological and behavioral effects of T would disappear. 7 days prior to being implanted with testosterone, subjects were switched from group housing on short-day photoperiod (8L:16D) to individual housing on long-day photoperiod (14L:10D). This involved moving the subjects to another room where they were exposed to four actively singing male canaries from another experiment. All subjects were in visual but not acoustic isolation from all other birds in the same room. The experimental subjects were split into four replicates with a schedule staggered by one day. Each replicate contained as far as possible similar numbers of birds from each group.

All subjects received a subcutaneous Silastic™ implant (Degania Silicone; internal diameter 0.76 mm, external diameter 1.65 mm, length 10 mm) which had either been filled with crystalline testosterone (Fluka Analytical, Sigma-Aldrich) or had been left empty depending on the experimental group (see experimental design section for explanation of groups). All Silastic™ implants had been pre-incubated in 0.9% saline at 37°C overnight and were inserted intraperitoneally via a small incision posterior to the last rib.

All experimental procedures complied with Belgian laws concerning the Protection and Welfare of Animals and the Protection of Experimental Animals, and experimental protocols were approved by the Ethics Committee for the Use of Animals at the University of Liege. The subjects had been exposed to natural daylight prior to their arrival in our lab at the University of Liege in early April. Upon arrival they were housed on short-day photoperiod (8L:16D) for 3 months to make sure they would be photosensitive. They were

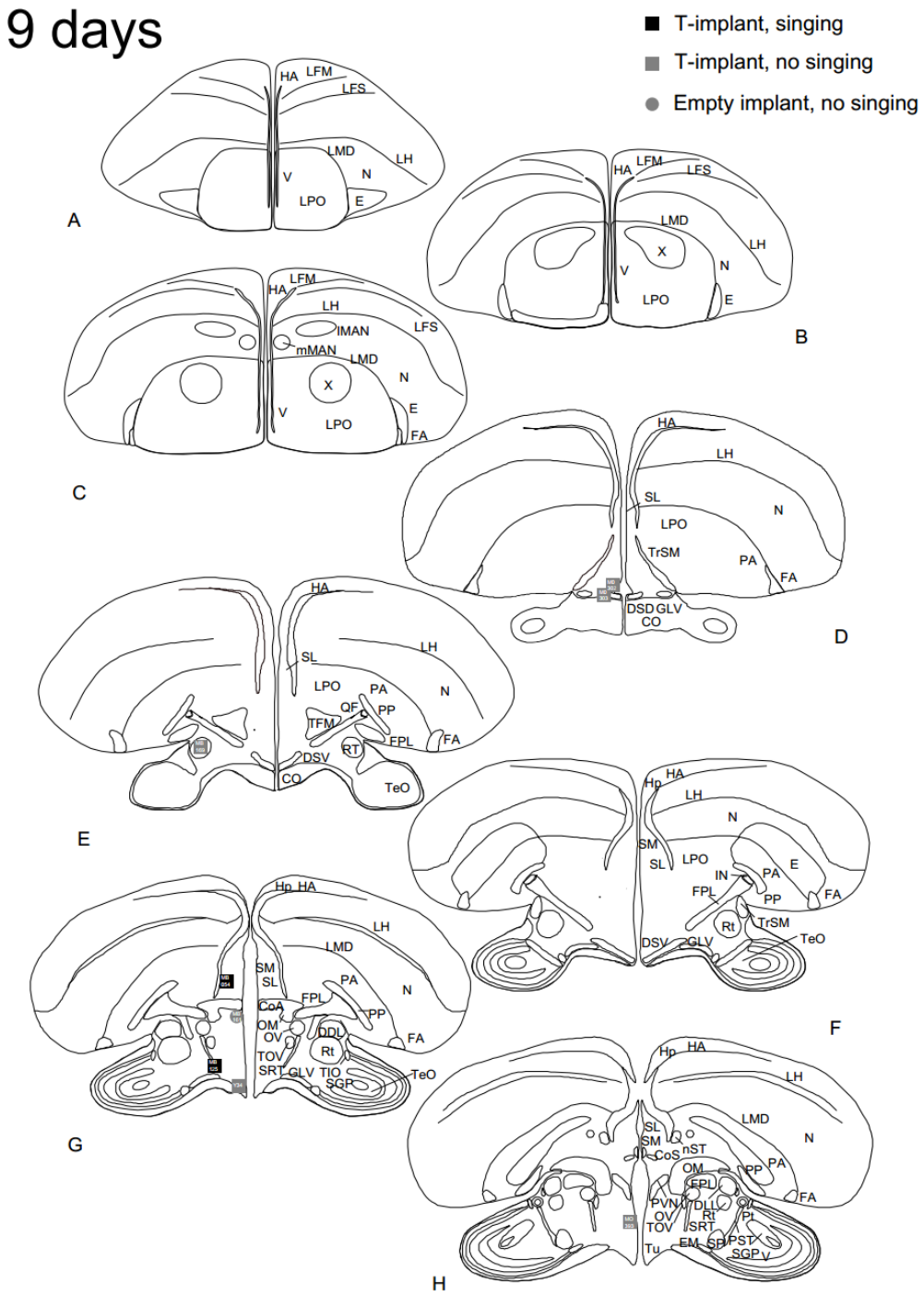
housed in single-sex groups of 5-6 and continuously had seeds, water, sand and cuttlebone available *ad libitum*. After 4 weeks of short-day photoperiod an evaluation of the molting status was completed giving a score of 1-4 to each bird, with 1 signifying that they did not start molting at all and 4 that most of the new feathers were fully regrown. The majority of the subjects had almost completed molt with a median molt score of 3.5 and a mean of 3.2 (SEM=0.2).

Experimental design

In order discriminate between the two hypotheses concerning how testosterone in POM can induce growth of the song control system - via trophic signaling between POM and SCS or via singing-activity feedback, we formed 4 groups of castrated subjects whose brains were collected at one of two time points, 9 or 25 days after the initiation of the treatments. The first group of subjects was treated with testosterone peripherally (PER-T) via a subcutaneous Silastic™ implant filed with T and received an empty implant in the POM, this group was a positive control group which would show the maximum HVC growth, all subjects in this group were sacrificed at the second time point. The second group (no T) was a negative control group where the subjects received an empty implant both peripherally and in the POM, some subjects in this group were sacrificed at each time point. The third and fourth groups received an implant filled with testosterone aimed at the POM and an empty implant peripherally. Brains of subjects in group 3 were collected on day 9 of treatment (POM-T 9 days), this was the day that followed the first instance of song observed from one member of this group, while brains in group 4 (POM-T 25 days) were collected 16 days after onset of singing to allow time for singing activity feedback to influence the plasticity of the song control system. Any difference in HVC volumes between the POM-T 9 days and POM-T 25 days groups would indicate the contribution of singing-activity feedback on HVC volumes. On the other hand, a lack of difference between these two groups, as well as a difference between POM-T 9 days and the no T group would indicate a contribution of trophic inputs from POM to HVC.

Several birds from the POM-T 9 days and POM-T 25 days groups were found to have a testosterone-filled implant either touching a ventricle or in a different region of the brain. In the former case they were assigned to two additional groups called ventricle-T

(VEN-T) 9 days and VEN-T 25 days. In the latter case they were assigned to the no T group. The VEN-T groups were considered separately because in these cases there was a substantial possibility that the testosterone was leaking into the ventricle and reaching the entire brain via the cerebrospinal fluid.



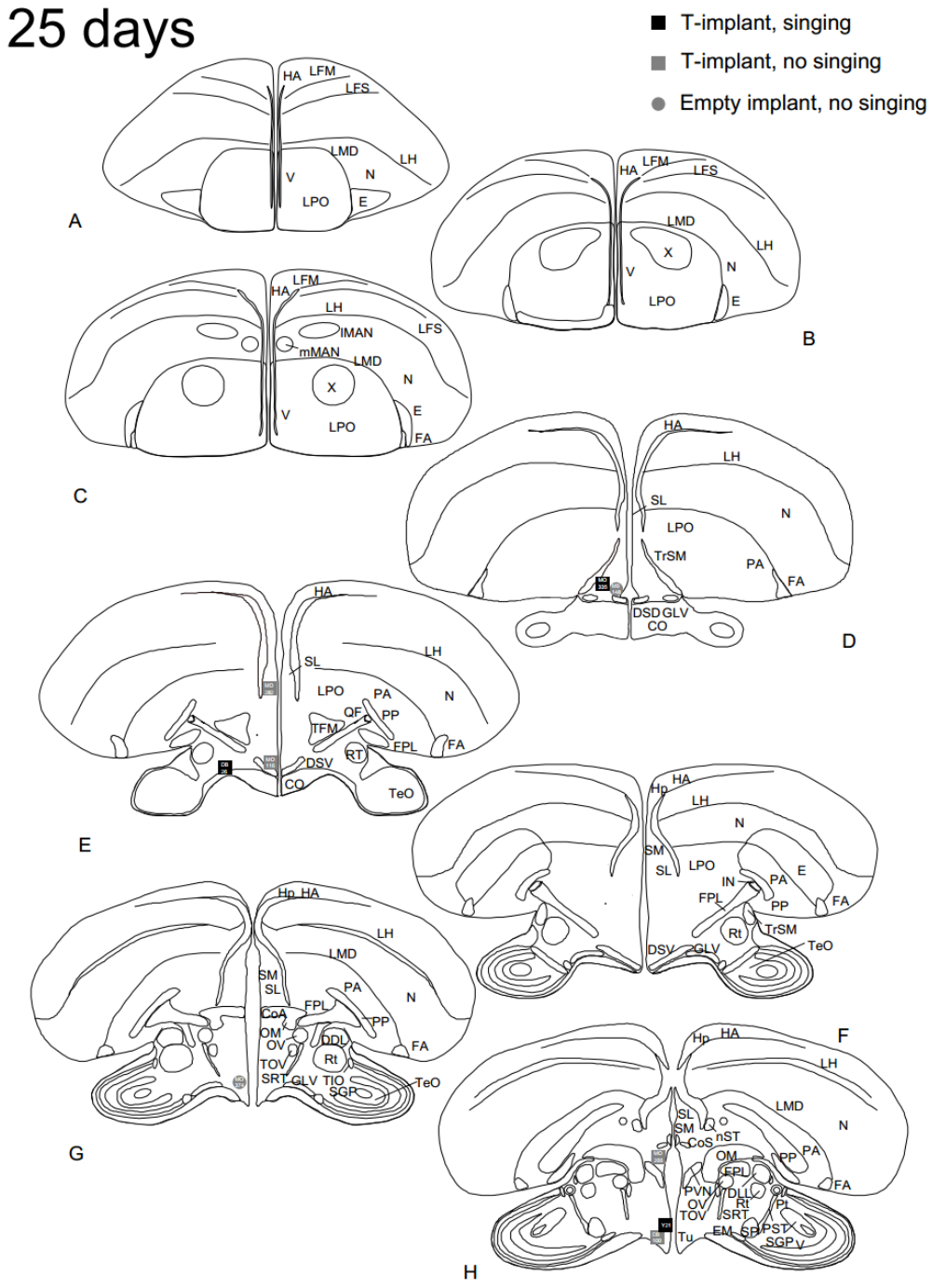


Figure 1. Schematic representation of location of brain implants in male canaries in two groups, with brain collection 9 days (upper panel, previous page) or 25 days (lower panel, this page) after surgery.

The brain implants were prepared, filled with crystalline testosterone and implanted into the POM following the procedure described in Alward et al (2013). Briefly, implants

were prepared using blunted 27 gauge needles filled over a length of 1 mm with crystalline T or left empty as a control. Under isofluorane anesthesia subjects were fixed in a stereotaxic apparatus with ear bars and a beak holder holding the head in a standardized position. The following stereotaxic coordinates were used to target the POM: dorsoventral: −6.5 mm from the dorsal surface of the brain; anterior–posterior: 2.2 mm from the rostral tip of the cerebellum; and medial–lateral: ±0.15 mm from midline. The skull immediately over this coordinate was removed with a micro-drill, the implant was lowered to the target coordinate and dental cement was applied around the implant, and the skin was sutured. The bird was placed under a heat lamp to recover until perching and then placed back in its home cage. The following day all subjects were injected intraperitoneally 5 times with a 50 mg/kg dose of bromodeoxyuridine (100 µl of 10 mg/ml BrdU dissolved in 0.9% saline with 28 mg/L NaOH) with two hours in between each injection. Immediately following the first injection, birds were placed in a new individual cage in a room where only subjects from the current study were present. The birds were monitored for song rate (see following section for details) during 9 or 25 days, after which they were sacrificed by transcardial perfusion.

Song rate observation

All song quantifications were carried out by direct observation, with the experimenter hidden from the birds' view, and all subjects were monitored simultaneously. It was possible to observe all birds simultaneously because overall the song rate of most subjects was very low. The number of songs performed per session was recorded. A song was defined as a sequence of syllables performed during a minimum of one second and with a minimum 0.5 seconds of silence preceding and following it. During the 7 days of long-day photoperiod preceding the implant surgery all birds were observed for singing during 10-15 minutes per day, only 1 bird (consequently allocated to the PER-T group) was observed to sing in this period. One day after the BrdU injections and continuing for the rest of the experiment, the observation period increased to 60 minutes per day.

Perfusion

On day 9 or 25 after the initiation of treatments, the subjects were caught and, within 3 minutes, a blood sample was collected from the brachial vein into microcapillaries and

stored on crushed ice until centrifugation. 1-2 hours later, the blood samples were centrifuged at 9000 g for 9 minutes, the supernatant plasma was collected and stored at -80°C until assaying. After blood sampling, the bird was weighed, the length of its cloacal protrusion was measured and the subject was deeply anaesthetized with 0.03 ml Nembutal (60mg/ml, Ceva). The subjects were perfused transcardially with 0.01 M phosphate-buffered saline (PBS, 1.43 g/L Na_2HPO_4, 0.48 g/L KH_2PO_4, 7.2 g/L NaCl) followed by 4% paraformaldehyde (PFA, 4.3 g/L NaOH, 40 g/L paraformaldehyde, 18.8 g/L $NaH_2PO_4.H_20$) to fix the brain. The brain was extracted from the skull and placed in a vial of PFA for overnight post-fixation. The syrinx was extracted and weighed, the absence of testicular material and presence of Silastic™ implants were verified. The following day brains were transferred to 30% sucrose. Upon sinking, the brains were frozen on dry ice and stored at -80°C until further use.

Testosterone Enzyme Immunoassay (EIA)

10 µl of plasma was diluted in 150 µl of deionized water (MilliQ) in glass test tubes, samples were kept at +4°C for 30 minutes, and 2 ml dichloromethane (a non-polar organic solvent) was added. Samples were vortexed and left immobile for 1-2 hours for separation. The organic phase was then moved to a new test tube and dried under nitrogen gas at 40°C. The dichloromethane extraction was repeated a second time and pooled extracts were kept at -20°C until the EIA assay. The average recovery rate was 80%. Testosterone concentrations were measured using an EIA kit (Cayman Chemicals) following the manufacturer's instructions. On the day of the assay, samples were re-suspended in 400 µl EIA buffer from the kit with 10% ethanol to improve recovery and placed on a shaker set to 1350 rpm for 1 hour. The assay was performed immediately after following the instructions provided with the kit, on a single plate. These assays have been previously validated for measuring testosterone in avian plasma (de Bournonville et al., 2016; Dickens et al., 2011). The minimum and maximum detection limit of the EIA, as determined by the lowest and highest concentration detected, were 5.20 pg/ml and 198.69 pg/ml respectively. The average intra-assay coefficient of variation was 19.3%.

Nissl staining and brain analyses

Brains were cut coronally into 4 series of 30 µm thick sections on a Leica cryostat. One series was collected on superfrost slides for performing Nissl staining, the other three were stored in anti-freeze at -20°C. The procedure for Nissl staining was the following: brain sections were pre-hydrated in Walpole buffer then stained in Toluidine blue for 90 seconds, washed twice in Walpole buffer for 15 minutes each, differentiated in a molybdate solution for 2.5 minutes, washed for 1 minute in deionized water and dehydrated for one minute each in 20%, 70%, 90% isopropanol, 2 minutes in 99% ethanol and 1 minute in xylene. The slides were coverslipped immediately using Eukitt as a mounting medium.

Nissl stained sections were examined for the location of needle track left by the implant under an Olympus (BH-2) light microscope. The implant was considered to contact the POM if the end of the needle track was within 300 µm of the borders of POM. HVC was visualized on the same microscope, in the same series of Nissl stained sections. Photomicrographs of every HVC section were taken at 4x magnification in left and right hemispheres. An outline was drawn around the perimeter of each cross-section of the nucleus using ImageJ v1.47v (National Institutes of Health) and the delimited area was measured by the program. The volume of the nucleus was calculated by summing the areas and multiplying by 120 µm, the distance between two successive sections in the series. The volume of the nuclei in each hemisphere was calculated separately and the average of the two measures was used for statistical analyses.

Statistics

Subjects from the T-POM 9 days and T-POM 25 days groups in whom the brain implant did not contact POM were not different from subjects in the no-T group in any measure. Therefore, these subjects were all pooled into one no-T group (n = 4). Two subjects (one from no-T group, one from T-POM 25 days group) were excluded from all analyses due to high circulating testosterone concentration which presumably indicated that their castration was not complete or more probably a piece of testis had regrown in the abdominal cavity and was not detected at autopsy. Further, one subject was removed from the analysis of syrinx mass due to damage of the syrinx during extraction. Molt

status, body mass, cloacal protrusion length, syrinx mass, plasma testosterone concentration, song rate and HVC volumes were compared across the six groups (PER-T, no-T, POM-T 9 days, POM-T 25 days, VEN-T 9 days and VEN-T 25 days) by one-way ANOVA using STATISTICA (Version 13). Differences were considered significant for $p<0.05$ and all data are represented here by their mean ± SEM.

Results

Physiological condition

An evaluation of molting was performed prior to the experiment to ensure that all subjects were photoregressed. A one-way ANOVA of the molt scores showed that the subjects assigned to different groups did not differ in their progression through molting (Fig. 2A, $F_{5,12} = 2.85$, $p = 0.539$). Body mass was measured on the day of brain collection to evaluate whether the treatment had a non-specific effect on the general condition of the subjects. No difference between groups was found in body mass (Fig. 2B, $F_{5,12} = 2.85$, $p = 0.064$).

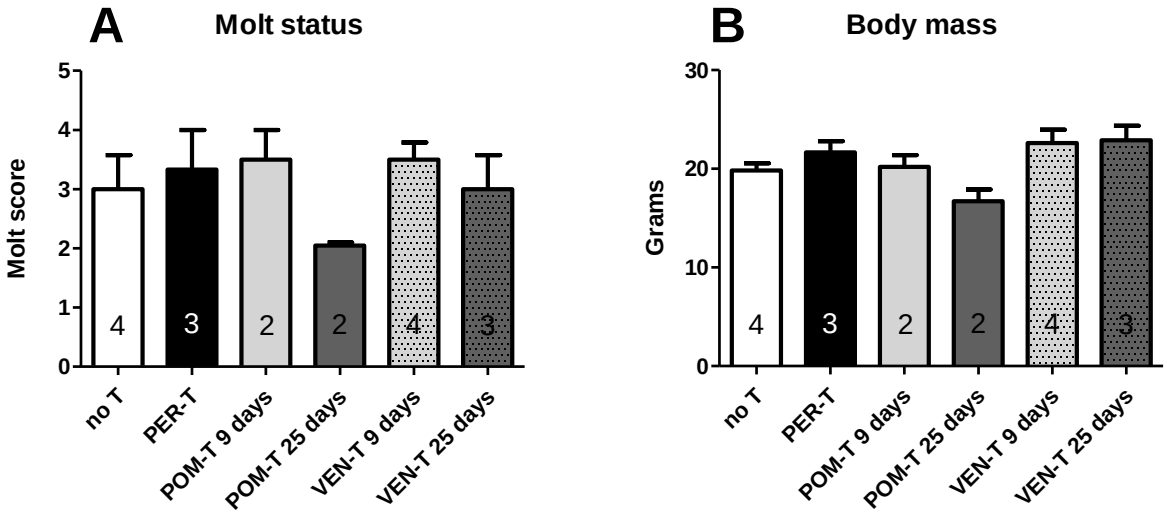


*Figure 2. The extent of molting (score from 0 to 4) of the experimental subjects 2 months before the treatment (**A**) and body mass on the day of brain collection (**B**) in castrated male canaries treated for 9 or 25 days with empty implants (no T), a peripheral testosterone implant (PER-T), a testosterone implant in POM (POM-T) or a testosterone implant touching a ventricle (VEN-T).*

Testosterone and androgen-sensitive measures

To ensure that castration was complete and testosterone was not leaking from the implants in the groups with no peripheral testosterone implant, circulating testosterone and two androgen-sensitive measures were compared between groups. As expected, testosterone concentration was elevated in the PER-T group (Fig. 3A). This was confirmed by a one-way ANOVA ($F_{5,12}$ = 4.75, p = 0.013), followed by a post-hoc test. PER-T subjects had significantly higher concentrations of testosterone than the no-T group, VEN-T 9 days group and VEN-T 25 days group. There was a trend for PER-T to have higher testosterone concentration than the POM-T 9 days and POM-T 25 days groups (p = 0.063 and 0.061, respectively). Surprisingly, there was no corresponding increase in cloacal protuberance length ($F_{5,12}$ = 1.87, p = 0.174) and syrinx mass ($F_{5,11}$ = 1.05, p = 0.435) even if the largest values of cloacal protuberance were observed in the PER-T group.

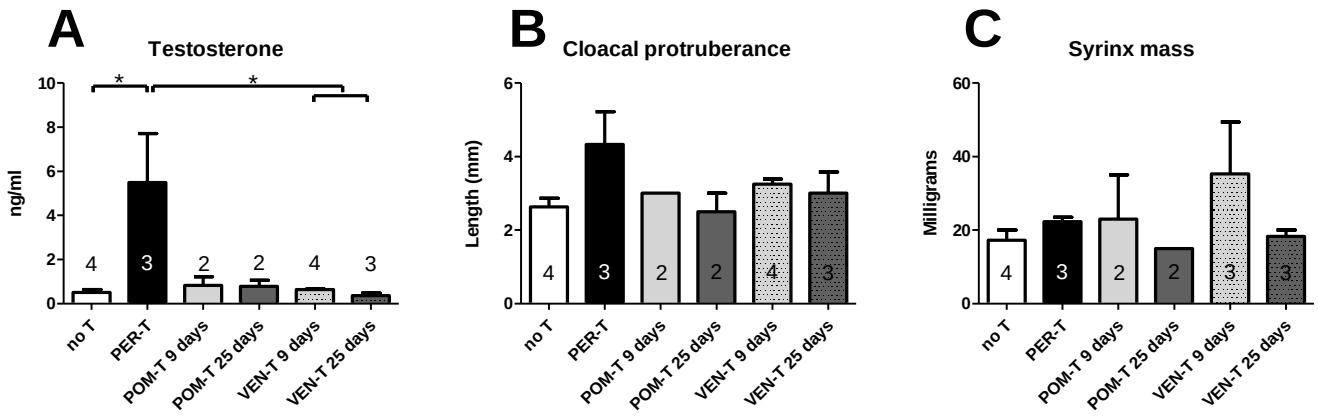


*Figure 3. Testosterone concentration (**A**), cloacal protuberance length (**B**) and syrinx mass (**C**), measured on the day of brain collection in castrated male canaries treated for 9 or 25 days with empty implants (no T), a peripheral testosterone implant (PER-T), a testosterone implant in POM (POM-T) or a testosterone implant touching a ventricle (VEN-T). *= p<0.05.*

Song rates

The total number of songs observed during the experiment was compared across groups as a measure of singing motivation. Although higher average numbers of songs were produced in the PER-T, POM-T 25 days and VEN-T 25 days groups compared to the other three groups, the ANOVA failed to reveal significant group differences, presumably

due to high variations with the three groups singing at high rate combined with the low numbers of subjects (Fig. 4, $F_{5,12} = 1.63$, $p = 0.227$).

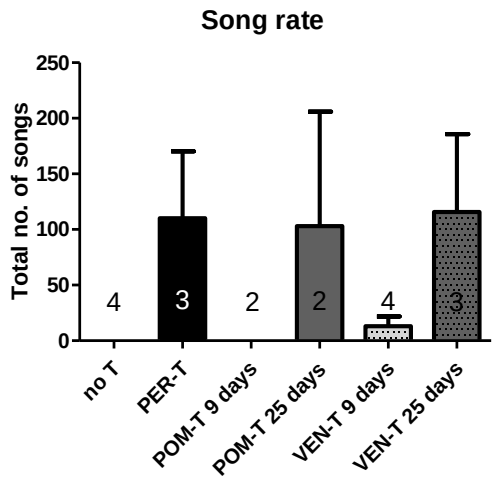


Figure 4. Total number of songs observed in castrated male canaries treated for 9 or 25 days with empty implants (no T), a peripheral testosterone implant (PER-T), a testosterone implant in POM (POM-T) or a testosterone implant touching a ventricle (VEN-T).

HVC volumes

The volume of HVC was not significantly different between groups (Fig. 5, $F_{5,12} = 0.49$, $p = 0.777$).

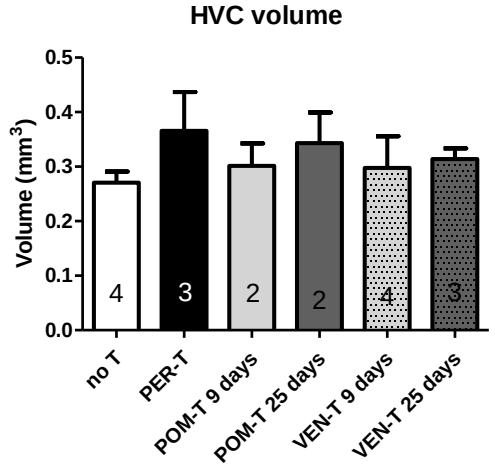


Figure 4. HVC volume observed in castrated male canaries treated for 9 or 25 days with empty implants (no T), a peripheral testosterone implant (PER-T), a testosterone implant in POM (POM-T) or a testosterone implant touching a ventricle (VEN-T).

Discussion

Photosensitive castrated male canaries were implanted with testosterone peripherally, in POM or in the ventricles for 9 or 25 days. Brains were collected at these two time-points that were chosen to ensure that one group had testosterone in the POM for as long as possible but no positive feedback of their own singing behavior, while the other had a substantial period of feedback from their own singing activity. The overall physical condition of the subjects was not different between groups, as confirmed by comparing their molting status prior to the experiment and their body mass at the end of the experiment, both of which did not differ between groups. Circulating testosterone concentration was elevated in the group that had been treated with testosterone systemically compared to the groups that had no testosterone implants and the two groups that had testosterone implants touching the ventricles. Additionally, there was a trend for peripherally implanted birds to have significantly higher circulating testosterone than the two groups with testosterone implants in POM, although this did not reach full significance, presumably due to the small sample size as well as some intra-group variation. There was no significant difference in the circulating testosterone concentration between the groups with no implants and the groups with implants either in the ventricles or in POM. Although it is possible and likely that the implants touching the ventricles released some testosterone into cerebrospinal fluid that could act in other brain regions, the quantity that escaped the brain into the general circulation was apparently not sufficient to be detected as being above the level of castrated control males at the time-points examined. Size of the cloacal protuberance, an androgen-sensitive measure (Appeltants et al., 2003; Tramontin et al., 2003), was somewhat elevated in the peripherally implanted group, however the small sample size and variability prevented the difference from becoming significant. There was no difference in the weight of the syrinx between the groups.

Birds systemically treated with testosterone started singing after 4-5 days of treatment, while the first songs in birds with implants in the ventricle were observed after 8 days of treatment and the only subject with an implant in POM that was observed to sing started to display the behavior after 6 days of treatment. These latencies between initiation of treatment and song activation are quite similar to what was reported by Alward

and colleagues (Alward et al., 2013) for PER-T and POM-T birds. The latency of song activation can vary from one individual to another, but it was surprising that within the VEN-T 25 days and even the PER-T group a few birds never started singing during the experiment. As a consequence of this inter-individual variation, the comparison of song rates did not identify statistically significant differences between groups, although it is clear that birds with no testosterone did not sing and that 9 days of testosterone treatment either in POM or in the ventricle is not sufficient for activating substantial singing behavior. Although illustrated by only a single subject, the data confirm the findings of Alward and colleagues (Alward et al., 2016c; Alward et al., 2013) that testosterone action in POM can be sufficient to induce high rates of singing behavior, providing further support for the role of POM in song motivation.

Alward and colleagues (Alward et al., 2016c; Alward et al., 2013) showed that implanting testosterone in POM not only increases the song rate, but also increases the volume of the song control nuclei HVC, RA and Area X. A moderate positive correlation between the song rate and volumes of these nuclei in individual birds led the authors to conclude that the increase in song control nuclei volume is attributable to the increased singing activity that the birds experience following T-POM implants, due to a positive feedback of singing on BDNF and neurogenesis in HVC (Li et al., 2000). However, an alternative explanation is that testosterone in POM triggers molecular changes that cause a growth of song control nuclei via trophic poly-synaptic inputs via DM, Gct, AVT and LoC (Riters and Alger, 2004). The current study tried to discriminate between the two explanations by collecting brains either at a time when subjects had time to sing only very few songs, or a time when the subjects already sang a substantial amount of songs, capable of causing trophic feedback on neurogenesis in HVC. Unfortunately, the number of subjects with testosterone implants actually located in POM was too low in the current study to make a conclusion about this question.

All the poly-synaptic connections between POM and SCN are almost exclusively ipsilateral (Appeltants et al., 2004, 2000; Ashmore et al., 2007; Riters and Alger, 2004). Therefore, any trans-synaptic effect on SCN growth due to T-action in POM should also be primarily ipsilateral. Alward and colleagues (Alward et al., 2016c; Alward et al., 2013)

do not find any lateralization of the growth of SCN volumes in subjects implanted with T in POM. Similarly, the expression of doublecortin, a marker of newborn neurons, is not lateralized in the HVC of birds with T in POM. Although not conclusive, these studies thus support the hypothesis that SCN volumes increase in response to T in POM is activity-dependent and due to the feedback of the increased singing behavior produced by these birds.

Chapter 6. Steroid-independent singing in castrated male canaries exposed to short days

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Abstract (247 words)

In seasonal songbirds such as canaries, singing behavior is predominantly under the control of testosterone and its metabolites. Increasing daylengths in spring activate singing via both photoperiodic and hormonal mechanisms. In this study, we show that surprisingly a large proportion of castrated male Fife fancy canaries maintained for four months under a short-day photoperiod began to sing at high rates. Singing rate was not correlated with the (low) circulating concentrations of the steroid. These singing castrated male canaries were then systemically treated with a combination of ATD, an aromatase inhibitor, and flutamide, an androgen receptor blocker or empty implants as a control to test whether this singing activity was steroid-dependent. Singing behavior was recorded for 27 days, the birds were then perfused and volumes of HVC and of the medial preoptic nucleus (POM) were measured, and ARO-ir neurons in POM were quantified. The treatment transiently and slightly decreased the number of songs detected per day and possibly increased the number of syllables per song, but did not affect any other measure of song. Multiple testosterone-dependent measures such as the volume of HVC and of the POM, as well as number of, average somal area and fractional area covered by ARO-ir neurons in POM were similarly not affected by the treatment. Several indirect indices suggested however that the ATD+flutamide treatment was effective for the whole duration of the experiment. We tentatively conclude that singing in castrated canaries induced by long-term exposure to short-days is largely sex steroid hormone-independent.

HIGHLIGHTS

- Long-term short-day photoperiod can activate singing in castrated male Fife canaries
- Singing rate in these birds did not correlate with the low circulating testosterone concentration
- Treatment with ATD+flutamide only marginally affected song and did not decrease measures of androgen-dependent structures
- Short-day singing and the underlying neuroplasticity are predominantly sex steroid hormone-independent

Introduction

Sex steroid hormones play a critical role in both the development and in seasonal changes of singing behavior in songbird species such as canaries. They exert these effects mostly by binding to androgen and estrogen receptors that are expressed within the network of brain nuclei dedicated to the control of singing behaviour (Bernard et al., 1999; Metzdorf et al., 1999), such as in the nucleus HVC (formerly high vocal center, now used as a proper name), as well as in sites involved in activating singing motivation, such as the medial preoptic nucleus (Bernard et al., 1999). In seasonal songbirds, the increasing day length initiates the growth of the testes resulting in an augmented testosterone production and other physiological changes, preparing the bird for breeding. In many species, the annual breeding terminates because birds become photorefractory and their reproductive system is no longer activated by the long-day photoperiod. Exposure to short-day photoperiods then restores photosensitivity for the following breeding season (for review see Farner and Wingfield, 1980). Both the lengthening photoperiod per se and the vernal rise in testosterone play a role in the seasonal changes in song and their relative contribution seems to vary from one species to another.

Implanting exogenous testosterone in male or female songbirds increases frequency and duration of singing in a number of species (Arnold, 1975a; Madison et al., 2015; Nottebohm, 1980a; Pröve, 1974). Castration causes a decrease in singing activity during breeding season (Arnold, 1975b; Nottebohm, 1980a; Pröve, 1974), although usually not in non-breeding season (Alvarez-Borda and Nottebohm, 2002; Pinxten et al., 2002). Testosterone regulates most aspects of song through a synergistic effect of its androgenic and estrogenic metabolites (reviewed in Ball et al., 2003).

Although in many species testosterone alone increases HVC volume even under short-day photoperiods to values typical of breeding conditions (Rouse et al., 2015; Sartor et al., 2005; Yamamura et al., 2011), HVC volume also increases in direct response to long-day photoperiods in castrated male American tree sparrows (Bernard et al., 1997). Furthermore, Robertson et al (2014) showed that castration and blockade of both androgen receptors and estrogen production during photostimulation in male white-throated sparrows did not decrease the size of the song control nuclei relative to untreated

photostimulated birds. Dloniak & Deviche (2001) similarly showed that photostimulation increases HVC volume even in castrated male dark-eyed juncos, compared to photosensitive or photorefractory castrated individuals. However, in this study only castrated males that were treated with testosterone sang at all, independently of their photoperiodic state.

Despite the important role of testosterone in song activation, some songbird species continue to sing during the non-breeding season when testosterone plasma concentrations are very low. Non-breeding season song often has a different function than breeding season song (Leitner et al., 2001a; Rost, 1990; Smith et al., 1997) and its mechanistic basis has been less studied. For example, European starlings sing throughout the year, but their songs are shorter in autumn than in spring (Riters et al., 2000). In this species, the relationship between the expression of opioid receptors (Kelm-Nelson and Riters, 2013) or dopamine signaling (DeVries et al., 2015; Heimovics et al., 2009; Heimovics and Riters, 2008) with singing behavior varies as a function of the season. Male song sparrows continue to sing in autumn but with greater variability compared to spring (Baker et al., 1984; Smith et al., 1997). Although during the non-breeding season the testosterone levels are very low and castration does not decrease non-breeding season song, this singing behavior is still partially under the control of sex steroid hormones. Song rate in non-breeding song sparrows is decreased by a combination of aromatase inhibition and androgen receptor blocking (Soma et al., 1999) and also by treatment with an aromatase inhibitor alone (Soma et al., 2000). Multiple lines of research converge to show that dehydroepiandrosterone (DHEA), an androgen produced by the adrenals is, in contrast to testosterone and estrogen, elevated during the non-breeding season in song sparrows (Soma and Wingfield, 2001) and that this steroid is activating the non-breeding season singing, likely via its neural metabolism to androgens and estrogens, but possibly via other mechanisms too (for review see Soma et al., 2014).

Canaries in the wild (Leitner et al., 2001a) as well as domesticated canaries (Voigt and Leitner, 2008) do continue to sing during non-breeding season, albeit with shorter song durations. However, in laboratory conditions, canaries are rarely observed to sing

under short-day photoperiod, especially following castration. We report here that castrated male Fife fancy canaries maintained on short-day photoperiod for an extended length of time start to dramatically increase their song rate after four months, despite having negligible circulating testosterone concentrations. We examined the contribution of sex hormones in this form of non-breeding condition singing behavior by treating half of the subjects with the inhibitor of aromatase androstatrienedione (ATD) combined with the anti-androgen flutamide, while continuing to monitor their singing. These treatments had minimal or no effect on all steroid-dependent measures that were quantified including singing rate. These data indicate that onset of active singing in photosensitive male canaries is possible in the absence of either photostimulation or circulating testosterone and that the role of sex hormones in singing under these conditions appears to be minimal.

Materials and Methods

Experimental animals

Sixteen male canaries of the Fife fancy breed used in this study were obtained from a colony maintained at the University of Antwerp, Belgium. They were born and had gone through a full breeding cycle in this colony before being transferred to our laboratory. All subjects had been on natural daylight during the months preceding their arrival in our laboratory at the University of Liege, Belgium, in late March. All experimental procedures complied with Belgian laws concerning the Protection and Welfare of Animals and the Protection of Experimental Animals, and experimental protocols were approved by the Ethics Committee for the Use of Animals at the University of Liege (Protocol number 926). In all housing situations food, water, baths, cuttlebone and grit were available *ad libitum*.

Experimental procedures

Upon arrival subjects were housed on long day photoperiod (16L:8D) for five months to make them photorefractory. During the first two months, subjects were housed in an aviary with a large mixed-sex group. Three recordings of molting status (see detail of method below) during this period showed no initiation of molting in these conditions. Since the socio-sexual interactions taking place in these conditions appeared to delay molting

and the development of photorefractoriness, males were switched to single-sex housing with 5-6 males per cage in a separate room with no visual contact and minimal acoustic contact with the females. Another six recordings of molting status then showed a slow but steady progression through molting in these conditions indicating that birds had become photorefractory at least partially and were photoregressing (for more details on molting see chapter 7 of this thesis). They were then moved to an intermediate photoperiod (12L:12D) for 2 months, to further push the photoregression.

Next, the photoperiod was changed to 8L:16D for five months to induce birds to become photosensitive again and prepare them for testing effects of testosterone on singing behavior under short days. Three weeks later all males were castrated following a procedure similar to that described in Sartor et al. (2005). Briefly, under isoflurane anesthesia each testis was removed via an ipsilateral incision posterior of the last rib. Testes were found to be regressed in all subjects. The incision was sutured, the subject was allowed to recover under a heat lamp until perching and then returned to their home cage.

To our surprise, after four months of short-day photoperiod (February), many of these birds were observed to sing at relatively high rates. They were thus transferred to single-housed sound-attenuated boxes for three days to record their singing during two hours every morning at the time of lights on. At that time, blood samples were collected to test via enzyme immunoassay whether their circulating testosterone levels were actually low. This was indeed the case, which confirmed that castration had been complete. After the three days of isolation in the sound-proof boxes, the subjects were returned to their previous group cages. We decided at that time to investigate whether this singing activity displayed by short day castrated males was nevertheless steroid-dependent, instead of testing effects of exogenous testosterone. Of the twenty-six males that were recorded, sixteen males sang at a high rate. The other 10 subjects were not included in the rest of the study because they were mostly singing at a low rate and would not have permitted to investigate whether inhibition of sex steroid action inhibits singing behavior. They were included in another experiment (see chapter 4 of this thesis).

One month later the sixteen behaviorally active males were distributed into a treatment (n= 8) and a control (n= 8) group, balancing the body mass and cage of origin

between the two groups and they were placed back in the sound-attenuated boxes for two days to obtain a new baseline recording of their singing behavior just prior to treatment onset. The following day they were implanted subcutaneously with four 10 mm Silastic™ implants (inner diameter: 1.47 mm, outer diameter 1.96 mm, Dow Corning, ref. 508-006), sealed with medical silicone (Medical Adhesive Silicone, Dow Corning) on both sides. For the control subjects all four implants were empty, for treated subjects two implants were filled with 1-4-6 androstatrien-3,17-dione (ATD, Steraloids, ref. A4100-000) and the other two filled with trifluoro-2-methyl-48-nitro-m-propionotoluidide (flutamide or FLUT, Sigma-Aldrich, ref. F9397-1G).

ATD is a steroidal aromatase inhibitor (Foidart et al., 1995b) that inactivates aromatase irreversibly (Numazawa and Tachibana, 1997) and additionally inhibits aromatase mRNA transcription (Foidart et al., 1995b). Flutamide is a non-steroidal reversible androgen receptor antagonist (Labrie, 1993; Neri et al., 1972). Several studies have used these compounds in songbird species either separately (e.g. Archawaranon and Wiley, 1988; Beletsky et al., 1990; Hegner and Wingfield, 1987; Schwabl and Kriner, 1991; Sperry et al., 2010; Walters and Harding, 1988), or in combination (e.g. Johnson and Bottjer, 1995; Moore et al., 2004; Robertson et al., 2014)

All implants were checked under a stereo-microscope to make sure they were well sealed and they were incubated in 0.9% NaCl at 37°C overnight before being inserted subcutaneously. After implanting the Silastic™ capsules the subjects were returned to the same sound-proof box as previously and their singing behavior was recorded daily during the 2 hours after lights on for twenty-seven days. On the 28th day blood samples were collected from the wing vein and jugular vein, the subjects were deeply anaesthetized and perfused with paraformaldehyde (see below for details).

Molting status recording

Molt was scored following the method described in (Dawson and Newton, 2004), which has previously been used to evaluate molt in canaries by Hurley and colleagues (Hurley et al., 2008). The eight primary feathers on the subjects' right wing were evaluated for molting – these were identified as being the eight first feathers starting from the lateral side of the wing. Molting of the other feathers on the wings and rest of the body were also

observed but were not quantified. A score of 0 was given to a feather that had not yet fallen out, 1 for a new feather that was ¼ regrown, 2 for a half-regrown feather, 3 when it was ¾ regrown and 4 for a fully regrown new feather. A total molt score for each subject on a given day was calculated by summing the molt index of all eight feathers and dividing this total by 32 to provide a final score ranging from 0 (no molt) to 1 (complete molt).

Song recording and analysis

Singing was recorded inside custom built sound-proof boxes for two hours daily immediately following lights-on (0900h). Sound was acquired from all 16 channels simultaneously via custom-made microphones (microphone from Projects Unlimited/Audio Products Division, amplifier from Maxim Integrated) and an Allen & Heath ICE-16 multichannel recorder. The sound file was acquired and saved as a .wav file by Raven v1.4 software (Bioacoustics Research Program 2011; Raven Pro: Interactive Sound Analysis Software, Version 1.4, Ithaca, NY: The Cornell Lab of Ornithology) at a frequency of 44100 Hertz. The sound files were analyzed by software specifically designed for canary song analysis designed by Ed Smith and Bob Dooling, University of Maryland at College Park. The program defines a vocalization as a song if it is at least one second long, is preceded and followed by at least 0.4 seconds of silence and is at least 30 dB above background noise. The program computed for each song defined in this way the following measures: number of syllables per song, song duration (in seconds), time vocalizing (in seconds), percent of time vocalizing (seconds), syllable average duration (in milliseconds), syllable standard deviation of duration (in milliseconds), syllable average root mean square (RMS) power level, syllable standard deviation of RMS power level, song average RMS power level, song total RMS power, song entropy, song bandwidth (in hertz), song centroid frequency (in hertz), song 1st, 2nd and 3rd quartile frequency. The average of these measures for all the songs produced by each bird in one day was used for further analyses. Additionally, for each day the number of songs detected, the song durations and the time vocalizing were summed to make a total per day. For these three measures, a zero value was attributed on days when no songs were detected. For all other measures the value attributed to days with no singing was the average of the preceding and following day from that bird, in order to do statistical

analyses that do not allow for missing values. To validate that the software was detecting songs correctly, ten recording sessions were quantified manually by visually counting the songs on the spectrograms generated by Raven Pro. The numbers of songs detected with the two methods were strongly correlated ($r^2_{153} = 0.98$, $p < 0.001$, see Fig.1).

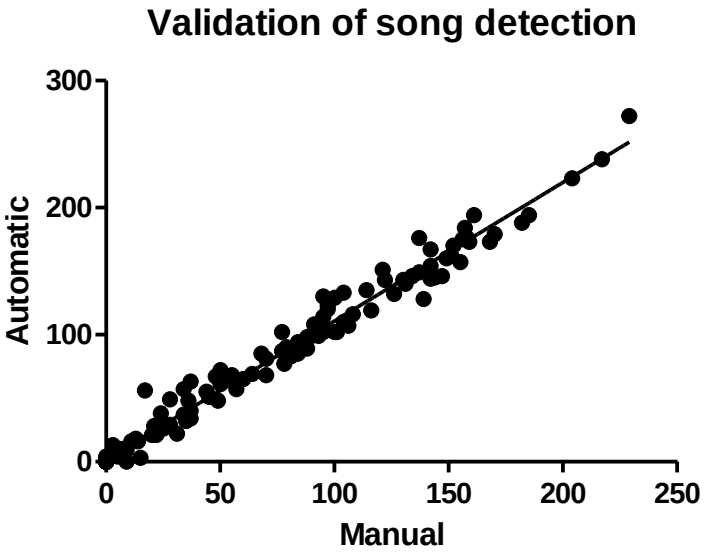

Figure 1. Validation of custom-built song analysis software. Regression of number of songs detected automatically by software with the number of songs detected manually by in the same recordings in 10 recordings from the 16 experimental birds.

Blood collection and hormone analysis

Blood samples of 150 µl were collected from the wing vein of all subjects during the transfer to sound-proof boxes for baseline recordings, just before placing the subcutaneous implants and on the day of brain collection. Wing vein blood collection was always performed within 3 minutes of catching the birds in their cage and within 90 minutes after lights on. In addition, just prior to perfusion a 100-200 µl blood sample was taken from the jugular vein. Blood was always collected into Na-heparinized micropipettes (Brand GMBH + CO KG, Wertheim, Germany) and any further blood flow was stopped by pressing cotton on the vein puncture. Blood was centrifuged at 9000 g for 9 minutes and the supernatant plasma was collected and stored at -80° C until further use.

Testosterone Enzyme Immunoassay

10 µl of plasma from each sample was diluted in 150 µl of ultra-pure water. Recovery samples were spiked with 20,000 CPM of tritiated-testosterone (Perkin-Elmer). All samples were extracted two times with 2 ml of dichloromethane. The organic phase was eluted into clean tubes, dried with nitrogen gas and stored at -20°C until further use. Average recovery rate was 72.8%.

Extracted samples were re-suspended in 400 µl Enzyme Immunoassay (EIA) buffer by vortexing for 30 seconds and shaking for 90 min at 1350 rpm. Re-suspended samples were assayed for testosterone concentration in two assays on three plates from a single Cayman Chemicals testosterone EIA kit (ref. 582701) following manufacturer's instructions. The minimum and maximum detection limit of the EIA, as determined by the lowest and highest concentration detected, were 4.79 pg/ml and 313.71 pg/ml respectively. The inter-assay coefficients of variation were 10.5 and 17.8% and the intra-assay variation for the plates were 8.7% and 13.5% respectively.

Confirmation of testosterone concentrations by GC/MSs

Androgens (dehydroepiandrosterone, androstenedione, testosterone, 5α-dihydrotestosterone, 3α5α-tetrahydrotestosterone) and estrogens (estrone, 17β-estradiol) concentrations were determined in plasma samples collected from the jugular vein of six control and six treated subjects by GC/MS according to the protocol described by Liere et al. (2000) with minor modifications. Briefly, steroids were extracted from individual plasmas by adding 10 volumes of methanol. Internal standards were introduced for steroid quantification: 2 ng of epietiocholanolone (for 5α-dihydrotestosterone, 3α,5α-tetrahydrotestosterone and dehydroepiandrosterone), 2 ng of ^{2}H5-testosterone (for testosterone), 2 ng of ^{2}H5-17β-estradiol (for 17β-estradiol, and estrone) and 5 ng of 19-nor progesterone (for androstenedione). Samples were purified and fractionated by solid-phase extraction with the recycling procedure (Liere et al., 2004). The steroid sulfates-containing fraction was directly derivatized with 20 µl heptafluorobutyric anhydride (HFBA) in 100 µl anhydrous acetone for 30 min at 20°C and analyzed by GC/MS. The unconjugated steroids-containing fraction was filtered and further purified and fractionated by high performance liquid chromatography (HPLC) as previously described (Hertig et al., 2010; Labombarda et al., 2006). Three fractions were collected from the

HPLC system: 5α/β-dihydroprogesterone were eluted in the first HPLC fraction (3-10 min) and were silylated with 50 µl MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide)/NH_4I/DTE (1000:2:5 vol/vol/vol) for 15 min at 70°C. The second fraction (10-31 min) contained pregnenolone, progestagens, androgens, estrone and 17β-E2 was derivatized with 25 µl HFBA and 25 µl anhydrous acetone for 1h at room temperature. Corticosterone, cortisone, cortisol and estriol were eluted in the third HPLC fraction (31-45 min) and derivatized with 50 µl HFBA and 25 µl anhydrous hexane for 1h at 80°C. All the fractions were dried under a stream of N_2 and resuspended in hexane for GC/MS analysis.

Calibration and biological samples were analyzed by GC/MS with an AS 3000 autosampler (ThermoFisher Scientific, USA). The Focus GC gas chromatograph is coupled with a DSQII mass spectrometer (ThermoFisher Scientific, USA). Injection was performed in the splitless mode at 250°C (1 min of splitless time) and the temperature of the gas chromatograph oven was initially maintained at 50°C for 1 min and ramped between 50 to 200°C at 20°C/min, then ramped to 285°C at 10°C/min and finally ramped to 350°C at 30°C/min. The helium carrier gas flow was maintained constant at 1 ml/min during the analysis. The transfer line and ionization chamber temperatures were 300°C and 220°C, respectively. Ionization was performed by electronic impact with electron energy of 70 eV. Derivatized steroids were identified by their retention time and two diagnostic ions in single ion monitoring (SIM) (**Table 1**). Quantification was performed according to the major diagnostic ion, called the quantification ion. The detection thresholds for all the screened steroids in plasma are reported in **Table 2**.

Table 1- Parameters used for steroid identifications and measurements by GC-MS

Steroids (Molecular weight)	Derivatized steroids (molecular weight)	Retention time (min.)	Diagnostic ions (*m/z*)
3α5α-tetrahydrotestosterone (292)	3α5α- tetrahydrotestosterone - 3,17- HFB_2 (684)	12.54	455 and **470**
***** $^2H_5$-Testosterone (293)	$^2H_5$-Testosterone, 17-HFB_2 (685)	**13.16**	**682-685**
Testosterone (288)	Testosterone-3,17-HFB_2 (680)	13.20	665 and **680**
***** $^2H_5$-17β-estradiol (277)	$^2H_5$-17β-estradiol-3,l7-HFB_2 (669)	**13.57**	**667-669**

17β-estradiol (272)	17β-estradiol-3,17- HFB_2 (664)	13.60	451 and **664**
***** Epietiocholanolone (290)	Epietiocholanolone-HFB (486)	**14.90**	**442 and 486**
Dehydroepiandrosterone (288)	Dehydroepiandrosterone-3-HFB (484)	15.71	255 and **270**
Δ4-androstene 3,17-dione (286)	Δ4-androstene 3,17-dione-3-HFB (482)	15.74	467 and **482**
Estrone (270)	Estrone-3-HFB (466)	16.17	422 and **466**
5α-dihydrotestosterone (290)	5α- dihydrotestosterone-17-HFB (486)	16.50	414 and **486**
***** 19 nor-progesterone (300)	19 nor-progesterone-HFB (496)	17.25	**481 and 496**

The diagnostic ions in bold face served for quantification

*: Internal standards

Table 2- GC/MS detection threshold in avian plasma

Steroids	ng/ml
3α5α-tetrahydrotestosterone	0.001
Testosterone	0.002
17β-estradiol	0.002
Dehydroepiandrosterone	0.010
Δ4-androstene 3,17-dione	0.010
Estrone	0.020
5α-dihydrotestosterone	0.005

Estradiol Enzyme Immunoassay

15 or 30 µl of plasma was diluted in 2ml MilliQ water (depending on volume available). Recovery samples were spiked with 200 pg/ml estradiol diluted from the stock solution supplied in the EIA kit and were treated the same as the unspiked experimental samples. C18 columns (Sep-Pak C18 Vac cartridge, Waters) were first primed with 1ml ethanol and washed with 2 ml MilliQ water. After loading the 2ml of diluted sample, the columns were again washed with 2ml MilliQ water and dried. Steroids were eluted with 1ml of 90% methanol (HPLC-grade) and dried at 40°C under a nitrogen gas flow, the walls of the tubes were rinsed with 500 µl HPLC-grade ethanol and re-dried under nitrogen gas. Average recovery was 72.9%. Dried samples were stored at -20°C overnight.

Extracted samples were re-suspended in 180 µl EIA buffer with 0.7% ethanol, vortexed for 30 seconds and shaken for 90 min at 1350 rpm. Re-suspended samples

were assayed for estradiol concentration on three plates from a Cayman Chemicals estradiol EIA kit (ref. 582251) following manufacturer's instructions. The minimum and maximum detection limit of the EIA, as determined by the lowest and highest concentration detected, were 9.17 pg/ml and 691.23 pg/ml respectively. The inter-assay variation was 12.6% and the intra-assay variation for the 3 plates was 26.4, 15.9, 21.1% respectively.

A parallelism validation, performed within the same assay, showed that sample dilution decreased the estradiol detected in parallel with the standard curve dilution (see Fig. 2) and the coefficient of variation of the sample assayed at different dilutions was 7.3%, confirming that the assay is specifically measuring estradiol in our canary plasma samples.

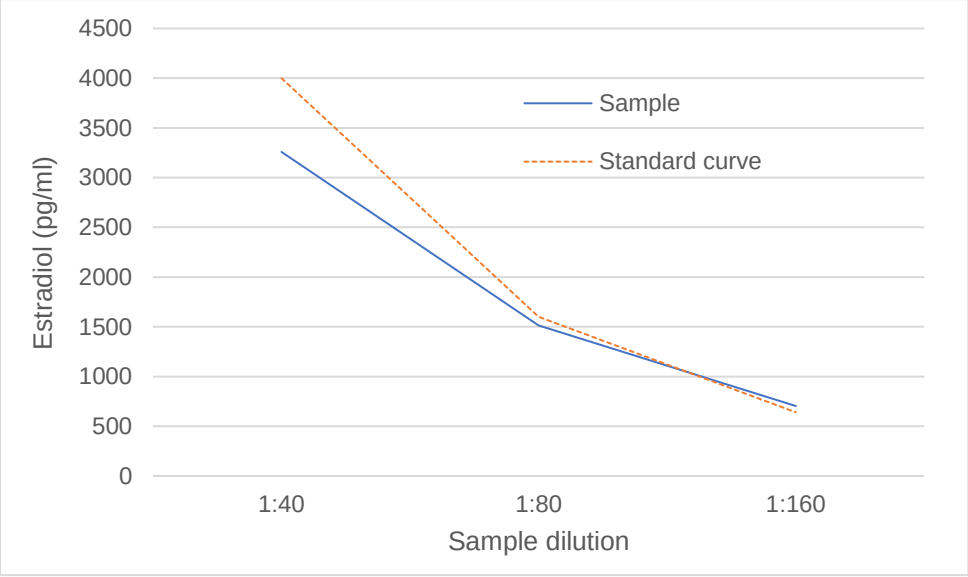

Figure 2. Parallelism of estradiol detection of sample at different dilutions by the estradiol EIA-kit used for assaying experimental samples.

Brain collection and processing

28 days after the implantation of Silastic™ capsules, subjects were weighed, the length and width (in millimeters) of their cloacal protuberance was measured, a blood sample was taken from the wing vein and then birds were anaesthetized with ~0.03ml of Nembutal. Once reflexes had stopped, a blood sample was taken from the jugular vein and immediately after the birds were perfused intracardially with phosphate-buffered saline (PBS, 1.43 g/L Na_2HPO_4, 0.48 g/L KH_2PO_4, 7.2 g/L NaCl) to remove blood and

immediately after with 4% paraformaldehyde (PFA, 4.3 g/L NaOH, 40 g/L paraformaldehyde, 18.8 g/L $NaH_2PO_4.H_20$) to fix the brain. After perfusion, the brain was immediately extracted from the skull and post-fixed overnight in 15 ml PFA.

The syrinx was extracted and weighed, the presence of implants and, when relevant, presence of drug inside the implants was confirmed. On the following day, brains were transferred to 15 ml of 30% sucrose solution (15.6 g/L Na_2HPO_4, 1.5 g/L KH_2PO_4, 300 g/L sucrose). Once the brains had sunk to the bottom of the vial they were frozen on dry ice and stored at -80° C until used. Brains were cut coronally on a cryostat into 30 µm-thick sections. The sections with the preoptic nucleus were collected separately into one series – these included all sections between the start of tractus septopallio-mesencephalicus (TSM) and 240 µm posterior to the anterior commissure (AC), while the rest of the brain was cut into four series. Sections were stored in anti-freeze (0.01M PBS with 10 g/L polyvinylpyrrolidone, 300 g/L sucrose, and 300 ml/L ethylene glycol) at -20°C until further use.

Brain sections staining

Nissl staining

One series of sections was mounted on Superfrost slides, dried at least overnight, and Nissl-stained with toluidine blue. After differentiation in Walpole buffer and molybdate, they were dehydrated in a series of increasing isopropanol concentrations, 99% ethanol and xylene and coverslipped using Eukitt as a mounting medium.

Aromatase immunohistochemistry

A second series of brain sections was stained by immunohistochemistry for aromatase. Washes were performed with Tris-buffered saline (TBS) or TBST (TBS with 0.2% Triton X-100). The blocking sera and antibodies were diluted in TBST with 5% normal goat serum (NGS) and 1% bovine serum albumin (BSA), this solution will be referred to as TBST-NGS-BSA. Sections were washed three times for five minutes to remove antifreeze and after all other steps except for the blocking serum. Endogenous peroxidases were inhibited by incubating the sections in 3% hydrogen peroxide in a solution of 50% methanol for 20 minutes. Sections were blocked in TBST-NGS-BSA solution for 60 minutes and incubated in primary antibody for one hour at room temperature and

overnight at 4°C (1:10,000 rabbit anti-quail aromatase antibody, a kind gift from Prof. Harada, Fujita Health University, Toyoake, Japan). This antibody has been especially developed and validated for quail (Foidart et al., 1995a) and also validated in songbirds (Balthazart et al., 1996a). On the following day, sections were blocked again in TBST-NGS-BSA solution for one hour, and incubated with the secondary antibody for two hours at room temperature (1:200 goat anti-rabbit biotinylated, DAKO, ref. E0432). The binding was amplified by incubating sections in ABC kit solution (both solution A and B at 1:400, Vectastain Elite PK-6100 2001). The binding sites were revealed by incubating for 10 minutes in 0.04% DAB with 0.012% H_2O_2 diluted in TBS. Sections were mounted from TBS with gelatin onto glass slides, dried overnight, immersed in xylene for 10 minutes and coverslipped with Eukitt mounting medium.

Microscopy and image analysis

HVC volume reconstruction in Nissl-stained sections

To reconstruct HVC volumes, photomicrographs were taken of each section in the series containing the nucleus, in both the left and right hemispheres with a Leica DMRB FL.100 microscope connected to a Leica DFC 480 color camera at a magnification of 5x using the same light settings for all pictures. An outline was drawn around the perimeter of each cross-section of the nuclei using ImageJ v1.47v (National Institutes of Health) and the delimited area was measured. In the few cases that a section was missing, the area was estimated by taking the average of the two sections immediately rostral and caudal to it. The volumes of nuclei were calculated by summing the areas and multiplying by 120 µm, the distance between two successive sections in the series. The volume of the nuclei in each hemisphere was calculated separately and the average of the two measures was used for statistical analyses.

Aromatase

Aromatase staining was analyzed on a Leica DMRB FL.100 microscope connected to a Leica DFC 480 color camera. Photomicrographs of each medial preoptic nucleus (POM) in the series were taken at 10x magnification using the same light settings for all pictures, starting from the most rostral section containing aromatase-immunoreactive (ARO-ir)

cells ventral of the TSM and finishing at the section containing the full extent of the AC. However, since a substantial proportion of brains had damage in at least one of sections with TSM, these sections were excluded from the quantification of POM volume for all brains. The cluster of ARO-ir cells that correspond to the POM was delineated and measured with ImageJ FIJI (National Institute of Health) and the volume of the nucleus was calculated by adding these areas in all sections and multiplying by 60 µm, the thickness between two consecutive sections.

The photomicrograph representing the middle section of the POM in the rostro-caudal axis was additionally analyzed for cellular level changes. Within an 852 x 852 µm square surrounding the POM (this square fully included the largest POM cross-section from all subjects) the number of aromatase-expressing neurons, the percentage of area covered by aromatase staining and the mean somal area of the ARO-ir neurons were quantified. Briefly, images were converted to 8-bit, a grey-value threshold was set to include all clearly visible aromatase-expressing neurons, but exclude all background. All particles over 30 μm^2 in area and over 0.15% circularity (circularity = 4π x area/$(perimeter)^2$, with a value of 1.0 indicating a perfect circle) were counted and measured. Additionally, the ARO-ir neurons in the same square were manually counted on the raw, non-thresholded image as a control.

Statistical analyses

A t-test was used to determine whether birds who had started singing (n = 16) were different in testosterone concentration and cloacal protuberance area (length x width) from the birds who did not sing (n = 10). A linear regression was preformed to verify whether testosterone or cloacal protuberance area correlated with the total duration of singing detected in these twenty-six birds.

Two-way repeated-measures ANOVA with treatment and time as main factors was used to analyze the following measures collected one month before the experiment, on the day of implantation of Silastic™ capsules and on the day of brain collection: body mass, cloacal protuberance area (length x width), testosterone and estradiol measured from the wingvein by EIA. A two-way repeated-measure ANOVA was also used to analyze testosterone and estradiol measured by EIA from the wing versus jugular veins,

with treatment as a between-subjects factor and vein as a within-subjects factor. Whenever a significant interaction between factors was found, Tukey's post-hoc tests were performed. A t-test was used to test for differences between the groups in syrinx mass, testosterone, androstenedione, 5α-dihydrotestosterone, 3α5α-tetrahydrotestosterone, dehydroepiandrosterone, 17β-estradiol and estrone concentrations according to GC/MS, HVC volume, POM volume, number of ARO-ir neurons, their average somal area and the % cover by ARO-ir material in the rostro-caudally middle section of POM. Linear regression was used to test for correlation between the testosterone and estradiol detected by EIA in the wing vein versus the jugular vein separately in the treated and control subjects. A two-way repeated-measures ANOVA was also used to analyze the song features, however in this case when a significant interaction was found a planned-comparisons test was used to verify differences between the two groups at specific time points.

A one-way ANOVA was used to compare the cloacal protuberance area, syrinx, POM volume, ARO-ir neuron number, area and % cover in the subjects of the current study with castrated photostimulated males implanted with testosterone-filled (CX+T) or empty (CX) Silastic™ implants from previous studies. A t-test was used to compare the HVC volumes of subjects in the current study with males implanted with testosterone.

For estradiol measurements among the 64 samples (4 per bird) 4 data points are missing due to insufficient plasma. All 64 samples were included in the analysis of testosterone by EIA, however many values for the treated birds after treatment exceeded the limits of the standard curve therefore likely are not accurate. One bird was excluded from measurements of POM volume and ARO-ir neuron properties due to damage to the hypothalamus during brain extraction from the skull. Additionally, one more bird was excluded from the analyses of ARO-ir neuron properties due to poor labeling and non-specific labeling of blood vessels. One bird was completely excluded from all song analysis because almost no songs were detected on any day from this bird. For all song measures day 1 was excluded from all analyses because no songs were detected from any bird on this day. On day 11 no song recording was made due to a technical problem. For all but three song measures (number of songs, total song duration and total vocalizing duration) two birds and the first 10 days of recording were excluded because a very low

number of songs were detected in both these cases. For six song measures (average number of syllables per song, average syllable duration, standard deviation of syllable duration, average song RMS power, average syllable RMS power and standard deviation of syllable RMS power) for the days where no songs were detected, an average value of the previous and following days for that measure was used.

A paired t-test was used to test whether the dependent variables that were considered here were lateralized. Specifically, we looked for the presence of differences between the left and right hemisphere in the volume of HVC and POM, the number and area of ARO-ir cells and the fractional area covered by these cells in POM. These tests showed that none of these measures were lateralized except for number of ARO-ir neurons ($t_{13} = 2.72$, $p = 0.018$) and % cover by ARO-ir material ($t_{13} = 2.24$, $p = 0.043$). Furthermore, using the values for each hemisphere separately to analyze the group differences gave very similar results therefore an average of the two hemispheres was used. The magnitude of these differences between sides was small and concerned two tests out of many.

These differences are thus not further considered. All statistical analyses were performed using STATISTICA and differences were considered significant for $p<0.05$. All data are represented here by their mean ± SEM.

Results

Hormone-behavior correlations during initial observations

The birds included in this experiment (n=16) and those who did not sing at all initially (n=10) were recorded one month before the experimental treatment, to establish baseline singing rates and balance the subjects across the two groups according to song rate. These groups were formed based on manual counting of songs on the spectrograms made from three days of baseline recording, the non-singing birds were not observed to sing at all during this period, while singing birds sang between 26 and 195 times. At the same time a blood sample was collected to measure testosterone to confirm that their castration was complete. Despite the recordings showing high rates of singing in a subset of birds, testosterone concentrations were basal in all birds (mean ± SEM = 0.58 ± 0.06 ng/ml) with the exception of one bird measured at 1.65 ng/ml. Furthermore, testosterone

concentrations did not differ between singing and non-singing birds (Fig. 3A, $t_{24} = 0.74$, $p = 0.467$). The total song duration detected (as identified by song analysis software, which is a more precise measure than the manual counting of songs) over these three days of recording were regressed against the concurrent concentrations of testosterone (Fig. 3B), but no correlation was evident ($r^2_{24} = 0.02$, $p = 0.470$). Similarly, there was no difference in cloacal protuberance area between singers (9.40 ± 0.79 mm^2) and non-singers (11.78 ± 1.59 mm^2, $t_{24} = 1.49$, $p = 0.149$) and no correlation between cloacal protuberance area and total song duration ($r^2_{24} = 0.01$, $p = 0.656$, data not shown).

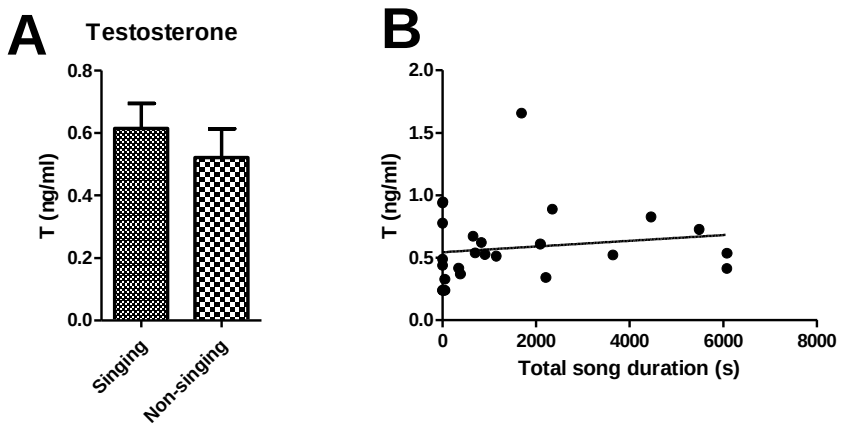

*Figure 3. Plasma testosterone concentrations in male canaries maintained for 4 months on short-day photoperiod that sang at high rates (n =16) or remained silent (n=10), all data are represented here by their mean ± SEM (**A**). Correlation between the testosterone concentrations and total song duration during three days of recordings in the same birds **(B).***

The rest of this presentation will focus on the 16 birds singing at a high rate that were treated with ATD and flutamide or kept as controls.

Morphological measures

Body mass was not different between control and treated subjects either before or after treatment ($F_{1,28} = 1.60$, $p = 0.226$, see Fig. 4A), however there was a slight decrease in body mass of all subjects over time, presumably due to the mild stress of handling and social isolation ($F_{2,28} = 31.01$, $p < 0.001$), with no interaction between the two factors ($F_{2,28}$

= 2.08, p = 0.143). A post-hoc analysis of the significant time effect showed that all three timepoints were different from each other.

The area (length x width) of the cloacal protuberance was not significantly different between treatment groups ($F_{1,28}$ = 0.22, p = 0.649, Fig. 4B) or time points ($F_{2,28}$ = 2.52, p = 0.098) and there was no interaction between the factors ($F_{2,28}$ = 1.16, p = 0.328). The cloacal protuberance areas of these subjects were compared to castrated, photostimulated testosterone-implanted males (henceforth called CX+T males) that were singing at similarly high rates as the subjects of the current study as well as their controls – castrated photostimulated males implanted with empty Silastic™ capsules (CX males). A one-way ANOVA showed the cloacal area of the three groups was significantly different ($F_{2,24}$ = 16.38, p < 0.001), a post-hoc test showing that the CX-T group had significantly larger cloacal protuberance areas (26.50 ± 4.64 mm^2) than both the CX controls (12.90 ± 4.29 mm^2) and the subjects of the current experiment (7.78 ± 0.67 mm^2), with no difference between the latter two groups.

The syrinx mass on the day of brain collection was also not different between the groups (t_{14} = 0.57, p = 0.581, Fig 4C). A one-way ANOVA comparing the subjects of the current study with CX+T and CX males showed that syrinx mass of the three groups was significantly different ($F_{2,24}$ = 8.38, p = 0.002), a post-hoc test showing that the CX-T group had significantly heavier syrinx (30.33 ± 3.46 mg) than both the CX controls (20.98 ± 3.41 mg) and the subjects of the current experiment (19.44 ± 0.82 mg), with no difference between the latter two groups.

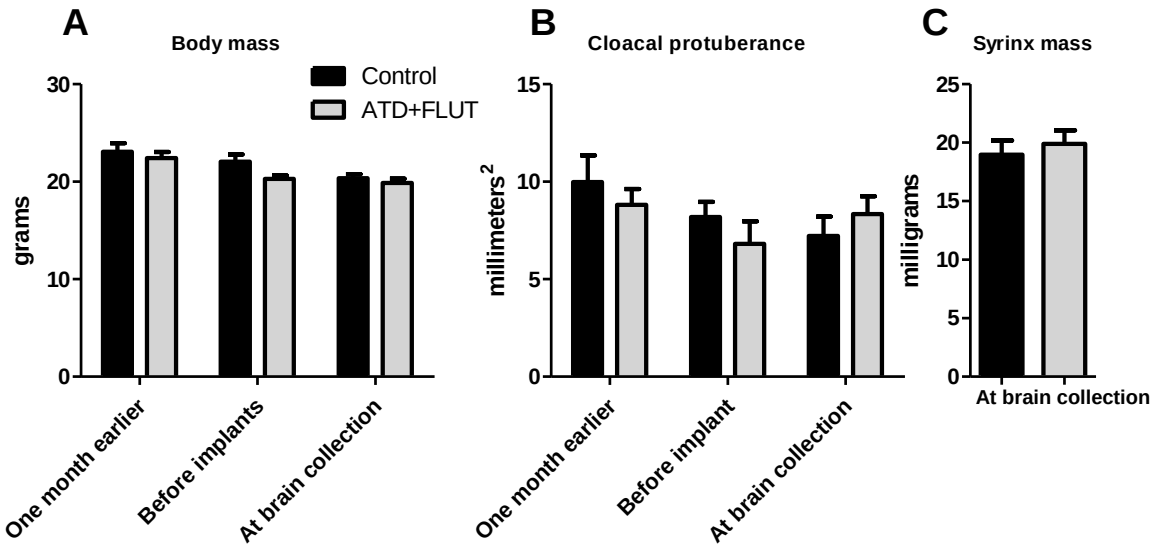

*Figure 4. Morphological measures. Body mass (**A**) and cloacal protuberance (**B**) of subjects one month prior to onset of treatment, just before treatment and on the day of brain collection in control and treated subjects. Syrinx mass of subjects in the two groups at end of the experiment (**C**).*

Song behavior

The number of songs detected per day was analysed in a repeated-measures ANOVA with treatment group as an independent factor. The main effect of treatment was not significant ($F_{1, 338} = 2.14$, $p = 0.167$). There was a significant effect of time ($F_{26,338} = 11.51$, $p < 0.001$) and a significant interaction between the two factors ($F_{26,338} = 2.24$, $p < 0.001$, see Fig. 5A). Planned-comparison post-hoc tests showed that treated subjects were significantly different from controls on day 18 of the treatment with an alpha-value below 0.05 and there was additionally a statistical tendency with alpha values between 0.05 and 0.1 on days 16, 17, 19, 20, 21, 22, 24 and 25. On all these days the treated group sang on average fewer songs than the control group.

The same analysis performed on total song duration recorded on each day detected no main effect of group ($F_{1,338} = 0.00$, $p = 0.952$), a significant effect of time ($F_{26,338} = 11.16$, $p < 0.001$) but no interaction ($F_{26,338} = 1.03$, $p = 0.430$, see Fig. 5B). A similar pattern of results was also detected in the analysis of the total time vocalizing: no effect of group ($F_{1,338} = 0.05$, $p = 0.824$), a main effect of time ($F_{26,338} = 11.98$, $p < 0.001$), but no interaction ($F_{26,338} = 1.03$, $p = 0.430$, see Fig. 5C).

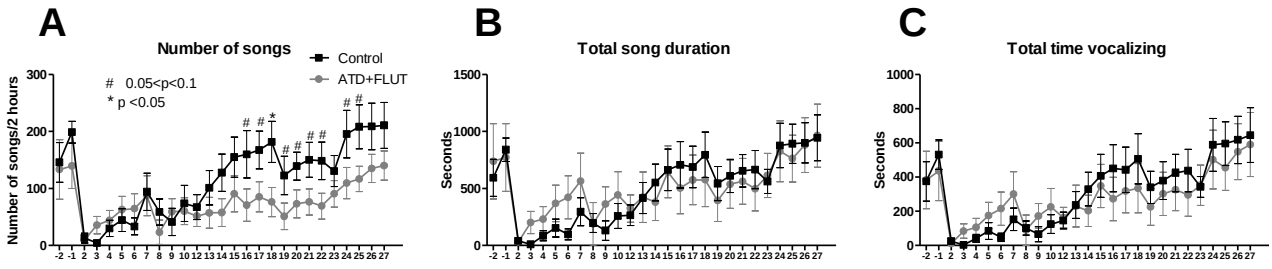

*Figure 5. The song rate (**A**), total song duration (**B**) and total time vocalizing (**C**) detected on days -2, -1 and days 2-27 of the treatment in the control and treated groups.*

The other measures of song were also analyzed with a repeated-measures ANOVA with groups as an independent factor, however the first 11 days of the experiment and 2 subjects were eliminated because no songs were detected. The analysis of **number of syllables per song** showed a main effect of treatment ($F_{1,187} = 7.22$, $p = 0.021$), a main effect of time ($F_{17,187} = 2.95$, $p < 0.001$) and an interaction ($F_{17,187} = 1.98$, $p = 0.014$, see Fig. 6A). Planned-comparison post-hoc tests showed that there was a difference between treated subjects and controls on all days between day 12 and day 27 of the treatment. A similar analysis of the **average syllable duration** showed no main effect of treatment ($F_{1,187} = 2.14$, $p = 0.172$), a main effect of time ($F_{17,187} = 5.35$, $p < 0.001$) and no interaction ($F_{17,187} = 0.70$, $p = 0.803$, see Fig. 6B). The **standard deviation of syllable duration** showed no main effect of groups ($F_{1,187} = 2.83$, $p = 0.121$), a main effect of time ($F_{17,187} = 3.94$, $p < 0.001$) and no interaction ($F_{17,187} = 0.89$, $p = 0.589$, see Fig. 6C). The **average song RMS power** showed no main effect of groups ($F_{1,187} = 1.47$, $p = 0.250$), a main effect of time ($F_{17,187} = 7.60$, $p < 0.001$) and no interaction ($F_{17,187} = 0.89$, $p = 0.583$, see Fig. 6D). The **syllable RMS power** also showed no main effect of treatment ($F_{1,187} = 0.61$, $p = 0.453$), a main effect of time ($F_{17,187} = 8.31$, $p < 0.001$) and no interaction ($F_{17,187} = 0.71$, $p = 0.790$, see Fig. 6E). Similarly, the **standard deviation of the syllable RMS power** showed no main effect of groups ($F_{1,187} = 1.16$, $p = 0.304$), a main effect of time ($F_{17,187} = 8.29$, $p < 0.001$) and no interaction ($F_{17,187} = 0.81$, $p = 0.679$, see Fig. 6F).

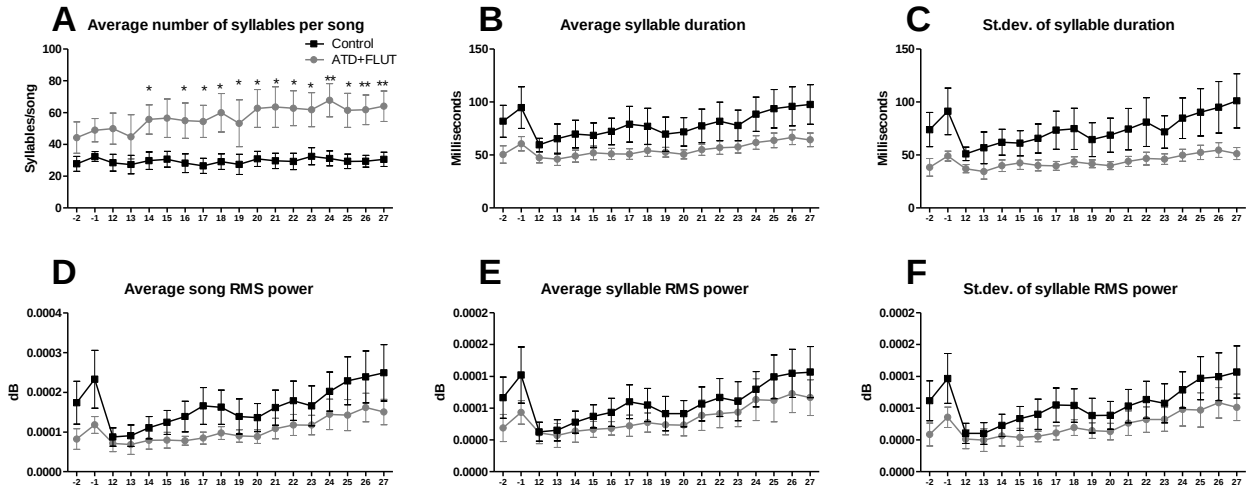

*Figure 6. Average number of syllables/song (**A**), average syllable duration (**B**) standard deviation of syllable duration (**C**), average song root mean square power (**D**), average syllable root mean square power (**E**), standard deviation of syllable root mean square power (**F**) detected on days -2, -1 and days 12-27 of the treatment in the control and treated groups.*

An additional 10 measures of singing behavior were treated similarly to the six measures discussed in the previous paragraph, however, since the graphs showed no obvious difference between the groups, these measures were not analyzed statistically. The evolution in time of these measures is however shown in the supplementary materials (see Fig. S1).

A commonly used measure of quality of song that has been shown to be modulated by sex steroid hormones (Alward et al., 2016c; Alward et al., 2013; Meitzen et al., 2007) is the coefficient of variation of various measures. To be sure that we were not missing more subtle effects of the treatment on song stereotypy over time we analyzed the coefficient of variation of song bandwidth, entropy and duration. This was done across days (1 pre-treatment bin of two days and 3 post-treatment bins of 8-9 days each) and within days (1 day before treatment and 3 days after treatment, evenly spaced across the experimental duration, see Fig. S2). However, these analyses did not show any group differences, therefore the corresponding statistical analyses are not presented here. The graphs representing the changes of coefficient of variation of these measures are presented in the supplementary materials.

Neuroplasticity

Subjects treated with ATD+FLUT did not have different HVC volumes compared to control subjects (t_{14} = 0.73, p = 0.478, see Fig. 7A). The POM volumes were also not different between these two groups (t_{13} = 0.43, p = 0.674, see Fig. 7B). The number of ARO-ir neurons in the middle section of the POM in the rostro-caudal axis was not different between groups (t_{12} = 0.23, p = 0.821, Fig. 7C) and likewise, no difference was found in the somal area of these ARO-ir neurons (t_{12} = 0.05, p = 0.964, Fig. 7D) nor in the % area covered by ARO-ir material (t_{12} = 0.03, p = 0.976, Fig. 7E).

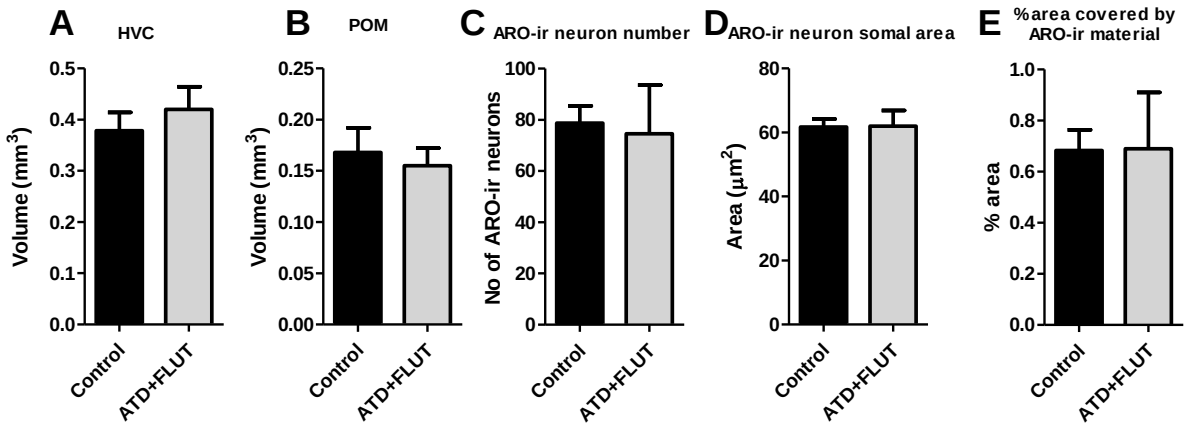

*Figure 7. HVC volume (**A**), POM volume (**B**), number of (**C**) and average somal area of (**D**) ARO-ir neurons, % area covered by ARO-ir material (**E**) in middle section of POM, measured in control and treated group.*

Compared to CX+T males of previous experiments in our laboratory the subjects of the current experiment had a large HVC volume (CX+T: 0.32 ± 0.02 mm^3, current study: 0.40 ± 0.03 mm^3, t_{28} = 2.28, p = 0.031). The average POM volumes of subjects in the current study (0.19 ± 0.05 mm^3) was compared to CX+T males (0.22 ± 0.04 mm^3) and CX males (0.10 ± 0.03 mm^3) from previous experiments. The one-way ANOVA showed a significant effect of group ($F_{2,22}$ = 13.61, p < 0.001). A post-hoc test showed that subjects of the current study had significantly larger POM volumes than the CX males but not different CX+T males. The number of ARO-ir neurons in one section of POM was significantly different across these three groups ($F_{2,21}$ = 4.62, p = 0.022). The post-hoc test indicated that this measure was not statistically different in the subjects of the current study (76.89 ± 8.65 neurons) from either the CX+T (113.60 ± 4.06 neurons) or CX (60.17

± 8.30 neurons) groups, but these last two groups were statistically different from each other, with a higher number of neurons in CX+T. The average somal area of the ARO-ir neurons was also different in the three groups ($F_{2,21} = 4.14$, $p = 0.031$). A post-hoc test showed that the CX+T group (73.45 ± 1.25 μm^2) had statistically larger ARO-ir neurons than the subjects of the current group (61.82 ± 2.41 μm^2) and showed a tendency to have larger neurons than the CX group (62.39 ± 1.07 μm^2, $p = 0.070$). The three groups were also different in the extent to which ARO-ir material covered the POM section analyzed ($F_{2,21} = 5.18$, $p = 0.015$). A post-hoc test showed that the CX+T group (1.15 ± 0.03%) had a higher percent cover than both the CX group (0.52 ± 0.08%) and the subjects of the current group (0.69 ± 0.10%), the latter two did not differ from each other.

Sex steroids plasma concentrations

Testosterone measured by EIA revealed a greatly elevated concentration of testosterone in subjects treated with ATD+FLUT when measured after onset of treatment, while all other plasma samples contained relatively low levels of testosterone, below what is typically seen in breeding condition (see Fig. 8A). A repeated-measures ANOVA of the testosterone detected in the wingvein by EIA at two time points before treatment and once after treatment, showed a significant effect of group ($F_{1,28} = 22.25$, $p < 0.001$), of time ($F_{2,28} = 23.30$, $p < 0.001$) and an interaction between these two factors ($F_{2,28} = 23.10$, $p < 0.001$). A post-hoc test showed that there was a significant difference in testosterone concentration between the two groups on the day of brain collection only.

Comparing the two samples collected from the wing and jugular veins on the day of brain collection showed an effect of groups ($F_{1,14} = 17.59$, $p < 0.001$), of the vein of origin ($F_{1,14} = 20.10$, $p < 0.001$) and an interaction between these factors ($F_{1,14} = 20.22$, $p < 0.001$). The post-hoc tests showed a difference between treated and control subjects for the jugular samples ($p = 0.016$) and a more pronounced difference was found in the wing vein ($p = 0.001$). On the other hand, when samples collected from the jugular vein at time of brain collection were measured with GC/MS both control and treated subjects showed very low concentrations of testosterone, well below concentrations found in breeding conditions and no difference between the two groups was found ($t_{10} = 0.50$, $p = 0.630$, see Fig. 8B).

Surprisingly, no difference was found between groups in the concentration of estradiol measured by EIA in the wingvein ($F_{1,20} = 0.19$, $p = 0.676$); there was also no effect of time ($F_{2,20} = 1.62$, $p = 0.224$) and no interaction ($F_{2,20} = 2.03$, $p = 0.158$, see Fig. 8C). The jugular and wing vein concentrations were also associated with no main effect of treatment ($F_{1,13} = 0.33$, $p = 0.574$), no main effect of vein ($F_{1,13} = 0.87$, $p = 0.369$) and no interaction ($F_{1,13} = 0.01$, $p = 0.998$). GC/MS analysis similarly showed that estradiol levels in the jugular vein were not different between groups ($t_{10} = 0.23$, $p = 0.825$, see Fig. 8D).

The GC/MS assay did not detect any difference between groups in the concentrations of estrone (control: 0.03 ± 0.01, treated: 0.02 ± 0.01, $t_{10} = 0.41$, $p = 0.689$), or 5α-DHT (control: 0.4 ± 0.03, treated: 0.03 ± 0.02, $t_{10} = 0.20$, $p = 0.845$). Androstenedione, dehydroeipandrosterone and 3α5α- tetrahydrotestosterone were below detection limit in all subjects.

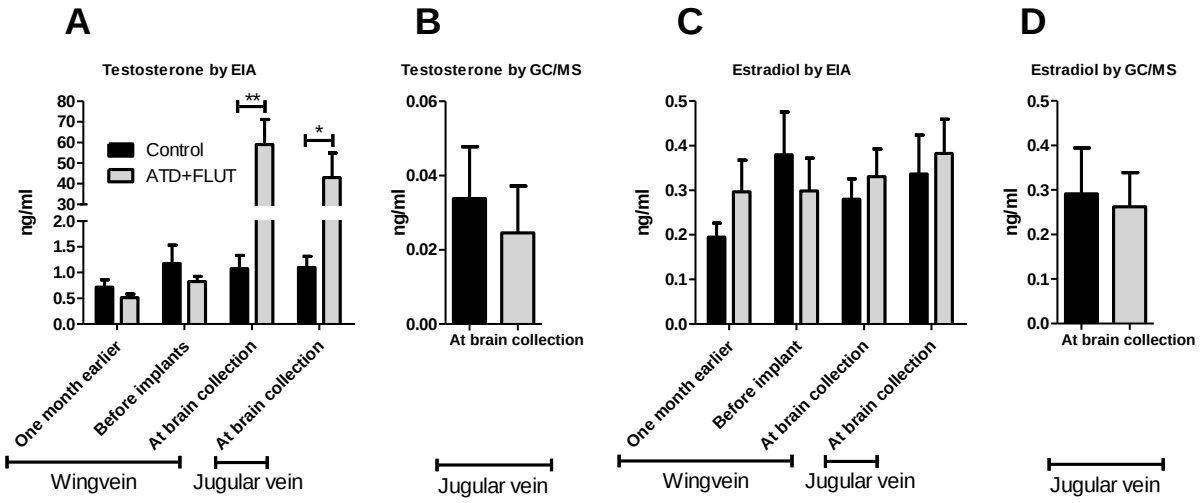

*Figure 8. Testosterone (**A**) and estradiol concentrations (**C**) as measured by EIA in samples collected during baseline recordings one month before treatment, on the day of implantation of Silastic™ capsules and on the day of brain collection from the wing vein and on day of brain collection from the jugular vein of control and treated subjects and testosterone (**B**) and estradiol concentrations (**D**) as detected by GC/MS in jugular vein samples collected on day of brain collection from control and treated subjects.*

We wondered whether the extremely high “testosterone” concentrations identified

by the EIA in the ATD-FLUT group and the discrepancy between the results of the two types of assays for testosterone could be explained by a cross-reactivity of the EIA either with the drugs themselves or with a metabolite of the drugs. We therefore assayed a range of doses of these two drugs in the testosterone EIA. No cross-reactivity with flutamide was identified in the testosterone assay, but a low cross-reactivity of the kit with ATD was found. ATD cross-reacted in a non-linear manner in the range of 0.13 – 0.61% in our measures. We also asked Cayman Chemical, the company providing the assay kit, whether they had information about this potential cross-reactivity and they indicated that in their hands the ATD cross-reactivity in this EIA was 0.197%. Interestingly a strong correlation between the "testosterone" concentrations in the wing and jugular vein was detected by the EIA in treated subjects ($r^2_6 = 0.91$, $p < 0.001$, see Fig. 9A), while no such correlation was found in the corresponding measures in control subjects ($r^2_6 = 0.08$, $p = 0.505$, see Fig. 9B). This might suggest that an exogenous compound was at the origin of this high correlation.

We extended these tests of specificity to the estradiol EIA kit but found no cross-reactivity of ATD in this assay (0.008 – 0.009% detected in our own assays, 0.003 reported by Cayman Chemicals). Correlatively estradiol concentrations in the jugular and wing vein, as measured by EIA, showed no correlation for either treated ($r^2_6 = 0.21$, $p = 0.253$) or control subjects ($r^2_5 = 0.285$, $p = 0.217$).

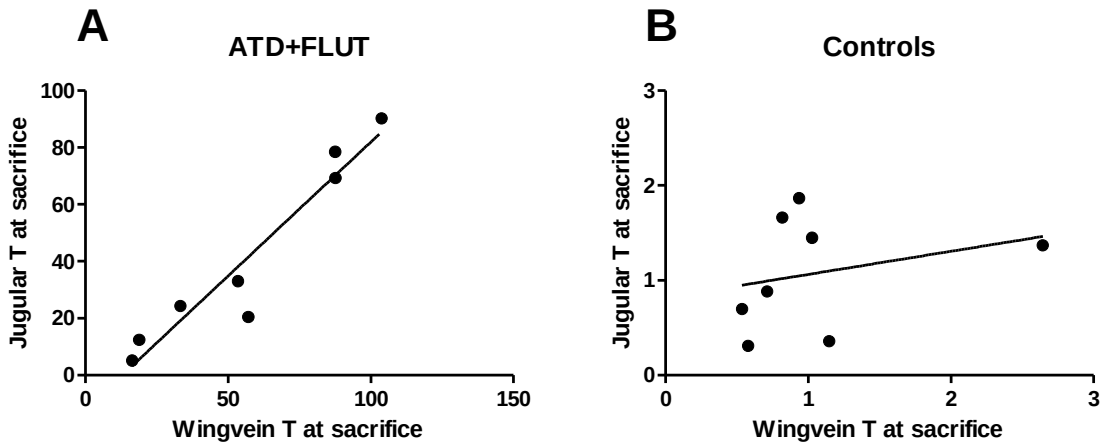

*Figure 9. Correlation of 'testosterone' concentrations as detected by EIA between jugular and wing veins in treated (**A**) and control (**B**) subjects.*

Discussion

This study identified an unexpected high rate of singing in a large proportion of castrated male canaries that had been kept for several months under short days. This singing activity was associated with very low basal testosterone concentrations and there was no difference in testosterone concentration between birds singing at a high rate and birds that were mostly inactive. We then tested whether this singing activity was dependent on sex steroids that would be produced in the brain by treating birds with an aromatase inhibitor combined with an antiandrogen, but this only very moderately and transitorily affected the behavior. We therefore suggest that the singing observed in short day castrated canaries is largely steroid-independent but several aspects of this conclusion require proper evaluation.

Hormones and morphological measures

The levels of circulating testosterone were basal at all sampling time points in control birds and in the pre-treatment samples of ATD+FLUT subjects, below the level usually seen in breeding condition in this strain of canaries (Iserbyt et al., 2015). The EIA detected, however, extremely high levels of testosterone in plasma from ATD+FLUT birds after treatment, well above the physiological level observed in male canaries during the breeding period. The jugular vein samples from the day of brain collection were also analyzed by gas chromatography/mass spectrometry, which detected in these samples basal levels of testosterone in both control and treated birds. The inflated values from the EIA for treated birds thus suggest that the testosterone antibodies in the EIA kit cross-reacted with ATD or flutamide present in the plasma of those subjects, or alternatively with a metabolite or another downstream signalling molecule of one of these two drugs. Flutamide is not likely to cross-react by itself with the testosterone assay since it is a non-steroidal compound. Indeed, specificity tests identified no cross-reactivity at all with flutamide, but there was a modest cross-reactivity of ATD with the testosterone EIA. However since this cross-reactivity was low (well below 1%), it is unlikely that the high levels of testosterone detected (around 50 ng/ml) could be accounted for by cross-reactivity with ATD alone because this would imply that concentrations of ATD in the blood of the treated subjects should be in the range of several micrograms per milliliter,

which is unlikely given the total quantity of ATD implanted in each bird one month earlier was about 20 mg and release from Silastic™ implants is very slow.

Interestingly there was in the treated subjects a strong correlation between the "testosterone" detected by EIA in the jugular and the wing veins, while in contrast no correlation between these two measures was found in control subjects. This discrepancy is specific to testosterone, the concentrations of estradiol detected by EIA in the wing vein were not correlated to concentrations detected in the jugular vein in either the control or the treated subjects. This suggests that endogenous concentrations of testosterone and estradiol are significantly affected by the brain as shown previously for estradiol in zebra finches (Schlinger and Arnold, 1992), but that another compound was "freely" circulating in the body of ATD+FLUT subjects that was not altered during the passage of blood through the brain.

The results of the testosterone EIA are thus not useful for determining the real concentrations of testosterone circulating in the subjects treated with ATD and flutamide, however, they do provide an indirect confirmation that the drugs, or at least one of them, were released from the Silastic™ capsules and were still present at the end of the experiment. Overall, the wing veins of treated subjects contained a higher concentration of pseudo-testosterone as detected by EIA, this difference was significant in a two-way repeated measures ANOVA with group and veins as factors. More work should however be done to reach a better understanding of the nature of the cross-reacting compound.

Surprisingly, the estradiol levels were not different between the control and treated groups in either the wing or jugular veins and did not change substantially over the different time points measured. The specificity of the estradiol EIA in canary plasma was demonstrated with a parallelism and only a few samples were outside of the sensitivity of the assay, therefore it is unlikely that a floor effect explains the lack of difference between groups. The GC/MS assay showed a similar range of concentrations and confirmed that estradiol levels were not different between groups. It is possible that the estradiol was not decreased by ATD at the timepoint examined because over time a compensation mechanism had restored initially depressed levels of estradiol. Nevertheless, it remains

a possibility that the ATD was not successful in inhibiting aromatase and this could explain the weak effect of the treatment seen on singing behavior.

Furthermore, the GC/MS assay did not identify a difference in concentration between groups in either estrone or 5α-DHT and found below detection limit levels of 3α5α-THT, DHEA and androstenedione. This suggests that it is unlikely that these hormones are important in regulating song rate under the current photoperiodic conditions. The low levels of DHEA found in the current study will be further discussed in the final section of the discussion.

The treatment did not have an effect on body mass, although this measure slightly decreased over time, presumably due to the stress of handling and social isolation. The drugs similarly did not change the area of the cloacal protuberance and mass of the syrinx, both androgen sensitive organs. Comparing the cloacal protuberance area and syrinx mass of all male canaries in this study with previous experiments conducted in our lab using the same strain of canary indicates that these organs were small in the subjects of this study, similar to castrated males and much smaller than in testosterone-implanted males (Ghorbanpoor et al., unpublished). Together these data confirm that indeed circulating testosterone concentrations were basal in all subjects throughout the experiment, despite presence of high rates of singing behavior.

Singing behaviour

A substantial decrease in singing behavior was seen in the first week following implantation of the Silastic™ capsules, probably due to stress from handling, Silastic™ capsule implantation and isolation. The singing rates started to restore back to baseline levels in the second week and in the third week a difference between the control and treated groups developed, which was close to being significant by planned-comparison tests on some recording days (one day at $p<0.05$, several days at $p<0.10$). In the final week, the singing rates of the treated group started to increase and reach the level of the control group, possibly due to compensation which has been shown previously for flutamide (Fusani et al., 2007) and ATD+FLUT (Adkins-Regan and Leung, 2006).

Despite the difference on some days in the number of songs between the groups, the total song duration and time vocalizing did not differ on any day between the groups, presumably because the treated birds surprisingly sang on average longer songs. This fact is also suggested by slightly longer average song durations of the songs produced by the treated group, although this effect does not reach significance (see figure S2 in supplementary materials). This assumption is further confirmed by the greater number of syllables per song on all days from day 14 till the end of the experiment in the songs produced by the treated subjects compared to the controls. It is possible, however, that this effect is driven by a subset of birds in the treated group that tended to sing more syllables per song even prior to treatment, because there is a statistically not significant but visible difference between the two groups in this measure already during baseline recordings (days -2 and -1).

A higher number of tours (a series of repetitions of the same syllable) per song were found to be produced by female canaries treated with testosterone and fadrozole (another aromatase inhibitor), than by females treated with testosterone alone (Fusani et al., 2003). In the current study the method of song analysis did not discriminate between the syllables of different tours, so it is not possible to compare our result directly with this observation from Fusani and colleagues, however the higher number of syllables per song detected in the songs of the treated group of the current study could be consistent with a higher number of tours per song.

Although many other qualitative measures of song were analyzed in the recordings made in this experiment, no other differences between groups were detected. Similarly, when the coefficients of variation of song duration, bandwidth and entropy within four recording days from different weeks of the treatment and across four bins of all days were analyzed, no differences between the groups were observed either. The manipulation of testosterone action in HVC has been shown to modulate the coefficient of variation of song duration in Gambel's white-crowned sparrows (Meitzen et al., 2007) and of song bandwidth and entropy in canaries (Alward et al., 2016c), however, both these experiments were performed with birds maintained on long-day photoperiod. Globally, the weak and transitory effect of the pharmacological treatment on singing behavior

suggests that the singing observed in castrated male canaries that were kept under short days presumably in a photosensitive status for several months is not strongly regulated by sex hormone action. The possibility that the lack of effect is due to a failure or ATD and flutamide to exert its intended pharmacological effects is considered later in this discussion.

Neuroplasticity

In line with the weak effects of the blockade of aromatase and androgen receptors on singing behavior, there was no change of volume of HVC, a nucleus involved in both production and feedback/maintenance of song (Bottjer and Johnson, 1997; Simpson and Vicario, 1990). A previous study using lower doses of both ATD and flutamide in male canaries similarly found no difference in HVC volume between the control group and subjects treated with these two drugs (Johnson and Bottjer, 1995), but detected larger HVC volumes in subjects treated with testosterone, which confirmed the sensitivity to sex hormone action of the birds involved in that study. In comparing the current experiment with previous studies performed in our lab using the same strain of canaries, it can be noted that the volumes of HVC in the current study are even larger than in male canaries stimulated with testosterone (Shevchouk et al., 2017a). Although surprising in light of the low levels of testosterone in these birds, this fact is congruent with the high levels of singing that the subjects displayed.

Although located outside the song control system itself, the medial preoptic nucleus (POM) has been shown to play a role in the control of singing rate and thus singing motivation (for a review see Alward et al., 2017). Since the only effect of the treatment on behavior we observed was on song rate, we decided to investigate whether neuroendocrine changes in the POM could underlie this effect. There was no difference in the volume of POM between groups. Similarly to what was seen for the volume of HVC, the POM volume of both the groups in the current study were significantly larger than that in castrated males and not different from castrated testosterone-treated males from previous experiments performed in similar conditions in our laboratory, tentatively suggesting that both testosterone and singing play additive roles in determining POM volume. Previous studies have suggested that measuring properties of ARO-ir neurons

in the middle section of POM can be a more sensitive measure than the volume of POM. However, we found no difference in the number of ARO-ir neurons, their average somal area or the % cover by ARO-ir material suggesting that these variables also were not markedly affected by the treatment in the present birds. Unlike the POM volume, the measures of ARO-ir neurons in the brains of the subjects of this experiment were more similar to what is seen in castrated males and decreased compared to castrated testosterone-treated males. This discrepancy suggests that while POM volume can be driven by singing feedback or other unidentified signalling that was increased in the current experiment, the number, size and extent of cover by aromatase-expressing neurons in POM is more critically dependent on circulating testosterone concentrations.

Efficiency of pharmacological treatments

In view of the absence of effect of the treatment on all measures of neuroplasticity that were considered and of the weak effects on the singing behavior of the canaries in the current study, one could of course question whether the ATD+FLUT treatments did in fact produce the expected endocrine effects. Given the presence of a compound that cross-reacted with the testosterone EIA in the plasma of the treated birds, it is unlikely that the drugs did not diffuse from the Silastic™ implants, neither that their diffusion was finished before the end of the experiment. This is also confirmed by the strong correlation observed between the “testosterone” detected by the EIA in the wing and jugular veins. In control subjects there was no such correlation, suggesting that the concentrations of real testosterone is regulated differentially across these two veins and it is another compound, likely ATD or one of its derivatives, that was present in proportional concentrations in these two compartments of the blood circulation. Admittedly these observations do not demonstrate that both drugs were present in the circulation but this is extremely likely based on previous literature.

The effectiveness of ATD and flutamide as aromatase inhibitor and androgen receptor blocker has been shown across a wide range of songbird species. The combination of these two drugs (administered subcutaneously and using the same or lower doses than the one we used) has been shown to inhibit sex hormone-dependent behaviors successfully in zebra finches (Adkins-Regan and Leung, 2006; Tomaszycki et

al., 2006), European stonechats (Canoine and Gwinner, 2002), spotted antbirds (Hau et al., 2000), rufous-collared sparrows (Small et al., 2015), dark-eyed juncos (Tonra et al., 2011), song sparrows (Soma et al., 1999) and great tits (Van Duyse et al., 2005). Many more studies have shown inhibition of behavior in songbirds by one of the two drugs (e.g. Sperry et al., 2010; Strand et al., 2008). This suggests enough homology in both the aromatase and androgen receptor genes between songbird species that it is likely these drugs work across the clade. The inhibition of aromatase by ATD has been quantified in zebra finch brain tissue (Wade et al., 1994) and the inhibition of sexual behavior by ATD in zebra finches has been shown to work specifically via inhibition of estrogen as the inhibited behaviors are restored by the co-administration of estradiol. Flutamide has been shown to decrease androgen receptor mRNA in canary kidney and testis, although the study found an upregulation of the transcript in the liver (Nastiuk and Clayton, 1994). The results of all studies cited in this paragraph thus give confidence that the pharmacological treatment used in the current study was effective and therefore that the singing behavior observed is indeed largely steroid-independent.

It must also be noted that the absence of effect on the cloacal protuberance and syrinx are not surprising since the testosterone levels were very low in both groups and indeed in these subjects, regardless of the drugs, the size of these organs did not differ from that found in castrated individuals but were significantly smaller than testosterone-treated individuals. The absence of a decrease in estradiol levels in the treated group is concerning but could be explained by a compensation mechanism.

Significance

One hypothesis explaining why some songbirds sing during the non-breeding season when sex hormone levels are low is that androgen and estrogen receptors and/or the aromatase enzyme are upregulated, making the song control nuclei more sensitive to sex steroid hormone action. This idea is supported by data showing in canaries a higher expression of ERα during autumn than spring (Fusani et al., 2000), likely due to estrogen having a negative feedback effect on its own receptor (Lauber et al., 1991; Lisciotto and Morrell, 1993). Additionally, a study on spotted antbirds, a tropical suboscine that displays territoriality year-round, found during the non-breeding season a higher expression of

ERα in POM, of AR in nucleus taeniae and a tendency towards higher expression of aromatase in nucleus taeniae, compared to non-breeding season (Canoine et al., 2006). However, the results of the current study do not support this hypothesis because blocking androgen receptors and aromatase activity left the singing behavior observed in photosensitive male canaries largely intact: there was only a slight and transient decrease in the number of songs detected per recording session. Our data suggest instead that singing behavior can be activated largely independently of sex steroid hormone action, possibly via activation of other neurochemical systems that have been shown to play a role in singing behavior such as catecholamines (Appeltants et al., 2003; Lynch et al., 2008; Maney and Ball, 2003), nonapeptides (Goodson et al., 1999; Voorhuis et al., 1991) or opioids (Kelm-Nelson et al., 2013; Riters et al., 2014). Under normal photoperiodic conditions all of these systems are regulated by testosterone, but it is conceivable that steroid-independent photoperiodic cues or other unidentifed cues can similarly regulate this signalling.

Contrary to the present findings, Soma and colleagues (1999) reported that songs produced in response to a simulated territorial intrusion by wild song sparrows decrease in rate after 30 days of treatment with ATD+FLUT, using the same doses of the two drugs as done here. The same results were obtained both during autumn and winter, and during both periods the song sparrows had low levels of circulating testosterone, similar to the subjects in the current study. The divergence of findings suggests that short-day singing in song sparrows is based on a different mechanism from the singing triggered by long-term exposure to short-days in castrated canaries. Indeed in the studies of Soma and colleagues, the song sparrows were found to have high circulating concentrations of DHEA that was metabolized in the brain into testosterone and estradiol, two sex steroids that were responsible for the activation of singing. In contrast, the GC/MS assays performed here did not detect measurable amounts of DHEA in the circulation of the canaries of the current study.

After four months of exposure to a short-day photoperiod, the majority of which (over three months) was in the absence of testes, male Fife canaries started to sing at high rates. The song activation was clearly not based on any photoperiodic cues, however

it is not clear if it was triggered by an internal or an external stimulus. It might be relevant to mention that these canaries started to sing in February which is the very beginning of the season for reproduction in Belgium. Although the birds could not technically detect the increasing daylength outside the laboratory since the photoperiod was fixed to 8L:16D in their housing that has no window, it is possible that they detected subtle changes in some other seasonal cue. Although the room was maintained at a more or less constant temperature, the outside temperature does partially influence the internal temperature and other cues such as the humidity might change seasonally. Independent of whether the cue that triggered song activation was internal or external, the fact that only a subset of birds started to sing indicates that there is individual variation in the ability to detect or react to this cue and it would be very important to identify the basis of this individual variation.

Supplementary figures

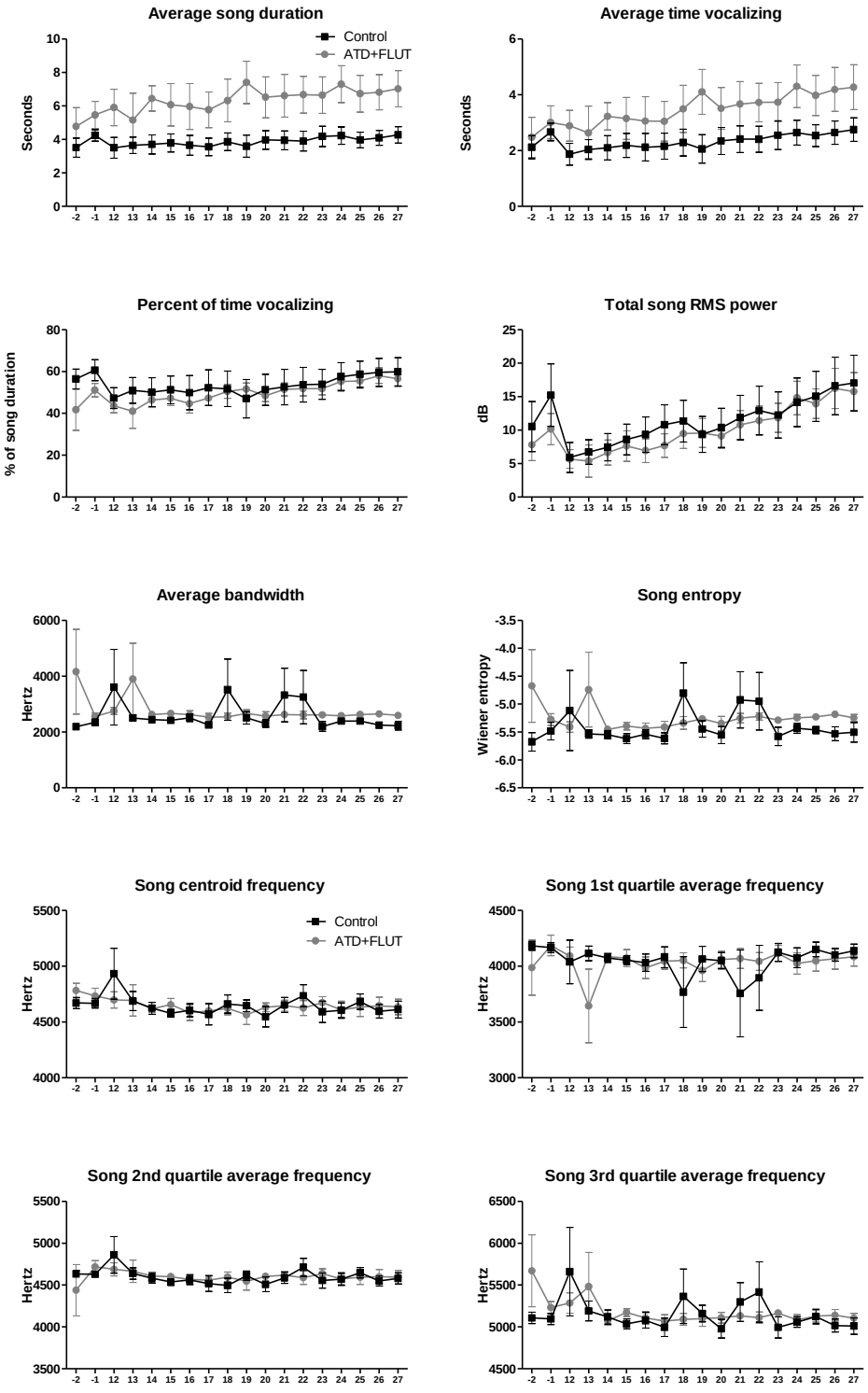

Figure S1. Evolution over time of other song parameters measured in song recordings of control and treated castrated male canaries maintained on 8D:16L for 5 months.

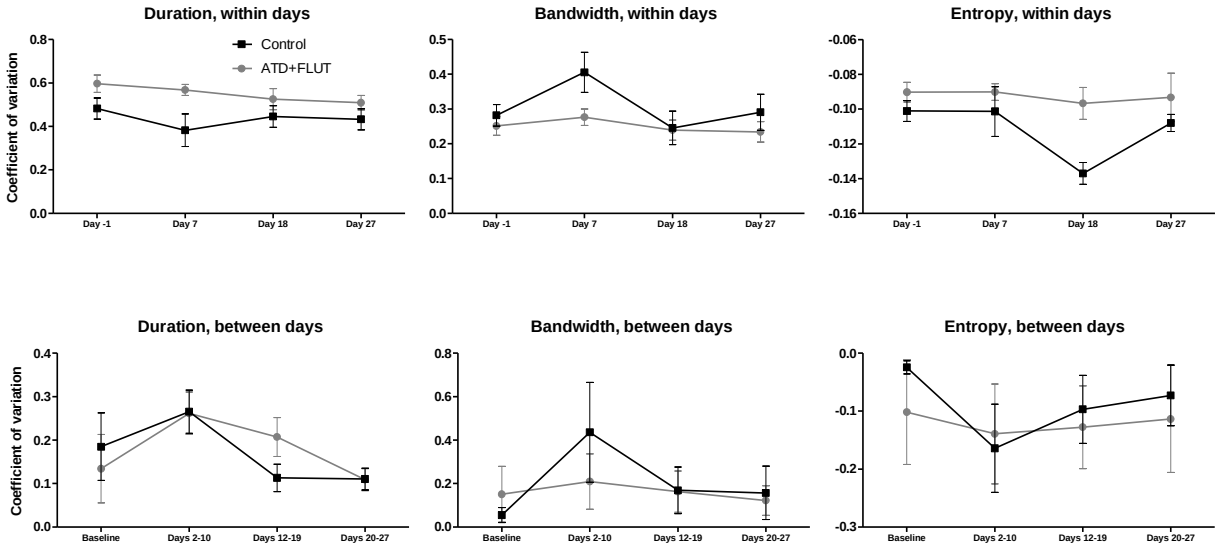

Figure S2. Coefficient of variation within days (top panel) and across days (bottom panel) in the duration, bandwidth and entropy of songs recorded from control and treated groups.

Chapter 7. Characterization of the responses to changes in photoperiod in the Fife fancy canary strain

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Key words: canary; molting, photoperiodism; songbirds; intra-species differences

Abstract

Seasonal birds in the temperate zone initiate breeding in response to photostimulation and most species cease to respond to long days at the end of breeding season, entering a photorefractory phase. Short daylengths dissipate photorefractoriness, inducing the ability to respond again to increasing photoperiod with gonadal growth. Some species reveal their photorefractoriness only when the daylength starts to decrease; the same daylength that initially stimulated gonadal growth now causes gonadal regression. They are said to display relative photorefractoriness. Other species in contrast undergo absolute photorefractoriness, they regress their gonads even under increasing photoperiods. Border canaries are clearly photoperiodic and show absolute photorefractoriness while American Singer canaries seem to have partially lost these photoperiodic responses. The photoperiodic responses of the Fife fancy strain of canaries have not been previously investigated and this information is important to properly design experiments on the seasonal control of singing behavior and its underlying neuroplasticity. Male and female Fife canaries were maintained on long-day photoperiod while regularly evaluating ovarian growth in females and molting score, cloacal protuberance (CP) length and body mass in both sexes. In both males and females body mass and molting score increased over time while CP length decreased, however molting was not complete after 5 months of photostimulation. Females had a larger body mass initially, shorter CP always and progressed through molting at an even pace while in males molting score increased in a step-wise fashion, lagging behind females at some time-points. In addition, females displayed robust ovarian growth after three weeks of photostimulation. Overall, these data suggest that Fife canaries are photoperiodic as evidenced by measures of gonadal development but the molting response suggest that they only display relative photorefactoriness.

Introduction

Seasonal songbirds such as canaries use day-length as a predictive cue to anticipate the onset of the forthcoming breeding season and adjust their physiology accordingly. Annual events such as breeding and molting are associated with substantial metabolic costs and therefore must be precisely timed to optimize fitness. Feathers are necessary for flight as well as insulation, and therefore for survival. Since feathers wear out, they need to be replaced each year which, like reproduction, imposes significant energetic demands (Farner et al., 1983). Both reproduction and molt are timed to occur during the period of food abundance, molting usually starting immediately after breeding (Dawson et al., 2001).

Increasing daylengths during spring stimulate the secretion of gonadotropin-releasing hormone (GnRH), which in turn leads to the gonadal maturation necessary for breeding (Dawson et al., 2002). Short days induce a decrease in GnRH secretion which leads to gonadal regression, but in many species this regression takes place before the end of the summer long day period. The same long photoperiod that stimulated the growth of the gonads with a certain delay now causes their regression through a process called photorefractoriness. During this photorefractory phase, even 24-hours of light cannot stimulate growth of the gonads (Hamner, 1968). The detail of the mechanisms controlling the onset of photorefractoriness under a photoperiod that was previously stimulating is yet unknown but clearly involves thyroid hormones (Dawson et al., 1986). Short-day photoperiods reinstates the sensitivity to light, the state of photosensitivity (Lofts and Coombs, 1965; Steel et al., 1975).

A subset of seasonally breeding birds, such as the Japanese quail, do not become photorefractory if continuously exposed to photostimulating long days but they will start gonadal regression under slightly decreasing photoperiods that are nevertheless largely longer than the daylength that initially initiated gonadal growth (Robinson and Follett, 1982). Since the daylength inducing this onset of regression is longer than the daylength initiating gonadal growth in the spring, the breeding season in these species is

asymmetrically distributed with respect to the annual changes of the photoperiod. They are said to show relative photorefractoriness, as opposed to absolute photorefractoriness.

Male and female canaries of the Border strain have small gonads when maintained on 8L:16D but exhibit a robust gonadal growth when transferred to 16L:8D (Storey and Nicholls, 1978). Furthermore, after 6 weeks of long-day photoperiod they show a spontaneous gonadal regression (Follett et al., 1973; Hurley et al., 2008; Storey and Nicholls, 1978, 1976), indicating that this strain of canary belongs to the group of photoperiodic seasonal breeders that shows absolute photorefractoriness. Border canaries initiate molting around the time that the gonads are regressing and complete molting about 5 weeks later. GnRH immunoreactivity in the preoptic area is high during photostimulation, low in photorefactory individuals and intermediate in photosensitive birds (Hurley et al., 2008).

In contrast, studies of wild canaries have demonstrated their flexibility in the onset of gonadal development that starts as early as 6 weeks prior to the annual time point when the daylength reaches 12L:12D, which is classically considered as the minimal stimulatory daylength (Leitner et al., 2003). This is likely because the dissipation of photorefractoriness by short-days leads by itself to an increased hypothalamic GnRH expression (Hurley et al., 2008); this puts the photosensitive individuals in a state of readiness to breed during which cues other than photoperiod also can trigger gonadal development.

Photostimulation of the American Singer strain of canaries has been shown to cause an increase of male gonads size followed by a regression, suggesting absolute photorefractoriness (Bentley et al., 2003). However, females of this strain did not show significant changes of gonad size when exposed to different photoperiodic conditions. Furthermore, both males and females showed no significant difference of the molt score across the three photoperiodic conditions, although the number of birds molting was significantly smaller in the photostimulated group than in the photosensitive or photorefractory groups. If molting occurred immediately following gonadal regression in this canary strain, the molt score would be expected to increase in photorefractory individuals only. Furthermore, unlike what was seen in Border canaries, hypothalamic

GnRH immunoreactivity was not different between photoperiodic states, suggesting that American Singer canaries might have become a relative rather than absolute photorefractory species or even have partially lost their aptitude to show photoperiodic responses in general, presumably due to prolonged domestication.

These two strains of canaries thus show divergent patterns of seasonal neuroplasticity related to hypothalamic GnRH expression, as well as of molting and female gonadal growth. These different photoperiodic responses could have implications for future investigations of neuroplasticity of the song control system, a major focus of our laboratory. Anecdotal evidence originating mainly from amateur breeders suggests that the Fife fancy canary strain, which we work with, is closely related to the Border strain of canaries, however no study has formally investigated whether the Fife fancy canary strain shows the robust photoperiodic responses that have been identified in Border canaries. We investigated here the effect of prolonged exposure to long-day photoperiods on photoregression in male and female Fife canaries by measuring molting, cloacal protuberance (CP) length and body mass, as well as the effect of photostimulation in females (the sex that seems less photoperiodic in American Singer canaries) on CP length, ovary mass and ovary area in order to evaluate photoperiodicity in this strain of canary.

Materials and methods

Experimental subjects

Thirty-nine female and thirty-two male canaries of the Fife fancy breed were used in the evaluation of photoregression and twenty-six females for the photostimulation study, all obtained from a colony maintained at the University of Antwerp, Belgium. They were born and had gone through a full breeding cycle in this colony, during this time they took part in experiments where behavior was measured, but did not receive any invasive manipulations. All subjects had been on natural daylight during the months preceding their arrival in our laboratory at the University of Liege, Belgium, in late March for the photoregression study and in January for the photostimulation study. All experimental procedures complied with Belgian laws concerning the Protection and Welfare of Animals and the Protection of Experimental Animals, and experimental protocols were approved

by the Ethics Committee for the Use of Animals at the University of Liege (Protocol number 926).

Photoregression study

Experimental procedures

Upon arrival (March 27th) subjects were housed on long day photoperiod (16L:8D) for five months to evaluate the subjects' transition through photoregression as measured by the molt score, body mass and CP length. During the first two months, the subjects were housed in an aviary with a large mixed-sex group. The first three measures of molt (see next paragraph for details), body mass and CP length were performed during this period. They were subsequently changed to single-sex housing - males were in cages with 5-6 individuals per cage and the females remained in the same aviary as before. The males and females were housed in separate rooms with no visual contact and minimal acoustic contact between them. Another 6 sets of measures were collected during this period. In all housing situations food, water, baths, cuttlebone and grit were available *ad libitum*.

Evaluation of molting

Molt was scored following the method described in Dawson and Newton (2004), which has previously been used to evaluate molt in canaries by Hurley and colleages (2008). The eight primary feathers on the subjects' right wing were evaluated for molting – these were identified as being the eight first feathers starting from the lateral side of the wing. Other feathers on the wings and rest of the body were also observed for molting but were not quantified. A score of 0 was given to a feather that had not yet fallen out, 1 for a new feather that was ¼ regrown, 2 for a half-regrown feather, 3 when it was ¾ regrown and 4 for a fully regrown new feather. A total molt score for each subject on a given day was calculated by summing the molt index of all eight feathers and dividing this total by 32 to provide a final score ranging from 0 (no molt) to 1 (complete molt).

Photostimulation study

Upon arrival subjects were housed on a short-day photoperiod (8L:14D) for at least 11 days in same-sex groups of 10 per cage. Since the subjects also took part in another study that investigated HVC neurogenesis, they were injected with Bromodeoxyuridine (BrdU) solution 5 times during the same day and received a subcutaneous 10 mm-long Silastic™ implant that was kept empty and sealed on both sides with medical silicone. Another group not discussed here was treated in parallel with implants filled with testosterone. 2-3 weeks after arrival the subjects were single-housed and the photoperiod was changed to 14L:10D. A blood sample was collected from their wing vein once during the short-day photoperiod (baseline) and once during the long-day photoperiod on the day of brain collection. CP length, ovary weight, length and width (multiplied to give ovary area) were also measured on the day of brain collection – 1, 2, 9 and 21 days after onset of long-day photoperiod. Brain collection and processing are not relevant to the current study and will not be described here.

Statistical analyses

In the photoregression study, the molt score, CP length and body mass were compared between males and females by a two-way repeated-measures ANOVA with sex and time as factors. When a significant interaction was found a planned-comparison post-hoc test was used to compare males and females with each other on specific days. Additionally, the molt scores of males and females on the final observation day were compared to the maximum score of 1 with a one-sample t-test, to test statistically whether the molting was complete in either sex. A simple t-test was used to compare the CP length on the last day of testing in the current experiment with the CP length of castrated males from other studies. In the photostimulation study, CP length, ovary mass and ovary area were compared by a one-way ANOVA with time-points as the main factor. When a significant effect was detected, Tukey's post-hoc tests were used to locate the significant differences. All statistical analyses were performed using STATISTICA and differences were considered significant for $p<0.05$. All data are represented here by their mean ± SEM.

Results

The thirty-nine female and thirty-two male canaries were inspected for progression of molting of the eight primary feathers a total of 9 times. The first three times (10/04, 24/04 and 11/05) no feathers had fallen out and therefore the molting score was zero for all birds. During this period all subjects were housed together in a mixed-sex group in a large aviary. Since no molting was observed on these days, these results were not included in any statistical tests.

The subjects were then separated by sex on 05/06 and were simultaneously inspected for molting. At this time a subset of subjects had initiated molting of the primary feathers. Starting from this molting test there was a gradual increase of the molt score, with both males and females progressing to about 40% of full molt in the three months up to the end of August (see Fig. 1A). A two-way ANOVA of the effect of time and sex on the molt score showed a significant effect of time ($F_{5,340} = 282.37$, $p < 0.001$). There was also a trend for sex to have an effect on molt score ($F_{1,340} = 3.18$, $p = 0.079$) and there was a significant interaction between these two factors ($F_{5,340} = 5.37$, $p < 0.001$). Planned-comparison tests showed that the molt score of females was significantly larger than in males (molt more advanced) on July 22nd ($p < 0.001$) and there was also a statistical trend towards on August 26th ($p = 0.074$). No other difference between sexes was observed. Note also that males and females did not complete their molt during the five months of photostimulation, as confirmed by a one sample t-test comparing the molt scores to the maximum possible value of 1 (females: $t_{38} = 27.08$, $p < 0.001$; males $t_{30} = 31.01$, $p < 0.001$).

A two-way ANOVA showed that the body mass changed in all subjects over time ($F_{8,536} = 37.18$, $p < 0.001$), slightly increasing from the beginning to the end of the observations (see Fig. 1B). There was no main effect of sex ($F_{1,536} = 0.43$, $p = 0.514$), however, sex and time interacted significantly in their effect on body mass ($F_{8,536} = 3.55$, $p < 0.001$). A planned-comparison test showed that females had a significantly higher body mass on April 10th and 24th and on 11th May whereas males had a significantly higher body mass on 19th June. Note that males and females were separated on 5th June and breeding stopped soon after this time-point.

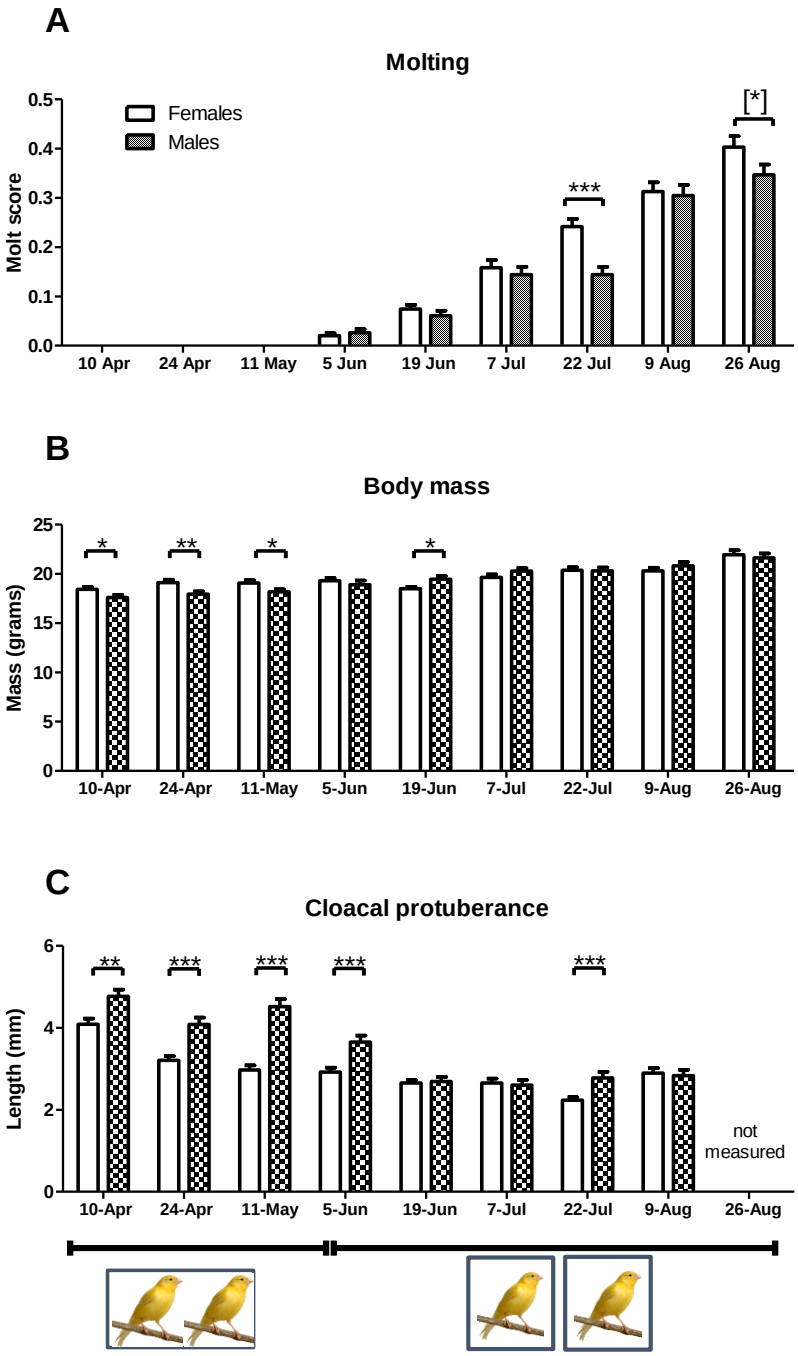

*Figure 1. Molt score (**A**), body mass (**B**) and CP length (**C**) in males and females maintained on 16L:8D photoperiod in a mixed-sex group for the first 2 months (represented by male and female canary inside a single box at the bottom of the figure) and single-sex groups for the following 3 months (represented by male and female canaries in individual boxes). * = p<0.05, **= p<0.01, ***= p<0.001, [*] = 0.5<p<0.1 for measures in females versus males measured on the same day. All data are represented here by their mean ± SEM.*

The length of the CP was significantly affected by time as confirmed by a two-way ANOVA ($F_{7,469} = 59.03$, $p < 0.001$), with an overall decrease being seen in both sexes over time (see Fig. 1C). The ANOVA also showed a significant sex difference ($F_{1,469} = 51.63$, $p < 0.001$), with an overall longer CP in males than females. There was also a significant interaction between these two factors ($F_{7,469} = 9.87$, $p < 0.001$). The post-hoc tests showed that CP length was longer in males than females on April 10th, and 24th, May 11th, June 5th and July 22nd. On all these days, the CP was longer in males than females. In general, during the 2nd half of the experiment the CP of males seemed to be fully regressed. We compared the CP length measured in males during the last observation of the current study with the CP length of castrated males from other studies (n= 21, mean ± SEM = 2.60 ± 0.17) and indeed, there was no significant difference between these two groups ($t_{49} = 1.08$, $p = 0.286$).

Photostimulation study

Photosensitive female Fife canaries were stimulated with 14L:10D photoperiod and CP length, ovary mass and ovary area were measured on days 1, 2, 9 and 21 of photostimulation (see Fig. 2). CP length was significantly affected by time, significantly increasing at later time-points ($F_{3,22} = 3.89$, $p = 0.023$). Post-hoc tests showed that the CP was significantly longer in females measured on day 21 than in those measured on day 2 of photostimulation. Ovary mass was also significantly increased over time ($F_{3,22} = 3.15$, $p = 0.046$). A post-hoc test showed that ovary mass was higher on day 21 than on day 1 of the treatment. Ovary area (length x width) was significantly different across time-points ($F_{3,22} = 10.48$, $p < 0.001$), with a post-hoc test indicating that on day 21 ovary area was larger than all other time-points, with no difference between the latter.

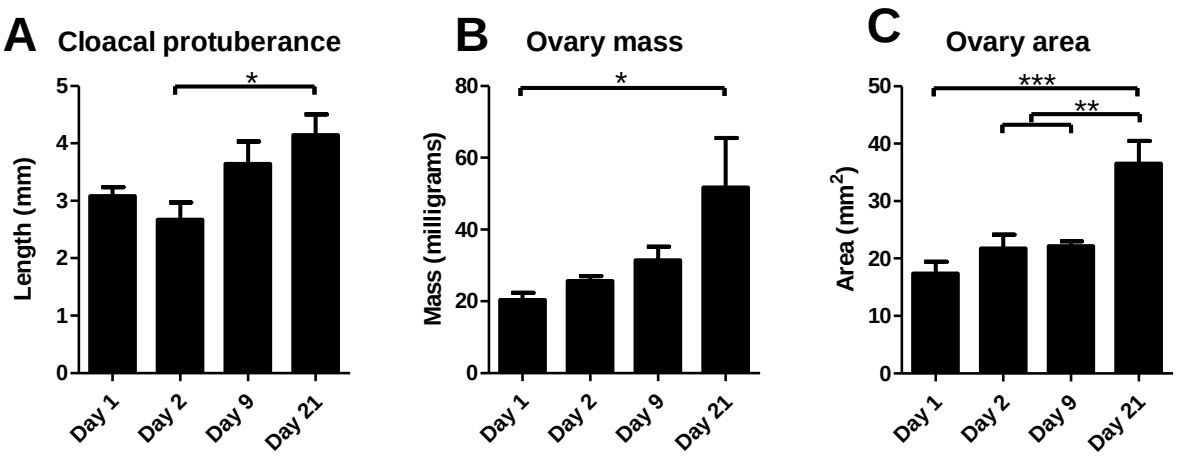

*Figure 2. CP length (**A**), ovary mass (**B**) and ovary area (**C**) in photosensitive female Fife canaries measured on day 1, 2, 9 and 21 of photostimulation. * = p<0.05, **= p<0.01, ***= p<0.001. All data are represented here by their mean ± SEM*

Discussion

The current study was designed to investigate whether Fife fancy canaries display robust photoperiodic responses like Border canaries (Hurley et al., 2008), or have partially lost this response, like American Singers canaries (Bentley et al., 2003). Measurements made over five months of photostimulation indicated that Fife fancy males show signs of gonadal regression (decreasing CP length) as expected in a photoperiodic species. In addition, exposure to long days progressively induces molting although this molt was still only partial after 5 months contrary to what is seen in birds showing absolute photorefractoriness. Additionally, photostimulation of females led to a robust growth of the ovaries after 3 weeks of long-day photoperiod.

The clearest indication of photoperiodism in the current study was the robust and relatively rapid increase in ovary mass and size in females. After three weeks of photostimulation there was a clear difference in the ovary size as compared to baseline values in short days. In American Singer female canaries follicular volume was not different during the photosensitive, photostimulated and photoregressed phases (Bentley et al., 2003). Although the two studies report different measures of female gonadal

changes, it seems likely that the gonadal growth is more responsive to photoperiodic changes in female Fife canaries than in American Singers.

The length of CP is sensitive to circulating androgens (Appeltants et al., 2003; Tramontin et al., 2003). During photostimulation and treatment with testosterone of male juncos the CP increases with a delay about one week (Tonra et al., 2011), although this has not been investigated, it is likely that the decrease of CP during photoregression also follows the decrease in testosterone concentrations (due to gonadal regression) with a delay. After 10 weeks of 16L:8D a decrease of CP length was observed here, which seemed to reach two weeks later its minimum size and stabilize to values that are not different from those observed in castrated male Fife canaries. The observations were presumably started too late to witness the increase of CP length in response to photostimulated gonadal growth, especially since the canaries were exposed to gradually increasing natural daylength before their arrival at our lab so that they had probably fully recrudesced gonads by that time. The pattern of CP length decrease observed while birds were being exposed to a stable long-day photoperiod is indicative of an absolute photorefractory response, not requiring any decrease in photoperiod to induce photoregression. Together with the robust ovarian growth observed in females, this supports the conclusion that Fife canaries are indeed photoperiodic. The length of CP in females was smaller than in males at all time-points as expected given the sex difference in androgen concentrations but it also decreased over the course of observations suggesting the development of photorefractoriness in this sex also. The slightly longer CPs observed in females at the start of the experiment was possibly related to the fact that they were egg-laying at that time and produced higher concentrations of sex steroids. Breeding and egg-laying then ended when males were removed from the females' aviary.

Both males and females started molting after approximately 10 weeks of 16L:8D and approximately at the same time as the males started to decrease CP length. This timing was consistent with what has been reported in Border canaries, who started molting after 11 weeks of 14L:10D (Hurley et al., 2008). CP size is a marker of testosterone concentrations but, as discussed, most probably its decrease lags by a few days or even weeks behind the decrease of circulating testosterone. Thus, molting started

probably after gonadal regression as would be expected in a photoperiodic species. More surprisingly, the pace of molting was much slower than what has been shown in Border canaries. Hurley and colleagues (Hurley et al., 2008) reported that molting was almost complete by 16 weeks of 14L:10D, whereas in the current study after 5 months of 16L:8D molting was less than halfway to being complete. Unfortunately, in this study the effect of decreasing the photoperiod on molting was not examined, but the prolonged duration of molting under a long-day photoperiod suggests that Fife canaries are possibly not showing the full signs of physiological regression under long days and might need a decreasing photoperiod to complete molting.

There was a sex difference in the pattern of molting, with females on average increasing their molt score in a steady fashion, while males showed a step-wise pattern of increases, with no increase for one month followed by a big increase on the next one, catching up to the molt score of the females. Due to these different patterns, the molt score was different between males and females on some days but not on others. This difference is possibly due to different hormonal influences in males and females. Molting is inhibited by both androgens and estrogens in canaries (Farner et al., 1983; Takewaki and Mori, 1944) and in other avian species it is also possibly modulated by progesterone (Adams, 1956; Juhn and Harris, 1956; Shaffner, 1955) and prolactin (Farner et al., 1983; Kobayashi, 1953), but the precise role of these hormones and how they influence the process of molting in males and females has not been studied.

Body mass was initially higher in females but this was reversed after the termination of breeding. In general body mass increased in both males and females over the five months of observation. Photostimulation has been shown to increase body mass in several avian species (Boon et al., 2000; Morton et al., 2004; Tonra et al., 2011). Therefore, the increase in body mass seen in both males and females in the current experiment might be another confirmation of the sensitivity to changes in photoperiod of Fife canaries. The social conditions likely did not induce the onset of either molting or the decrease in CP length as both seemed to have been initiated on 5th June which is the day when the males and females were separated. It can however not be excluded that the

separation of the birds from congeners of the other sex did in fact increase the rate of molting and CP regression thereafter.

In conclusion, the data presented here clearly demonstrates that Fife fancy canaries are photosensitive and also supports the idea that this breed displays absolute photorefractoriness as indicated by the induction of molt and decrease in CP reflecting inhibition of gonadal activity. Molting started after gonadal regression, however, three months later it was less than half-way to being complete. In nature if the molting would continue at this pace it would only be completed during the winter, which would not be adaptive. It thus appears that the full molt can only take place if the birds are exposed to a decreasing photoperiod, which would be a characteristic of species displaying relative and not absolute photorefractoriness. Taken together, these data suggest that Fife fancy canaries are photosensitive but display mixed signs of absolute and relative photorefractoriness.

General discussion

The results presented in this thesis enlarge our understanding about how sex hormones, social environment, photoperiod and sex of the bird modulate the plasticity of brain regions that control singing. Here we will discuss the contributions made by this thesis regarding the plasticity of song control volumes, HVC neurogenesis, POM plasticity and song behavior. Finally, we will summarize the knowledge gained specifically about sex differences, the role of photoperiod and some methodological concerns raised by the experiments presented in this thesis.

Song control system plasticity

Nuclei volumes

The vernal increase in testosterone influences many aspects of song control system neuroplasticity and in this thesis we have explored the time course of some of these changes. We investigated the increase in volume of three song control nuclei in female canaries (chapter 3) at four time-points following the onset of photostimulation and testosterone treatment compared to photostimulated but untreated females. The volume of HVC in the treated females was increased relative to controls at 21 days of treatment, but there was a trend for an increase already at 9 days. For RA and Area X there was no trend at 9 days, but at 21 days of treatment the testosterone-treated birds had significantly larger volumes. Thus, we confirmed in canaries the delayed increase of the volumes of RA and Area X relative to HVC that had been shown in song sparrows (Smith et al., 1997). Testosterone has a trans-synaptic trophic effect from HVC to Area X and RA: lesioning HVC in testosterone-implanted sparrows prevents the increase of RA and Area X volumes, while implanting testosterone stereotaxically near HVC, but not near RA, increases the volume of RA and Area X (Brenowitz and Lent, 2002, 2001). Our study suggests that the mechanism of RA and Area X volume growth in female canaries is likely also to involve a trans-synaptic effect of testosterone and/or estradiol acting in HVC. On the other hand, compared to what has been shown in male Gambel's white-crowned sparrows, the progression of HVC growth is different in female canaries. In the former, HVC volume is already at the maximum size by 7 days of treatment, while our study showed that although HVC had started to grow by 9 days of treatment, there was a further

increase between this timepoint and 21 days. Based on our experiment, we cannot know whether three weeks is enough for the female HVC volume to increase to its maximum extent.

Although we were not able to investigate the time course of song control volume changes in male canaries due to their unexpected singing behaviour (chapter 6), we showed that two days of photostimulation and testosterone treatment is not sufficient to increase the volume of HVC above the level of photostimulated control males (chapter 4). In contrast, the transfer of male white-crowned sparrows to a short-day photoperiod and removal of their testosterone implant significantly decrease HVC volume after only one day (Thompson et al., 2007). Most likely the divergence of time-scales reflects the differential mechanisms involved: a decrease of inter-neuronal space followed later by a decrease of neuron number for the regression, while the addition of new neurons might be necessary for HVC growth. Species differences might however also contribute to explain this discrepancy.

In chapter 1 we showed that when exposed to an intermediately short photoperiod males housed with another male, a female or alone do not have a different volume of HVC, in contrast to what had been demonstrated in male canaries housed in a long-day photoperiod (Boseret et al., 2006). In that study the males housed with a female had a larger HVC volume than males housed with another male. Another study has housed male canaries alone or with a female on long-day photoperiod and found no significant difference in their HVC maximum areas (Alward et al., 2014).

The importance of the photoperiod for song control nuclei plasticity has also been demonstrated in chapter 6. Castrated male canaries housed on a short-day photoperiod for five months who had started singing profusely had larger HVC volumes than photostimulated testosterone-implanted male canaries singing at similar rates that had not been maintained on a short-day photoperiod for a similarly long period. This suggests that sex steroid hormones is not the single factor controlling the growth of song control nuclei. This conclusion is further supported by a lack of decrease of HVC volume in subjects treated with a combination of an androgen antagonist and an aromatase inhibitor. This result is similar to what has been shown in European starlings:

photostimulated males treated with the same two drugs did not have a significantly decreased volume of HVC, RA or Area X (Robertson et al., 2014). Since the male canaries in chapter 6 had been actively singing for at least one month, it is possible that the large volume of their HVCs was due to activity-driven feedback of singing on HVC neurogenesis, but the causality of these two observations cannot be established from our study. The question of activity-dependence of song control nuclei growth was also addressed in chapter 5, however the low number of subjects and low hit rate of testosterone implants in POM prevented us from reaching any conclusion.

HVC neurogenesis

An important form of plasticity in HVC is the replacement of neurons. The addition of new neurons mediates the increase in volume of this nucleus and their survival is increased by testosterone. In this thesis, the time course of changes during testosterone treatment was investigated in female canaries (chapter 3). The time course allowed us not only to analyze the effect of testosterone but also map in more detail the sequence of events in the early life of new neurons. From day 1 to day 2 after mitosis, we observed a slight increase of BrdU-labeled cells in the VZ, likely indicating divisions between these two time points. Subsequently, from day 2 to day 21 there was a decrease in the number of BrdU cells in the VZ and a concomitant increase inside HVC. The decrease of BrdU-labeled cells in the VZ reflects a combination of migration away from VZ and loss of BrdU label over time due to multiple divisions. The first BrdU-labeled cells appeared in HVC 1 day after mitosis, however the appearance of double-labeled BrdU-doublecortin neurons followed one day later. This suggests that there is a non-neuronal population of newborn cells that migrates more rapidly to HVC than newborn neurons or perhaps is even born inside HVC. Alternatively, it could be that doublecortin is only expressed two days after mitosis. Comparing the numbers of single and double-labeled cells suggests that the majority of newborn cells entering HVC are neurons and the non-neuronal cells do not increase over time, as has been also shown by Alvarez-Buylla and Nottebohm (1988). A decrease from day 9 to day 21 of fusiform DCX neurons while the number of round DCX neurons increases in this period is consistent with a transition of newborn neurons from one morphology to the other.

Testosterone treatment had no effect on number of proliferating cells, confirming what has been previously shown (Barker et al., 2014; Brown et al., 1993; Rasika et al., 1994). The recruitment of DCX neurons born one day before the treatment (labelled by BrdU) into HVC was increased by testosterone at all time-points from 2 days of treatment onwards, as was also the total fusiform neurons in HVC. However, testosterone did not affect the density of round DCX neurons. Yamamura and colleagues (Yamamura et al., 2011) also report an effect of three weeks of testosterone on fusiform but not round DCX neurons in the HVC of female canaries, while in males an effect of testosterone has been shown on both morphologies of DCX after four weeks (Balthazart et al., 2008). One explanation of the discrepancy is that newborn neurons mature more slowly in the female HVC; therefore the majority of round DCX neurons even at 3 weeks were born before the testosterone treatment and their survival was not affected by it. A positive effect of testosterone on the number of fusiform DCX neurons at time points as early as 2 days suggests that testosterone and its metabolites protect newborn neurons from cell death not only during their integration and differentiation in HVC, as has been shown previously (Rasika et al., 1994), but also during the early migration phases. In this early phase testosterone could be acting via pathways involving n-Cadherin (Barami et al., 1994), NgCAM (Williams et al., 1999) and IGF-1 (Jiang et al., 1998) which have been shown to regulate early newborn neuron migration in the songbird brain.

In chapter 1 we also identified an increased density of both fusiform and round DCX neurons in females as compared to males in an equivalent social context, a finding that has also been reported in starlings (Hall and MacDougall-Shackleton, 2012), brown-headed cowbirds and red-winged blackbirds (Guigueno et al., 2016). This observation is in line with the hypothesis that newborn neurons in the female HVC develop more slowly than in the male HVC. Females also had a higher density of older newborn neurons (BrdU-labelled, not cross-reacting with EdU) and a higher ratio of older newborn neurons to newer newborn neurons in the VZ and a trend toward the same pattern in HVC. On the other hand, the newer newborn neurons (EdU-labelled) were fewer in the female VZ than in the male. Overall, the data suggests that in females the newborn neurons seem to survive longer than in males.

Analyzing the effect of social context on HVC neurogenesis in males we observed that the younger newborn neuron (EdU-labeled) were increased in males that were housed with another male more than in those housed with a female. The same pattern was also seen in older newborn neurons (BrdU-labeled not cross-reacting with EdU), although it did not reach significance in that case. The ratio of older to younger newborn neurons was higher in males housed alone than males housed with a female. Finally, the total number of fusiform DCX neurons per HVC was lower in males housed with a female than in those housed with another male. Overall, our data suggest that both older and newer newborn neurons survived less in the males housed with a female. This result is unexpected given that previous studies have shown that males housed with a female have bigger HVCs (Boseret et al., 2006) and a higher density of round DCX neurons (Alward et al., 2014). The males housed with a female did not have a higher concentration of corticosterone during the treatment, therefore differential levels of stress do not explain this result. This group did have higher baseline levels of corticosterone compared to females, but this was not significantly different from the other male groups. This surprising result could be due to the different photoperiod used in this experiment compared to the two previous studies.

POM plasticity

The medial preoptic nucleus (POM) is a critical brain area controlling appetitive sexual behaviors (Balthazart and Ball, 2007; Panzica et al., 1996), including courtship song (reviewed in Alward et al., 2017). Lesions of POM in male starlings reduce the song output during the breeding season when male song often serves the purposes of attracting a female (Alger and Riters, 2006; Riters and Ball, 1999). The high density of androgen and estrogen receptors in this region (Bernard et al., 1999) suggested that testosterone could be acting in POM to modulate the song rate. Indeed after 7 days of treatment, testosterone implants into POM increase the rate of singing to the same level as systemic testosterone treatment in male canaries, while the quality of song remains unchanged (Alward et al., 2016c; Alward et al., 2013). We have investigated the time course of POM plasticity following testosterone treatment in order to confirm its role in song motivation and increase our understanding of the testosterone-induced changes that could mediate the increase in singing. Previous studies have shown that song rate increases within 5-6

days of treatment with exogenous testosterone (Madison et al., 2015), therefore the changes in POM would be expected to take place earlier than this time-point. In chapter 3 we showed that photostimulated females treated with testosterone had an increased volume of POM relative to photostimulated untreated controls after a single day of treatment; this volume peaked at 2 days of treatment and then remained stable for the rest of the experiment (until day 21). The POM volume of control photostimulated females did not increase over this time-period and always remained significantly lower than in the treated group. This rapid time course of POM volume growth in response to testosterone is in line with the role of POM in activating song behavior.

In males only one time point was investigated, but it was confirmed that as was seen in female canaries two days of testosterone-treatment is sufficient to significantly increase the volume of POM above the level observed in similarly castrated, photostimulated but untreated control male canaries (chapter 4). A rapid testosterone-induced increase of POM volume after just one day of treatment and before the activation of sexual behavior by the steroid, has also been demonstrated in male quail (Charlier et al., 2008). It is likely that the increase of POM volume in male canaries would be also evident following just one day of treatment, although this remains to be tested in future experiments. Further support for the role of POM in song motivation is provided by observing that the only control male canary that had been singing at high rates prior to the experiment, had a somewhat increased POM volume relative to the rest of the control group, although not as large as in testosterone-treated subjects (graph 7 in chapter 4). This suggests that testosterone and singing could have additive effects on POM volume, but further research should address this question more comprehensively.

Interestingly, although testosterone increased POM volume in both sexes, the magnitude of the increase was larger in males (chapter 4). Testosterone-treated males had twice larger POM volumes than testosterone-treated females, while in untreated subjects the two sexes did not differ. In Japanese quail the volume of the POM is larger in sexually mature males than in sexually mature females, it is increased by testosterone in gonadectomized birds however there is no sex difference in POM volume when males and females are exposed to the same concentration of testosterone (Panzica et al., 2001,

1996, 1987). We showed here that in canaries the sex difference in POM volume is apparently maintained despite equal concentrations of testosterone (see figure 4 in chapter 4). However, this sex difference should be confirmed in a future study that would include both testosterone-treated males and females within the same experiment.

Additionally, we investigated the changes in the number of neurons expressing aromatase, their somal area and the percentage of POM area covered by aromatase-immunoreactivity (ARO-ir). In females, the number of ARO-ir neurons and the percent area covered by ARO-ir material increased more dramatically than the POM volume, especially in the earlier time-points. In males, the magnitude of increase of the three measures was more similar, probably due to the higher magnitude of the increase of POM volume compared to females. Comparing between the male and female data for the day 2 time point also suggests that in males the increase of ARO-ir neuron somal area is more rapid than in females. These observations provide clues suggesting that the mechanism of POM volume growth is different between the sexes, which could account for the sex difference in POM volume seen in testosterone-treated individuals.

Although we showed that testosterone has a powerful effect on POM plasticity, chapter 6 presents some tentative evidence that sex steroid hormones are not absolutely necessary for POM growth accompanied by an increase in song rate. Castrated male canaries maintained on a short-day photoperiod had basal levels of testosterone, but spontaneously started to sing at high rates. Using a standardized method, we compared the POM volume of these subjects with those of subjects that had been under the same conditions but did not sing. The high-singing low-testosterone subjects had POM volumes significantly larger than low-singing control subjects (with similarly low levels of testosterone) and POM volumes not significantly different from testosterone-treated low-singing subjects (figure 4, chapter 4). Furthermore, when some of the high-singing low-testosterone subjects were treated with an inhibitor of aromatase and antagonist of the androgen receptor for 28 days, the POM volumes did not significantly decrease compared to untreated controls. Similarly, there was no decrease of number of ARO-ir neurons, their somal area or percent of area covered by ARO-immunoreactivity. These data suggest a steroid independent mechanism of POM volume growth and a correlation between

singing rates and POM volume, but cannot address the question of causality – did singing increase POM volumes or did the canaries start to sing because their POM volumes had increased due to another mechanism?

Song behavior

In addition to a relatively detailed examination of the effect of social and hormonal modulation of song system neuroplasticity, some of the experiments reported in this thesis also investigated the effect of the same factors on song behavior itself. In chapter 1 we confirmed that male canaries housed with a female display a markedly decreased rate of singing compared to isolated males (Alward et al., 2014) and compared to males housed with another male (Boseret et al., 2006). In our study, all three conditions were compared simultaneously, which showed that the isolated males sing at the highest frequency, significantly more than males with a female, while males housed with another male are intermediate in song rate, not differing significantly from either isolated males or males with a female. The male-male groups showed the highest variability in song rate, suggesting that the more complex social dynamics in these dyads make it more difficult to detect a clear effect of this social context on song rate. In chapter 2, two small-scale studies attempted to address this question. A mirror was placed in the cage of the experimental males but not the control males and the subjects likely perceived their reflection as another bird living in the same environment (Buckley et al., 2017; Henry et al., 2008). The first study in chapter 2 suggested that a mirror suppresses singing in males maintained on a short-day photoperiod, however a follow-up study conducted on long-day photoperiod did not replicate this result.

In chapter 6 we showed that prolonged exposure of castrated male canaries to a short-day photoperiod caused an activation of singing in a subset of birds despite basal levels of testosterone. This singing was not blocked by antagonizing androgen receptors and inhibiting aromatase, although the song rate was transiently decreased by the treatment. Despite the difference on some days in the number of songs between the groups, the total song duration did not differ on any day between the groups, likely because the treated birds sang slightly longer average songs with a greater number of syllables per song. Although many parameters of song quality were assessed, no effect

of the treatment was seen on any of these measures. Several indirect indices suggested that the treatment was effective for the whole duration of the experiment, therefore we tentatively conclude that singing in castrated canaries induced by long-term exposure to short-days is largely sex steroid hormone-independent.

The experiment reported in chapter 4 showed that in photostimulated visually and acoustically isolated male canaries two days of testosterone-treatment is not sufficient to activate singing, the only subject that sang in this period was already singing before the testosterone treatment. In chapter 5 photostimulated males systemically treated with testosterone started to sing after 4-5 days, while the subject who was successfully implanted with testosterone in POM started singing on day 6 of the experiment. These latencies were similar to what was reported by Alward and colleagues (Alward et al., 2013). In subjects who had testosterone-implants close to and likely leaking into a ventricle, song activation took on average 10 days. The slight delay in the ventricle-implanted birds relative to POM-implanted birds could be due to the potentially lower concentration of testosterone reaching POM in the former group. One subject in the 25-day brain collection group with a testosterone implant touching the ventricle did not start singing, however the implant was touching the lateral ventricle, from where testosterone is less likely to reach the POM, while the other subjects had the implants close to either the cerebral aqueduct or the subarachnoid space. Although the low sample size in all groups prevents us from concluding anything, these data suggest that song activation timing depends on the concentrations of testosterone reaching and acting in POM.

Sex differences

Songbirds exhibit some of the most extreme sex differences in brain and behavior of all vertebrates (MacDougall-Shackleton and Ball, 1999). In this thesis sex differences have been addressed wherever possible. As discussed earlier, chapter 1 demonstrated that females have a higher density of doublecortin-positive newborn neurons in HVC, a higher proportion of older newborn neurons than newer newborn neurons surviving and fewer proliferating cells labeled 10 days earlier in the ventricular zone than males. Chapter 3 does not compare males and females directly, however juxtaposition of the data reported in this chapter with previous studies suggests that newborn neurons could be developing

more slowly in the HVC of female canaries than in the male HVC. In chapter 4, a comparison of POM volume growth in males is made with the equivalent data in chapter 3, showing that despite equivalent concentrations of plasma testosterone, treated males have twice larger POM volumes than females. In chapter 7, a sex difference is shown in cloacal protuberance length (M>F) and body mass (F>M) in photostimulated condition, but not in photoregressed condition. Furthermore, the extent of molting is different between sexes at some time-points. A careful examination of the molting patterns shows that while in females the molt score increases over time in a gradual fashion, the molt score of males increases in a step-wise fashion, with periods of no change followed by an increase of a bigger magnitude, causing the molt score of males and females to diverge and converge at different times. Together these data indicate that even when placed in similar endocrine conditions male and female canaries still differ in a number of traits suggesting that either these traits are organized by early hormone action or they depend on intrinsic genetic differences. This topic could be the subject of exciting additional research.

Photoperiod

Many effects of season on song behavior and neuroplasticity are mediated by changes in sex steroid hormones, however some sex hormone-independent photoperiodic effects have also been reported (Bernard et al., 1997; Bernard and Ball, 1997; Robertson et al., 2014). Although not directly assessing the effect of photoperiod, the results reported in chapter 1 when compared to previous studies suggests the existence testosterone-independent effects of photoperiod. While we report no difference in HVC volumes between social conditions and a decreased rate of survival of older newborn neurons in males housed with a female compared to males housed with a male (figure 7 in chapter 1), Boseret and colleagues (2006) showed that HVC volumes were larger in males housed with a female. An important difference between our study and that of Boseret is the photoperiod the birds were exposed to, suggesting that the social effect on HVC plasticity could be gated by photoperiod.

In chapter 6 we report photoperiod-induced, sex hormone-independent effects on song behavior and song system neuroplasticity. Additionally, we also present data

concerning photoperiodic effects that are most likely sex hormone-dependent. In chapter 3 we used one-way repeated measures ANOVA to probe for photostimulation-induced changes in intact female canaries. Although no changes in the neuroplasticity were evident, the photostimulation caused an increase in ovary mass, cloacal protuberance and syrinx mass. Interestingly, there was also a transient increase in testosterone concentrations in intact untreated females one day after onset of long-day photoperiod, with a significant difference in the concentration on this day compared to day 21 of photostimulation.

In chapter 7 we investigated the process of photoregression in male and female canaries by exposing them to long-day photoperiod for five months. After approximately two months both males and females started molting and around the same time the length of the cloacal protuberance started to decrease in males. Over the five months there was a progressive increase in body weight in both sexes. All three measures indicate that the long-day photoperiod had induced a transition into photoregression, suggesting that a decrease in daylength is not necessary for this strain of canaries to photoregress. The reduction in cloacal protuberance length in males was completed by approximately 11 weeks of photostimulation. At this time point the length of male cloacal protuberance was not significantly different from castrated male canaries from a different experiment and after this there was no further decrease. On the other hand, the molting in both males and females was not complete after the five months of photostimulation. In Border canaries molting takes approximately 11 weeks (Hurley et al., 2008) so that this extended period of molting identified in our study suggests that the molting response of Fife canaries reflects a relative rather than absolute photorefractoriness: full molt probably requires a decrease of photoperiod to be completed, although this was not directly investigated.

Methodological concerns

In addition to theoretical contributions to the field of songbird neuroendocrinology and neuroplasticity, this thesis has addressed multiple methodological issues. In chapter 1 we warned about the potential problems associated with the use of 5-ethynyl-2'-deoxyuridine (EdU) as a marker of proliferation. At the time when we conducted the experiment it was not well known that EdU can be toxic to the organism at longer survival periods. During

the experiment, we noticed that in the days following EdU injection, the injected birds were not looking healthy: their feathers were puffed up and some were sleeping with the head turned around in the night sleeping position. Around the time when our experiment ended a direct confirmation of the toxicity of EdU to cells that have incorporated this thymidine analog was published – the majority of cells that incorporate EdU undergo apoptosis within 72 hours (Ponti et al., 2013). In our study the organismal symptoms disappeared after 1–2 days but the cellular toxicity might have remained. This marker should thus be used during *in vivo* studies only when the tissue of interest will be collected within 24 hours after injection. Furthermore, our data suggest that the cross-reactivity of the BrdU antibody with EdU is dose-dependent. When the brain is collected 24 hours after exposure to EdU, most cells that have incorporated EdU are still surviving, including the ones with a high concentration of EdU. Under these conditions, the cross-reactivity of BrdU antibody was 87.6%. However, when the EdU was injected further away in time from the time of brain collection, most of the cells with a high concentration of EdU had presumably died but some cells that had incorporated less EdU were probably still surviving. The cross-reactivity under these conditions was on average only 43.7% in the VZ and 42.8% in HVC. This dose-dependent cross-reactivity should be kept in mind when EdU and BrdU are both injected to study multiple populations of proliferating cells.

In chapter 3 testosterone was administered subcutaneously to female canaries in Silastic™ implants. This method of hormone administration was chosen because it has been shown to induce stable concentrations of steroid hormones over a long duration in multiple avian species (Desjardin and Turek, 1977; Turek et al., 1976). In our study, we assayed plasma testosterone at 1, 2, 9 and 21 days of treatment and observed a sharp decrease in the concentration from the first day of treatment to day 2 and a further decrease until day 21. A similar pattern of decreasing testosterone concentrations in female canaries implanted with Silastic™ implants over time has been shown (Fusani, 2008; Ko et al., 2015), however it remains unclear whether this phenomenon also happens in male individuals and in other species; this should be investigated in future studies.

When assaying by a testosterone enzyme immunoassay (EIA, Cayman Chemical) the plasma of individuals treated systemically with the steroidal aromatase inhibitor ATD and non-steroidal androgen receptor antagonist flutamide, extraordinarily high concentrations of 'testosterone' were detected. However, an assay of the same samples with gas chromatography/mass spectrometry showed basal concentrations of testosterone, suggesting a cross-reactivity of the EIA with an unknown compound in the plasma of treated subjects. A direct test of cross-reactivity of ATD and flutamide with the EIA showed only a modest cross-reactivity with ATD and none with flutamide, suggesting that at least a part of the 'testosterone' signal given by the EIA should be a metabolite that somehow results from this treatment, rather than one of these two pharmacological agents. This methodological issue concerns multiple published articles that have reported high concentrations of 'testosterone' following treatment with the same two drugs (Moore et al., 2004; Small et al., 2015; Van Duyse et al., 2005) and should be investigated further.

Finally, this thesis reports the validation and results generated by a new canary song analysis software (chapter 6). Software for analyzing zebra finch song has been developed and used extensively for over a decade (Tchernichovski et al., 2000). However, the song of other songbird species, including canary song, is significantly more complex and up to now has been analyzed either completely manually (for example Leitner et al., 2001a) or in a semi-automated manner: the experimenter pre-selects sections of recordings that represent songs and uses a software to analyze specific parameters of these recording segments (Alward et al., 2013; Madison et al., 2015). In chapter 6 we show some validations of a program for analyzing canary song in a fully automated way, developed by our collaborators at the University of Maryland. We report a strong correlation between the number of songs detected by the software and manually by visual inspection of the spectrogram ($r^2 = 0.98$), indicating that the software is correctly detecting songs. Furthermore, we used this software to analyze a large quantity of song recordings, which would take weeks of the experimenters' time to analyze manually. From this analysis, we were able to show that treatment of male canaries maintained for five months on short-day photoperiod with an aromatase inhibitor and androgen receptor blocker transiently reduces song rate, possibly increases the average number of syllables per song, but does not affect other song parameters. Further validations of the software

are underway but will not be reported in this thesis. Once fully validated, the software can be used to reliably and automatically analyze canary song with minimal time-expenditure on the part of the experimenters.

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