Molecular profiling of sex-specific development of song and the song control nucleus HVC of songbirds

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Abbreviations

17β-HSD	Protein symbol of the 17β-hydroxysteroid dehydrogenase
3α-HSD	Protein symbol of 3α-hydroxysteroid dehydrogenase
3β-HSD	Protein symbol of 3-β-hydroxysteroid dehydrogenase
5α-DHT	5α-dihydrotestosterone
5β-DHT	5β-dihydrotestosterone
AF-1	Activation function 1
AKR	Aldo-keto reductases
AKR1C	Gene symbol of 3α-hydroxysteroid dehydrogenase
AKR1D1	Gene symbol of 5β-reductase
AR	Gene symbol of the androgen receptor
AR	Protein symbol of the androgen receptor
ARE	Androgen response element
ATAD3	Protein symbol of ATPase family AAA domain containing 3A
ATAD3A	Gene symbol of ATPase family AAA domain containing 3A
BDNF	Brain derived neurotrophic factor
CBf	Cordon bleu female
CBG	Protein symbol of corticosteroid-binding globulin
CBm	Cordon bleu male
Cf	Breeding canary female
Cf(iso, NB)	Non-breeding canary female, social isolated
Cf(NB)	Non-breeding canary female
CfS	Non-breeding spontaneously singing female canary
ChIP	Chromatin immunoprecipitation
ChIP-chip	Chromatin immunoprecipitation DNA microarray
ChIP-Seq	Chromatin immunoprecipitation sequencing
Cm	Breeding canary male
Cm(NB)	Non-breeding canary male
COMT	Protein symbol of catechol o-methyl transferase
CMM	Caudal mesopallium
CNS	Central nervous system
CYP	Cytochrome p450 enzymes
CYP11A1	Gene symbol of cytochrome p450 cholesterol side chain cleavage enzyme
CYP17	Protein symbol of steroid 17α-hydroxylase/17,20 lyase
CYP17A1	Gene symbol of steroid 17α-hydroxylase/17,20 lyase
CYP19A1	Gene symbol of aromatase
d	Day
DHEA	Dehydroepiandrosterone
DLM	Medial part of dorsolateral thalamus
DM	Dorsomedial intercollicular nucleus
DMRT1	Doublesex and mab-3 related transcription factor 1
DNA	Deoxyribonucleic acid
ENT	Entopallium
ERE	Estrogen response elements
ERα	Protein symbol of the estrogen receptor α
ERβ	Protein symbol of the estrogen receptor β
ESR1	Gene symbol of the estrogen receptor α
ESR2	Gene symbol of the estrogen receptor β
FDR	False discovery rate
FWf	Forest weaver female
FWm	Forest weaver male
GO	Gene ontology
GPCR	G-protein coupled receptor
h	Hour
HSD	Hydroxysteroid dehydrogenase enzymes

HSD17B	Gene symbol of the 17β-hydroxysteroid dehydrogenase
HSD3B	Gene symbol of 3-β-hydroxysteroid dehydrogenase
HVC	Used a proper name
LMAN	Lateral magnocellular nucleus of anterior nidopallium
mAR	Membrane-bound androgen receptor
MCA	Multi_channel_analyser
mRNA	Messenger ribonucleic acid
NCM	Caudomedial nidopallium
Nif	Nucleus interface of nidopallium
NLS	Nuclear localization signal
nXllts	Tracheasyringeal portion of 12th motor nucleus
P450scc	Protein symbol of the cytochrome P450 cholesterol side chain cleavage enzyme
Pam	Nucleus parambigualis
PC	Principal component
PCA	Principal component analysis
POM	Medial preoptic nucleus
RA	Robust nucleus of arcopallium
Ram	Nucleus retroambigualis
RNA	Ribonucleic acid
SDR	Short chain dehydrogenase/reductases
SERPINA6	Gene symbol of corticosteroid-binding globulin
SHBG	Gene symbol of sex hormone-binding globulin
SHBG	Protein symbol of sex hormone-binding globulin
SRD5A2	Gene symbol of 5α-reductase
STAR	Gene symbol of the steroidogenic acute regulatory protein
StAR	Protein symbol of steroidogenic acute regulatory protein
STRING	The Search Tool for the Retrieval of Interacting Genes/Proteins
SULT1E1	Gene symbol of sulfotransferase
T	Testosterone
TSPO	Gene symbol of translocator protein
TSPO	Protein symbol of translocator protein
UTR	Untranslated region of a mRNA sequence
VDAC	Protein symbol of voltage dependent anion channel 1
VDAC1	Gene symbol of voltage dependent anion channel 1
VEGF	Vascular endothelial growth factor
W	Sex chromosome W
WGCNA	Weighted gene correlation network analysis
X	Area X
X	Sex chromosome X
Y	Sex chromosome Y
Z	Sex chromosome Z
ZFm	Zebra finch male

Summary

Singing of songbird species is a behavior that integrates multiple sensory inputs and motor outputs, which primarily reply on interconnected neural circuits in the avian brain, the song control system. The nucleus HVC is of particular interest because of its integrative roles in the song control system. In the majority of Northern temperate songbird species, males sing predominately, and they often use their songs to attract female mates. In contrast, most Northern temperate female songbirds sing either rarely or only in certain contexts. The characteristics and functions of female songs are less clear. In the tropics, many species of females sing regularly and depending on species, their song complexity is less or comparable to that of males. However, the HVC volume is greater in males than females in all songbird species that have been examined.

In the first experiment of this thesis (p. 43), I described song features of spontaneously and rarely singing female canaries (*Serinus canaria*), a Northern temperate songbird species. I observed higher blood testosterone concentrations and greater HVC volume of singing females than that of non-singing females. The results suggest female canary singing is testosterone-dependent.

Subcutaneous testosterone implantation induces singing in female canaries. In the second experiment (p. 65), I implanted female canaries with testosterone for six time periods (T1h, T3h, T8h, T3d, T7d and T14d) and studied changes of gene expression in the HVC. I observed approximately 2,600 genes regulated by testosterone after one hour and the regulation was dynamic throughout the experimental time window. I investigated putative biological functions of testosterone-regulated genes in the six time points by gene ontology (GO)-term enrichment analysis, and showed that the enrichment of angiogenesis began at T1h and the enrichment of neurogenesis began at T3h, with both processes continuing until T14d.

Furthermore, genes associated with "GABA" and "spine" were enriched in T3d birds when the birds started singing, while the number of genes associated with "nervous system development" was highest in T14d birds, when the HVC volume was significantly greater than controls. Finally, using approaches integrating gene expression, HVC volume, circulating testosterone levels, and song characteristics, I identified a potential master regulator of testosterone-regulated changes.

Male canary songs vary seasonally. Breeding season songs are longer, louder, and more complex than non-breeding season songs. Non-breeding males implanted with testosterone sing songs resembling that of breeding season songs. In the third experiment (p. 81), I studied gene expression in the HVC of seven canary groups, females and males of breeding season and of non-breeding season, non-breeding season females and males treated with testosterone, and spontaneously singing female canaries. Hierarchical clustering and principal component analysis (PCA) showed that circulating testosterone levels and sex were the predominant variables associated with variation in the HVC transcriptomes. Comparison between natural singing canaries with testosterone-induced singing canaries of the same sex revealed large differences in the HVC transcriptomes. Moreover, the intersection of natural and testosterone-induced singing females shared little resemblance with males in terms of genes. GO-term enrichment analysis suggested functional overlap between sex-specific gene networks. However, although strong transcriptional changes in HVC correlate with the transition from non-singing to singing in both sexes, the type of transcriptional changes are sex-specific.

In the fourth experiment (p. 89), I studied sex differences in HVC gene expression between three songbird species: the canary, the blue-capped cordon bleus (*Uraeginthus cyanocephalus*), and the forest weavers (*Ploceus bicolor*). Cordon bleu females sing regularly with female-specific songs, whereas forest weaver females sing songs identical to males. I found substantial sex differences in HVC gene expression in all three species, and sex-biased genes differed between species. Surprisingly, the majority of sex-biased

genes were on autosomes instead the sex chromosome Z. These results provide further evidence for sex differences in brain structure at the molecular and cellular levels in sexually reproductive animals.

Introduction

It is a scientific consensus that birds had evolved from a group of theropod dinosaurs that originated during the Mesozoic Era and survived the mass extinction event 65 million years ago (Chiappe, 1995). Today, Aves is one of the most diverse taxonomic class in the animal kingdom that comprises approximately 10,500 extant species in the world (Gill and Donsker, 2015). The human fascination with birds also has an ancient origin. Prehistoric caves, such as the ones in Australia and France, were found with bird drawings on the walls. Birds appear in a large body of literature across many cultures: some praised the incredible ability in predicting the beginning of breeding season of migratory birds, while others admired the melodious songs of nightingales or canaries. All these appreciations are based on the accumulation of many centuries' observations of birds. Ever since the emergence of the fields of taxonomy and biogeography, the taxonomic lineages and geographical distributions of birds have been well documented for the majority of bird species around the planet. In recent years, the development of computers and software has allowed a more precise description on sound characteristics of birdsong. The well-documented natural history and behaviors of birds and the recent development of bioinformatics technologies and databases have provided the platform for scientists to address key questions, such as interplay between environmental changes, animal behavior, and gene regulation.

I. Songbirds are important animal model systems

More than half of the extant bird species are categorized in the order Passeriformes. This order includes three suborders: Acanthisitti, Tyranni (suboscines), which comprises ~1,100 species, and Passeri (oscines or songbirds), which comprises ~4,500 species (Barker et al., 2004; Gill et al., 2010; Prum et al., 2015). Fundamental features that distinguish the songbirds from the suboscines and most other avian taxa are 1) their vocal organs, the syrinxes, which are more complex in terms of the syringeal musculature (Amador et

al., 2008; Ames, 1971); 2) their songs appear to be learned by imitation (Kroodsma, 1984; Kroodsma and Konishi, 1991); and 3) they have a specialized neural circuit called the song control system that is responsible for vocal production and learning, which is not found (Gahr, 2000) or less developed in the suboscines (de Lima et al., 2015; Liu et al., 2013).

I. 1. Song control system is a complex neural circuit responsible for song control

The song control system consists of two interconnected neural pathways (Figure 1): the anterior forebrain pathway is required for song learning, and the posterior motor pathway is responsible for song production (Bolhuis and Gahr, 2006; Bolhuis et al., 2010; Farries, 2006; Nottebohm et al., 1976; Wild, 2004). The posterior motor pathway contains the nucleus HVC in the caudal nidopallium, which innervates the robust nucleus of the arcopallium (RA). RA projects to the brainstem vocal motor nucleus, the trachea-syringeal portion of the 12th motor nucleus (nXllts), which innervates the syrinx and trachea, the major vocal and respiratory organ, respectively (Figure 1). RA also projects to hindbrain nuclei, coordinating respiratory activity with song production, including the nucleus retroambigualis (Ram) and nucleus parambigualis (Pam;(Schmidt and Martin Wild, 2014). The anterior pathway, as the posterior one, begins from the HVC, which projects to Area X in the striatum (Figure 1). Area X innervates the medial part of the dorsolateral thalamus (DLM) that projects to the lateral magnocellular nucleus of the anterior nidopallium (LMAN). LMAN projects to RA and Area X. Thus, the anterior forebrain pathway contributes an indirect connection between HVC and RA.

Both anterior learning and posterior motor pathways originate in the HVC. In addition, HVC receives and integrates the auditory inputs from several brain nuclei, such as the field L complex, the caudal mesopallium (CMM), and nucleus interface of the nidopallium (NIf) (Prather, 2013; Shaevitz and Theunissen, 2007). Moreover, HVC is involved in frequency modulations of songs (Halle et al., 2003),

generation of motif sequences (Hahnloser et al., 2002), and sexual preferences to conspecific song displays (Del Negro et al., 1998). In sum, HVC serves as a crucial nucleus in the song control system.

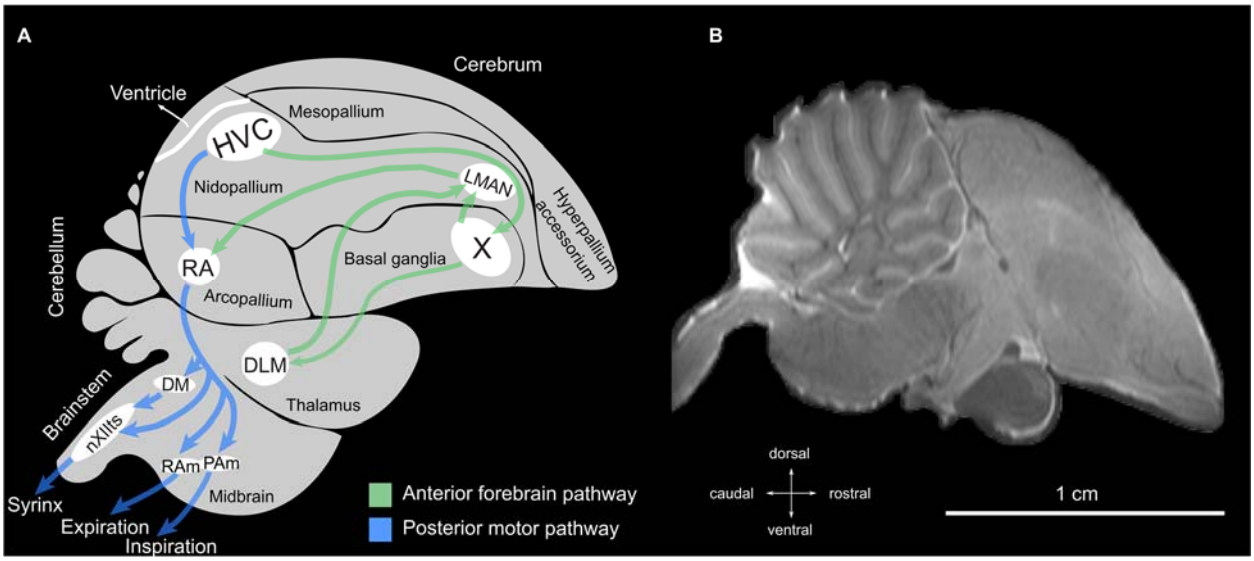

Figure 1. Song control system.

A, a simplified scheme shows the brain and song control system of songbirds. B, a parasagittal view of an MRI image of a male European starling shows the medial part of the brain. Image is courtesy of Dr. Van Meir. Abbreviations: HVC: initially hyperstriatum ventrale pars caudale, now acronym used as proper name; LMAN: lateral magnocellular nucleus of the anterior nidopallium; RA: nucleus robustus arcopallialis; X: Area X; DLM: dorsal lateral nucleus of the medial thalamus; DM: dorsomedial intercollicular nucleus; nXIIts: motonucleus of tracheosyringeal part of the XIIth cranial nerve; RAam: nucleus retroambigualis; PAm: nucleus parambigualis.

I. 2. Birdsong is sexually dimorphic or monomorphic depending on species

Birdsong tends to be long, complex vocalizations (Catchpole and Slater, 1995). It is a behavior that involves intensive integration of sensory input, motor output, and various contexts mediated by the song control system (Wild, 2004). The functions of male song are diverse, including territory defense, mate attraction, mate solicitation, (Byers et al., 2010; Catchpole and Slater, 1995; Drăgănoiu et al., 2002; Kroodsma and Byers, 1991; Searcy and Andersson, 1986), and pair bond formation in species where the male duets with its female (Wickler and Seibt, 1980). Song quality, such as song complexity and/or production rate, is thought to show an individual's quality (Byers et al., 2010; Drăgănoiu et al., 2002). Therefore, male song

likely bears fitness consequences. In many northern temperate species, females do not sing regularly or sing only in a specific context (Gahr, 2014). However, females of many species sing, especially in species dwelling in the tropics or the southern temperate zone. In fact, recent studies surveyed a total of 1,314 songbird species and reported that females sing in at least 656 species belonging to some 32 avian families, giving a 49.9% probability of observing female songs among the surveyed species (Odom et al., 2014; Webb et al., 2016). These observations stimulated and supported the view that female singing might be widespread and ancestral to songbirds (Garamszegi et al., 2007; Odom et al., 2014). The low abundance of female singing among species of the northern hemisphere might reflect multiple independent evolutionary losses of female song from an ancestor (Garamszegi et al., 2007; Odom et al., 2014; Price et al., 2009). The functions of female song have been postulated in association with territorial defense, mate attraction, mate guarding, and coordination of breeding activities (Arcese et al., 1988; Baptista et al., 1993; Gahr and Güttinger, 1986; Langmore, 1998; Slater and Mann, 2004).

While in general females sing shorter songs and at a lower rate among the female-singing species, females of some species can sing songs identical to those of conspecific males (comparable complexity). For example, forest weavers, *Ploceus bicolor* (Gahr et al., 2008), African bush shrikes, *Laniarius funebris* (Gahr et al., 1998a), bay wrens, *Thryothorus nigricapillus* (Brenowitz et al., 1985; Levin, 1996) and red-backed fairy-wrens, *Malurus melanocephalus* (Schwabl et al., 2015) all do so. Females of other species can sing at equal or even higher rate than conspecific males like Streak-Backed Orioles, *Icterus pustulatus* (Hall et al., 2010). The species differences in the loss or preservation of female song imply that species-specific selection pressures against or favoring female singing are likely life history-dependent. For example, changes in breeding grounds from tropical to temperate zones have been associated with the (evolutionary) loss of female songs in some New World oriole species, such as *Icterus cucullatus* and *Icterus galbula* (Price et al., 2009). Furthermore, a composite of life-history features, including monogamous social system, dispersed nests, and sedentariness have been proposed to explain the preservation of female song among the species of the Icteridae family (Price, 2009). Interestingly, no single trait was consistently associated

with evolutionary changes in female song across the Icterid phylogeny when each life history trait was considered separately (Price, 2009). These data provide strong evidence for the association between ancestral changes in life history and losses (or preservations) of female song in avian phylogeny (Price, 2009; Price et al., 2009).

I. 3. Song control nuclei are strictly sexually dimorphic

Surprisingly, anatomical sexual differences are evident in the song control system of all songbird species that have been examined. These phenomena held true even for species in which females sing comparable songs as, or at higher rates than, their males. The male-biased sex differences of the song control nuclei include gross anatomical properties like larger volumes (e.g., HVC, RA and nXIIts) and higher neuron numbers (Brenowitz et al., 1985; Gahr et al., 1998a; Gurney and Konishi, 1980; Nottebohm and Arnold, 1976; Schwabl et al., 2015), as well as microscopic anatomical properties like a more complex dendritic arborization of the RA (DeVoogd and Nottebohm, 1981; DeVoogd et al., 1988; Gurney, 1981) and the HVC neurons (Nixdorf et al., 1989). Intriguingly, the extent of these anatomical sex differences decrease yet retain when comparing the males and females of species with great similarities in their songs, such as duetting species forest weavers (Gahr et al., 2008; Gahr et al., 1998a). Interspecies comparative work has established the positive correlation of the relative HVC volume and relative song repertoire size (Gahr et al., 1998a; Hauber et al., 1999). Work controlling for phylogenetic relationships at the species level supports the relationship (Devoogd et al., 1993; MacDougall-Shackleton and Ball, 1999; Székely et al., 1996). Taken together, despite that in some species females sing comparable songs to their males, the neuroanatomical properties of song nuclei are tightly regulated in a male-biased fashion. Thus, there might exist some evolutionary constraints for female songbirds, and females in different species preserved distinct levels of song behavior. In order to understand the problem, comparative neuroanatomical data of evolutionarily old songbird families, such as the lyrebirds, would be helpful, since females and males of these species sing (Taylor, 1986).

I. 4. Neural plasticity of song control system is hormone-dependent

The observation that anatomical sex differences exist in the song control system across many songbird species suggests that sex hormones might play a role in neural anatomy. Indeed, administration of exogenous testosterone to females of many species increases the volumes of the song control nuclei. Examples are the canary (*Serinus canaria* (Bottjer and Dignan, 1988; DeVoogd and Nottebohm, 1981; Fusani et al., 2003a; Madison et al., 2015; Nottebohm, 1980)), the European robin (*Erithacus rubecula* (Dittrich et al., 2014)) and the starling (*Sturnus vulgaris* (Hausberger et al., 1995; Van Meir et al., 2004)). In addition, increasing circulating levels of testosterone elevate song rates of these females (Dittrich et al., 2014; Fusani et al., 2003a; Herrick and Harris, 1957; Leonard, 1939; Madison et al., 2015; Shoemaker, 1939). Thus, the effects of testosterone on both singing activity and the volumes of song control nuclei seem to be universal among female songbirds. This finding inspired investigations of such relationship in seasonal singing male songbirds. In many northern temperate songbird species, breeding is restricted to spring and early summer. The gradually lengthening photoperiod activates the avian reproductive system. Specifically, the gonad size and the circulating testosterone levels are greater during the breeding season than the non-breeding season (Nottebohm et al., 1987; Voigt and Leitner, 2008). In seasonal breeders, such as the canary, males sing more frequently, louder, longer, or more stereotyped songs in the breeding season than in the non-breeding season (Leitner et al., 2001a; Nottebohm et al., 1986; Voigt and Leitner, 2008). Notably, artificially increased circulating testosterone of non-breeding males mimic the characteristics of breeding songs, as well as anatomical properties of the song control nuclei (Gulledge and Deviche, 1999). For example, administering testosterone increases the volumes of RA in male dark-eyed juncos (*Junco hyemalis*) (Gulledge and Deviche, 1999) and the volumes of HVC in male European starling (*Sturnus vulgaris*) (Bernard and Ball, 1997), both held under non-breeding light schedules. Conversely, castration during the breeding season causes both HVC and Area X to shrink in adult male dark-eyed juncos (Gulledge and Deviche, 1998). Therefore, both male and female songbirds are under the influences of testosterone in terms of singing activity and neural properties of song control nuclei.

In summary, the fact that all songbird species possess a specialized neural circuit for song production highlights the importance of singing behaviors as a valuable skillset. On the basis of a conserved song control system (Nottebohm et al., 1976; Reiner et al., 2004; Wild, 2004), songbirds of different species exhibit distinct song characteristics, differences in sexual dimorphic singing, and the degree of hormone-dependent plasticity to singing varies (Gahr, 2014). Thus, the song control system provides a platform for comparative studies on various topics: for example, the neural and motor coordination for producing syllables with high repetition rates (Suthers et al., 2012; Suthers Roderick and Zollinger Sue, 2006), the anatomical and molecular basis of brain plasticity in response to hormone manipulation (Arnold et al., 1976; DeVoogd and Nottebohm, 1981; Devoogd et al., 1985; Fusani and Gahr, 2006; Fusani et al., 2001; Fusani et al., 2003a; Fusani et al., 2003b; Hartog et al., 2009; Louissaint et al., 2002; Nottebohm, 1980; Nottebohm and Arnold, 1976; Rasika et al., 1994a) (the second experiment of this thesis), and the molecular basis of sexually dimorphic singing (the third and fourth experiments of this thesis).

II. Sexual determination and differentiation of birds: basic concepts

Sexual reproduction, by which organisms of the same species reproduce by uniting two types of gametes, is evolutionarily ancient and phylogenetically ubiquitous (Bachtrog et al., 2014; Herpin and Schartl, 2015). Higher vertebrates, birds and mammals produce female and male gametes that carry sex-specific sets of chromosomes next to autosomes. Sex chromosomes evolved independently many times throughout the eukaryotes and even among vertebrates (Bachtrog et al., 2014; Irwin, 2018) (Figure 2). Most sex chromosome systems fall into two categories: ZW systems, as in the cases of birds, many snakes, butterflies, and moths, and XY system as in mammals, beetles, and fruit flies (Figure 2). In ZW systems, females are heterogametic (ZW) and males are homogametic (ZZ), whereas in XY systems males are heterogametic (XY) and females are homogametic (XX). In both systems, the sex chromosome that is only found in one sex (W or Y) is smaller and has less functional genes than the Z and X chromosomes, respectively (Irwin, 2018).

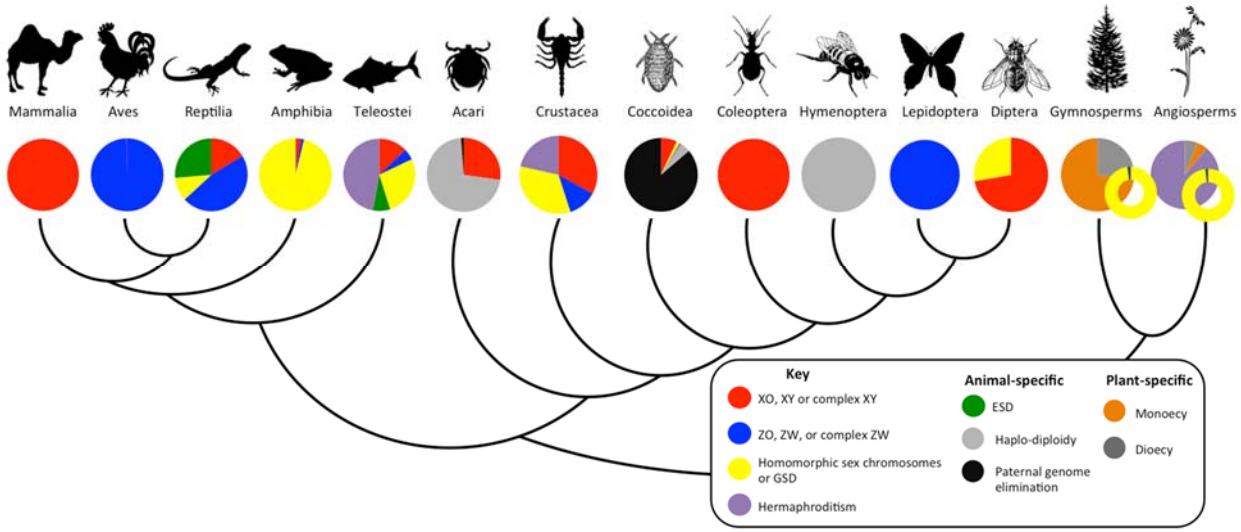

Figure 2. Diversity of sex determination systems for representative plant and animal clades.
Taken from (Bachtrog et al., 2014). GSD: genotypic sex determination; ESD: environmental sex determination.

Birds are a phylogenetic group in which sex is strictly determined genetically (Major and Smith, 2016), unlike other groups including sea turtles and crocodilians, in which sex is determined by the environment, e.g. by temperature during embryonic development (Figure 2) (Bachtrog et al., 2014). It should be noted that birds differ from mammals not only in the form of heterogamety, but also in activity of the sex chromosomes in the homogametic sex. In mammals, one of these sex chromosomes is widely inactive, while this is not the case in birds (Itoh et al., 2007; Mank, 2013). In relation, sex-specific gene expression profiles were detected in chicken embryos before gonad development and secretion of sex hormones, including 12-h post fertilization blastoderms and 23-h post-fertilization primitive streak (Ayers et al., 2013; Zhang et al., 2010). This implies that sexual differentiation takes place with a background of overall sex-specific expression of sex-chromosomal genes in various tissues besides the gonads.

In birds, the current best candidate 'sex determinant' gene is *DMRT1* (doublesex and mab-3 related transcription factor 1) (Major and Smith, 2016); *DMRT1* gene is required for male sex determination (Smith et al., 2009). This gene is located on the Z chromosome in all birds, including the ratites, the most basal bird order (Zhang et al., 2014). The gene dosage of *DMRT1* expression has been proposed for avian sex

determination (Major and Smith, 2016; Smith et al., 2009). Because *DMRT1* is Z-linked and the general phenomenon that gene dosage is generally not compensated in birds, the *DMRT1* expression is higher in male embryos than in female embryos. Such high *DMRT1* expression in male embryos likely activates the downstream gene cascades for testis development (Major and Smith, 2016). Knockdown *DMRT1* in early chicken embryos leads to feminization of the embryonic gonads in genetically male (ZZ) embryos (Smith et al., 2009). Conversely, overexpression of *DMRT1* in female gonads activates genes that are involved in testis development (Lambeth et al., 2014). Studies based on several model animal systems from worms to flies to mammals have revealed a great diversity of master sex-determining genes. On the other hand, the downstream effectors appear to converge on the regulation of a few common effectors (Herpin and Schartl, 2015). Indeed, *DMRT* gene family is one of the common downstream effectors in sex determination in phylogenetically diverse groups such as mammals, fish, frogs, and flies (Herpin and Schartl, 2015). Thus, bird presents an example that one (*DMRT1*) of the downstream effectors is "promoted" to become the master sex determiner (Herpin and Schartl, 2015).

The lower *DMRT1* dosage then activates gene cascades in which aromatase activity and estrogen synthesis are promoted, which plays a central role for the formation of ovaries. Aromatase is the rate-limiting enzyme in estrogen synthesis (see II. 2. Steroidogenesis), and its expression is female-specific in the embryonic gonad. Administration of aromatase blocker, such as fadrozole, into female chicken eggs prior to the gonadal sex differentiation induces development of the testes (Elbrecht and Smith, 1992; Smith et al., 2003; Vaillant et al., 2001; Vaillant et al., 2003). Most of fadrozole-treated genetically female chickens have at least one testis after hatching, and some of them continued to exhibit male phenotype (testes, spurs, bomb and wattle) along with male behavior after adulthood (Elbrecht and Smith, 1992; Vaillant et al., 2001). Conversely, administration of estrogens into genetically male eggs prior to gonadal sex differentiation can transiently feminize male gonads, which revert to a male phenotype after hatching (Scheib, 1983). In addition, the extent of effects of hormonal manipulation is species-dependent. For

example, all fadrozole-treated ZW zebra finches do not have a male plumage (Wade and Arnold, 1996), unlike fadrozole-treated genetically female chickens (Elbrecht and Smith, 1992).

Later during embryonic development, sex steroid hormones, likely originating from the developing gonads, play important roles for sexual differentiation of somatic tissues including the brain. For example, the development of singing behavior of female zebra finches can be masculinized, i.e., sing male-typical songs as adults, upon administration of testosterone or estradiol during the nestling period, while such treatments during later stages have no effect (Gurney and Konishi, 1980; Pohl-Apel and Sossinka, 1984). By contrast, testosterone-treated female canaries in adulthood can induce male-typical songs (Nottebohm, 1980). Thus, the sensitive period to hormonal manipulation seems to be species-dependent. It should be noted that these activities of gonadal hormones during later stages of development do not revert gonadal differentiation. Therefore, despite the fact that sex is determined genetically in birds, sex steroid hormones clearly play an important role in directing the development of sex typical phenotypes according to the genetic sex. However, this must happen in concert with sex differences in the gene dosage of genes not regulated by gonadal hormones (Gahr, 2004).

II. 1. Steroidogenic enzymes

All steroid hormones are synthesized from cholesterol by the enzymatic process called steroidogenesis, in various endocrine tissues that express cytochrome P450 cholesterol side chain cleavage enzyme (protein name P450scc; gene name *CYP11A1*). These tissues include adrenal cortex, Leydig cells of the testes, and theca cells of the ovarian tissues (Edelmann et al., 1999; Greaves et al., 2014; Tsutsui et al., 2006). Notably, recent studies showed that vertebrates including fish, birds, and mammals can produce steroid hormones in the nervous system, either by *de novo* synthesis from cholesterol or from local metabolism of steroid intermediates produced in the periphery (Diotel et al., 2018; London et al., 2010; Schlinger and Callard, 2005; Soma et al., 1999; Soma et al., 2003a; Tsutsui et al., 2006). The steroid hormones synthesized in the

central nervous system (CNS) are also called neurosteroids. Importantly, these steroids can modulate brain homeostasis, functions, and plasticity. Together, neurosteroids and peripherally-produced steroids have pleotropic effects on many target tissues.

The steroidogenic enzymes are essentially classified into two groups, the cytochrome P450 (CYP) enzymes and the hydroxysteroid dehydrogenase (HSD) enzymes (Greaves et al., 2014; Miller and Auchus, 2011). CYP enzymes are heme-containing oxidative enzymes, including cytochrome P450 cholesterol side chain cleavage enzyme (protein name P450scc; gene name *CYP11A1*), steroid 17α-hydroxylase/17,20 lyase (CYP17; *CYP17A1*) and aromatase (*CYP19A1*). In contrast, HSD are either the short chain dehydrogenase/reductases (SDR) or aldo-keto reductases (AKR) (Greaves et al., 2014; Miller and Auchus, 2011).

Several isoforms (in case of humans, type 1, 2 and 3) of 17β-hydroxysteroid dehydrogenase (17β-HSD; *HSD17B*) and 5α-reductase (*SRD5A2*) are examples of SDR enzymes. The 17β-HSD composes a family of enzymes that catalyze the conversion between the low-active 17-keto steroids and the highly active 17b-hydroxy steroids. The members of 17β-HSDs differ in their substrate preference and expression sites. Reactions catalyzed by 17β-HSDs are bi-directional, the predominant action *in vivo* depends on local factors like pH and the concentration of cofactors (Greaves et al., 2014). Nevertheless, some isozymes are preferential oxidases, whereas others are preferential reductases in humans. Although at least 14 human isoforms of 17β-HSD have been identified (Miller and Auchus, 2011; Saloniemi et al., 2012), less (nine) 17β-HSD isoforms were found in the zebra finch genome assembly (London and Clayton, 2010).

Members of AKR include 3α-hydroxysteroid dehydrogenase (3α-HSD, *AKR1C*), 3-β-hydroxysteroid dehydrogenase (3β-HSD; *HSD3B*) and 5β-reductase (*AKR1D1*). 3β-HSD is a family of up to seven enzymes, each derived from a unique gene. There are two human isoforms of 3β-HSD, 3β-HSD1 (*HSD3B1*) predominately is expressed in the placenta, breast, liver, and brain, whereas 3β-HSD2 (*HSD3B2*) is

expressed in the adrenal cortex, Leydig cell, and theca cells (Greaves et al., 2014). However, only one 3β-HSD has been identified in the zebra finch genome (*HSD3B1*) (London and Clayton, 2010).

The expression and enzymatic activities of the steroidogenic enzymes (P450scc, CYP17, 3β-HSD, 17β-HSD aromatase, 5α-reductase and 5β-reductase) as well as a wide variety of steroid intermediates have been detected in the brains of multiple bird species, including quail, zebra finch, song sparrows and canaries (Fusani et al., 2001; London et al., 2006; Metzdorf et al., 1999; Schlinger and Callard, 2005; Soma et al., 2003b; Soma and Wingfield, 2001; Soma et al., 2002; Tsutsui et al., 2006; Vockel et al., 1990a; Vockel et al., 1990b). These data provide strong support for *de novo* steroid synthesis in the brains of avian species. However, whether testosterone can be produced *de novo* from cholesterol in brain cells needs to be seen. Furthermore, it should be noted that functional analysis of most steroidogenic enzymes is missing for bird species, with zebra finches being a partial exception.

II. 2. Steroidogenesis

The first and rate-limiting step in steroidogenesis involves translocation of cholesterol from the outer membrane to inner membrane of the mitochondria. A complex responsible for the transport of cholesterol is composed of the steroidogenic acute regulatory protein (protein name StAR; gene name *STAR*), translocator protein (TSPO; *TSPO*), and several other proteins, including the voltage dependent anion channel 1 (VDAC; *VDAC1*) and the ATPase family AAA domain containing 3A (ATAD3; *ATAD3A*) (Diotel et al., 2018). P450scc, the first steroidogenic enzyme, converts cholesterol into pregnenolone in the inner mitochondrial membrane (Figure 3), and then pregnenolone can be converted into progesterone by 3β-HSD or into 17-hydroxypregnenolone by CYP17 in the smooth endoplasmic reticulum (Figure 3). Then, CYP17 can convert progesterone into 17-hydroxyprogesterone, while 3β-HSD can convert 17-hydroxypregnenolone into 17-hydroxyprogesterone. Subsequently, CYP17 can covert 17-hydroxypregnenolone to dehydroepiandrosterone (DHEA) and covert 17-hydroxyprogesterone to

androstenedione. Thereafter, 17β-HSD can transform DHEA to androstenediol or transform androstenedione to testosterone (Figure 3). In the case of androstenediol, it can be converted to testosterone by 3β-HSD. Testosterone is the major androgen in the circulatory system. Testosterone can be converted to the major estrogen 17β-estradiol by aromatase (*CYP19A1*), to a bioactive androgen 5α-dihydrotestosterone (5α-DHT) by 5α-reductase, or to an inactive androgen 5β-dihydrotestosterone (5β-DHT) by 5β-reductase (Figure 3).

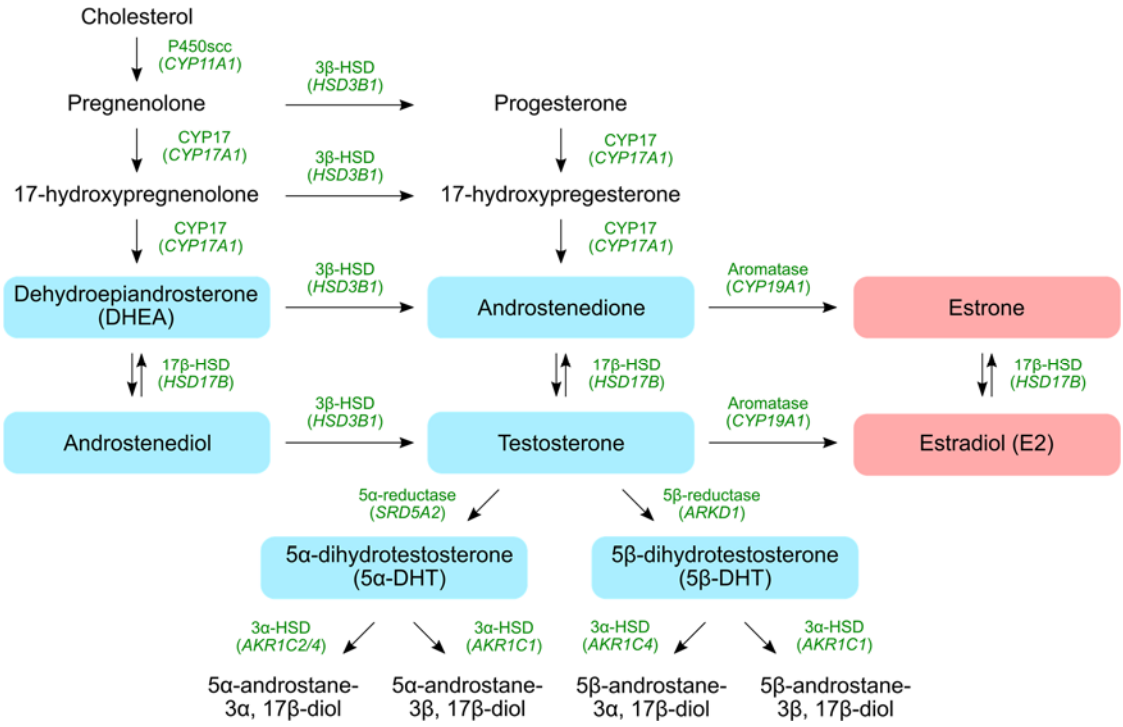

Figure 3. A simplified diagram depicting steroidogenic enzymes (green texts; gene names are shown in italic) and major steroid metabolites (black texts) in the blood.

Metabolites in blue boxes are androgens or precursors of androgen, whilst pink boxes enclose estrogens (Diotel et al., 2018; Miller and Auchus, 2011; Penning, 2010; Tsutsui et al., 2013).

In females, testosterone is produced in the ovary as a precursor of estrogens (Nitta et al., 1991). In relation, testosterone is highest in the chicken hen about 6-8 hours before ovulation (Etches and Cheng, 1981; Robinson et al., 1988). However, maximal concentrations of circulating testosterone (range: 50 pg – 3.8 ng/ml) of female birds are 4-10 times lower than in male conspecifics during the breeding season

(Ketterson et al., 2005), and are around or below the detection limits during the non-breeding periods (Ketterson et al., 2005). Concentrations of circulating estrogens in female songbirds are highest during the nest-building and the egg-laying period, with concentrations between 100 – 700 pg per ml blood plasma and are low throughout the rest of the year (Gwinner et al., 1994; Schwabl, 1992; Schwabl et al., 1980; Silverin et al., 1986; Wingfield and Farner, 1975).

II. 3. Steroid degradation pathways

The degradation of steroids eventually involves conjugation with sulphate by sulfotransferases or glucuronide by UDP-glucuronosyltransferases to reduce their hydrophobicity, allowing for excretion (Greaves et al., 2014; Thomas and Potter, 2013). Steroid sulfates may also be hydrolyzed back to the native steroid by steroid sulfatases (Miller and Auchus, 2011). Notably, recent studies indicated that steroid sulfates may not simply be inactivated forms of steroid and might serve specific hormonal roles or as storage of steroids (Miller and Auchus, 2011).

Circulating testosterone can be broken down in the liver by 3α-HSD or 3β-HSD to yield tetrahydrosteroids (Figure 3). These tetrahydrosteroids can be further conjugated with sulphate or glucoronide for excretion (Penning, 2010). Recent evidence showed that glucuronidation and sulfonation may precede the formation of tetrahydrosteroids (Penning, 2010). In the estrogen degradation pathway, the first step is hydroxylation. The hydroxyl group can then be further sulfated, glucuronidated, or methylated. Sulfotransferase (*SULT1E1*) catalyzes the sulfonation and has a high affinity for estrogen, while the catechol O-methyl transferase (COMT) carries out methylation of estrogen (Thomas and Potter, 2013).

II. 4. Steroid transportation in the bloodstream

Once steroid hormones are synthesized, they can be released into the bloodstream, where they can either travel freely (unbound) or bound to carrier proteins, including albumin, sex hormone-binding globulin

(SHBG; *SHBG*), and corticosteroid-binding globulin (CBG; *SERPINA6*). These carrier proteins regulate the equilibrium of unbound and bound fractions of circulating steroid hormones. Albumin non-specifically binds to all classes of steroids, but its high concentration in the plasma buffers the steroid levels. In contrast, SHBG binds to major androgens and estrogens with high affinity and specificity in mammals, and CBG preferentially binds to the glucocorticoids and progesterone (Hammond, 2016). However, there is no indication of SHBG presence in the avian genomes and plasma (Malisch and Breuner, 2010). Instead, the avian CBG binds to androgens (testosterone and DHT) with physiologically-relevant affinity, but essentially [illegible] (Deviche et al., 2001; Malisch and Breuner, 2010).

Upon arrival at target tissues, the bound steroids have to be released from carrier proteins, and then enter the tissues by simple diffusion and there exert their physiological roles. Steroids can also be transported back to the bloodstream by efflux transporters, for example P-glycoprotein, also known as multidrug resistance-associated protein. A chicken copy of multidrug resistance-associated protein has been described (Edelmann et al., 1999). This chicken protein was expressed in various tissues, including brain, liver, and theca cells of the ovarian tissues and was able to transport estradiol (Edelmann et al., 1999). Steroid hormone assays, such as the radioimmunoassay used in my study, quantify unbound fractions of circulating testosterone.

II. 5. Steroids in action: activation of the androgen receptor and estrogen receptors

Steroid hormones enter the target tissues and exert their physiological effects by binding to steroid receptors. Sex hormones, such as testosterone and 17β-estradiol, bind to their cognate receptors and can subsequently act via the classic genomic signaling pathway or the non-classical signaling pathway. Conventionally, the classic genomic pathway involves transcription and translation and may take several hours to days to cause hormonal effects. In contrast, the non-classical signaling pathways do not involve

transcription on the onset of activation and act on the scale of milliseconds to minutes. However, the non-classical pathways might ultimately act to modulate transcriptional activity. For the scope of this thesis, I will focus on the regulation of androgen and estrogen receptors.

II. 5. a. Classical genomic signaling pathway

In songbirds and mammals, there is only one androgen receptor gene (AR; *AR*), whereas there are two estrogen receptor genes (ERα; *ESR1* and ERβ; *ESR2*). The androgen receptor (*AR*) has high affinity to androgens, such as 5α-DHT and testosterone, in the low nanomolar range (Davey and Grossmann, 2016; Katoh et al., 2006). While testosterone is the major circulating androgen, 5α-DHT is biologically more active than testosterone, because it has approximately twice the affinity for the AR and an approximately five times faster dissociation rate with AR (Davey and Grossmann, 2016). 5β-DHT does not bind to the AR (Perusquía and Stallone, 2010).

As members of the nuclear receptor superfamily, the AR consists of an N-terminal domain, followed by an almost strictly conserved DNA-binding domain, an inter-domain hinge, and a C-terminal ligand-binding domain (Rastinejad et al., 2013) (Figure 4). In the absence of ligand, the AR is associated with heat-shock and other chaperone proteins located in the cytoplasm. Upon binding to the ligand-binding domain, a conformational change of the AR causes the dissociation of chaperone proteins and the exposure of the nuclear localization signal (NLS). The exposure of NLS leads to translocation of the androgen-bound AR to the cell nucleus where the ARs form a homodimer (Nadal et al., 2017), and act as a transcription factor. Another signal sequence located in the N-terminal domain is activation function 1 (AF-1), which is required for maximal activity of the AR (Davey and Grossmann, 2016). The DNA-binding domain of AR consists of two zinc fingers that recognize the androgen response elements (AREs) based on the highly conserved sequence (inverted repeats of the half site 5'-AGAACA-3' consensus sequence with a 3-nucleotide spacer), as well as the geometry of the DNA targets (Pihlajamaa et al., 2015; Rastinejad et al., 2013). The DNA-bound

AR dimer can interact with coregulators (co-activator or corepressor) using solvent-exposed surfaces such as activation function 2 and binding function 3, forming a complex that regulates gene transcription (Nadal et al., 2017; Pihlajamaa et al., 2015).

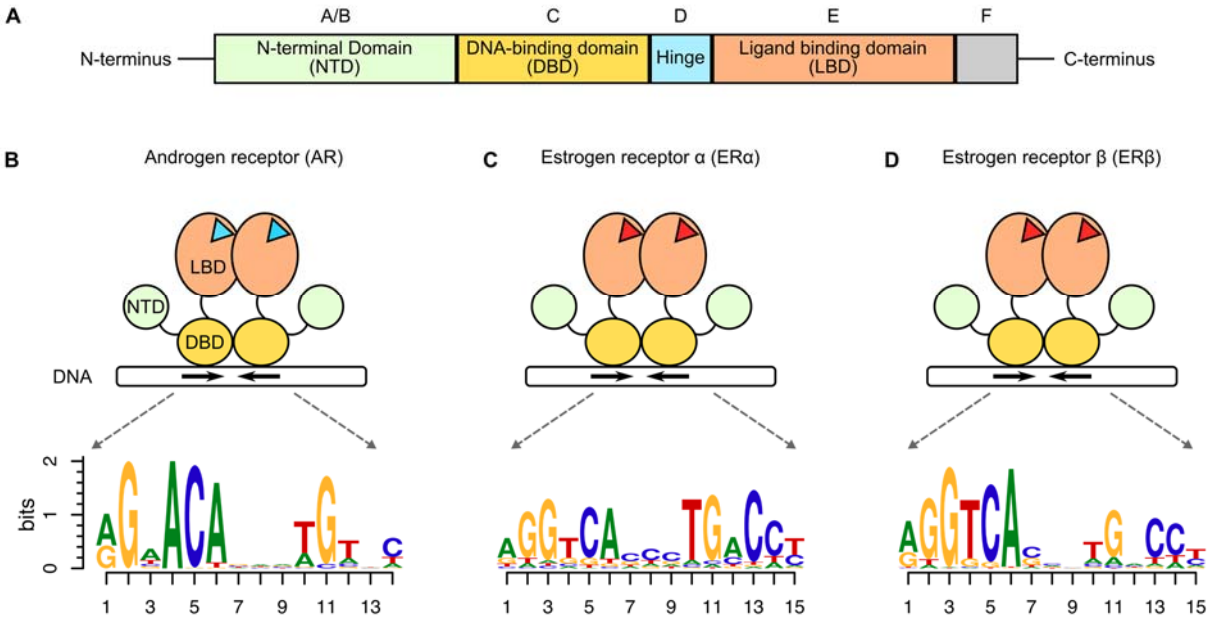

Figure 4. Schematic representation of androgen and estrogen receptors.

A, a scheme shows the six-region general structure of the nuclear receptor superfamily. Androgen receptor (AR), estrogen receptor α (ERα) and β (ERβ) shared this structure. The scheme was drawn after (Davey and Grossmann, 2016; Frankl-Vilches and Gahr, 2017; Rastinejad et al., 2013). B, a schematic representation of two androgen (blue triangle) binding ARs forming a dimer, which recognizes a DNA sequence bearing the androgen response element (ARE). The two black arrows represent the ARE that is composed of one half-site and its inverted repeat with a 3-bp spacer. The frequency matrix of AREs is shown. C-D, schematics of ERα and ERβ binding to a DNA sequence baring the estrogen response elements (EREs) upon binding to estrogens (red triangles). The frequency matrices of EREs are shown. The frequency matrices were downloaded from the JASPAR transcription factor binding profiles database (Khan et al., 2018) and visualized by motifStack R package (Ou et al., 2018).

ERα and ERβ are the products of *ESR1* and *ESR2*, respectively. Both types of ER have a six-region structure similar to the AR and contain defined functional domains that have considerable homology (Thomas and Gustafsson, 2011). ERs have high affinity to estradiol (Kon et al., 1980) and regulate gene transcription by binding to the estrogen response elements (EREs). The consensus ERE consists of a 5-bp

palindrome with a 3-bp spacer: GGTCAnnnTGACC (Figure 4). However, many natural EREs deviate substantially from the consensus sequence (Zhao et al., 2010a). ERs can also modulate gene expression by interacting with other DNA-binding transcription factors (Zhao et al., 2010a). Empirical results showed that ERα and ERβ bind to the same ERE motif (Figure 4), nevertheless, the two subtypes seem to work in a competitive manner: in the presence of ERα, ERβ occupied fewer sites than in the absence of ERα (Zhao et al., 2010a).

Studies using chromatin immunoprecipitation (ChIP) methods, such as ChIP-combined with tiling microarrays (ChIP-chip) or ChIP-sequencing (ChIP-Seq), demonstrated that while AREs can be found at various genomic elements, including core promoters, introns, intergenic regions, 5'-UTRs, 3'-UTRs, etc., the majority of AR-occupied AREs are in intergenic regions distal from the transcription start sites (Pihlajamaa et al., 2015; Takayama et al., 2007; Wilson et al., 2016). Similar results were found for estrogen response elements (EREs) (Bourdeau et al., 2004). By forming DNA loop structures, sex steroid receptor-bound enhancers interact with promoters of steroid-regulated genes. Cohesin or other coregulators can further stabilize DNA loops.

Notably, sex steroid receptor binding events are extremely tissue-specific. A ChIP-seq study comparing three androgen-responsive tissues, prostate, epididymis, and kidney, showed little overlap of AR-binding events (Pihlajamaa et al., 2014). Only 10% of ER-binding sites overlapped between a breast cancer cell line and an endometrial cancer cell line (Gertz et al., 2013; Pihlajamaa et al., 2015). Recent studies suggest that such tissue-specificity largely depends on pioneer factors, which are transcription factors that can bind to heterochromatin, leading to opening of the local chromatin structure. A ChIP-seq study showed three distinct pioneer factors (FOXA1, HNF4A and TFAP2A) were the most abundant in three different cell types and all co-localized with AR (Pihlajamaa et al., 2014). Evidence showed that depletion of FOXA1 and GATA2 attenuated binding of AR and ER and subsequent transcriptional activation (Pihlajamaa

et al., 2015). However, investigations into what pioneer factors work in conjunction with AR or ERs in brain tissues or non-mammalian tissues in general are lacking.

Interestingly, it has been suggested that the distribution and frequency of AREs and EREs in regulatory regions are species-specific in songbirds (Frankl-Vilches et al., 2015). The comparative work on two species of songbirds, one (canaries) being more hormone-sensitive than the other (zebra finches), demonstrated higher occurrences of AREs and EREs in regulatory regions (1 kb upstream from transcription start sites) of testosterone-responsive genes in canaries (Frankl-Vilches et al., 2015). Similar results were observed in rodents, where a comparison between rat and mice epididymis was made (Hu et al., 2010).

II. 5. b. Non-classical signaling pathway

More recently, it has been observed that the canonical sex hormone receptors (AR, ERα and ERβ) can also act as membrane receptors and induce very rapid effects (milliseconds to minutes) that are too fast for genomic actions (Davey and Grossmann, 2016; Hadjimarkou and Vasudevan, 2018; Rahman and Christian, 2007; Simoncini and Genazzani, 2003; Simoncini et al., 2004; Vasudevan and Pfaff, 2007). Instead of translocation to the cell nucleus, the canonical sex hormone receptors can locate to the plasma membrane and activate secondary messenger pathways. For example, in both Sertoli cells and macrophages, membrane-bound AR (mAR) can activate Gq protein-mediated phospholipase C signaling, resulting in membrane depolarization and Ca^{2+} influx in a testosterone-dependent manner (Benten et al., 1999; Loss et al., 2004; Von Ledebur et al., 2002). An alternative pathway has been reported for mARs, in which they activate MAPK3, leading to phosphorylation of CREB (a transcription factor) and following transcription of CREB-regulated genes, including EGR1 (Fix et al., 2004). Studies of mAR are largely *in vitro*, using cancer cell lines (Davey and Grossmann, 2016). Furthermore, the nature of mAR is not clear (see below) and the roles that mAR plays in normal physiological contexts remains to be discovered. In contrast to AR, more

studies focused on the non-classical rapid effects of ERs in neural tissues (Hadjimarkou and Vasudevan, 2018; Vasudevan and Pfaff, 2007). For instance, estradiol rapidly decreases the spontaneous firing of neurons in the medial preoptic areas (Kelly et al., 1977) and rapidly increases the firing rate of pituitary cells *in vitro* (Dufy et al., 1979). That social interactions and song playbacks rapidly increase production of neuroestrogens in an auditory brain region (NCM) of zebra finches was shown by *in vivo* microdialysis (Remage-Healey et al., 2012; Remage-Healey et al., 2008). However, agonists for ERα and ERβ do not mimic rapid estradiol actions in the NCM of male zebra finches, indicating the possibility of involvement of an alternative receptor (Remage-Healey et al., 2013) (see below). In anesthetized zebra finches, perfusing estradiol into NCM increased auditory-evoked firing rates and bursts in the same region within 30 minutes (Remage-Healey et al., 2012). However, most data suffer from the application of high pharmacological levels of testosterone or estradiol.

Recently, it has been shown that membrane receptors other than the canonical sex hormone receptors could mediate the effects of androgens and estrogens. In the case of androgens, GPRC6A, a member of class C G-protein coupled receptor (GPCR), has been identified as a membrane androgen receptor both *in vivo* and *in vitro*. GPRC6A stimulated ERK activity in a dosage-dependent manner by testosterone (Pi et al., 2010). On the other hand, G protein-coupled estrogen receptor 1 (GPER1), also a member of class C GPCR, binds to estradiol with high affinity and can couple to $G\alpha_s$ protein, leading to stimulation of adenylate cyclase and an increase in cyclic AMP levels (Hadjimarkou and Vasudevan, 2018). Investigation into the roles of these membrane receptors in songbird brains has shown that GPER1 is involved in auditory responsiveness in zebra finches in a sex-specific way (Krentzel et al., 2018).

II. 6. Expression of sex steroid receptors in the song control system

Because song features of songbirds, in some extent, are sex hormone-dependent (Gahr, 2014), the question about the possible sites of hormone regulation was raised. Using *in situ* hybridization or

immunohistochemistry techniques, both protein and mRNA expression of the AR were detected in the medial preoptic area in the hypothalamus in all examined bird species (Bernard et al., 1999; Fusani et al., 2000; Gahr, 2014; Metzdorf et al., 1999) (Figure 5). In fish and mammals, AR is also expressed in the hypothalamus (Diotel et al., 2018). In addition, AR expression was observed in the brainstem respiratory-vocal areas (nXIIts, RAm and PAm) and forebrain areas HVC, RA and LMAN in all examined songbirds (Alward et al., 2018; Bernard et al., 1999; Frankl-Vilches and Gahr, 2017; Gahr, 1990a, b; Gahr, 2000; Gahr, 2014). In Area X, AR expression was detected in some individuals of canaries (Gahr, 2006).

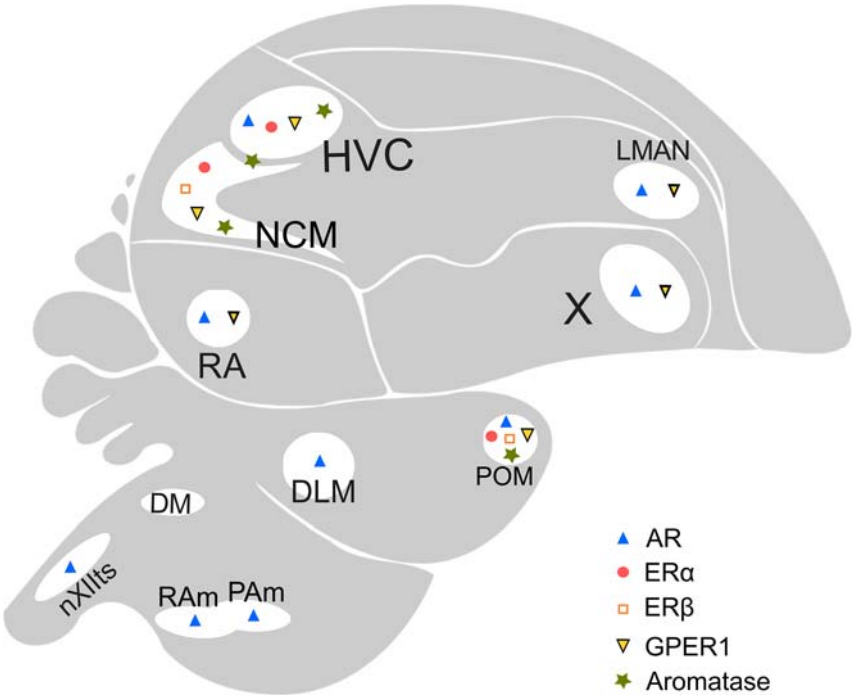

Figure 5. The bird brain and the brain nuclei involved in song control are shown.

This figure also illustrates the distribution of androgen receptor (AR), estrogen receptor α (ERα), estrogen receptor β (ERβ), G protein-coupled estrogen receptor 1 (GPER1) and aromatase. Abbreviations: HVC: initially Hyperstriatum Ventrale pars caudale, now acronym used as proper name; NCM: caudal medial nidopallium; LMAN: lateral magnocellular nucleus of the anterior nidopallium; RA: nucleus robustus arcopallialis; X: Area X; POM: medial preoptic nucleus; DLM: dorsal lateral nucleus of the medial thalamus; DM: dorsomedial intercollicular nucleus; nXIIts: tracheosyringeal part of the XIIth cranial nerve; RAam: nucleus retroambigualis; PAm: nucleus parambigualis. Drawn after data of (Acharya and Veney, 2012; Ball et al., 1999; Bernard et al., 1999; Fusani et al., 2000; Gahr, 1990a, b; Metzdorf et al., 1999; Saldanha and Coomaralingam, 2005; Vockel et al., 1990a; Vockel et al., 1990b).

In contrast to AR, ERα expression in songbird brain was only detected in the HVC and the dorsal surrounding of RA in canaries and zebra finches (Figure 5) (Ball et al., 1999; Bernard et al., 1999; Fusani et al., 2000; Gahr, 1990a, b; Gahr et al., 1993; Metzdorf et al., 1999; Saldanha and Coomaralingam, 2005). The distribution of ERα expression in the HVC is species-specific: i.e., 1) high expression throughout the HVC in canaries and East-African shrike, 2) high expression in the medial part of HVC in forest weavers and black red-start, and 3) no expression in the lateral part and low expression in the medial part of HVC in zebra fiches and Bengalese finches (Frankl-Vilches and Gahr, 2017). Of note, the population of ERα$^+$ cells is bigger in canaries (approximately 15% of the HVC neurons) than in zebra finches (~0.5%) (Gahr et al., 1987; Gahr et al., 1993).

Few studies investigated expression of ERβ and GPER1 (protein or mRNA). In adult European starlings, ERβ mRNA was detected in the caudomedial nidopallium (NCM) and the medial preoptic area, but not in HVC (Ball et al., 1999; Bernard et al., 1999) (Figure 5). In adult zebra finches, GPER1$^+$ cells were detected in the HVC, RA, LMAN, and NCM (Acharya and Veney, 2012; Krentzel et al., 2018) (Figure 5).

Aromatase is the enzyme converting testosterone to estradiol in the brain (see II. 2. Steroidogenesis). The expression of aromatase has been studied in detail. Its expression was detected at high levels in the caudo-ventral surrounding of the HVC, NCM, and medial preoptic area and at low levels in the HVC (Alward et al., 2018; Fusani et al., 2001; Metzdorf et al., 1999; Vockel et al., 1990a; Vockel et al., 1990b) (Figure 5).

Given that HVC is involved in various important aspects of song control (see I. 1. Song control system is a complex neural circuit responsible for song control) and it is the only song control nucleus expressing both AR and ESR1, it is likely that HVC is an important site for hormonal regulation (Herrmann and Arnold, 1991).

III. Aims of the thesis

This thesis focuses on understanding the molecular mechanisms of how testosterone influences song development in adult female canaries. Furthermore, the thesis will investigate whether sexually monomorphic singing behavior is a result of sexually monomorphic gene expression by testosterone manipulation in canaries or by comparative approaches, using multiple species of songbirds that differ in their degree of sex-specific singing.

The first experiment of this thesis (p. 43) aimed to quantitatively describe spontaneous female songs. The canary (*Serinus canaria*) is a northern temperate songbird species exhibiting stark sexual dimorphism in singing behavior. Male canaries sing regularly, especially in the breeding seasons, while a low percentage of females sing spontaneously on rare occasions. Shoemaker noted "one case out of about a hundred in these flocks an untreated female uttered a loud song" (Shoemaker, 1939). Few other anecdotal occasions were documented in later studies (Hartley et al., 1997; Herrick and Harris, 1957). Although spontaneous singing of female canaries has been known for at least several decades, only two studies, to my knowledge, have shown spectrograms of these songs (Pesch and Güttinger, 1985; Vallet et al., 1996), and no study has quantified spontaneous female songs. I screened 112 female canaries and found six females (5%) emitted spontaneous songs. I followed the development of these songs and showed that the song structures of each individual varied over time. Moreover, I found that the syllable repetition rate was one of the most important components leading to great individual differences in these songs, as suggested by principle component analysis. By comparing the blood plasma testosterone levels between singing and non-singing females, I found that the singing females had significantly higher testosterone levels. However, the spontaneous songs were most discernible from songs of testosterone-treated females and males by syllable repetition rate and song length. Thus, the data suggested a link between female singing and testosterone levels within physiological range. Nevertheless, high level of testosterone is required for eliciting high syllable repetition songs.

Introduction

Although spontaneous female canary song is rare, female canary songs can be easily induced within 3 to 5 days of testosterone implantation. Previous studies demonstrated that the estrogenic metabolites of testosterone activates VEGF pathway via VEGF receptor (KDR) leading to angiogenesis and an increased BDNF production in the HVC of female canaries within one week (Louissaint et al., 2002). BDNF is essential for female song development and for neuronal recruitment in the HVC, which peaks around two weeks after testosterone treatment (Hartog et al., 2009; Kirn et al., 1999). Under this framework, I studied the progression of testosterone effects on gene expression in the HVC. In the second experiment of this thesis (p. 65), I implanted testosterone in six groups of female canaries: three time periods for observing short-term effects (one, three and eight hours), and another three time periods for observing long-term effects (three, seven and 14 days). An additional control group was implanted with empty tubes. To confirm the systemic effects of testosterone, I measured the blood plasma testosterone, singing behavior, and the volumes of HVC. The blood testosterone levels responded to the treatment within one hour, 11 out of 12 birds sang by the fourth day, and the HVC volumes significantly increased after 2 weeks. To study the transcriptomic effects of testosterone, I performed microarray analyses on the HVC. I found that within one hour, HVC transciptomes responded to testosterone treatment; more than 14% of genes in the canary genome expressed differently than the control group. Few genes (0.3% of the genome) were constantly up- or down-regulated; instead, the majority of genes (52% of the genome) were regulated transiently. Moreover, Gene Ontology (GO)-term enrichment analysis showed that genes associated with angiogenesis were enriched as early as one hour after testosterone treatment, and that genes associated with neuronal recruitment were enriched after three days of the treatment. Furthermore, by using bioinformatics approaches to integrate blood testosterone levels, singing behavior, and gene expression data, I identified a hub gene (transcription factor SP8), which might be a potential master regulator of changes caused by testosterone.

In the third experiment of this thesis (p. 81), I investigated the sex differences in the HVC transcriptomes in canaries with or without testosterone treatment by microarray analyses. I found that

although females sang male-like songs after one month of testosterone treatment, the gene expression profiles were greatly different from singing breeding males and testosterone-treated males. In addition, the HVC transcriptomes of testosterone-treated females were greatly different from female groups, including two non-singing females (breeding and non-breeding) and the spontaneously singing females. This data suggested that although testosterone masculinized the HVC and singing of female canaries, there are alternative pathways for females to achieve similar song output using completely different gene sets.

Based on the results from the third part, transcriptomic sex differences existed even when the female and male birds exhibited similar singing behavior. In the fourth experiment of this thesis (p. 89), I extended the investigation into whether sex differences would exist in gene expression in the HVC by comparing three songbird species with distinct levels of sex differences in singing behavior. In addition, I questioned whether the extent of transcriptomic sex differences would correlate with the degree of sex differences in singing. The female and male forest weavers sing identical songs after pair formation (Wickler and Seibt, 1980). The female blue-capped cordon bleus sing shorter and less stereotyped songs than the males (Geberzahn and Gahr, 2011). In canaries, the majority of females do not sing regularly, while males sing elaborate songs in the breeding seasons (Leitner et al., 2001a; Leitner et al., 2001b; Voigt and Leitner, 2008). By comparing the HVC transcriptomes of the same species, I identified genes that were expressed differently between sexes (sex-biased genes) in each species. I found stark sex differences in gene expression in all three species, even in the forest weavers, in which birds sing identical songs. Counter-intuitively, I found that the majority of sex-biased genes were on autosomes, instead of sex chromosome Z. Furthermore, sex-biased genes differed depending on species. These results suggested that females and males of both monomorphic and sex-dimorphic singing species activate different gene networks in HVC, a key nucleus in the song control system. The data also suggested that the co-evolution of females and males might have species-specific trajectories. The comparative approach indicates that sex is an important variable even when the behavioral output of females and males is identical.

Finally, in the Discussion chapter, the results of this thesis are summarized and discussed in a boarder context, and questions left unanswered and possible future developments in tackling these questions are discussed.

Results

I. Experiment I

Spontaneous female canary songs

Songbirds (oscines) constitute approximately half of all extant bird species (Gill and Donsker, 2015) and live in diverse habitats. Male songbirds use song in various contexts such as mate attraction and territory defense (Catchpole and Slater, 1995; Kroodsma and Byers, 1991; Searcy and Andersson, 1986; Wickler and Seibt, 1980) and a recent study suggested that among 1,314 surveyed songbird species, females sing in at least 656 of them (Webb et al., 2016). Singing in females might be ancient and widespread (Odom et al., 2014), but among species of the northern temperate zone female song is more uncommon in general and, if present, is often restricted to a specific context or to a limited group of individuals. The patterns of female song have only been analyzed quantitatively in a few northern temperate species: the European robin (*Erithacus rubecula*) (Hoelzel, 1986), the European starling (*Sturnus vulgaris*) (Pavlova et al., 2005), the red-winged blackbird (*Agelaius phoeniceus*) (Beletsky, 1983; Yasukawa et al., 1987), the Northern Cardinal (*Cardinalis cardinalis*) (Yamaguchi, 1998, 2001), the white-crowned sparrow (*Zonotrichia leucophrys*) (Baptista et al., 1993) and the dark-eyed junco (*Junco hyemalis*) (Reichard et al., 2017). Additionally, occasional or individual female song was reported in a larger number of northern species (Langmore, 1998; Ritchison, 1983; Webb et al., 2016). Some of these reports are convincing but miss quantitative analysis of female song structure of, for example, the canary (*Serinus canaria*) (Pesch and Güttinger, 1985), the house wren (*Troglodytes troglodytes*) (Johnson and Kermott, 1990) and the song sparrow (*Melospiza melodia*) (Arcese et al., 1988)). Further studies report female song only anecdotally and lack clear methods to identify the sex of the singing individuals as in skylarks (*Alauda arvensis*) (Delius, 1965), chaffinches

(*Fringilla coelebs*) (Halliday, 1948), Eurasian reed warblers (*Acrocephalus scirpaceus*) (Kuschert and Ekelöf, 1981) and American dippers (*Cinclus americanus*) (Bakus, 1959).

The domesticated canary (*Serinus canaria*), a northern temperate songbird originally from wild populations of the North Atlantic islands (Leitner et al., 2001a), is an important model species for studying plasticity of birdsong and its underlying neural circuitry (for review see Chen et al. (2013)). A major component of this so-called song control system is HVC, an anatomically well-defined nucleus critical for many functions such as integration of auditory input and motor output as well as song pattern generation (Hahnloser et al., 2002; Nottebohm et al., 1976; Wild, 2004). The HVC of male canaries undergoes anatomical (Gahr, 1990a; Nottebohm, 1981) as well as transcriptional (Frankl-Vilches et al., 2015) seasonal plasticity, both processes being testosterone-sensitive. The song control system is present in both sexes and singing as well as a growth of HVC can be easily induced by testosterone treatment in females. Since the neuronal changes underlying testosterone-induced female singing are thought to be comparable to changes naturally occurring in males due to seasonality (Balthazart et al., 2010; Brenowitz and Lent, 2002; Devoogd et al., 1985; Nottebohm, 1980), female canaries are used to mimic hormone-dependent plasticity of birdsong and its neuronal correlates (Fusani et al., 2003a; Madison et al., 2015; Nottebohm, 1980; Shoemaker, 1939). To be able to reliably use female canaries as a model for testosterone-induced singing in songbirds, it is important to evaluate spontaneous female singing in this species. Although it is known that female canaries are able to sing without hormone treatment (Hartley et al., 1997; Herrick and Harris, 1957; Pesch and Güttinger, 1985; Shoemaker, 1939; Vallet et al., 1996), the structure of spontaneously uttered song has not been quantitatively described yet.

By screening continuous sound recordings of a large population of 112 female canaries, we were able to document spontaneous songs in six individuals. In order to describe the female song syntax, we developed a tool for categorizing song syllables computationally. We show that females are capable of producing song with a structural organization similar to male song but with more variation in quality. To

elucidate potential factors correlating with song development and to compared singing and non-singing females, we furthermore measured plasma testosterone concentration, HVC size and syrinx weight. We found that an increase in HVC volume of singing females and that song development in singing females is likely testosterone-dependent.

I. 1. A minority of female canaries sing spontaneously

We screened 112 female canaries among which six spontaneously started to sing (Figure 6a). We defined a sound segment as an individual song if the segment was longer than three seconds and did not contain a single pause of more than 1.5 seconds or contained less than three consecutive pauses longer than 0.4 seconds. All 112 recorded females produced calls. The singing females were monitored for at least 40 days each, while two of the six birds (bird 2 and 5) were monitored more than 200 days in total (Figure 6a). Each of the 112 females was observed for at least four weeks to screen for occasional singing. Nine non-singing females were included in this study.

I. 2. Daily singing rates

Song occurrences were low and varied daily. At least one fifth of recordings per individual were analyzed. The percentage of days with observed singing was variable, ranging from 8% to 77% depending on the individual (Table 1). Bird 3 sang least frequently and only produced seven songs on three different days during the recording period, therefore spending as little as 0.0078% of the day singing (Table 1). In contrast, bird 6 showed the highest singing activity with song produced on 77% of recorded days (Table 1). On these days, the bird produced 42 ± 47 songs (mean ± SD), an average singing time of 0.83% per day. In addition, preference of singing activity within a day varied among birds: bird 1 and 2 preferred to sing before noon, whereas bird 4 and 5 preferred the afternoon while bird 6 sang throughout the whole day (Figure 6b).

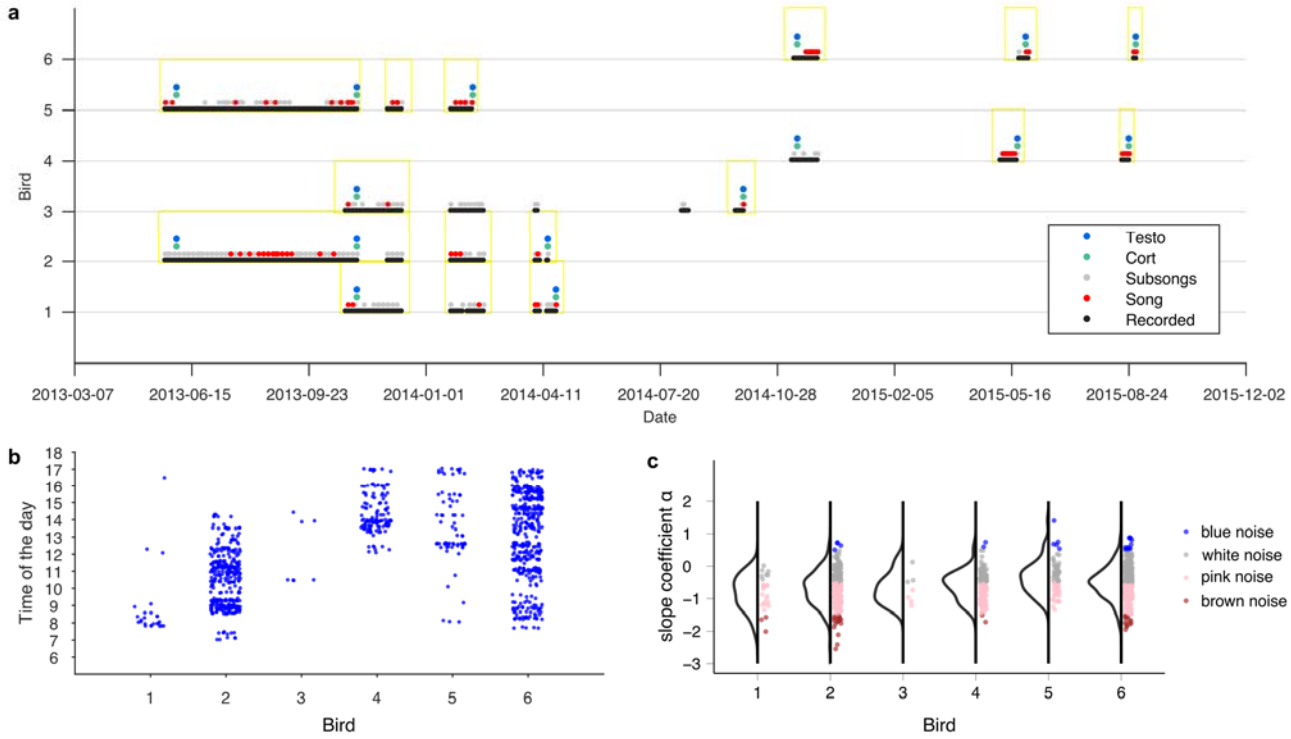

Figure 6. Female canary singing was infrequent and varied between individuals.

a, The time line of the recording periods. Six female canaries were recorded repeatedly in sound-attenuated boxes (black dots), other times they were housed in single-sex aviaries with other females. The occurrences of songs (see methods) and unstable songs were marked red and grey, respectively. The dates when blood samples for plasma testosterone and corticosterone measurement were taken were marked blue and green, respectively. Songs from different recording periods (encircled by yellow dotted-boxes) were analysed separately (see texts). b, Preference of singing activity of each bird. While bird 6 sang all day, bird 1 and bird 2 primarily sang in mornings, whereas bird 4 and bird 5 sang in afternoons. No songs were uttered before 6 am or after 6 pm, when the light was off. Each data point represents a song. c, The slope coefficient α (see method) reflects the temporal organization of syllables in a song. A white noise song indicates syllables were randomly organized, in contrast to a pink noise song, which might contain biological information. A blue noise song indicates short-interval of syllable rhythmic repeats, whereas a brown noise song indicates long-interval of syllable rhythmic repeats. Probability density functions estimated for each bird were depicted, showing the majority songs of bird 1, 2 and 3 were pink noise songs, while the majority songs of bird 4, 5 and 6 were white noise songs.

Table 1. Song occurrence and song rate of spontaneously singing female canaries.

Number days recorded: number of days of recording made; Number days analysed (% analysed): number of days of recording analysed (percentage of analysed days among recorded days); Number days with songs: number of days with song presence among the days that were analysed; Number songs: total number of songs observed; Song occurrence (%): chance of observing songs in an analysed day; Song rate (%): percentage of time spent singing among the days analysed (9-hour recording time daily); Number songs/day: number of songs in an analysed day when singing occurred.

Bird	Number days recorded	Number days analysed (% analysed)	Number days with song(s)	Number songs observed	Song occurrence (%)	Song rate (%)	Number songs/day		
							Mean ± SD	Min	Max
1	95	28 (30)	6	25	0.21	0.024	4.2 ± 4.2	1	12
2	221	64 (29)	18	331	0.28	0.069	18 ± 19	1	67
3	100	38 (38)	3	7	0.08	0.0078	2.3 ± 1.5	1	4
4	47	22 (47)	10	128	0.45	0.21	13 ± 13	1	44
5	202	42 (21)	16	71	0.38	0.066	4.4 ± 5.4	1	19
6	40	13 (33)	10	423	0.77	0.83	42 ± 47	1	167

I. 3. Between individual variation in song structure

The differences in overall song structure between singing females were conspicuous (Figure 7, Table 2). Power spectrum analysis showed that most (>55%) songs of bird 1, 2 and 3 had characteristics of pink noise, indicating that the song syllables were organized in a non-random fashion (See methods). In contrast, most (>40%) songs of bird 4, 5 and 6 had characteristics of white noise, indicating temporal organizations of song syllables were random (Figure 6c). Moreover, the songs of the same individual visually differed during subsequent recording periods (e.g. bird 2, Table 3). Therefore, we analyzed the song structure separately for songs recorded more than three weeks apart and compared the song structure observed during these recording periods within the same individual (Figure 6a).

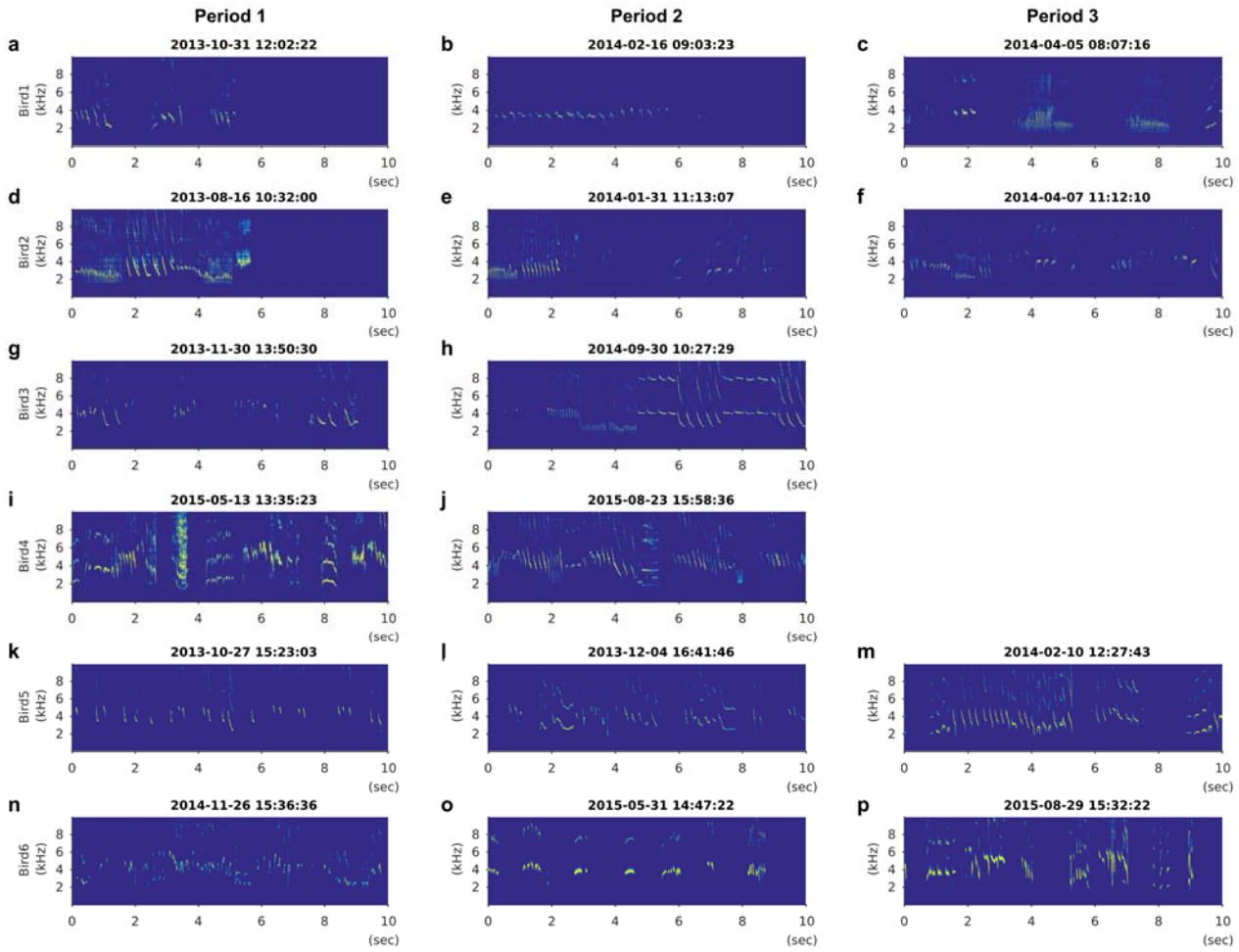

Figure 7. Female canary song structures changed over time.

The first 10 seconds of the longest song of each period of each bird is visualized in a sound spectrogram (sonogram). a-c, bird 1; d-f, bird 2; g-h, bird 3; i-j, bird 4; k-m, bird 5; n-p, bird 6.

Table 2. Summary table for measured song parameters (mean ± SD).

Bird	Number of songs analyzed	Song length (s)	Repetition rate (syl/s)	Number of syllables per song	Slope coefficient α	Syllable length (ms)	Syllable interval (ms)	Wiener entropy	Peak freqency (kHz)
1	25	8.8 ± 3.9	5.2 ± 1.7	46 ± 22	-0.82 ± 0.52	65 ± 68	127 ± 275	-9.2 ± 1.3	2.6 ± 0.53
2	301	4.3 ± 2.4	6.8 ± 2.0	30 ± 8	-0.66 ± 0.54	73 ± 57	90 ± 146	-9.6 ± 1.1	2.8 ± 0.72
3	7	10 ± 4.2	7.4 ± 4.2	84 ± 62	-0.68 ± 0.46	54 ± 57	67 ± 153	-8.6 ± 0.91	2.7 ± 1.1
4	128	6 ± 2.1	5.5 ± 1.0	28 ± 11	-0.60 ± 0.46	109 ± 88	79 ± 120	-9.4 ± 1.3	4.1 ± 1.2
5	71	13 ± 9.6	3.4 ± 0.93	45 ± 31	-0.36 ± 0.52	107 ± 74	201 ± 220	-10 ± 1.1	3.6 ± 0.76
6	423	8.2 ± 2.5	5.4 ± 0.96	30 ± 16	-0.52 ± 0.5	85 ± 72	100 ± 105	-10 ± 1.9	3.8 ± 1.1

Table 3. Summary table for measured song parameters (mean ± SD) in each recording period, and the average performance of the period with the highest average repetition rate each bird ("best" period).

Bird	Period	Number of songs analyzed	Song length (s)	Repetition rate (syl/s)	Number of syllables per song	Slope coefficient α	Syllable length (ms)	Syllable interval (ms)	Wiener entropy	Peak freqency (kHz)	Repertoire size	The "best" period
1	1	3	4.6 ± 0.97	3.4 ± 0.49	15 ± 2.5	-1.1 ± 0.47	119 ± 68	191 ± 277	-9.3 ± 1.6	2.9 ± 0.5	16	
	2	2	4.9 ± 2.6	3.1 ± 0.64	16 ± 11	-0.83 ± 0.74	127 ± 77	175 ± 147	-9.0 ± 1.5	3.3 ± 0.42	14	
	3	20	9.9 ± 3.7	5.7 ± 1.5	54 ± 17	-0.77 ± 0.52	61 ± 66	123 ± 278	-9.2 ± 1.3	2.6 ± 0.52	32	*
2	1	245	3.8 ± 0.62	8.3 ± 1.3	31 ± 6.3	-0.73 ± 0.51	75 ± 70	47 ± 41	-9.7 ± 1	2.7 ± 0.66	26	*
	2	50	4.4 ± 1.4	7.1 ± 1.7	30 ± 6.7	-0.55 ± 0.59	70 ± 55	81 ± 127	-9.4 ± 1.2	3.3 ± 0.8	46	
	3	6	9.1 ± 3.8	4.7 ± 2.4	37 ± 12	-0.44 ± 0.49	91 ± 70	156 ± 205	-8.8 ± 1.2	3.4 ± 0.86	38	
3	1	3	6.8 ± 3.3	3 ± 0.2	20 ± 9.8	-0.26 ± 0.34	95 ± 85	250 ± 374	-9.8 ± 1.2	4.1 ± 0.72	14	
	2	4	13 ± 3	11 ± 1.5	132 ± 25	-1.0 ± 0.2	49 ± 51	46 ± 81	-8.5 ± 0.76	2.5 ± 0.96	11	*
4	1	91	6.3 ± 3.2	4.1 ± 0.8	26 ± 13	-0.53 ± 0.48	149 ± 111	100 ± 135	-9.6 ± 1.3	3.8 ± 1.2	34	
	2	37	5.1 ± 1.9	5.7 ± 1.1	29 ± 12	-0.75 ± 0.36	104 ± 87	74 ± 123	-8.9 ± 1.3	4.3 ± 1.1	52	*
5	1	26	11 ± 5.8	2.5 ± 0.68	29 ± 15	-0.093 ± 0.6	89 ± 64	325 ± 276	-10 ± 1.1	3.6 ± 0.69	34	
	2	11	9.6 ± 5.6	2.7 ± 0.95	26 ± 18	-0.3 ± 0.42	86 ± 66	293 ± 270	-9.5 ± 1.2	3.5 ± 0.69	25	
	3	34	14 ± 11	3.7 ± 0.78	51 ± 34	-0.59 ± 0.36	115 ± 77	171 ± 195	-10 ± 1.1	3.6 ± 0.8	27	*
6	1	167	7.6 ± 9.7	4 ± 0.87	29 ± 37	-0.48 ± 0.52	106 ± 95	157 ± 211	-8.8 ± 1.3	4.3 ± 1.1	35	
	2	35	6.2 ± 2.9	3.8 ± 0.85	22 ± 9.1	-0.92 ± 0.49	103 ± 83	181 ± 275	-11 ± 2.2	3.9 ± 0.95	31	
	3	221	9.1 ± 7.3	5.6 ± 1.1	52 ± 44	-0.49 ± 0.47	79 ± 64	97 ± 126	-11 ± 1.7	3.6 ± 1.0	21	*
"best" period			6.9 ± 6.2	6.7 ± 1.9	42 ± 32	-0.59 ± 0.50	80 ± 69	86 ± 130	-10 ± 1.6	3.3 ± 1.0	28 ± 14	

We applied principal component analysis (PCA) to the medians and interquartile ranges of the parameters (song level parameters: song length, number of syllables in a song, syllable repetition rate, the slope coefficient α; syllable level parameters: syllable length, inter-syllable silent intervals (pauses), the peak frequency and the Wiener entropy) for each period of each bird (Figure 8a). Parameters that contributed the most to PC 1 (principal component 1) were median repetition rate and median number of syllables in a song, whereas the most relevant parameters to PC 2 were the interquartile range of song length and of inter-syllable interval. In other words, PCA suggests that the differences in repetition rate, the number of syllables in a song, the variations of song length and inter-syllable pauses contributed most to the distinction between songs of different singing periods of the six birds.

To evaluate the extent of song variation between singing female canaries and given that female song quality changed over time (see below), we chose the period with best song quality for analysis by using syllable repetition rate as an indicator. Syllable repetition rates were higher during stable periods than during unstable ones. In addition, PCA suggests the parameter that distinguishes song periods the most (Figure 8a). The average repetition rate during the best period of six birds was 6.7 ± 1.9 syllables per second (syl/s, Table 3 range: 1.1 to 12.5 syl/s). Bird 3 was the best singer among the six with an average repetition rate of 11 ± 1.5 syl/s and a maximum of 12.5 syl/s. Bird 2 was the second best with an average of 8.3 ± 1.3 syl/s and a maximum of 12.2 syl/s. The worst bird was bird 5 with an average of 3.7 ± 0.8 syl/s (Table 3).

I. 4. Within individual variation of song and song syntax

To estimate within-individual song variation, we calculated the center of mass for each bird using the coordinates of the different recording periods on the first six principal components, which explained over 90% of variance in the dataset (Figure 8b). Subsequently, for each bird we calculated the mean Euclidean distance between individual periods to the center of mass. This provides an approximate estimation for

variations between different song recording periods for each bird. We found that bird 4 had the smallest mean distance, implying the least change in song structure, whereas bird 3 had the greatest mean distance to the center of mass, implying the greatest change in song structure (Figure 7, mean Euclidean distance of females: 3 > 1 > 6 > 5 > 2 > 4). The PCA results reflected the visual impressions on the songs of the six individuals (Figure 7).

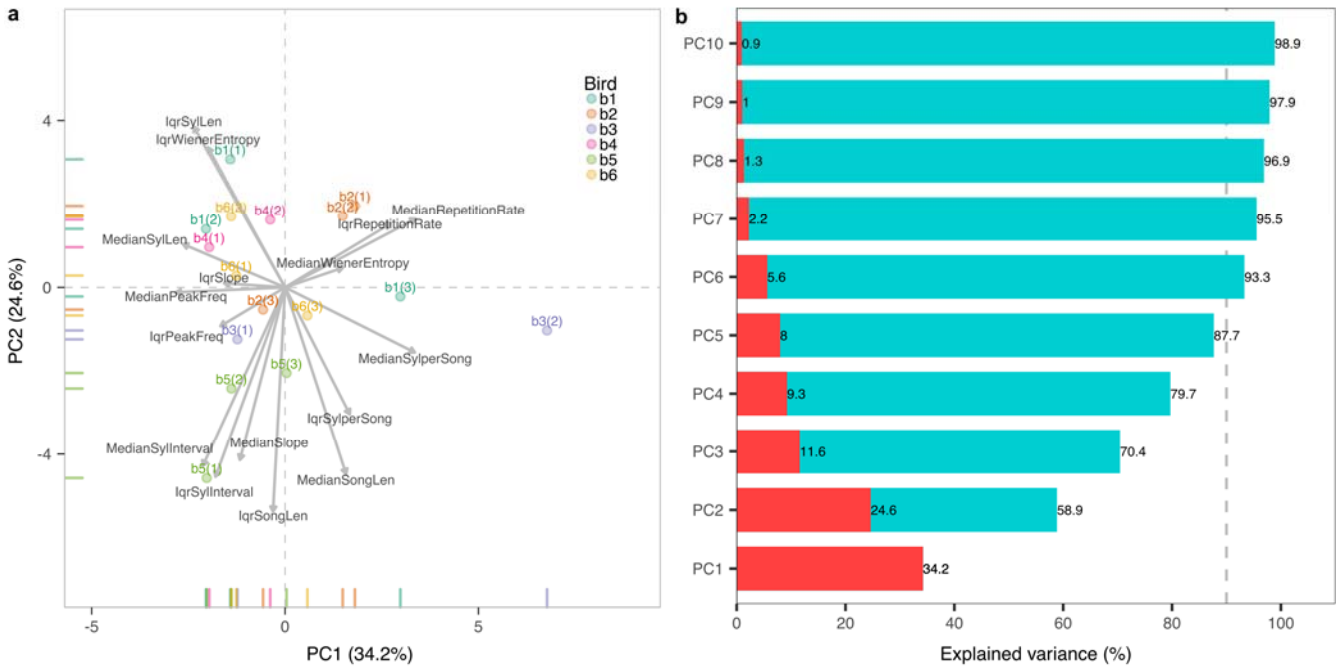

Figure 8. Song variation within individuals.

Principle component analysis (PCA) of the medians and interquartile ranges (IQR) of the measured parameters for each period of each bird. a, A visualization of the contributions the measured parameters to the first 2 principle components (PC1 and PC2, grey arrows) and the coordinates of the observations (in this case, recording periods). The most relevant parameters for PC 1 were median repetition rate, median number of syllable in a song, and median peak frequency, whereas the most relevant parameters to PC 2 were IQR of song length, IQR of syllable interval and median of song length. Data points from different birds were color-coded, and periods were indicated inside the parentheses. Abbreviations: SongLen: song length; Slope: slope coefficient α; SylLen: syllable length; SylperSong: number of syllables per song; SylInterval: inter-syllable interval; PeakFreq: peak frequency. b, A scree plot showing the first six PCs explained more than 90% of the data variance.

A separate PCA on the song-level parameters (song length, repetition rate, the number of syllables in a song, and slope coefficient α) showed that because of the high repetition rate and long song length, songs from period 2 of bird 3 were the most distinct from the songs from other birds, (Table 3 and Figure 9). Four songs of bird 2 differed significantly from its other songs, mainly because these songs showed a greater repetition rate. Similarly, about 20 songs of bird 6 were distinct, being significantly longer while showing a low repetition rate.

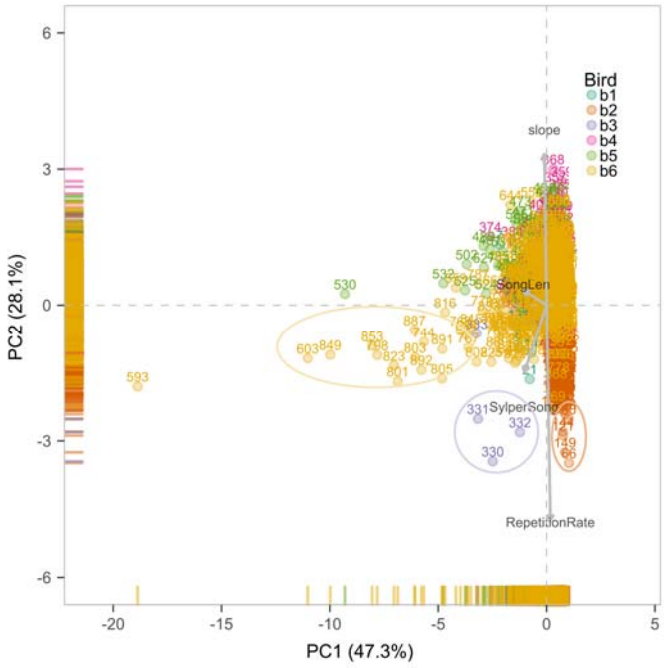

Figure 9. Principle component analysis (PCA) on song-level parameters (song length, repetition rate, the number of syllables in a song, and slope coefficient).

A visualization of the parameter contributions to the first 2 principle components (PC1 and PC2, grey arrows) and the coordinates of the observations (in this case, songs). The most relevant parameter for PC 1 was song length, whereas the most relevant parameter for PC 2 was repetition rate. Songs from period 2 of bird 3 were the most distinct from the songs from other birds (blue dots), indicating the songs were distinct on repetition rate and song length.

Given that the song structure changed overtime within individuals, we asked whether the syllable usage would alter as well. Therefore we performed syntax analysis on the syllables of two individuals (bird 3 and bird 2). The song structure of bird 3 changed most dramatically among the six individuals in terms of increased syllable repetition rate (Student's t test, $t = -10.15$, $df = 3.13$, $p = 1.7 \times 10^{-3}$), whereas those of bird 2 changed into the opposite direction (Kruskal-Wallis rank sum test followed by Bonferroni-Dunn post hoc test for effects of recording period on repetition rate, adjusted P values: 3.1×10^{-5}, 0.0034, 0.38, period 1-2, period 1-3, period 2-3, respectively). Songs of both animals were well-structured and syllables were relatively stereotypical.

Period 1 of bird 3 comprised three songs, consisting of 61 syllables that were sorted into 14 different clusters (SylSorter). The majority was assigned to cluster five and 28 (arbitrary numbers), while the syllables in cluster 28 were used to initiate songs. Clusters 54, 57, and 61 were used as final syllable of the songs (Figure 10 and Supplementary Figure 1). The average syllable repetition rate of the second recording period was more than threefold higher than that of the first recording period (Table 3). The 528 syllables of the second period were sorted into eleven clusters (Figure 11a, b, and Supplementary Figure 2). Songs were started with syllables from cluster 295, 143 and 296 (Figure 11c, d). Syllables in cluster 78 were primarily produced with a high repetition rate following a phrase of repetitive syllables in cluster 394 after initiation of the song. Syllables in cluster 78 ended the songs (Figure 11c, d).

We furthermore compared the syllables produced by bird 3 during different recording periods (Figure 10 and Figure 11). Cluster 78 of the second period was similar to cluster 28 of the first period (SSIM: 0.08) and both were used in the repetition of songs. Cluster 394 of the second period could be traced back to cluster 5 of period one (SSIM: 0.2533) which was used in the initial phrase of songs. Therefore, it is likely that bird 3 increased the song length between the periods by adding repetitions of pre-existing syllables.

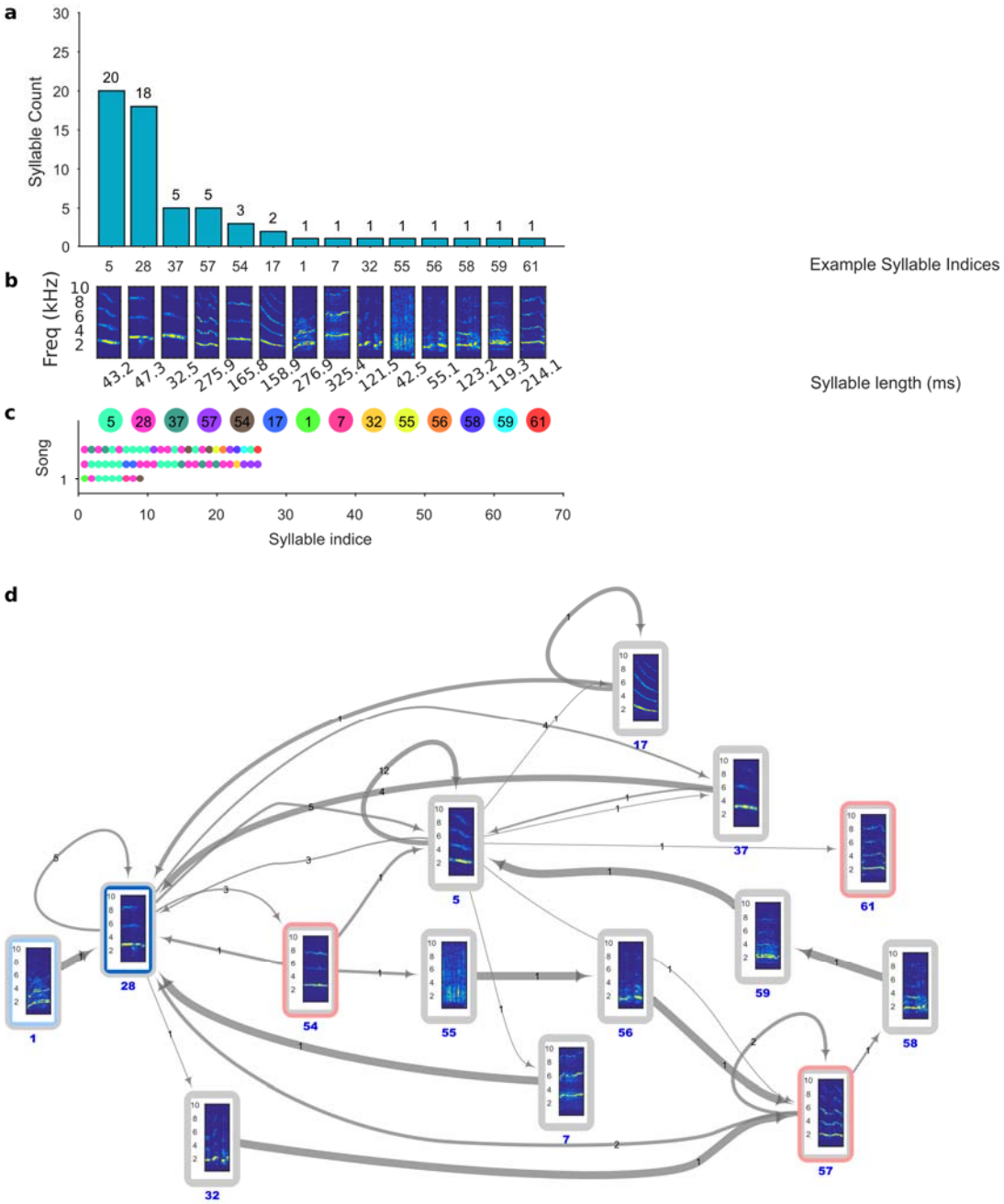

Figure 10. Syllable analysis of songs from period 1 of bird3.

SylSorter classified the 61 syllables into 14 clusters based on Structural Similarity Index. a, A histogram summarizes the occurrences of each cluster. b, Sound spectrograms of the representative syllables. c, Visualization of syllable composition of songs. Syllables were colour-coded according to the SylSorter assignment. d, A simplified network-based representation of song syntax. Each node represents a cluster type (blue texts), and is connected to the node which is the most likely to occur. The width of edges represents the probability to the next node; the number of occurrences was indicated as well. Clusters that were used as the first syllables of the songs were labelled in blue, while clusters that were used as the last syllables of the songs were labelled in red. Darker colours indicate higher probability of being the first (blue) or last (red) syllable.

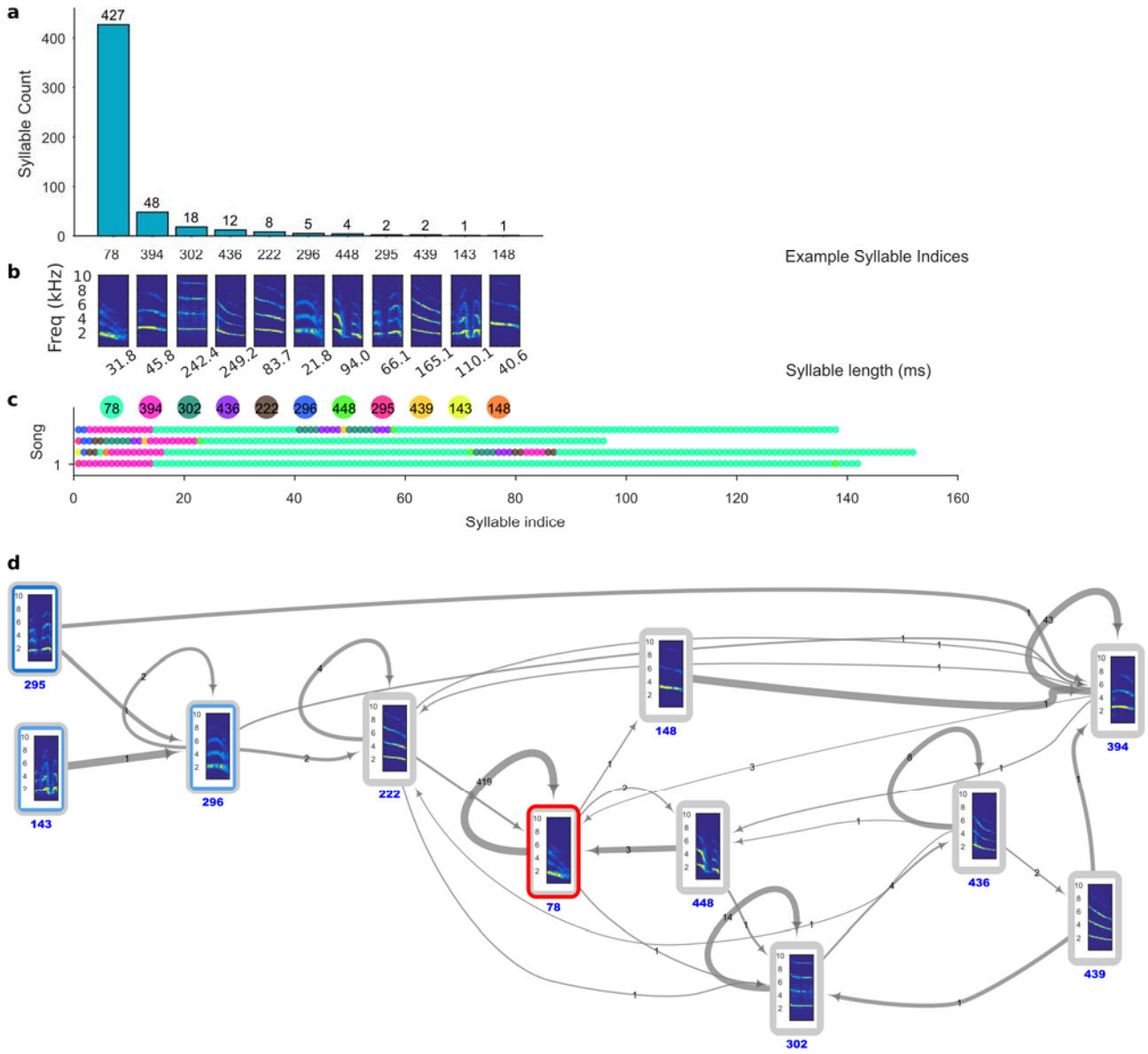

Figure 11. Syllable analysis of songs from period 2 of bird 3.

SylSorter classified the 528 syllables into 11 clusters based on Structural Similarity Index. a, A histogram summarizes the occurrences of each cluster. b, Sound spectrograms of the representative syllables. c, Visualization of syllable composition of songs. Syllables were colour-coded according to the SylSorter assignment. d, A simplified network-based representation of song syntax. Each node represents a cluster type (bold blue texts), and is connected to the node which is the most likely to occur. The width of edges represents the probability to the next node; the number of occurrences was indicated as well. Clusters were used as the first syllables of the songs were labelled in blue, while the cluster was used as the last syllable of the songs were labelled in red. Darker colours indicate higher probability of being the first (blue) or last (red) syllable.

We furthermore analyzed 50 songs of bird 2 from the first and the second recording periods, classifying 1,542 syllables from period 1 into 26 clusters in total (Figure 12a, b, Supplementary Figure 3). The songs produced during the first period were composed of four phrases with two different types of initial phrases: one starting with syllables in cluster 43 and repeated two to five times (34% of 50 songs), the other one starting with syllables from cluster 1050, followed by repeats of cluster 18 (18% of 50 songs). The second phrase of a song, comprised of cluster 46, was repeated four to ~12 times, followed by another phrase comprised of long repetitions of cluster 18 (~20 repeats), and a short ending phrase (one to eight repeats) comprised of syllables from cluster 854 (28%), 15 (22%) or 302 (16% of being the last syllable, Figure 12c).

Songs from the second recording period of bird 2 showed a lower repetition rate than those from period 1 (adjusted P value = 3.1×10^{-5}, period 1-2, Kruskal-Wallis followed by Bonferroni-Dunn post hoc test). The syllable types were less stereotypical, resulting in twice as many clusters (46) as in the first period (Figure 13 and Supplementary Figure 4). Initial syllables were sorted into three clusters, cluster 24 (14%), 17 (14%) and 137 (12% of being the first syllable). However, the syllables of 17 other clusters were also used to initiate song (Figure 13). Seven songs consisted of a phrase made up of syllables from cluster 1056, which was comparable to cluster 46 of period 1 (SSIM: 0.32). The most elongated phrase consisted of syllables in cluster 147 (Figure 13e), which was similar to cluster 18 of period 1 in sound spectrogram (SSIM: 0.62) and syllable usage. The ending syllables from period 2 were also less consistent, 25 different clusters were used in 50 songs (Figure 13e). Therefore, songs of bird 2 became less structured by losing syllable stability (increased syllable type number) and losing phrase pattern in the songs.

In summary, we found that female songs were comprised of two to five phrases. Their syllable repertoire grew larger from periods with a more stable song structure to structurally less stable periods. Syllable types used during stable periods were also found in unstable periods. Moreover, the composition of repertoires and the ordering of phrases remained the same between stable and unstable periods, but the

repetition rates in stable periods were higher than the ones observed during unstable periods (bird 3: Figure 10 and Figure 11; bird2: Figure 12 and Figure 13). This suggests that song produced during stable periods is elongated by increased repetition of syllables as well as of phrase sequences (Figure 12).

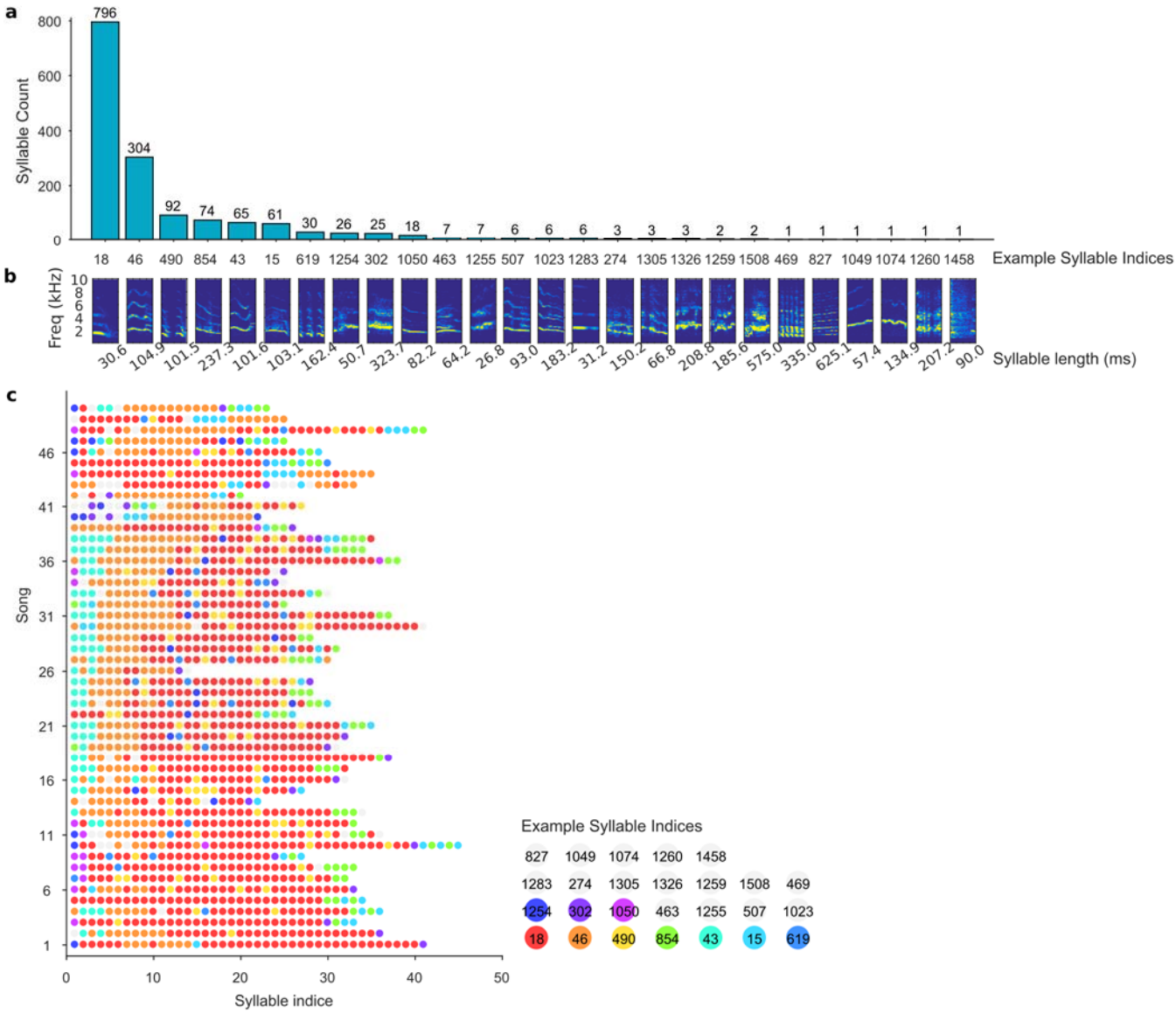

Figure 12. Syllable analysis of songs from period 1 of bird 2.

SylSorter classified the syllables from 50 songs into 26 clusters based on Structural Similarity Index. a, A histogram summarizes the occurrences of each cluster. b, Sound spectrograms of the representative syllables. c, Visualization of syllable composition of songs. Syllables were colour-coded according to the SylSorter assignment.

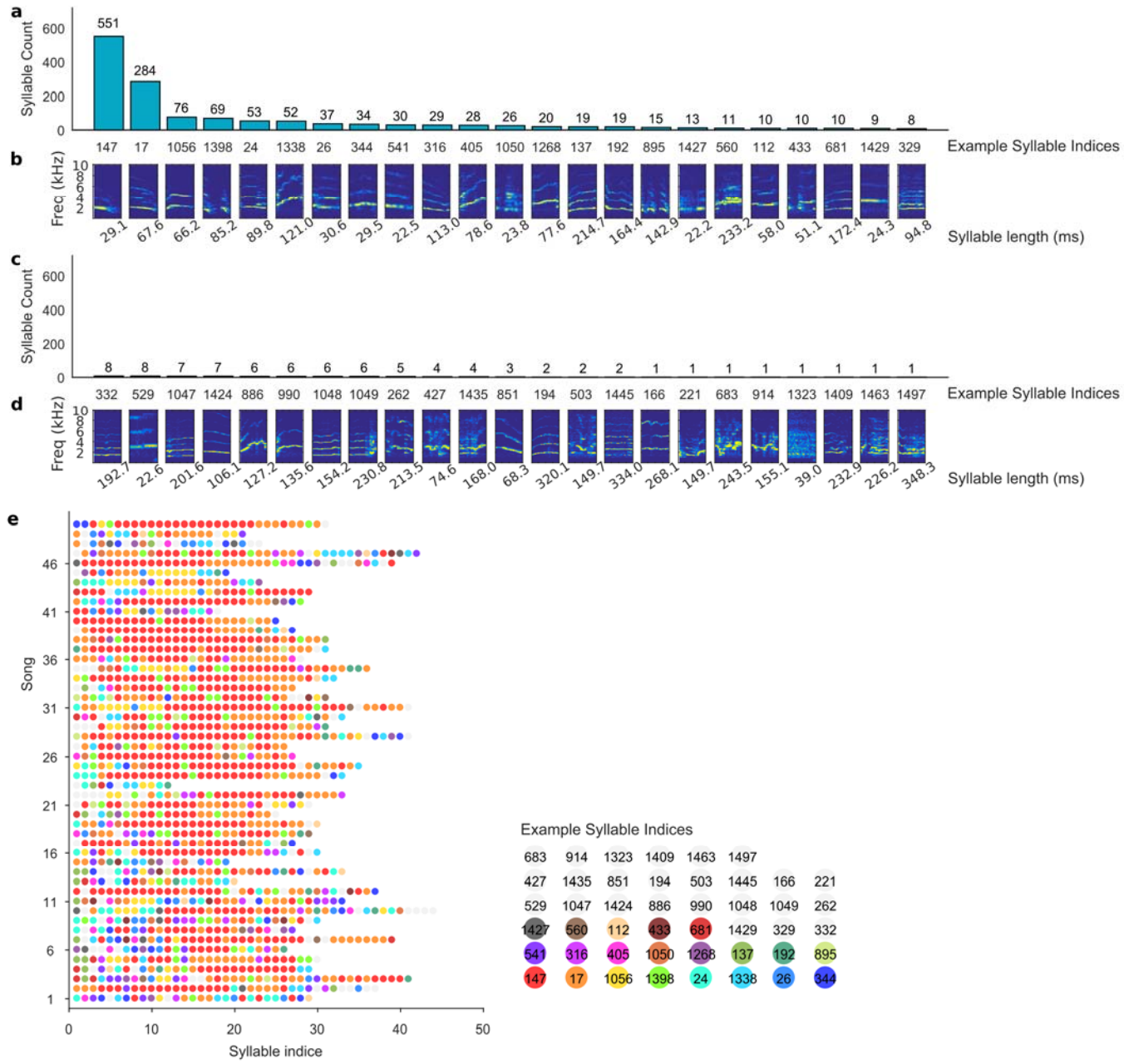

Figure 13. Syllable analysis of songs from period 2 of bird 2.

SylSorter classified the syllables from 50 songs into 46 clusters based on Structural Similarity Index. a, A histogram summarizes the occurrences of each cluster. b, Sound spectrograms of the representative syllables. c, Visualization of syllable composition of songs. Syllables were colour-coded according to the SylSorter assignment.

I. 5. Hormone levels, HVC-volumes and organ size of singing and non-singing females

Artificially administered testosterone strongly affects song development, syrinx size and HVC volume in adult female canaries (Fusani et al., 2003a; Madison et al., 2015; Nottebohm, 1980). Therefore, we asked whether HVC volume, the plasma testosterone level and other physiological measurements of the spontaneously singing females differed from those of non-singing female canaries kept under same conditions.

Testosterone levels and oviduct weight

We found that the plasma testosterone levels as well as the oviduct weight of singing females were significantly different from those of non-singing individuals (Figure 14a, b, plasma testosterone: singers 512 ng/ml, non-singers 36.7 ng/ml (mean), Mann-Whitney U = 55, P value = 4.8×10^{-3}, n = 15; oviduct weight: singers 194 mg, non-singers 18.5 mg, Mann-Whitney U = 54, P value = 4.0×10^{-4}, n = 15). The plasma testosterone level of bird 3 was extremely high, after excluding bird 3 the trend of differences of plasma testosterone between singing and non-singing birds remained the same (plasma testosterone: singers 66.6 ng/ml, non-singers 36.7 ng/ml (mean), Mann-Whitney U = 41, P value = 0.01, n = 14).

HVC volume and syrinx weight

HVC volume (Figure 14c, singers 0.176 mm^3, non-singers 0.0974 mm^3, Mann-Whitney U = 51, P value = 2.8×10^{-3}, n = 15) was significantly different between singers and non-singers. In contrast, brain weight (Figure 14d, singers 704 mg, non-singers 660 mg, Mann-Whitney U = 43, P value = 0.066, n = 15), body weight (Figure 14e, singers 20.5 g, non-singers 18.5 g, Mann-Whitney U = 42, P value = 0.088, n = 15) and syrinx weight (Figure 14f, singers 21.8 mg, non-singers 19.6 mg, Mann-Whitney U = 30, P value = 0.77, n = 15) did not differ significantly between singing and non-singing individuals.

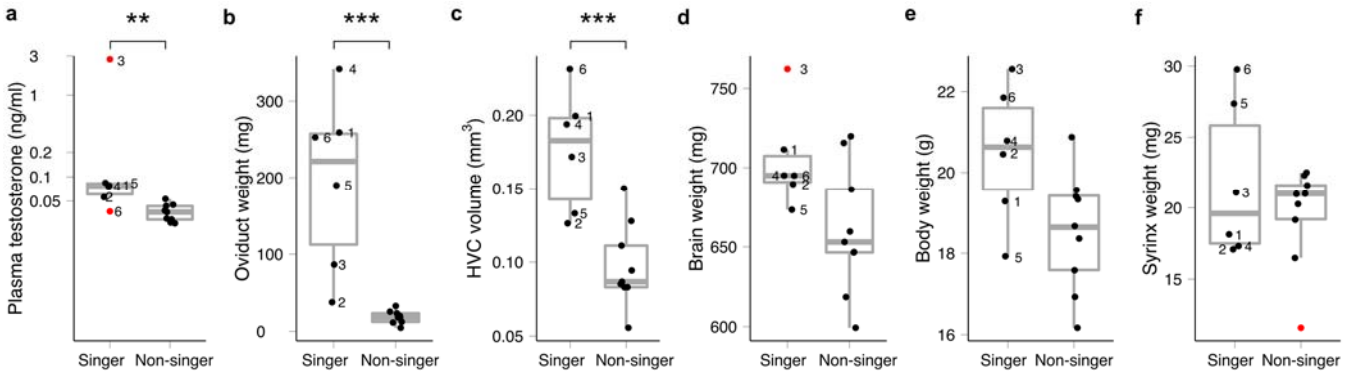

Figure 14. Physiological measurements of singing and non-singing female canaries.

a, Plasma testosterone levels on the day of sacrifice of singing and none-singing female canaries were statistically different between the two groups (Mann-Whitney Test, U = 50, P value = 4.8×10^{-3}). For singers, bird ids were indicated. Bird 3 had an extremely high testosterone level. b, Oviduct weight was statistically different between groups (Mann-Whitney Test, U = 54, P value = 4.0×10^{-4}). c, HVC volume of singing and non-singing female canaries. The HVC volume was statistically different between the two groups (Mann-Whitney Test, U = 51, P value = 2.8×10^{-3}). d, Brain weight was statistically different between groups (Mann-Whitney Test, U = 43, P value = 0.066). e, Body weight was not statistically different between groups (Mann-Whitney Test, U = 42, P value = 0.088). f, Syrinx weight was not statistically different between groups (Mann-Whitney Test, U = 30, P value = 0.77). The boxes indicate the 25th/50th/75th percentiles (bottom/middle/top bar), the extent of the whiskers indicate the most extreme values that are within 1.5 times the IQR (inter-quartile range) of the hinge. Outliers are labelled in red. **: P value < 0.001; *** P value < 0.0001.

Correlation of testosterone, HVC volume, syrinx weight and singing

We further investigated whether physiological features like HVC volume, and plasma testosterone levels might be correlated with parameters of the song structure. We calculated the Pearson's correlation coefficients between song parameters measured in the last period and the HVC volume and hormone levels on the day of sacrifice. We did not find a correlation between HVC volume and any song parameters. Normalization of HVC volume against body weight or brain weight did not influence these results. In contrast, the plasma testosterone levels were positively correlated with the mean syllable repetition rate (Pearson's correlation coefficient r = 0.94, FDR-adjusted P value = 0.04, Figure 15 and Supplementary Table 6) and the mean of the number of syllables per song (Pearson's correlation coefficient r = 0.96, FDR-adjusted P value = 0.04, Figure 15, and Supplementary Table 6). The brain weight was positively correlated with the mean syllable repetition rate (Pearson's correlation coefficient r = 0.98, FDR-adjusted P value =

0.01, Figure 15 and Supplementary Table 6). Syrinx weight was positively correlated with the variance of the number of syllables per song (Pearson's correlation coefficient r = 0.97, FDR-adjusted P value = 0.02, Figure 15 and Supplementary Table 6).

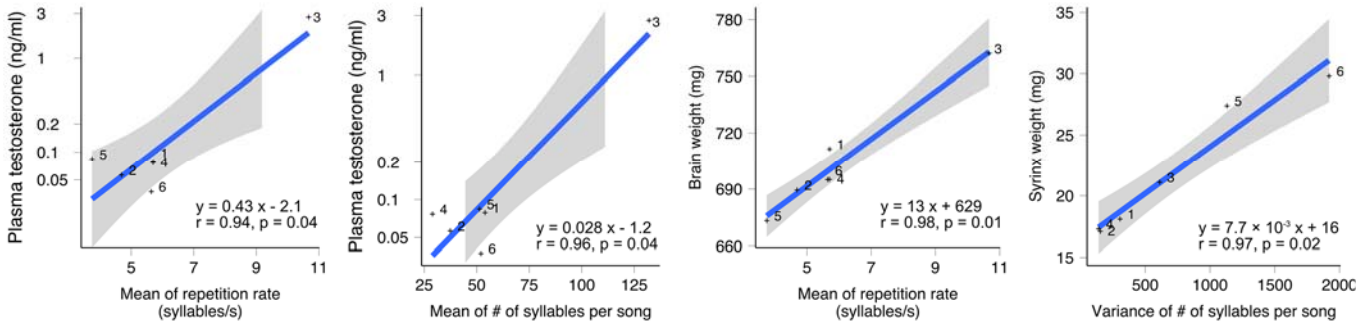

Figure 15. Correlations between song parameters and physiological measurements.

a, The plasma testosterone levels were positively and significantly correlated with the mean of syllable repetition rate. b, The plasma testosterone levels were positively and significantly correlated with the mean of the number of syllable in a song. c, The brain weights were positively correlated with the mean of syllable repetition rate. d, The syrinx weights were positively and significantly correlated with the variance of the number of syllable in a song. The equation for the regression line, Pearson's correlation coefficient and FDR-adjusted P value are shown in the bottom right portion of each correlation graph. See Supplementary Table 6 for all correlation comparison.

I. 6. Song structure comparison between spontaneously singing and testosterone-induced singing female canaries

Despite the low natural occurrence of song in female canaries, it can be easily induced by testosterone treatment (Fusani et al., 2003a; Madison et al., 2015; Nottebohm, 1980; Shoemaker, 1939). To compare spontaneously occurring song to that produced by testosterone-treated females, we only considered songs produced during the high quality period with highest average repetition rates and presented the data as mean ± SEM (n = 6) as in the literature (Table 4). It was shown that the average length of song produced by testosterone-treated females was approximately 7 ± 2 s (Fig. 4A in (Fusani et al., 2003a)), which is similar to that observed by us in spontaneously singing females (6.9 ± 2.5 s, Table 4). However, with an average syllable repetition rate of 6.7 ± 0.79 s and a maximum repetition rate of 12.5 Hz (Table 4), songs of

spontaneously singing females were significantly slower compared to testosterone-stimulated females which are able to produce songs with repetition rates higher than 20 Hz (Fusani et al., 2003a; Hartog et al., 2009).

Average repertoire size of testosterone-treated females ranges from 4 to 14 (Fusani et al., 2003a; Hartley et al., 1997; Hartog et al., 2009; Nottebohm, 1980), whereas spontaneously singing females ranged between 11 and 52 during high repetition rate periods (Table 3). This suggests that songs of naturally singing females are less stereotyped than those of testosterone-induced females.

Table 4. Song structure comparison of spontaneously singing female canaries to males and testosterone-induced singing females.

Sources: wild male canaries (Leitner et al., 2001a); domesticated male canaries (Voigt and Leitner, 2008); testosterone-induced females (Fusani et al., 2003a); Max repetition rate of repeated syllable types of domesticated breeding males (Leitner and Catchpole, 2007); repertoire size of testosterone-induced females (Hartog et al., 2009).

Group	Spontaneously singing females	Breeding males		Non-breeding males		Testosterone-induced female
		Wild	Domesticated	Wild	Domesticated	
Song length (s)	6.9 ± 2.5	8.9 ± 0.5	8.6 ± 1.02	6.2 ± 0.7	5.40 ± 1.02	7 ± 2
Sample size	6	9	7	9	7	8
Repertoire size (syl)	28 ± 5.6	69 ± 8	27 ± 2	67 ± 7	27 ± 4	8.2 ± 3.1
Number of syllables per song (syl)	42 ± 13	-	-	-	-	-
Max syllable repetition of repeated syllable types (syl)	127	37	139	25	52	-
Repetition rate (syl/s)	6.7 ± 0.79	-	-	-	-	-
Max repetition rate (syl/s)	12.5	-	-	-	-	-
Max repetition rate of repeated syllable types (syl/s)	14.6	25	17	-	-	> 20

I. 7. Song structure comparison between spontaneously singing females and male canaries

Songs naturally produced by females were comprised of two to five phrases (Figure 11 and Figure 12) with an average song length of 6.9 ± 2.5 s (Table 3). The average length of male song produced during the breeding season ranges around 9 s (wild canaries: 8.9 ± 0.5 s, n = 9 (Leitner et al., 2001a); domesticated canaries: 8.6 ± 1.02 s, n = 7 (Voigt and Leitner, 2008)), whereas the average song length during the non-breeding season ranges around 6 s (wild canaries: 6.2 ± 0.7 s, n = 9 (Leitner et al., 2001a); domesticated canaries: 5.40 ± 1.02 s, n = 7 (Voigt and Leitner, 2008)).

The average syllable repetition rate of females was 6.7 ± 0.79 syl/s with a maximum repetition rate of 12.5 syl/s (Table 4). Considering the highly repetitive phases composed of only one syllable type, bird 3 produced the highest repetition rate of 14.6 syl/s. It was shown that the maximum repetition rate of wild canary males during the breeding season was 25 syl/s, and a minimum of 17 syl/s is required for triggering copulation solicitation displays of female canaries (Amy et al., 2015; Vallet and Kreutzer, 1995).

The average female repertoire size was 28 ± 5.6 (syllable types, Table 4), which represents the lower bound of the male repertoire size. Depending on the strain of canaries, breeding stage and classification method, the repertoire size of male canaries ranges from 25 up to ~180 (Güttinger, 1985; Leitner et al., 2001a; Nottebohm et al., 1987; Voigt and Leitner, 2008).

I. 8. Conclusion

By screening a large quantity of female canaries we could show that approximately 5% of the individuals in our facilities spontaneously produced song, despite song frequency was generally low (Table 1). We provide an in-depth description of female canary song, and showed that songs were very variable between individuals as well as within individuals over time, differing in terms of repetition rate, number of syllables

per song and song length (Figure 8). Although the overall female song organization was similar to the general organization of song observed in male canaries, the quality of female song was highly variable and in general did not reach the male levels (Table 4). We showed that female singers had elevated testosterone levels and an increased in HVC volume than female non-singers (Figure 14), and showed that frequency modulation of song might be correlated to plasma testosterone levels (Figure 15). Thus, spontaneously singing female canary demonstrated a natural range of behavioral and neural plasticity in a northern temperate songbird species.

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II. Experiment II

Time-lapse transcriptomic profiling of the brains of adult female canaries from the onset of testosterone-induced singing behavior

Birdsong is a complex behavior that involves intensive integration of sensory inputs, motor outputs and various contexts (Wild, 2004). The functions of birdsong are diverse but include mate attraction (Byers et al., 2010; Drăgănoiu et al., 2002) and pair bond formation (Wickler and Seibt, 1980). In many northern temperate species, females either do not sing regularly or sing only in a specific context (Gahr, 2014). For example, spontaneous songs of female canaries (*Serinus canaria*) were rarely observed (Hartley et al., 1997; Herrick and Harris, 1957; Pesch and Güttinger, 1985) and their song complexity is incomparable to male songs (Table 4). Known for decades, testosterone has both prolonged and pronounced effects on the behaviors and brain anatomy of female canaries (Hartog et al., 2009; Herrick and Harris, 1957; Louissaint et al., 2002; Nottebohm, 1980; Shoemaker, 1939). Specifically, systemic administration of testosterone to adult female canaries induced the production of male-quality songs (Herrick and Harris, 1957; Leonard, 1939; Shoemaker, 1939) and an enlargement of the brain nuclei involved in song control (Fusani et al., 2003a; Madison et al., 2015; Nottebohm, 1980). However, only few studies have documented testosterone-dependent transcriptomic changes in songbird brains (Dittrich et al., 2014; Frankl-Vilches et al., 2015; Larson et al., 2015; Peterson et al., 2013; Thompson et al., 2012).

The premotor forebrain nucleus HVC is a sensorimotor integration center, and the only song control nucleus expressing both androgen and estrogen receptors (Frankl-Vilches and Gahr, 2018; Gahr, 2001) within the song control system (Hahnloser et al., 2002; Nottebohm et al., 1976; Wild, 2004). Previous studies demonstrated that angiogenesis take place in adult female canaries HVC following testosterone treatment within a week and that the mechanism of this process in mediated by estradiol (Louissaint et al., 2002). Yet, the transcriptomic effects on the onset of testosterone-driven singing behavior as well as the

progression of genetic regulating molecular events that accompany such anatomical changes in the HVC and song control system remain to be examined.

In order to investigate short-term and long-term responses of the HVC transcriptome after testosterone treatment, adult female canaries were implanted with testosterone for six time periods (short-term effects: T1h, T3h, and T8h; long-term effects: T3d, T7d, and T14d, Figure 16A and Table 5). The gene expression changes of the HVC were studied by performing exon-level microarray analyses (Affymetrix) of HVC and testosterone-regulated genes were associated with singing behavior and anatomical changes of HVC. Our results indicate that adult female canary HVC is highly responsive to testosterone and the testosterone effects on gene expression were both rapid and long-lasting. Our investigation of the dynamics of gene expression after testosterone treatment elucidated the progression of molecular and cellular events in the HVC required for the development of singing behavior and fully-differentiated songs.

Table 5. Experimental groups and sample sizes.

				Sample size (n)			
Group	Sex	Singing	Tissue sampled	HVC volume	Microarray	Brain weight	Blood plasma testosterone, body weight and oviduct weight
CON	Female	No	HVC	6	5	6	6
T1h	Female	No	HVC	6	6	6	6
T3h	Female	No	HVC	5	5	5	5
T8h	Female	No	HVC	9	5	8	9
T3d	Female	No	HVC	6	6	9	9
T7d	Female	Yes	HVC	6	6	6	6
T14d	Female	Yes	HVC	6	6	6	6

CON: control; HVC: used as a proper name.

II. 1. Testosterone implantation acutely and persistently elevated plasma testosterone levels

To confirm for the effect of our systemic hormone manipulation on the blood testosterone level, we performed radioimmunoassay on samples both before and after the hormone implantation (Figure 16B). Striking increase of the testosterone level was detectable in blood plasma of implanted birds after one hour. Plasma testosterone levels at this time point were at least 17-fold above the baseline (17 to 88 ng/ml, baseline < 1 ng/ml), and remained high among the short-term treatment groups (T1h, T3h, and T8h). Subsequently, the testosterone level decreased and was only 10-fold above the baseline by the end of day 14.

II. 2. Testosterone implantation induced singing within 4 days

To establish the development timeline of testosterone-induced singing, we monitored the birds for singing activity before and during the hormone implantation experiment. Birds of the short-term groups and T3d were either calling or silent while no song was emitted. Five out of six birds of the T7d group and all T14d birds emitted songs (Table 6). On average the first song was emitted 3.9 ± 1.7 days (mean ± SD) after implantation, similar to males of the non-breeding season that were implanted with testosterone (Sartor et al., 2005).

In the T14d group song length was significantly longer than in the T7d group (T7d: 5.9 ± 3.7 s; T14d: 7.2 ± 6.1 s, mean ± SD, Mann-Whitney Test, U = 1593000, P value = 0.015). Likewise, syllable repetition rate of the T14d group was significantly higher than of the T7d group (T14d: 7.1 ± 2.9 syllables/s (syl/s); T7d: 6.3 ± 2.0 syl/s, mean ± SD, Mann-Whitney Test, U = 1959500, P value < 2.2×10^{-16}). The maximum syllable repetition rate of the T14d group was 26 syl/s. These results were in agreement with another previous study (Fusani et al., 2003a).

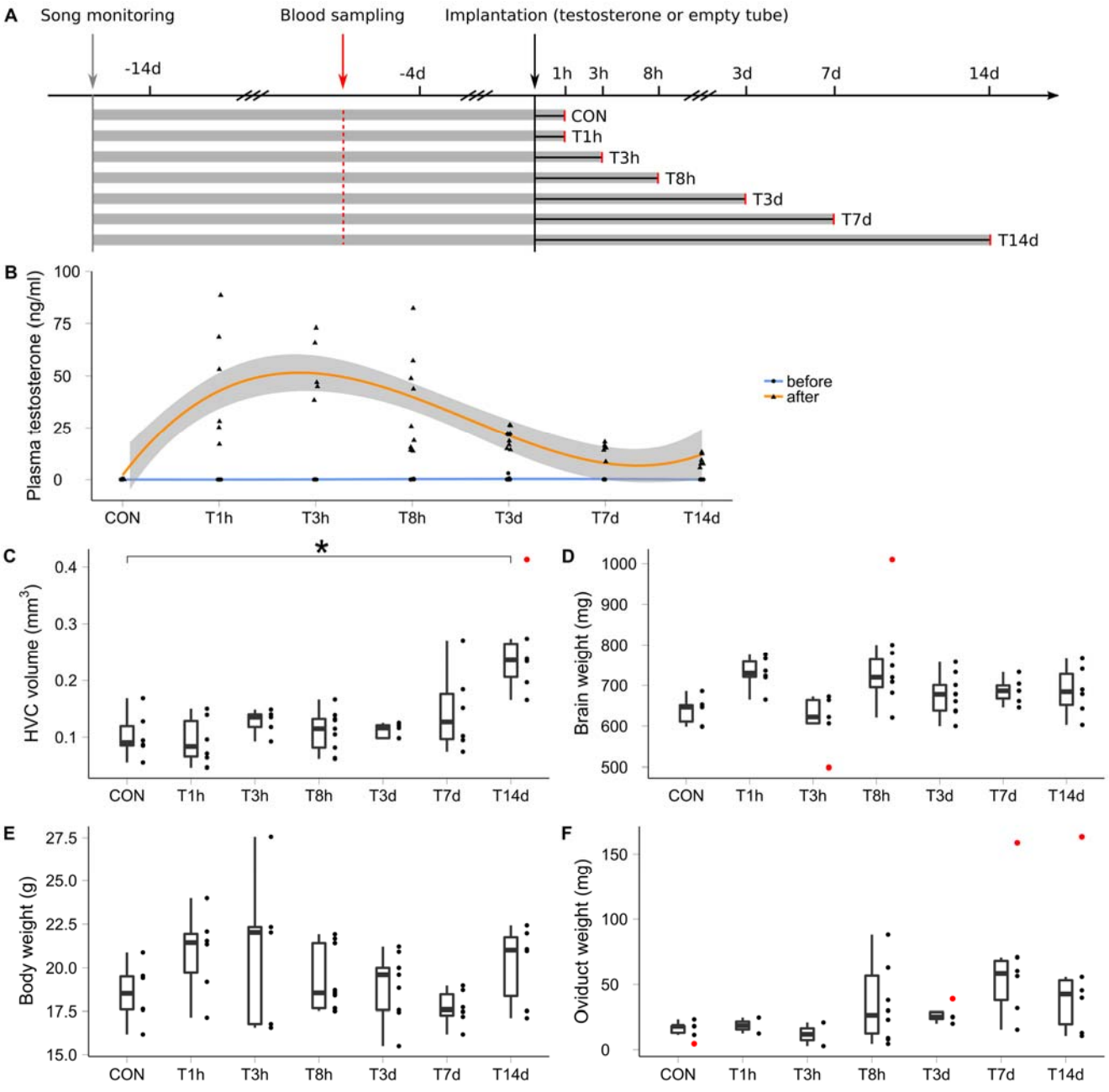


Figure 16. Effects of testosterone on physiological measures of adult female canaries.

A, An illustration indicates the experimental setup. Vertical red lines indicate the 2 time points for blood plasma testosteorne sampling. B, Blood plasma testosterone concentrations before and after implantation. The curves were plotted by fitting a third order polynomial equation. Grey shaded area indicating 95% confidence intervals. CON: birds implanted with empty tubes; T: testosterone-treated birds. C, HVC volume; D, brain weight; E, body weight; and F, oviduct weight of control and testosterone-treated animals. The boxes in B-E indicate the 25^{th}/50^{th}/75^{th} percentiles (bottom/middle/top bar), the extent of the whiskers indicate the most extreme values that are within 1.5 times of the inter-quartile range (IQR) of the hinge. Outliers are labeled in red. *: adjusted P value <0.05, Kruskal-Wallis followed by Bonferroni-Dunn post hoc test.

Table 6. Summary table for measured song parameters (mean ± SD).

Group	Bird	Number of songs	Daily song rate (‰)	Song length (s)	Repetition rate (1/s)	Number of syllables per song	Slope coefficient α	Syllable length (ms)	Syllable interval (ms)	Wiener entropy	Peak freqency (kHz)
T7d	1	36	0.19 ± 0.25	6.7 ± 3.4	5.6 ± 0.99	37 ± 18	-0.76 ± 0.62	112 ± 110	63 ± 141	-10 ± 1.7	3.1 ± 0.83
T7d	2	0	-	-	-	-	-	-	-	-	-
T7d	3	696	5.4 ± 3.3	5.9 ± 3.7	6.3 ± 2	36 ± 22	-0.31 ± 0.57	85 ± 61	76 ± 237	-14 ± 1.7	2.8 ± 0.33
T7d	4	1	0.0044 ± 0.012	2.2 ± 0	1.4 ± 0	3 ± 0	-0.19 ± 0	130 ± 127	839 ± 1097	-11 ± 1.6	3.5 ± 0.59
T7d	5	3	0.023 ± 0.030	2.8 ± 0.37	3.7 ± 0.39	10 ± 2.1	-0.65 ± 0.39	99 ± 99	188 ± 179	-11 ± 1.4	2.7 ± 0.97
T7d	6	1	0.0062 ± 0.014	2.2 ± 0	2.8 ± 0	6 ± 0	-0.38 ± 0	122 ± 55	286 ± 329	-11 ± 1.6	3.5 ± 0.39
T14d	1	7	0.024 ± 0.021	6.6 ± 8.9	3.8 ± 1.2	32 ± 57	-0.83 ± 0.61	62 ± 42	132 ± 229	-10 ± 1.4	4.3 ± 0.87
T14d	2	100	0.26 ± 0.22	16 ± 12	5.3 ± 1.3	90 ± 78	-0.3 ± 0.36	83 ± 70	92 ± 237	-9.6 ± 1.3	2.4 ± 0.73
T14d	3	751	1.9 ± 1.3	12 ± 7.6	11 ± 3.5	129 ± 95	-0.85 ± 0.56	47 ± 58	39 ± 183	-10 ± 1.6	3.1 ± 0.8
T14d	4	1461	4.5 ± 5.9	3.3 ± 1.2	7.7 ± 1.4	26 ± 10	-0.75 ± 0.55	104 ± 81	25 ± 52	-13 ± 0.91	3.7 ± 0.52
T14d	5	1629	3.6 ± 3.0	7.7 ± 4.8	5.1 ± 1.5	41 ± 30	-0.46 ± 0.51	98 ± 67	90 ± 111	-11 ± 1	3.7 ± 0.65
T14d	6	145	0.41 ± 0.54	11 ± 6.4	6.9 ± 1.6	78 ± 51	-0.46 ± 0.48	58 ± 32	76 ± 104	-12 ± 2.3	3.2 ± 0.52

II. 3. Testosterone implantation increased the HVC volume within two weeks

Analyzing the response of the HVC volume to testosterone, we found a significant increase in T14d birds (Figure 16C, CON: 0.103 ± 0.039 mm^3 (mean ± SD); T14d: 0.253 ± 0.087 mm^3, Kruskal-Wallis followed by Bonferroni-Dunn post hoc test, adjusted P value = 0.021). The HVC volumes of T7d were not significantly different from those of controls' (T7d: 0.146 ± 0.073 mm^3, Kruskal-Wallis followed by Bonferroni-Dunn post hoc test, adjusted P value = 1), nor were they different from those of T14d (Kruskal-Wallis followed by Bonferroni-Dunn post hoc test, adjusted P value = 0.904). This is likely due to the large variations of T7d HVC volumes. Testosterone treated birds were not significantly different from controls in terms of brain weight, body weight and oviduct weight (Figure 16D-F). In summary, we consolidated previous findings on testosterone-increased HVC volume in female canaries (Fusani et al., 2003a; Hartog et al., 2009; Madison et

al., 2015; Nottebohm, 1980; Rasika et al., 1994b), and inferred that the effects on gross morphology of HVC were delayed for at least 2 weeks relative to plasma testosterone increase.

II. 4. Testosterone implantation drastically altered the HVC transcriptome

In order to understand the effect of testosterone on HVC transcriptome, we studied the differential gene expression of six testosterone-treated groups against the controls by performing microarray analyses. Remarkably, the HVC transcriptome was affected by testosterone after the first hour of treatment (Figure 17A). The expression of more than 2,600 genes was significantly different between T1h birds and controls. This correlated with the highly elevated level of plasma testosterone detected in the T1h group (Figure 16B). The number of differentially expressed genes was reduced to approximately 1,400 genes in T3h birds, subsequently increased to approximately 2,500 from T8h to T7d, and rose to approximately 8,000 genes at T14d (Figure 17A). The microarray data, (T14d, Figure 17C), coincide with previous study on the mRNA elevation of a vascular endothelial growth factor receptor (*KDR*) and brain-derived neurotrophic factor (*BDNF*) that were reported after 2 weeks implantation (Louissaint et al., 2002). In addition, we verified the microarray results with RNAScope® *in situ* hybridization assay (Wang et al., 2012). The quantification results of the staining of several, including androgen receptor (*AR*), 17β-hydroxysteroid dehydrogenase (*HSD17B12*), 5α reductases 2 (*SRD5A2*) and aldehyde dehydrogenase 1 family member A2 (*ALDH1A2*) were congruent with the microarray (Figure 18). These genes were selected because of their involvement in testosterone metabolism.

A, Number of HVC genes differentially expressed between control and testosterone-treated birds. The proportions of transcription factors (TFs) were indicated in darker colors. B, The majority of differentially expressed genes was transiently regulated. We categorized the differentially expressed genes to four classes based on their temporal regulation patterns: 1) genes being constantly up-regulated, 2) genes being constantly down-regulated, 3) genes being up- or down-regulated after all testosterone-treatment periods, 4) genes not being regulated after at least one testosterone-treatment period. C, A heatmap showing expression levels of genes of sex steroid receptors (blue), genes with known functions in steroidogenesis (grey), genes previously identified playing roles in testosterone-induced angiogenesis (red) and neuronal recruitment (green). LFC: log fold change (base 2).

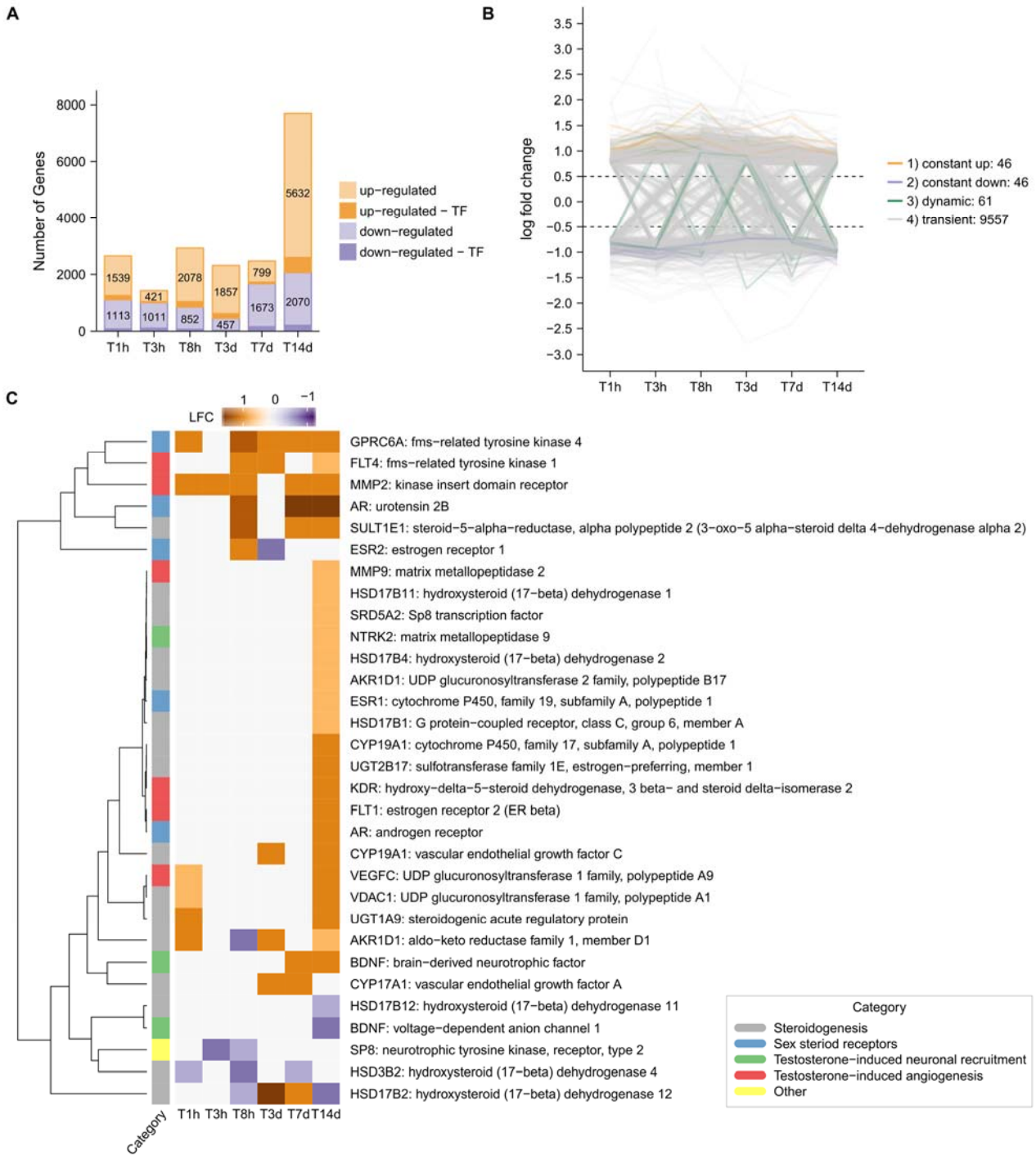


Figure 17. Testosterone rapidly and persistently altered the adult female canary HVC transcriptome, although individual genes were regulated in a transient manner (see the left page for legend).

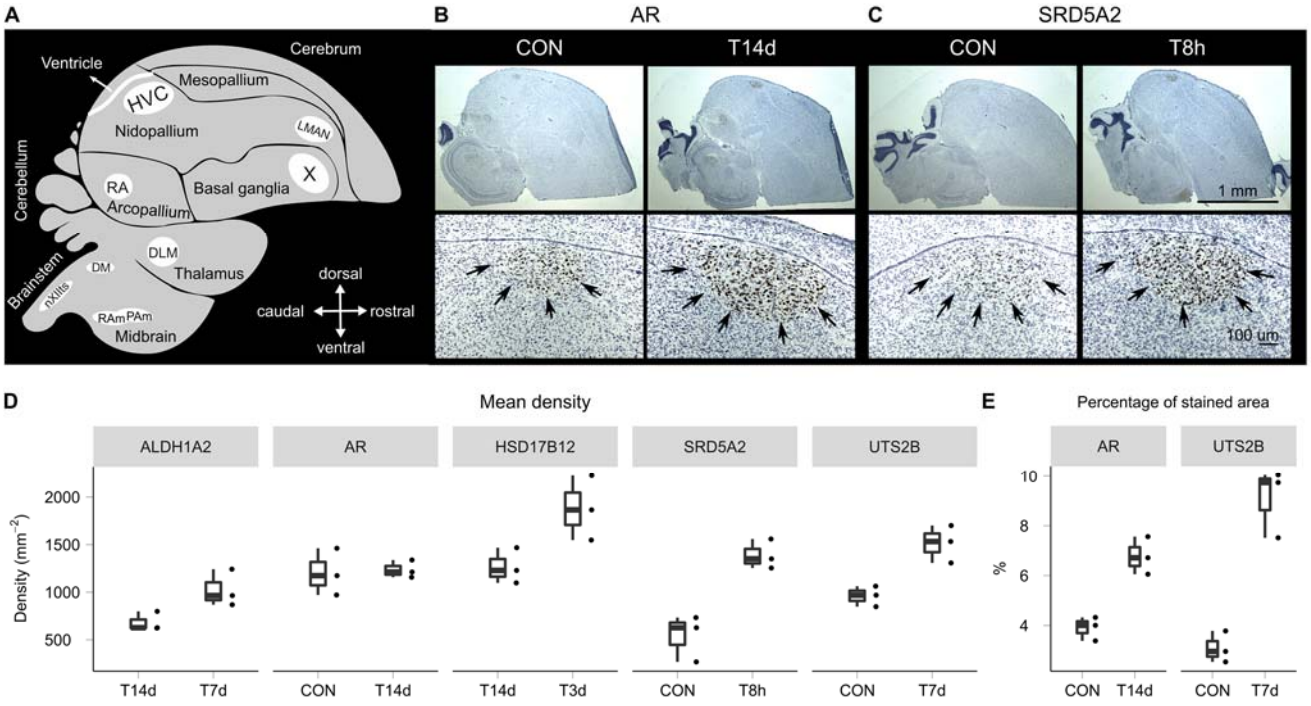


Figure 18. Quantification of mRNA expression of five probes in the HVC by RNAScope® *in situ* hybridization.

A, Diagram of the song bird brain with the labels of the song control nuclei. Microphotographs of the brain (upper panel, 1.25x) and HVC (lower panel, 5x, arrow indicated) of *in situ* hybridization for B, androgen receptor (*AR*) and C, 5α-reductase 2 (*SRD5A2*) in controls and treated birds. D, Quantifications of mean density of accumulated chromogenic particles in HVC were significantly different ($p < 0.05$) between the two time points checked for each probe, except for *AR* and *UTS2B* (Mann-Whitney U Tests). E, Quantifications of *AR* and *UTS2B* as the percentage of the stained area versus the HVC region in the sections show statistical differences between the tested time points (Mann-Whitney U Tests, $p < 0.001$). *ALDH1A2*: aldehyde dehydrogenase 1 family member A2; *AR*: androgen receptor; *HSD17B12*: 17β-hydroxysteroid dehydrogenase; *SRD5A2*: 5α reductases 2; *UTS2B*: urotensin 2B.

II. 5. Testosterone-induced genes were associated with endothelial cell and projection neuron

We used Gene Ontology (GO)-term enrichment analysis to assess potential biological processes influenced by testosterone in the HVC at different time points. After one hour of testosterone treatment, enrichment in processes like circulatory system development (GO:0072359), cell development (GO:0048468) and neuron development (GO:0048666) were noticeable, whereas neurogenesis (GO:0022008) was enriched after

three hours (Figure 19A). Because all of these processes continuously enriched in the later time points, and because most of the genes were transiently-regulated by testosterone (Figure 17B), these results suggested that testosterone activates different groups of genes that work combinatorially and successively to accomplish the long-term testosterone-associated biological processes.

To elucidate testosterone effects in the HVC at cellular level, we refined our analyses to search-terms associated with cell types, types of neuronal protrusion and biosynthesis of neurotransmitters and neurotransmitter receptors. More specifically, we searched for 35 different terms using the "Genes and gene products" tool of the AmiGO website (Ashburner et al., 2000; Consortium, 2017), and downloaded the gene lists associated with the search-terms (see methods, Supplementary Figure 5). Subsequently, we performed enrichment analysis on the time point gene sets using Fisher's exact test and summarized the results in Figure 19B. We observed enrichments of "endothelial cell" in T3h and T7d birds. Enrichment of "smooth muscle cell" was observed at a later stage (3 days after treatment) than "endothelial cell." "Astrocyte" and "oligodendrocyte" were briefly enriched in the up-regulated genes at T3h, whereas "interneuron" was enriched in the up-regulated genes at T14d. It appears that testosterone did not regulated genes associated with microglia within the time window of our study. Conversely, both up- and down-regulated genes associated with projection neuron were enriched in almost all time points.

We further asked which neuronal processes were associated with the testosterone-sensitive genes. Interestingly, the term "axon" was enriched in the up-regulated genes as soon as the birds underwent the first hour of testosterone treatment. The up-regulated genes at T3h were associated with the term "myelin," corresponding to the enrichment of "oligodendrocytes" associated genes. Enrichment of "dendrite" was observed in T8h, T3d and T7d birds, and enrichment of "spine" was observed later in T3d birds (Figure 19B). In the class of down-regulated genes, the terms "axon," "myelin," "dendrite," and "spine" were enriched in almost all time points (Figure 19B). The fact that both up- and down-regulated genes were

enriched of these search-terms at the same time points suggests testosterone might cause morphological changes in the HVC neurons by elevating expression of certain genes while suppressing others.

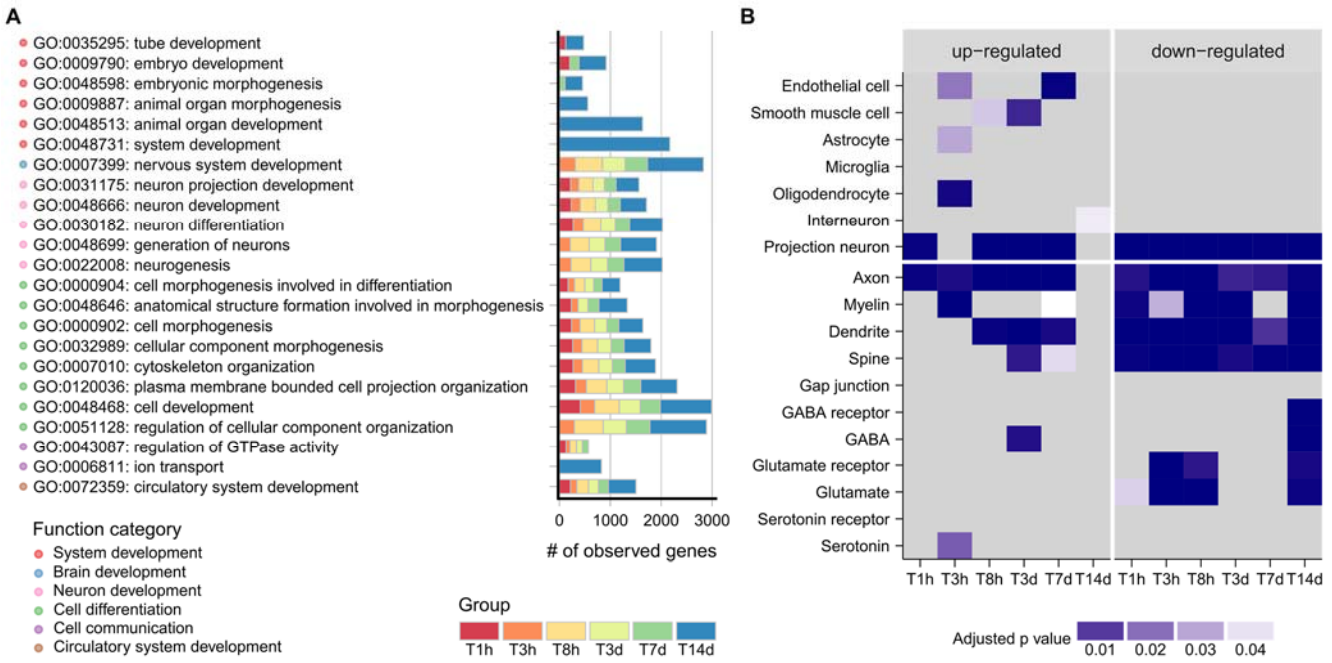


Figure 19. Enrichment analyses predicted potential biological processes under the influence of testosterone in the HVC.

A, GO-term enrichment analysis (biological process) of the differentially expressed genes indicated the progression of testosterone effects in the HVC. The top ten most likely occurring GO-terms (least Bonferroni-adjusted p values) of each time point were shown and classified to six functional categories. The numbers of observed genes (accumulative) were visualized in a stacked bar plot. The results suggested many processes such as GO:0072359 circulatory system development, GO:0048468 cell development and GO:0048666 neuron development take place after one hour of testosterone treatment and be persistent for at least two weeks. B, Search term enrichment analyses focused on genes associated with the major cell types, neuronal protrusion types and biosynthesis of neurotransmitters and neurotransmitter receptors in the HVC (see Supplementary Figure 5 for the results of all search terms). The results suggested that testosterone influenced several cell types at specific time points such as endothelial cells, smooth muscle cells and oligodendrocytes, while projection neurons were constantly under the influence of testosterone during the time window of the experiment.

In order to evaluate whether testosterone changes neuronal activities of electrical or chemical synapses, we performed similar analyses for additional search-terms including “gap junction,” eight neurotransmitters and their receptors (Figure 19B, Supplementary Figure 5 and Supplementary Table 2). We found no indication of testosterone effect on the enrichment of “gap junction.” However, the terms

"glutamate" and "glutamate receptor" were enriched in the down-regulated genes at T3h, T8h and T14d. The four subunits of AMPA receptors (*GRIA1, GRIA2, GRIA3,* and *GRIA4*) and two AMPA receptor interacting proteins (*GRIP1* and *GRIP2*) were all down-regulated at T3h, and three AMPA receptor subunits were down-regulated at T14d (*GRIA2, GRIA3,* and *GRIA4*; Supplementary Table 2). The genes associated with the inhibitory neurotransmitter "GABA" were enriched in up-regulated genes at T3d, but later (T14d) "GABA" and "GABA receptor" were enriched in the down-regulated genes, including four of the six isoforms of theα subunit (*GABRA1*, *GABRA2*, *GABRA4,* and *GABRA5*), two β subunit (*GABRB2* and *GABRB3*) and two γ subunit isoforms (*GABRG1* and *GABRG2*) of $GABA_A$ receptor (Supplementary Table 2).

Collectively, these results suggest that 1) plasma testosterone elevation was relevant to the gene expression changes in multiple cell types including endothelial cells and neurons, 2) morphological changes in the HVC caused by testosterone might take place as short as one hour after treatment, 3) testosterone transiently-regulated genes associated with neuronal morphology throughout the experimental time window, 4) the electrophysiological properties might be under the influence of testosterone because the expression of both excitatory and inhibitory neurotransmitters and their receptors were regulated by testosterone.

II. 6. Identification of key genes for testosterone-induced anatomical and behavioral changes

We used weighted gene correlation network analysis (WGCNA, (Langfelder and Horvath, 2008, 2012)) to identify functional units of gene co-expression network that are relevant for testosterone-induced morphological and behavioral changes such as increase of HVC volume and singing activity. WGCNA calculates network adjacency matrix based on correlation of gene expression data and then assigns genes into modules (sub-networks within the overall network) based on topological overlap matrix from a given network adjacency matrix. The adjacency value has a range between 0 and 1 that is an approximate

indicator for connection strengths among the nodes (genes) in the network: a pair of genes that are highly correlated has adjacency value of 1, indicating the pair is likely to co-express at a given time.

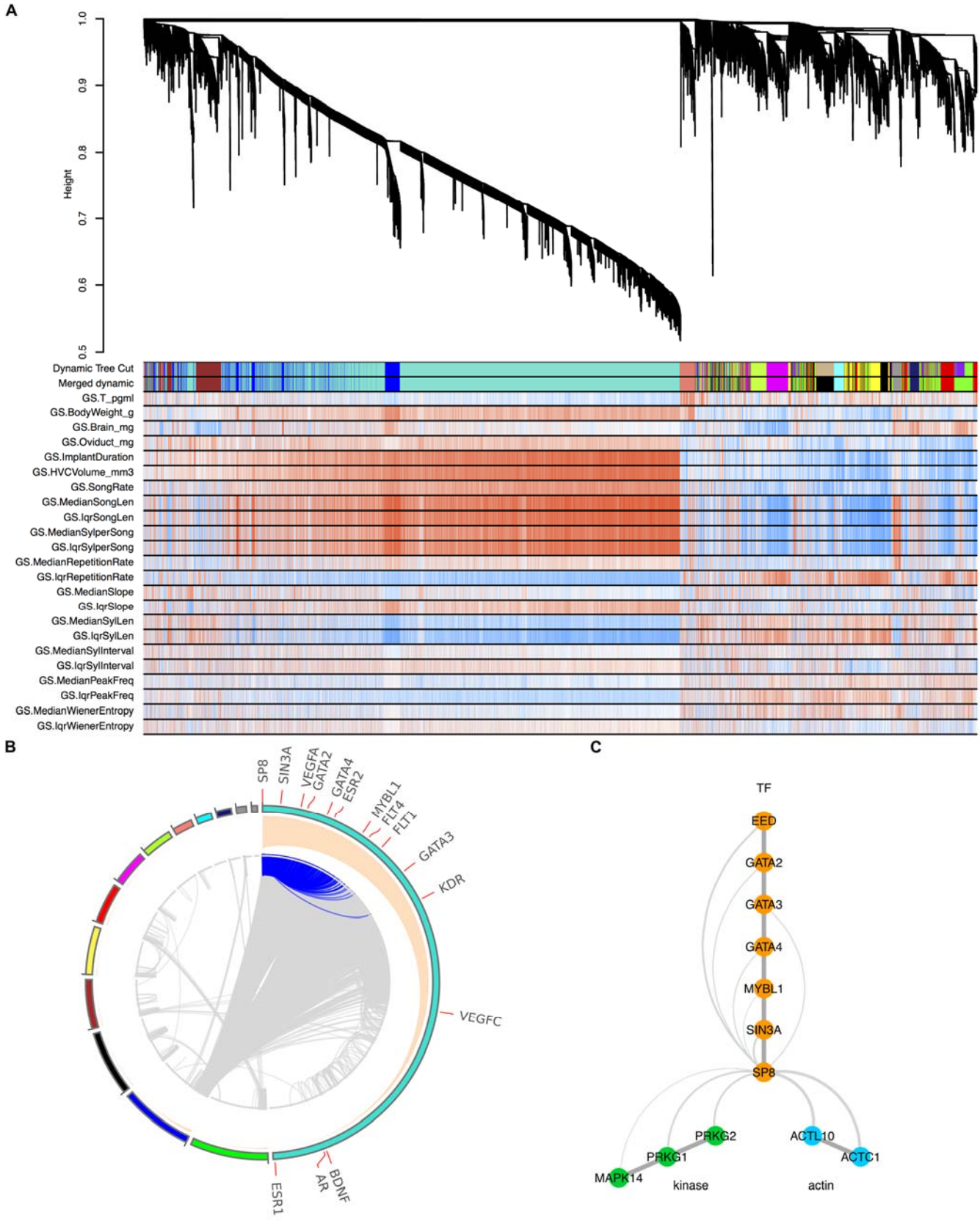
We constructed an overall network composed from all the HVC samples using WGCNA (Table 5). In the overall network, 12,360 genes were assigned into 14 modules named as distinct colors (Figure 20). We found that turquoise module was positively and significantly correlated with the period of testosterone implant, HVC volume, song rate, song length (median and inter-quartile range, IQR) and the number of syllables in a song (median and IQR, Figure 20A, Pearson correlation and Bonferroni correction for multiple comparisons, see also Supplementary Figure 6). Eight other modules were significantly correlated with song-related parameters, among these eight modules five were enriched for neuronal properties (Supplementary Figure 6B). For example, yellow module was enriched for the search-terms "dendrite," "glutamate," and "myelin", whereas brown module was enriched for "axon," "myelin," "dendrite," and "spine." In addition, salmon module was positively correlated with plasma circulatory testosterone concentrations (Supplementary Figure 6A) and was enriched for "endothelial cell." Next, we examined the hub genes that were well-connected with other members in a given module (genes with the greatest intramodular connectivity). Remarkably, transcription factors were frequently the top 10 densely connected genes in ten out of 14 modules, while in four out of the ten modules (turquoise, red, salmon and yellow) transcription factors are the most connected genes.

Turquoise module

Turquoise module was the biggest module (6,480 genes, see Supplementary Table 3) and with the greatest average intramodular connectivity among the 14 modules (Supplementary Figure 7), meaning members of this module are more likely to connect with each other than members of other modules. Although members of turquoise module were not enriched for any cell types we checked (Supplementary Figure 6B), GO-term enrichment analysis showed it was highly associated with anatomical structure morphogenesis (GO:0009653, adjusted p value = 8.2×10^{-17}). Members of turquoise module (Figure 20B) include brain-

derived neurotrophic factor (*BDNF*), vascular endothelial growth factor (*VEGFA* and *VEGFC*) and VEGF receptors (*KDR, FLT1,* and *FLT4*), insulin like growth factor 1 (*IGF1*) and matrix metallopeptidases (*MMP2* and *MMP9*). The aforementioned members have been associated with testosterone-induced angiogenesis and neuron recruitment (Hartog et al., 2009; Kim et al., 2008; Louissaint et al., 2002; Rasika et al., 1999; Rasika et al., 1994a). In addition, 20 enzymes in steroidogenesis such as hydroxysteroid dehydrogenases (*HSD3B2* and *HSD17B12*), 17α-hydroxylase (*CYP17A1*), aromatase (*CYP19A1*), and 5α reductases 2 (*SRD5A2*), as well as steroid receptors such as estrogen receptors (*ESR1* and *ESR2*), androgen receptor (*AR*) and G protein-coupled receptor family C group 6 member A (*GPRC6A*) that mediates the non-genomic effects of androgens (Pi et al., 2010), were assigned to this module (Supplementary Table 3).

The most connected gene of the turquoise module was *SP8* (greatest intramodular connectivity, Figure 20B), a zinc-finger transcription factor required for survival of pallial progenitor cells during corticogenesis (Zembrzycki et al., 2007). To evaluate the probability that the gene product of *SP8* might regulate transcription of the fellow members in the turquoise module, we selected the genes co-expressed with *SP8* (adjacency value > 0.5, n = 1,151) in the turquoise module, and submitted the promoter sequences of these genes to the overrepresented transcription factor binding sites tool of Genomatix software suite (Genomatix-overrepresented TFBS tool) (Cartharius et al., 2005; Genomatix; Quandt et al., 1995; Sui et al., 2005). The promoter sequences (1,000 bp upstream from the transcription start site) were based on the canary genome assembly published by Frankl-Vilches et al., 2015 (available at http://public-genomes-ngs.molgen.mpg.de/cgi-bin/hgGateway?db=serCan1). Genomatix-overrepresented TFBS tool identified that SP8 binding sites (V$SP1F) were enriched in the input sequences and potentially exist in the promoter sequences of 625 genes, including *VEGFA* and *ESR2* (Supplementary Table 4).


A
Height
1.0
0.9
0.8
0.7
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0.5
Dynamic Tree Cut
Merged dynamic
GS.T_pgml
GS.BodyWeight_g
GS.Brain_mg
GS.Oviduct_mg
GS.ImplantDuration
GS.HVCVolume_mm3
GS.SongRate
GS.MedianSongLen
GS.IqrSongLen
GS.MedianSylperSong
GS.IqrSylperSong
GS.MedianRepetitionRate
GS.IqrRepetitionRate
GS.MedianSlope
GS.IqrSlope
GS.MedianSylLen
GS.IqrSylLen
GS.MedianSylInterval
GS.IqrSylInterval
GS.MedianPeakFreq
GS.IqrPeakFreq
GS.MedianWienerEntropy
GS.IqrWienerEntropy
B
SP8
SIN3A
VEGFA
GATA2
GATA4
ESR2
MYBL1
FLT4
FLT1
GATA3
KDR
VEGFC
ESR1
AR
BDNF
C
TF
EED
GATA2
GATA3
GATA4
MYBL1
SIN3A
SP8
PRKG2
PRKG1
MAPK14
kinase
ACTL10
ACTC1
actin

Figure 20. Identification of key genes relevant for testosterone-driven changes.

A, An unsupervised clustering method (dendrogram), WGCNA, classified genes into modules and merged similar modules to form 14 modules (sub-network within the overall network) and calculated Gene significance (GS) between expression level of each gene and physiological traits (including plasma testosterone concentrations (T), and weights of body, brain and oviduct), the HVC volume and song measurements by Pearson correlation. Genes with positive GS are shown in reds, while genes with negative GS are shown in blues. Iqr: inter-quartile range.B. a circus plot visualises the entire WGCNA gene network by arranging the 14 modules (color-coded) in the outer ridge of the circle. The adjacency matrix is indicated in grey; the adjacencies of SP8, a zinc-finger transcription factors, are indicated in blue. The number of neighbours of each gene is indicated in orange, and within each module, genes were sorted by the number of neighbours. SP8 was with the highest number of neighbours in the turquoise module. C, Proteins that might interact with SP8 to regulate gene expression in the HVC. Orange circles: transcription factors; yellow: transcription cofactors; green: kinases; blue: actins. The widths of the connections represent the confidence levels (with a minimum of 0.4) given by STRING database.

Transcription factors can regulate gene expression in a combinatorial fashion (Reményi et al., 2004). Therefore, in addition to the identification of potential downstream targets of SP8, we proceeded to identify the potential interacting partners of SP8 protein, by submitting the list of 1,151 genes to Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database. The database identified 11 proteins that SP8 might interact with based on bioinformatics evidence (Figure 20C), including two transcription cofactors EED and SIN3A, and four transcription factors; three of the four belong to the GATA family (*GATA2*, *GATA3* and *GATA4*). We subsequently evaluated the probability that SP8 forms transcription factor complex with GATA using module search function of the Genomatix-overrepresented TFBS tool. Binding sites for complex V$GATA-V$SP1F were enriched in the promoter sequences of 1,151 genes and were potentially present in 169 submitted genes, which includes *VEGFA* and *ESR2* (Supplementary Table 5).

II. 7. Conclusion

In this study, we studied the time course of gene expression responses in the HVC, a brain nucleus within the song control system responsible for sensorimotor integration (Hahnloser et al., 2002; Nottebohm et al., 1976; Wild, 2004) of adult female canaries implanted with testosterone. Furthermore, we investigated the link between HVC transcriptomes and progression of testosterone effects on singing behavior and song

nucleus anatomy. We found that the plasma concentrations were elevated within one hour by subcutaneous testosterone implantation (Figure 16A) and these elevated concentrations last for at least two weeks. We detected more than 2,600 genes respond to the treatment within one hour and pertinent differences on gene expression for subsequent time points (Figure 17A). We observed the testosterone-induced singing behavior occurred within four days in the treated female canaries and their song performances improved over time as both song length and syllable repetition rate increased (Table 6). Result of the bioinformatic analysis revealed up-regulated genes in T3d birds to be highly associated with the search-terms "spine" and "GABA" (Figure 19B). In addition, we found that HVC volume was significantly greater than that of the control animals already after two weeks of testosterone treatment. At the same time point, we identified the greatest number of regulated genes associated with nervous system development (GO:0007399) (Figure 19A). We found that more than 9,000 testosterone-responsive genes that were regulated transiently rather than constantly, indicating the dynamic regulation of gene expression by testosterone (Figure 17B). Furthermore, we associated the transcriptomic data with various physiological and behavioral data and applied several bioinformatics strategies to identify gene modules relevant for these changes and the master regulator for testosterone-driven angiogenesis and neuron recruitment. Finally, we identified *SP8*, a zinc-finger transcription factor, which was the most well-connected gene in the module that was highly correlated with testosterone-driven changes (Figure 20). Our investigation elucidated the sequence of molecular and cellular events in the HVC that necessitate the development of female canary's singing behavior and fully-differentiated songs.

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III. Experiment III

Testosterone doesn't make you male

The canary (*Serinus canaria*) is a Northern temperate songbird species in which males predominately sing. Male canaries sing more elaborate songs in the breeding seasons than in the non-breeding seasons. Specifically, the male songs in the breeding seasons are on average longer and the syllable repetition rate is higher than the songs in the non-breeding seasons (Leitner et al., 2001a; Leitner et al., 2001b; Voigt and Leitner, 2008). The songs from the breeding seasons are sexually attractive to females, suggesting a reproductive function of the "fast-frequency modulated songs" (Leitner et al., 2001a). The overall temporal pattern of a male canary song depends on circulating testosterone levels (Hartley and Suthers, 1989; Heid et al., 1985; Leitner et al., 2001a; Nottebohm et al., 1987).

In contrast to males, female singing is rare but it has been observed (in the first experiment, and (Hartley et al., 1997; Herrick and Harris, 1957; Pesch and Güttinger, 1985; Shoemaker, 1939; Vallet et al., 1996)). The occurrence of spontaneous female songs is also testosterone-dependent (Figure 14). Moreover, male-quality songs are reliably inducible by systemic administration of testosterone to non-singing adult female canaries (Fusani et al., 2003a; Herrick and Harris, 1957; Leonard, 1939; Madison et al., 2015; Nottebohm, 1980; Shoemaker, 1939). Although spontaneous female singing is possible, the song quality of spontaneous female songs on average is very different from male songs and to testosterone-induced female songs (Table 4 and (Vallet et al., 1996)). Furthermore, spontaneous female songs are less likely to induce a sexual response from females than testosterone-induced female songs (Vallet et al., 1996). In addition, when given the choice, females preferred male songs over songs of testosterone-treated female songs (Vallet et al., 1996).

In songbirds, the song control system, a set of interconnected neural circuits, is responsible for singing. The premotor nucleus HVC is a center of sensorimotor integration in the song control system and

controls temporal patterns of songs (Hahnloser et al., 2002; Nottebohm et al., 1976; Wild, 2004). In addition, the HVC is the only brain nucleus expressing both androgen and estrogen receptors within the song control system (Frankl-Vilches and Gahr, 2018; Gahr, 2001). Intriguingly, the volume of HVC is male-biased (greater in males) in all songbird species that have been examined, including canaries (Brenowitz et al., 1985; Gahr et al., 2008; Gahr et al., 1998a; Gurney and Konishi, 1980; Hall et al., 2010; Lobato et al., 2015; MacDougall-Shackleton and Ball, 1999; Nottebohm and Arnold, 1976). Testosterone implantation increases the volume of HVC in both male and female canaries (Fusani et al., 2003a; Madison et al., 2015; Nottebohm, 1980). Nevertheless, HVC volume of female canaries implanted with testosterone is still smaller than that of male canaries (Nottebohm, 1980). Thus, testosterone is clearly crucial for song production and enlargement of HVC in both male and female canaries; however, a limit of testosterone-induced alteration exists that prevents female canaries to reach male levels of song characteristics and HVC volume.

In order to investigate the fundamental sex differences in the HVC, we studied the transcriptomes from HVC tissue in seven groups of canaries (Table 7) - breeding females and males, non-breeding females and males, non-breeding females and males treated with testosterone and a group of spontaneous singing non-breeding females - by performing exon-level microarray analyses (Affymetrix) on the micro-dissected HVCs. Among these seven groups, four groups sang: breeding males, spontaneously singing non-breeding females, non-breeding females treated with testosterone, and non-breeding males treated with testosterone (Table 7). Thus, we investigated transcriptional sex differences in the HVC between singing females and males. Our results indicate that HVC gene expression is largely discernible by sex. In addition, our results also suggest that although testosterone mimics male-like HVC anatomy and singing in female canaries, it could not mimic male-like HVC gene expression. Testosterone could also not mimic breeding male HVC gene expression in non-breeding males.

III. 1. HVC transcriptomes are largely discernible by sex but not singing

In order to evaluate the similarity of HVC transcriptomes between the 7 canary groups, we calculated Spearman's rank correlation coefficient σ between each pair of groups and calculated the distances between groups based on the coefficient σ. The result indicated that the HVC transcriptomes of breeding and non-breeding males are the most similar (Figure 21A). In addition, 3 female canary groups were assigned to a separate branch, including both singing and non-singing non-breeding females and breeding females. The testosterone-treated females and males were assigned to another branch, suggesting that testosterone failed to induce the HVC transcriptomes of breeding birds.

We applied principal component analysis (PCA) to the HVC transcriptomic data of these 7 canary groups in order to reveal which variables explain the most variation in the data. PCA decomposes a multivariate dataset in a set of successive orthogonal principal components (PCs) that explain a maximum amount of the variance. Thus, by calculating correlation coefficient between the PCs and the variables (plasma testosterone levels, HVC volume, sex and singing), we identified the variables that are highly correlated with the most important PCs. PC1 explained 32% of the data variance (Supplementary Figure 8), and was strongly correlated with blood plasma concentrations (Pearson's r = -0.57, Bonferroni-adjusted p = 1.0×10^{-4}, Supplementary Table 6). Sex and the HVC volume were strongly correlated to the PC2 (Sex: Pearson's r = -0.91, Bonferroni-adjusted p = 4.4×10^{-16}; HVC volume: Pearson's r = -0.73, Bonferroni-adjusted p = 2.1×10^{-7}), which explained 19% of the data variance (Supplementary Figure 8). In contrast, singing was strongly correlated to PC5 (Pearson's r = 0.58, Bonferroni-adjusted p = 6.3×10^{-5}), which contributed 3.7% of the data variance (Supplementary Figure 8). Taken together, the hierarchical clustering and PCA results suggested that the circulating testosterone levels and sex identity but not singing phenotype dominate gene expression patterns in the HVC.

Table 7. Experimental groups and sample sizes.

Group	Sex	Singing	Tissue	Sample size
Breeding	Female	No	HVC	6
	Male	Yes	HVC	6
Non-breeding	Female	Yes	HVC	6
	Female	No	HVC	6
	Male	No	HVC	6
Non-breeding + testosterone	Female	Yes	HVC	6
	Male	Yes	HVC	6

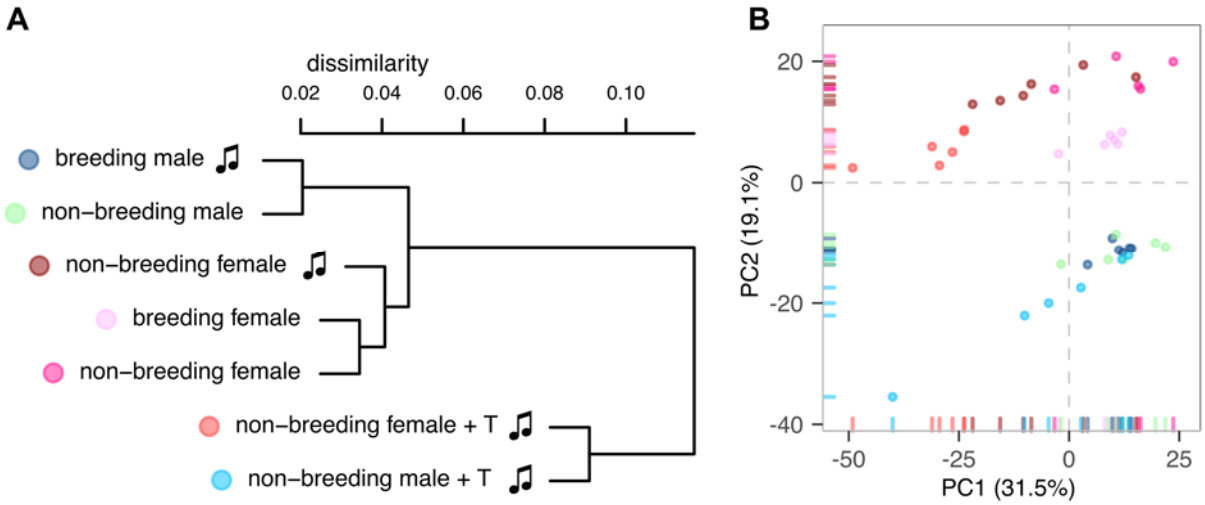

Figure 21. Testosterone and sex identity are the major determinants of HVC gene expression patterns in canaries.

A, Hierarchical clustering of HVC transcriptomes of seven canary groups, showing testosterone-treated animals were the least similar to the un-treated canaries; among those, males and females were clustered on separate branches. T: testosterone. B, PCA analysis of HVC transcriptomes of seven canary groups distinguished male birds from female birds. Each point is color-coded by group (see A) and represents a HVC sample of a bird.

III. 2. Testosterone did not mimic the HVC transcriptomes of natural singing birds

To investigate molecular mechanisms underlying singing phenotype, we calculated differential gene expression of the HVC transcriptomes of the two groups of singing females against the non-singing non-breeding females. Similarly, differential gene expression of the HVC transcriptomes of the two groups of singing males was calculated against the non-singing non-breeding males. Since microarray detects mRNA of genes that were being transcribed at the time of animal sacrifice, the differentially expressed genes between singing and non-singing groups might represent the actively transcribed genes in the singing birds. A substantial amount of genes were expressed differentially between singing and non-singing birds (Figure 22A, non-breeding females: 4,125 genes; non-breeding females treated with testosterone: 7,702 genes; breeding males: 3,359 genes; non-breeding males treated with testosterone 5,106 genes). Both testosterone-treated groups had higher numbers of differentially expressed genes than the naturally singing groups of the same sex (Figure 22A). More than 62% of the differentially expressed genes of the naturally singing birds overlapped with the testosterone-treated birds of the same sex. Thus, although testosterone induced singing in females and males, the majority of testosterone-responsive genes (female: 67%; male: 57%) might not be essential for the singing phenotype.

III. 3. The majority of differential genes between singing and non-singing birds are sex-specific

Next, we examined whether the overlapped gene sets of female signing groups are similar to the overlapped gene sets of the males (Figure 22A, Supplementary Table 7, Supplementary Table 8 and Supplementary Table 9). The Venn diagram indicates that approximately 25% of female-specific genes and 29% of male-specific genes were shared by both sexes. Moreover, the females expressed 358 more genes than the males, suggesting that more genes needed to be activated in females than males during the transition between non-singing and singing. We annotated chromosomal location to the differential genes

(female-specific, male-specific and sex-shared) based on information from zebra finches, because such information is not yet available for the canary. Using Fisher's exact test, we found that chromosomes 1 and 7 were enriched in the sex-shared gene list (Supplementary Table 10). Chromosomes 5, 6, 13 and 17 were enriched exclusively in the female-specific gene list, whereas chromosomes 2, 3, 4, 8 and Z were enriched exclusively in the male-specific gene list (Supplementary Table 10). This might imply sex-specific and sex-shared "hot spots" for singing.

III. 4. The sex-specific genes are functionally overlapped

To understand putative biological functions of the female-specific, male-specific and sex-shared genes, we performed gene ontogeny (GO)-term enrichment analysis (Figure 22B and Supplementary Table 11). Interestingly, the results suggested that female-specific and male-specific gene lists largely overlapped functionally. GO-terms such as nervous system development (GO:0007399), neuron development (GO:0048666), cellular component assembly (GO:0022607) and intracellular signal transduction (GO:0035556) were sex-shared. Female-specific GO-terms were mainly related to cellular maintenance such as organonitrogen compound metabolic process (GO:1901564) and phospholipid metabolic process (GO:0006644), while male-specific GO-terms were axon development (GO:0061564), DNA replication (GO:0006260), cell migration (GO:0016477) and blood vessel development (GO:0001568). In sum, singing females and males expressed genes associated with important functions, including nervous system development and intracellular signaling pathways. However, male physiology, such as active transcription, axon development and supplementary nutritional supply from blood flow, might provide better support for HVC during singing performance than female physiology could.

A, Venn diagrams comparing two female singing groups and two male singing groups indicating the transcriptomes differed. Another Venn diagram comparing the intersection of female singing groups and male singing groups showing the majority genes were not sex-shared; approximately 75% of female intersection was specific in female groups while 71% of male intersection was specific in male groups. B, GO-term enrichment analysis of female-specific, male-specific and sex-shared gene lists derived in A. The results suggest functional overlap between female- and male-specific gene sets.

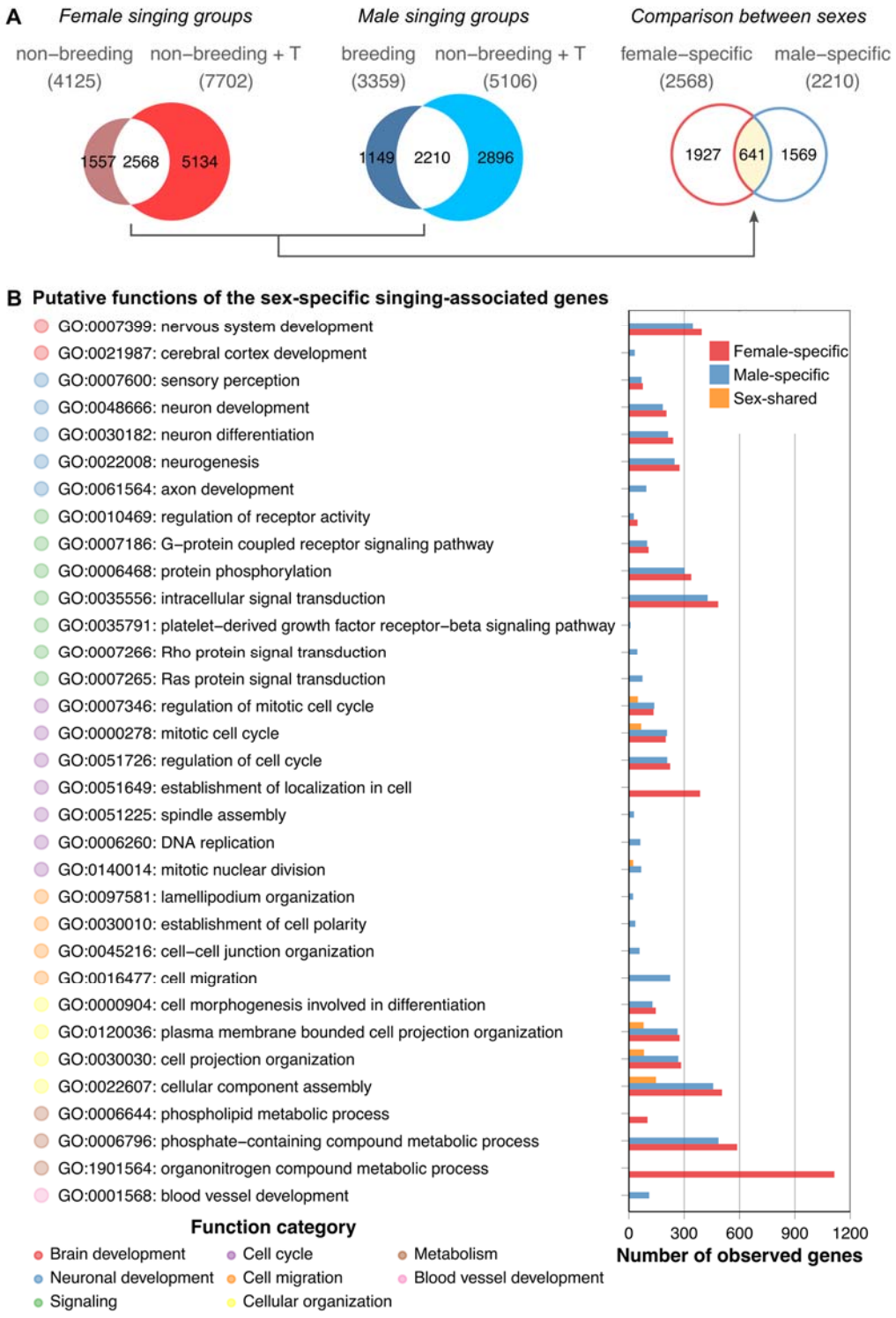

Figure 22. HVC transcriptomes of natural singing canaries are dissimilar from testosterone-treated canaries (see the left page for legend).

III. 5. Conclusion

In this study, we investigated sex differences in gene expression patterns in the HVC of canaries. We found that sex was the major contributor of transcriptomic variation. Although testosterone reliably induces singing in both female and male canaries, it cannot imitate the transcriptomic landscape of natural singing birds. Our result suggested female and male canaries rely on different gene networks for singing, however, sex-specific gene networks are functionally overlapped.

Acknowledgements

We thank Stefan Leitner for providing breeding canaries, Roswitha Brighton and David Witkowski for maintaining the colony, Wolfgang Goymann and Monika Trappschuh for RIA of plasma testosterone. Furthermore, we express our appreciation to Vincent Van Meir and Glenn Cockburn for their comments on previous versions of this manuscript and to the International Max Planck Research School for Organismal Biology for training and support.

IV. Experiment IV

Singing female songbirds express female-specific gene networks in their song control systems.

Behavioral sex differences are likely governed by sex differences at various levels of brain organization (Yang and Shah, 2014) and gene regulation (Gershoni and Pietrokovski, 2017; Yang et al., 2006). The link between sex-specific gene expressions in brain regions and unique sexual traits is emerging in mammals (Yang and Shah, 2014) but has not yet been extensively studied in other vertebrates.

Singing of songbirds (oscine passerines) is an adaptive behavior used in various contexts (Wickler and Seibt, 1980). The degree of sexually different singing varies greatly across songbirds: in some species females never or rarely sing (Nottebohm and Arnold, 1976), whereas in others females sing just as males do (Brenowitz et al., 1985; Gahr et al., 2008). All songbird species that have been studied possess a homologous neural circuit for song control: the song control system (Nottebohm et al., 1976; Wild, 2004). A major component of the system is the HVC, which is anatomically well-defined and critical for generating song pattern (Hahnloser et al., 2002).

Comparative analyses have offered puzzling insights into the neurobiological basis of natural variation in singing. For example, the HVC volume has been shown being larger in males than in conspecific females, regardless of the degree of sexually different singing (Brenowitz et al., 1985; Gahr et al., 2008; Gahr et al., 1998b; Hall et al., 2010; Lobato et al., 2015; Nottebohm and Arnold, 1976; Schwabl et al., 2015). Various sexually different neuronal phenotypes in the song nuclei have been proposed to correlate with sex-specific singing in some species but not in others (DeVoogd et al., 1988; Gahr et al., 1998b). A meta-analysis did not identify sex as a major factor for differential gene expression in songbird brains (Drnevich et al., 2012). The data raise the fascinating question of whether sex-specific brain transcriptomes underlie the sex-specific behaviors.

Birds are particularly interesting because the expression of Z-chromosomal genes is not equal between the sexes (incomplete sex chromosome dosage compensation) (Itoh et al., 2007; Mank, 2013; Naurin et al., 2011). Female are the heterogametic sex (ZW), and males are the homogametic sex (ZZ) (Mank, 2013). The Z-chromosome harbors ~700 genes (Yates et al., 2016), including ~45 transcription factors (this study), while the W-chromosome contains only ~40 genes (Frankl-Vilches et al., 2015; Smeds et al., 2015). Therefore, "default" sex-specific gene expression is possible. Indeed, transcriptome studies of entire brains or large brain parts detected sex differentially expressed genes (Itoh et al., 2007; Naurin et al., 2011; Wolf and Bryk, 2011). Thus, genotype-dependent gene dosage likely influences the functions of somatic tissues, namely the brain (Agate et al., 2003; Gahr, 2003; Zhao et al., 2010b). The existence of an imbalanced expression of Z-chromosomal genes in the behavioral-controlling neurons suggests that such brain functions should always be sexually different.

In this study, we used a custom-made exon microarray to study the HVC transcriptomes of three songbird species that show strikingly different degrees of sexually different singing (Figure 1): forest weavers (FWs) (*Ploceus bicolor*), blue-capped cordon bleus (CBs) (*Uraeginthus cyanocephalus*), and canaries (*Serinus canaria*). FW mates learn to sing identical duets during pair-bond formation (Wickler and Seibt, 1980). In CBs, both sexes sing, although the female songs are usually shorter and less complex than male songs (Geberzahn and Gahr, 2011). In contrast to the FWs and CBs, male canaries sing more elaborate songs in the breeding season than in the non-breeding season (Voigt and Leitner, 2008). Thus, female and male canaries show very different vocal behavior in the breeding season but less in the non-breeding season. Further, singing has been observed in some female canaries in the non-breeding season (Pesch and Güttinger, 1985), which would further reduce or reverse the vocal sex differences. The remarkable divergence of sex-specific singing and a homologous song control circuit across songbird species offer the unique opportunity to define the genetic sexual similarities and differences that contribute to the evolution and sexual specificity of female and male singing.

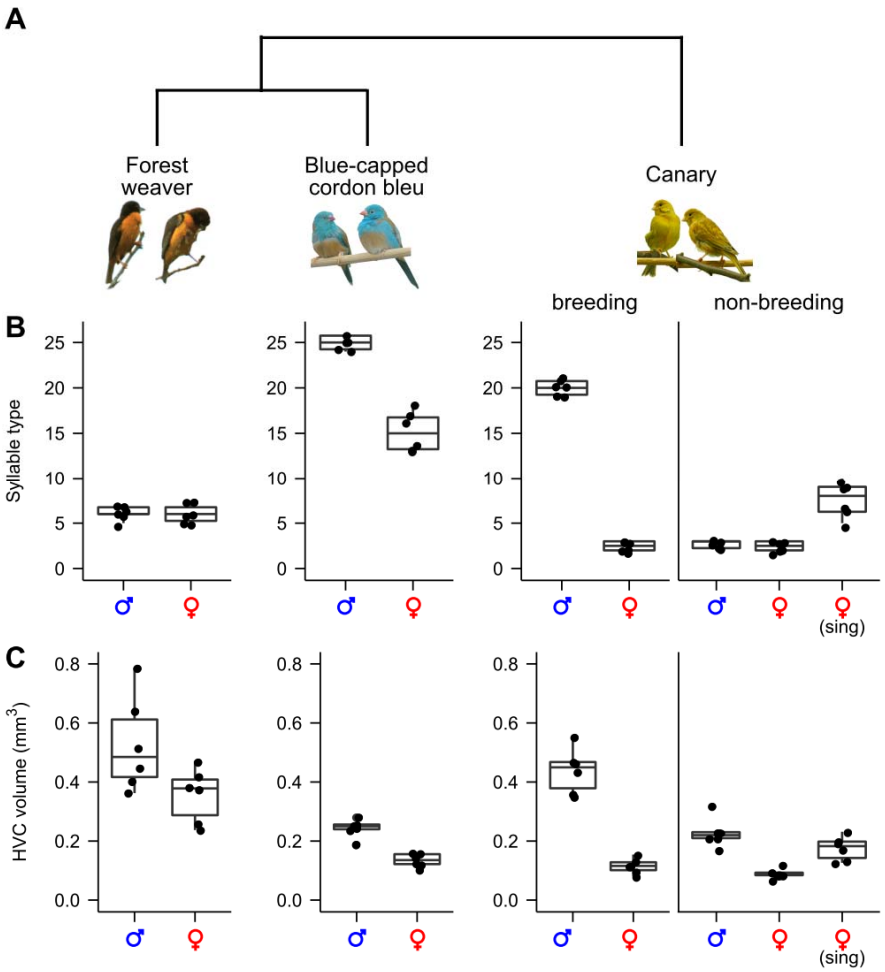

Figure 23. Songbirds vary in the degree of sex differences in singing.

A, Phylogenetic relationships (Christidis, 1986; Jetz et al., 2012) of the three songbird species studied (forest weavers (FWs), blue-capped cordon bleus (CBs), and canaries). **B,** Syllable repertoire. The FW males and females shared their repertoires; the CB males sang slightly more different syllables than females. The breeding male canaries produced many more syllables than the breeding females, non-breeding males and non-breeding females. A small number of female canaries sang under non-breeding conditions. **C,** The Nissl-defined HVC volumes of the reproductively active males of the three species were larger than those of the corresponding females (Mann-Whitney U Test, FW: W = 8, P value = 0.010; CB: W = 0, P value = 2.2×10^{-3}), consistent with the literature (Gahr et al., 2008; Lobato et al., 2015; Nottebohm and Arnold, 1976). In non-breeding (labelled non-breeding) canaries, the Nissl-defined HVCs of males were also larger than those of the non-singing females but similar to those of the singing female canaries (One-way ANOVA followed by Tukey's test, F = 19.83, degree of freedom = 3, adjusted P values: $Cm_{(NB)}$-$Cf_{(NB)}$ = 4.5×10^{-5}; $Cm_{(NB)}$-CfS = 0.079; CfS-$Cf_{(NB)}$ = 4.0×10^{-3}). The box indicates the 25th/50th/75th percentiles (bottom/middle/top bar), and the extent of the whiskers indicates the most extreme values that are within 1.5 times the IQR (inter-quartile range) of the hinge (as per R package ggplot2/geom_boxplot). See Table 8 for sample sizes.

Table 8. Description of experimental groups and sample sizes.

Detailed information about the experimental groups is summarized, including sample size, species, sex, tissue sampled and whether birds were in breeding condition and were singing.

Group	Species	Sex	Breeding	Singing	Tissue sampled	Sample size
FWm	Forest weaver	Male	Yes	Yes	HVC, ENT[1]	6
FWf	Forest weaver	Female	Yes	Yes	HVC, ENT	6
CBm	Cordon bleu	Male	Yes	Yes	HVC, ENT	6
CBf	Cordon bleu	Female	Yes	Yes	HVC, ENT	6
Cm	Canary	Male	Yes	Yes	HVC, ENT	6
Cf	Canary	Female	Yes	No	HVC, ENT	6
$Cm_{(NB)}$	Canary	Male	No	No	HVC, ENT	6
$Cf_{(NB)}$	Canary	Female	No	No	HVC, ENT	6
CfS	Canary	Female	No	Yes	HVC, ENT	6
$Cf_{(iso, NB)}$	Canary	Female	No	No	HVC, ENT	6
ZFm[2]	Zebra finch	Male	Yes	Yes	HVC, ENT	6

[1]HVC: used as a proper name; ENT: entopallium

[2]Published data (Frankl-Vilches et al., 2015).

IV. 1. Sex differences in HVC gene expression are species specific but always sexually different

To examine whether HVC gene expression is sex specific and whether such transcriptional sex differences are similar across species, we compared the transcriptomes of male and female conspecifics during the breeding season in three songbird species (Figure 23 and Table 8): FWs, CBs, and breeding canaries (C, see

Methods). Due to the low RNA yield isolated from isolated HVCs (~150 ng) and the limited amount of biological replicates, we hybridized each HVC sample separately to an exon microarray chip, a strategy that has been successfully used and validated for cross-species hybridizations (Dittrich et al., 2014; Frankl-Vilches et al., 2015), to increase the sensitivity and statistical confidence. RNA-Seq could result in higher stochastic variability, particularly for genes with low expression levels, than microarray (Liu et al., 2011; Nazarov et al., 2017). In addition, extant reference genomes and annotations in public databases are currently available for only one of the three study species (canary) (Frankl-Vilches et al., 2015). To correct any possible technical bias resulting from cross-species hybridization onto the zebra finch microarrays, we analyzed the microarray data using a within-species design (male-to-female, see Methods). The transcriptional sex differences were quantified by the number of sex-biased genes, i.e., genes that showed significant differential expression between the sexes, using stringent settings (see Methods). This procedure identified significant genes for which the expression in one sex was 1.25- to 12.6-fold higher than that in the other sex (FW: 1.57 ± 1.09; CB: 1.50 ± 1.09; C: 1.69 ± 1.08, mean ± sd). We considered only those genes with at least 1.41-fold higher expression in one sex ($|\log_2\text{-fold change}| \geq 0.5$) for further analysis, a typical threshold for expression differences that has been reported previously for brain tissue (Mank et al., 2007; Nätt et al., 2014; Naurin et al., 2011).

The HVC transcriptome of FWs was the least sexually different (1,358 genes; 11% of all detected genes); however, considerable transcriptional sex differences were still present in this species, in which the two sexes sing identical songs (Figure 24A, Supplementary Table 12 and Supplementary Table 13). The HVC transcriptome of breeding canaries was the most sexually different (5,177 genes; 43% of all detected genes), while the HVC transcriptome of CBs was intermediate (2,970 genes; 25% of all detected genes). This species comparison of reproductively active songbirds shows that vast transcriptional sex differences are present in an important song control region in each songbird species, regardless of the degree of sex differences in singing.

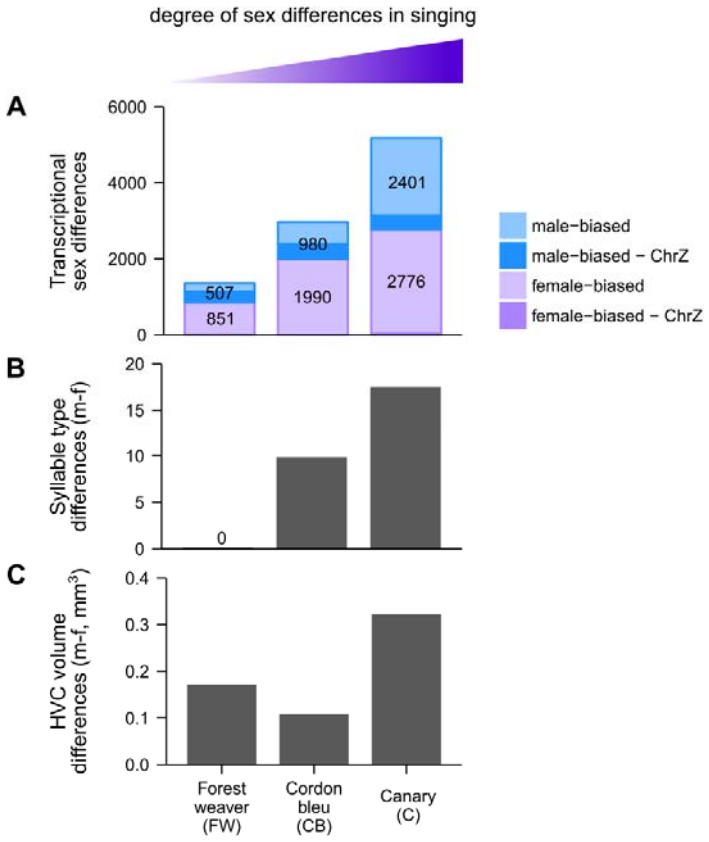

Figure 24. Transcriptional sex differences in the HVC are present in the three species studied.

A, Although transcriptional sex differences based on the numbers of sex-biased genes in each species were smaller in forest weavers (FW) than in cordon bleus (CBs) and canaries (C), they still involved more than 1,300 genes despite the sexually identical singing in FWs. The sum of the autosomal and Z-chromosomal male-biased (blue) and female-biased genes (pink) was included. See Supplementary Table 12 and Supplementary Table 13 for complete gene lists. **B,** Sex differences in the number of different syllables of each species: the FWs were not sexually different, male CBs sang more syllables than female CBs, and female canaries did not sing during the breeding season. **C,** Sex differences in the HVC volumes of FW, CB and C birds during the breeding season.

IV. 2. The degree of sex-biased gene expression in canaries varies seasonally

To investigate the link between the degree of transcriptional sex differences and the degree of sexually different singing within a species, we used canaries as a model system. Male canaries sing more stereotyped songs in the breeding season than in the non-breeding season (Voigt and Leitner, 2008), and the female canaries used in this study do not sing unless specified (Figure 23). Here, we compare singing

male and non-singing female canaries during the breeding season (Cm and Cf, respectively) with non-singing males and non-singing females during the non-breeding season ($Cm_{(NB)}$ and $Cf_{(NB)}$, respectively). The number of sex-biased genes in the canary HVC differed drastically according to the season: HVC-transcriptional sex differences were larger between males and females in the breeding season (Cm vs Cf) than between males and females in the non-breeding season ($Cm_{(NB)}$ vs $Cf_{(NB)}$), which was consistent with the seasonal increase and decrease in the vocal differences of canaries (Figure 3A, "season", Figures 2-1, 2-2). Nevertheless, fundamental transcriptional sex differences remained, even between non-singing female and non-singing male canaries ($Cm_{(NB)}$ vs $Cf_{(NB)}$).

To further evaluate the possibility that sex-specific HVC transcriptomes inform the sexual dimorphism of singing behavior within a species, we considered a group of singing female canaries (CfS) for comparison with singing male canaries.

IV. 3. Singing female canaries (CfS) express large female-specific transcriptomes in their HVCs

Although female canary song is rare, we found that ~5% of the females sang occasionally in our facilities when housed singly in non-breeding short-day conditions (CfS). On average, female songs were less structured than those of males and contained fewer stereotyped syllables (Figure 23 and Table 4). Although these females began singing in an artificial social environment, we studied their song control system to investigate whether this behavior activated "male-typical" pathways for song development. Surprisingly, the amounts of sex-biased genes differed more strongly between singing males and singing females (Figure 25A, "Singing females", Cm vs CfS, Supplementary Table 12 and Supplementary Table 13) than between non-singing males and singing females ($Cm_{(NB)}$ vs CfS). Thus, large transcriptional sex differences appeared when female canaries sang spontaneously, i.e., the gene networks in the HVC of singing females of all three species were not male-like.

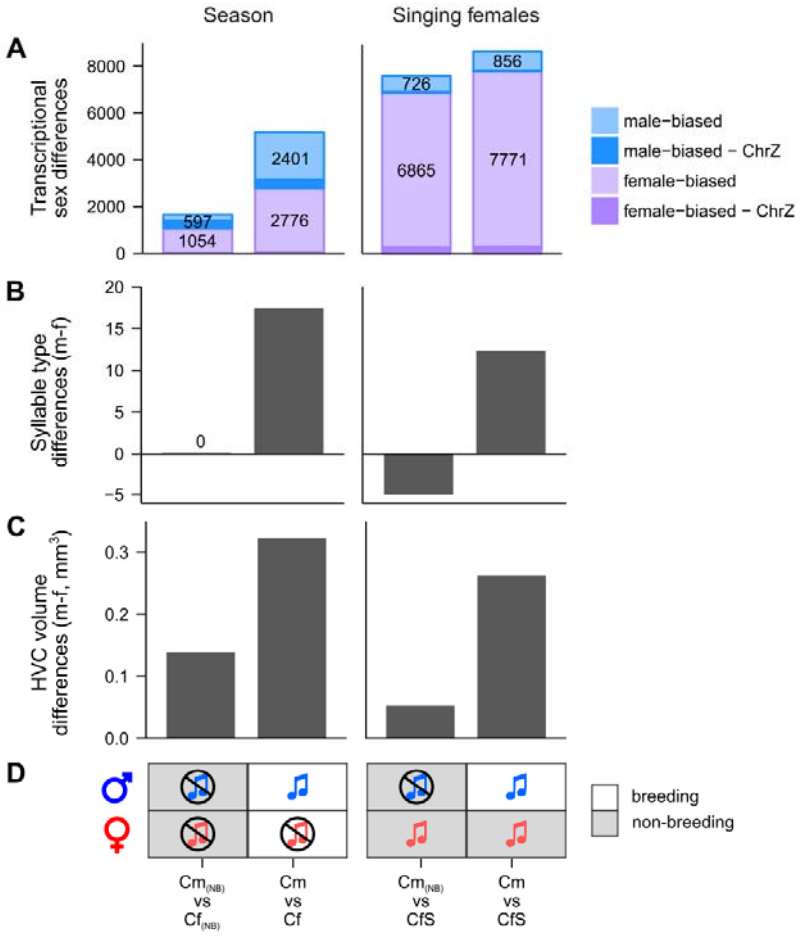

Figure 25. Transcriptional sex differences in the HVC of canaries are context-dependent.

A, Seasonality affected the magnitude of transcriptional sex differences in the HVC of canaries (left panel). The sum of autosomal and Z-chromosomal male-biased (blue) and female-biased genes (pink) was included, see Supplementary Table 12 and Supplementary Table 13 for complete gene lists. When non-breeding singing females (CfS, right panel in A) were compared with either non-singing ($Cm_{(NB)}$) or singing (Cm) male canaries, the magnitude of the transcriptional sex differences drastically increased, even though the differences between singing CfS and Cm were smaller than those between Cf and Cm (see Figure 24B). The sex differences in the syllable types (**B**) and HVC volume (**C**) of each comparison (**D**) are depicted. Abbreviations: $Cm_{(NB)}$: non-breeding male canaries; $Cf_{(NB)}$: non-breeding female canaries; Cm: breeding male canaries; Cf: breeding female canaries; CfS: non-breeding singing female canaries.

IV. 4. Transcriptional sex differences in HVC are autosomal and sex-chromosomal

To investigate whether singing females and males of each species compensate for gene dosage differences in the HVC, we calculated the female-to-male (F:M) expression ratios between singing females and males in

each of the three species described above: FW: FWm vs FWf; CB: CBm vs CBf; and singing canaries (CS: Cm (singing male canaries) vs CfS). The F:M ratios of all detected autosomal genes were approximately 1, whereas the F:M ratios of all detected Z-chromosomal genes were less than 1, indicating generally higher expression in males. Moreover, the F:M ratios of autosomal and Z-chromosomal genes were significantly different (Figure 26C, Mann-Whitney U test). Previous reports that studied large brain regions (Itoh et al., 2007; Naurin et al., 2011) already showed transcriptional sex differences, particularly for Z-chromosomal genes. However, by studying a defined brain area (the HVC), we found many more such genes and detected many more sex-biased autosomal genes than were found in the previous reports (Figure 26C). In contrast to the findings in the two species with regularly singing females (FW, CB), female singing canaries showed higher levels of gene expression than those in males for a large number (260) of Z-chromosomal genes (Figure 26 A and E).

To identify specific genomic regions that mediate sex differences in the HVC across species, we compared singing males and females of each species. We visualized the sex-biased genes according to their chromosomal location retrieved from the Ensembl database (taeGut3.2.4) (Yates et al., 2016). Sex-biased genes were located not only on the Z-chromosome but on all autosomes (Figure 26 A and B). FW female-biased genes were enriched on chromosomes 1A, 2, and 3 (Fisher's exact test, Bonferroni-adjusted P = 0.028, 4.4×10^{-5}, 2.9×10^{-3}, respectively). Enrichment was detected on chromosome 3 for CB female-biased genes and on chromosome 2 for CS female-biased genes (Fisher's exact test, Bonferroni-adjusted P = 5.1×10^{-3}, 9.1×10^{-4}, respectively). Thus, female-biased genes were in general enriched on autosomes in a species-specific manner. Most (> 94%) of the ~700 protein-coding genes (Yates et al., 2016) of the Z-chromosome do not have a homologue on the W-chromosome, which contains ~40 genes (Frankl-Vilches et al., 2015; Smeds et al., 2015). Therefore, the male-biased expression of Z-chromosome genes was not explained by a technical short-coming, i.e., the lack of W-chromosome genes in our custom exon microarrays. In summary, sex-biased expression in the HVC concerned both the autosomes and the Z-chromosome and was widely species specific in gene dosage regulation and genomic locations.

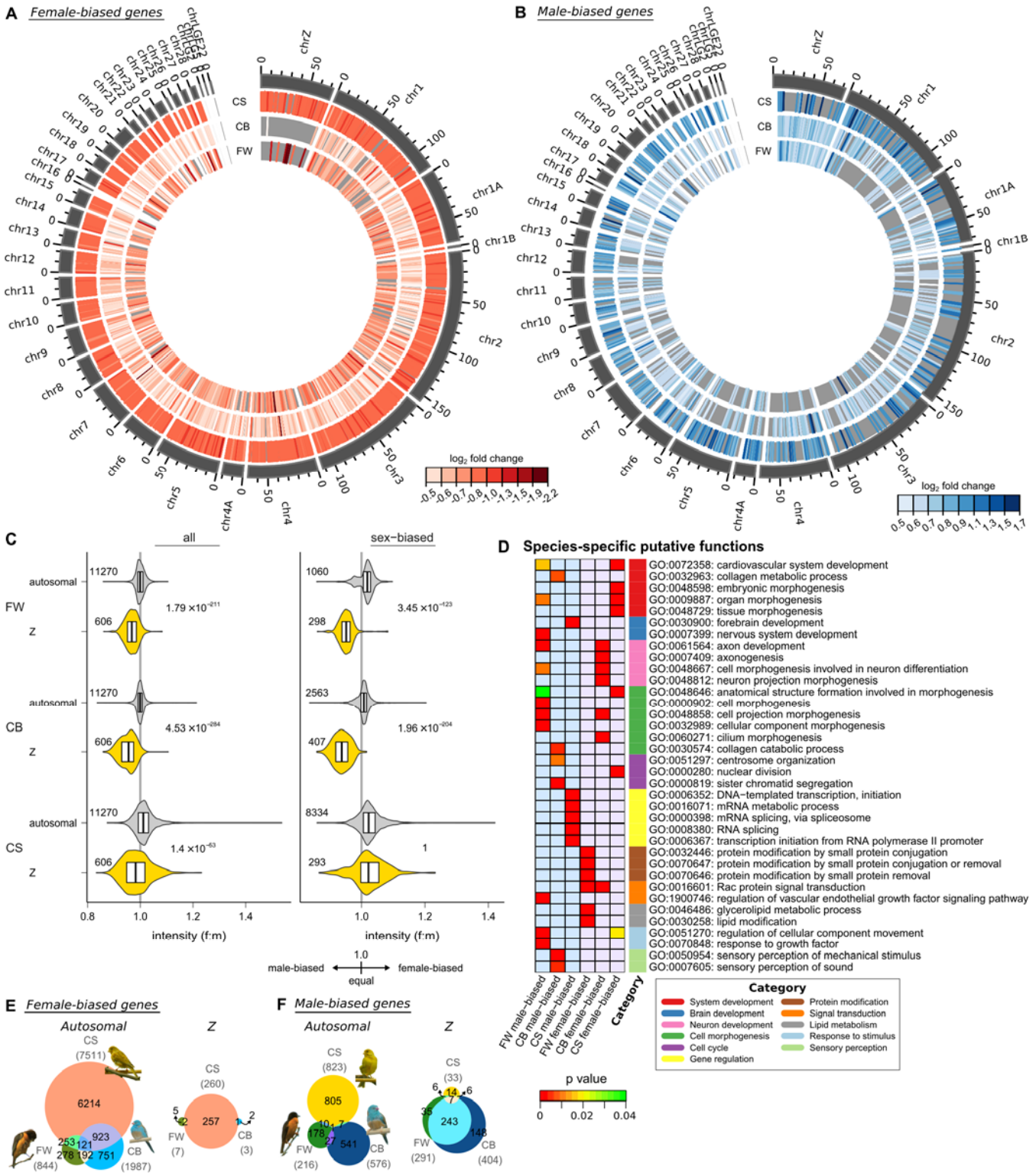

Figure 26. Transcriptional sex differences in the HVC are not restricted to sex chromosomes and are species-specific.

Female-biased (A) and male-biased (B) genes were located on the Z-chromosome and on all autosomes. The red and blue scales indicate up-regulated expression levels in females and males, respectively. C, Box and violin plot showing the median and distribution of female-to-male expression ratios for all detected genes (all) and for the sex-biased genes. The autosomal (grey) and Z-chromosomal (yellow) female-to-male ratios were significantly different in forest weavers (FWs), cordon bleus (CB) and breeding male and female canaries (not shown) but not in singing canaries (CS, Mann-Whitney U test, Bonferroni-corrected P values are indicated). The box indicates the 25th/50th/75th percentiles (bottom/middle/top bar). D, Heatmap of GO-term enrichment analysis predicting the biological functions of sex-biased genes (autosomal + Z). The colour codes represent the Bonferroni-adjusted P values and the 11 annotation categories of the GO terms (only the GO terms with the six highest probabilities from each GO-term enrichment output are shown; see Supplementary Table 14 for complete results). There was no functional overlap across all species of male- or female-biased genes. Venn diagrams of female-biased (E) and male-biased (F) genes in the HVC showing that the female- and male-biased genes were species-specific. (FW: forest weaver male-to-female comparison; CB: cordon bleu male-to-female comparison; CS: singing canary males (Cm) to singing canary females (CfS)).

IV. 5. Singing-relevant genes and pathways: general and species-specific patterns

Singing by both sexes has been suggested to be the ancestral state in songbird evolution and to have been lost in the females of some families or species (Odom et al., 2014). Therefore, we assumed that all singing individuals, regardless of species and sex, would express a set of ancestral genes for song production in the HVC.

First, comparing the male-biased HVC genes across species (FW, CB, CS), we found that many male-biased genes, particularly autosomal genes, were species-specifically expressed, although male-biased Z-chromosomal genes greatly overlapped between FW and CB (Figure 26F). The females shared few (autosomal: 121) or no (Z-chromosomal: 0) female-biased genes across species (Figure 26E). As shown by the Venn diagrams (Figures 4E and 4F), the putative biological functions of the sex-biased genes rarely overlapped across species (Figure 26D and Supplementary Table 15).

A second approach to the question of general species- and/or sex-specific genes related to singing was the study of the HVC-specific transcriptomes of the males and females of the three species. To this end, HVC gene expression was normalized with respect to the entopallium (ENT) to identify HVC-specific genes

expressed by each group (henceforth called HVC_{spec}). Like the HVC, the ENT is located in the forebrain, but it is not part of the song control system. Instead, the ENT is the avian analogue of the primary visual cortex of mammals (Wylie et al., 2015). Similar to the above intra-species male-to-female comparison of HVC, this procedure accounted for potential species differences in the microarray hybridization. Since HVC_{spec} genes are differentially expressed inside and outside the HVC, these genes are likely to be relevant to the song control function of HVC. The hierarchical clustering of gene co-expression networks of HVC_{spec} genes categorized the experimental groups by species (Figure 27A), and the resulting hierarchical pattern was similar to the phylogenetic relationships of our study species (Jetz et al., 2012). To identify both commonly expressed and sexually different HVC_{spec} genes, we categorized the HVC_{spec} genes into female-specific genes (756), male-specific genes (435), and commonly expressed genes (1,342) of the six singing groups (both sexes of FWs and CBs, breeding male canaries, non-breeding female singing canaries; Figure 27B, Supplementary Table 15, Supplementary Table 16 and Supplementary Table 17). Principal component analysis (PCA) of the 1,342 commonly expressed HVC_{spec} genes (Figure 27 E and 5F) showed clear species-specific regulation even for this group of genes. In summary, HVC_{spec} gene expression was species specific and sex specific within each species.

A, Hierarchical clustering of HVC_{spec} expression in FWs, CBs, the different canary groups, and zebra finch males (ZFm) (Frankl-Vilches et al., 2015). The HVC gene expression was compared with that of the ENT (entopallium, visual area) from the corresponding group to generate HVC-specific (HVC_{spec}) gene expression. HVC_{spec} expression was clustered by species. **B,** HVC_{spec} genes were categorized as female-specific, male-specific, or as commonly expressed genes by comparing the transcriptomes of the six singing groups, see Supplementary Table 15, Supplementary Table 16 and Supplementary Table 17 for gene lists. Note the higher number of HVC_{spec} genes expressed in a female-specific manner. **C,** Putative functions of the female- and male-specific HVC_{spec} genes. Many female-specific genes were related to brain and neuron development. See Supplementary Table 19 and Supplementary Table 20 for raw output of GO-term analysis. **D,** The putative functions of the commonly expressed HVC_{spec} genes. **: Bonferroni-corrected P values < 0.01. See Supplementary Table 18 for the raw output of GO-term analysis. **E,** PCA analysis of the commonly expressed HVC_{spec} genes showed distinct clusters, suggesting that singers carried species- and sex-specific molecular signatures. **F,** The PCA projections of animal groups on PC3 to PC6. Note that the opposite sexes of canaries were projected on the opposite ends of PC3. Similarly, that the opposite sexes of FW and CB were projected on the opposite ends of PC4 and PC6, respectively. Abbreviations: FWm: forest weaver males; CBm: cordon bleu males; Cm: breeding singing male canaries; FWf: forest weaver females; CBf: cordon bleu females; CfS: non-breeding singing female canaries.

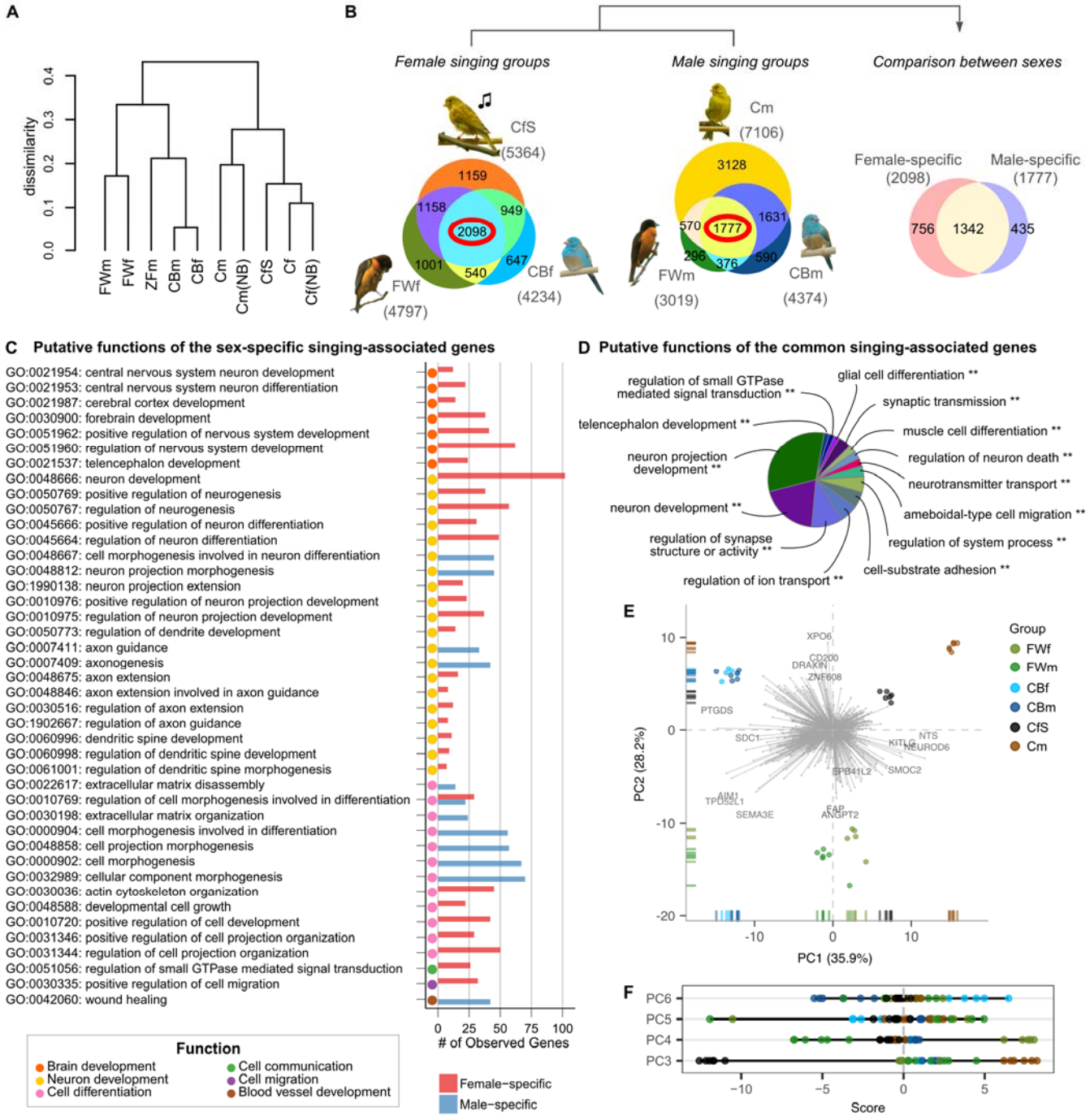

Figure 27. Singing-related genes and functional pathways are sex-specific (see the left page for legend).

We performed GO-term enrichment analysis to predict the putative biological functions of the three categories of HVC_{spec} genes. The commonly expressed HVC_{spec} genes were enriched in processes associated with forebrain development, neuron projection development, and synaptic transmission and its regulation, as well as with small GTPase signaling (Figure 27D and Supplementary Table 18). The female-specific genes were enriched in a wide range of processes, which mainly contribute to brain and neuron development, e.g., forebrain development, neurogenesis, neuron projection and dendritic spine development (Figure 27C and Supplementary Table 19). In contrast, male-specific genes were only related to axonal projections (Figure 27C and Supplementary Table 20). Thus, the female-specific HVC_{spec} genes were associated with a wider span of biological (neural) functions than that of the male-specific genes.

IV. 6. Conclusion

We investigated the relationship between transcriptional and behavioral sex differences by studying the major song nucleus HVC of three songbird species with distinct levels of sexual differences in singing behavior. We found large-scale transcriptional sex differences in our inter- and intra-species (canaries) analysis, regardless of sexually identical or different singing (Figure 24 and Figure 25). This suggests that the transcriptional sex differences in a major song control brain region do not predict the sexual dimorphism of singing. Instead, fundamental transcriptional sex differences are present in the HVC. The gene network analysis of these sex-specifically expressed genes indicated various neuronal functions, strongly supporting the notion that the observed transcriptional sex differences occur in HVC neurons and are behaviorally relevant (Figure 27C). Furthermore, the differences in HVC transcriptomes between singing female canaries (CfS) and singing females of other species suggest several independent mechanisms that lead to the differentiation of singing.

Acknowledgements

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Discussion

In the present thesis I showed that testosterone implantation successfully induced singing in adult female canaries (Experiment II). In addition, circulating testosterone levels were higher in a rare group of spontaneously singing female canaries than in a group of non-singers. Thus, it is clear that testosterone plays a role in the natural induction of female canary singing (Experiment I). Moreover, I investigated in detail the gene regulation and molecular progression in the HVC during the testosterone-triggered song development in female canaries (Experiment II). Further, by comparing the gene expression profiles in the HVC of singing female canaries with singing male canaries, I showed that transcriptomes of both, spontaneous and testosterone-driven singing females, are different from the song of reproductively active males (Experiment III). Thus, this result implied that gene regulation in the HVC is fundamentally different between the two sexes. I further explored this idea by comparing sex differences in the gene expression of the HVC of three songbird species and found large sex differences in the HVC transcriptomes in all three species (Experiment IV). These results provide further evidence for sex differences in brain structure at the molecular and cellular levels in sexually reproductive animals (McEwen and Milner, 2017; Portman, 2017; Ratnu et al., 2017). In the following, I first discussed specifically for each experiment, and then provided a general discussion, and future outlook.

I. Spontaneous female canary songs

I. 1. A minority of female canaries sings spontaneously

Approximately 5% of female canaries in our animal facilities produced song spontaneously. Such an abundance of singing females was observed by previous studies (Hartley et al., 1997; Herrick and Harris, 1957; Pesch and Güttinger, 1985; Shoemaker, 1939) but has never been quantified. The low abundance of

singing females is furthermore comparable to other species of the northern hemisphere such as the song sparrow (Arcese et al., 1988), the indigo bunting, and the rufous-sided towhee (Nolan, 1958). Song production of female canaries was observed in non-breeding conditions (this study; (Pesch and Güttinger, 1985)). However, since three of six singing females showed enlarged reproductive tracts, female singing in this species might occur in the post-reproductive as well as in the reproductive season (as suggested by (Pesch and Güttinger, 1985)). Similarly, females of other species have been reported to sing in several periods of the year (Johnson and Kermott, 1990; Langmore, 1998; Ritchison, 1983; Yasukawa et al., 1987).

Another similarity between female canaries and females of other species producing song is the obliquity of song production. Birds often stayed quiet for the whole day and if singing occurred, three of six females sang less than four songs on average a day (Table 1). In total, all birds spent less than 1% of their day singing (Table 1). Such low song occurrence was already reported for females of northern temperate zone, such as the Northern cardinals (Ritchison, 1986), and the white-crowned sparrow (Baker et al., 1984; Baptista et al., 1993). However, in the tropics and the southern temperate female song is common in term of prevalence in female population and song occurrence (Odom et al., 2014; Slater and Mann, 2004). Based on observations in non-northern temperate species, it is suggested that female singing is ancient and widespread (Odom et al., 2014), and the low female song occurrence in northern temperate is likely due to evolutionary losses in multiple species from an ancestor that had both female and male song. The example of New World blackbirds showed that a composite of life-history traits, including monogamy mating system, dispersed nesting, and sedentary behavior, was correlated with loss of female song (Odom et al., 2015; Price, 2009; Price et al., 2009). Although female song is not lost in canaries, the low prevalence and occurrence imply factors that suppress female song exist. Identification of such factors would explain the low prevalence and occurrence of female songs in canaries, and in songbirds in general.

I. 2. Song structure comparisons between spontaneously singing female, testosterone-treated female and male canaries

Discussion

Because of its rare occurrence, the natural song of female canaries has not yet been analyzed in depth. Long-term observations allowed song comparisons of naturally singing females not only with testosterone-treated females, but also with male canaries in different seasons. In addition, to estimate the repertoire size of each singing female, we developed SylSorter, a computer protocol to semi-automatically classify the syllables of an individual into clusters based on SSIM index (Wang and Bovik, 2002, 2009). Syllable repertoire size is generally an indication of song complexity (Güttinger, 1985). Female repertoire size was only described in one study so far (Hartley et al., 1997). The reported repertoire size of 17 syllables for a spontaneously singing female based on visual inspection (Hartley et al., 1997) agrees with our results showing a repertoire size between 11 up to 52 syllable types per individual (Table 3).

The songs of domesticated male canaries are organized in long sequences of syllables called phrases or tours, which are repetitions of identical syllables, and non-repeat syllables transitioning phrases (Leitner et al., 2001a; Madison et al., 2015; Markowitz et al., 2013). The songs of all spontaneously singing females had a similar general structure (Figure 7), but with less syllable types than male canaries. When comparing spontaneous female song during the period with the highest average repetition rates to male songs, it appears that the average song length of the spontaneous female song is intermediate between the length of male song during the breeding and the non-breeding season (Leitner et al., 2001a; Voigt and Leitner, 2008). In addition, the average song length of spontaneous singing females was comparable to that of testosterone-induced singing females. However, the average syllable repetition rate of testosterone-induced singing females was higher than that of spontaneously singing females (Table 4). Similarly, the maximum repetition rate observed in a female canary was 14.6 syl/s, lower than observed in both breeding and non-breeding male canaries. The so-called sexy syllable of a male canary requires a minimum of 17 syl/s to attract a female (Amy et al., 2015; Vallet and Kreutzer, 1995). Elevated levels of circulating testosterone within physiological range might be a reason for the production of spontaneous female singing. However, the levels of plasma testosterone were in general lower than that of breeding males and testosterone-treated females (Fusani et al., 2003a; Madison et al., 2015; Voigt and Leitner, 2008). This

might explain the observation that spontaneous female song is clearly discernible from songs produced by males during the breeding season and from song produced by females treated with testosterone. When given a choice, a female canary prefers songs of testosterone-treated female over spontaneous female songs, in addition, she prefers male songs over testosterone-treated female songs (Vallet and Kreutzer, 1995). The exact function of spontaneous female songs is unclear, however, female attraction is unlikely.

I. 3. Mechanisms of female spontaneous singing

What are the factors enabling some females to sing but not others? To answer this question, we compared seven physiological measurements between spontaneously singing and non-singing females kept in social isolation during song monitoring. To which extent social isolation contributes to singing in female canaries is currently unknown. However, social isolation *per se* might not be the major cause for singing, because the majoriety of females did not sing. In contrast, oviduct weight and plasma testosterone concentration were found statistically different between the two groups (Figure 14). In female birds, the ovarian tissue is the primary source of circulating testosterone (Johnson, 1986). Thus, female singing might be dependent on plasma testosterone levels and might be a by-product of active ovarian tissue. Our spontaneously singing females were kept in single-sexed aviaries when the birds were not being monitored for song; they did not hear or see a male canary during the experiment. High level of female-female competition for mates has been associated with female song occurrences (Langmore, 1998). It is likely that the lack of male contact and constantly housed under short-day length (non-breeding condition) might drive these female birds to be “ready for mating” all the time, thus explaining an increase in oviduct weight and plasma testosterone level.

While plasma testosterone levels of female singers were generally significantly higher than levels observed in non-singers, the plasma testosterone level of one bird (bird 3) was high enough to be comparable to breeding male canaries (Voigt and Leitner, 2008). Since that individual’s syllable repetition

rate was also the highest and was close to that of non-breeding males, the high level of plasma testosterone of this bird might be biological relevant. Plasma testosterone concentration was positively correlated with the mean of syllable repetition rate and the mean of the number of syllables in a song. The significance of these correlations passed a FDR test for multiple comparisons (Figure 15a-b). Correlations between testosterone level and repetition rate were already observed in male canaries before (Heid et al., 1985) and are mediated by estrogenic activity (Fusani et al., 2003a). Unfortunately, blood estrogen concentrations were not measured. Because behaviorally active estrogens are likely derived from testosterone in the forebrain of the canary (Schlinger and Arnold, 1991) and because oviduct weight and plasma testosterone concentration were higher in these females, an increase in estrogen level would be expected. In contrast, although testosterone generally increases song rate in male and female songbirds (for review (Gahr, 2014)), we did not find a correlation between plasma testosterone and song rate of spontaneously singing female canaries (Supplementary Table 6). Similarly, such correlations were lacking in males of red-winged blackbirds (Harding et al., 1988; Johnsen, 1998).

Elevated testosterone levels have been shown to increase the volume of the song control nucleus HVC (Madison et al., 2015; Nottebohm, 1980) of adult canaries and change the anatomy of the syrinx in adult zebra finches (Bleisch et al., 1984; Bleisch and Harrelson, 1989; Luine et al., 1980). Similar effects were observed in adult female European starlings (Hall and MacDougall-Shackleton, 2012) and female zebra finches (Wade and Buhlman, 2000). We observed statistical differences of HVC volume between female singers and non-singers (Figure 14c), but did not find difference in syrinx weights (Figure 14f). Correlation between the variance of syrinx weight and the number of syllables in a song suggests that the weight of syrinx musculature is associated with the variability of frequency modulation of song. Testosterone-induced changes might occur on the molecular, neurochemical and ultrastructural level in the syrinx (Bleisch et al., 1984; Bleisch and Harrelson, 1989; Luine et al., 1980) as well as in other parts of the adult vocal control system (e.g. (Devoogd et al., 1985; DeVoogd et al., 1991)). It seems that relatively small increases of testosterone are sufficient to trigger singing and increase HVC volume, but a larger increase of

testosterone production, as in bird 3, might lead to elevating syllable repetition rates (Fusani et al., 2003a; Leitner and Catchpole, 2007). More observations of singing females will be necessary to get a more detailed insight in the mechanisms underlying song development in adult female canaries.

II. Time-lapse transcriptomic profiling of the brains of adult female canaries from the onset of testosterone-induced singing behavior

II. 1. Testosterone implantation perturbed steroid homeostasis in the HVC

Testosterone has been associated with courtship behaviors including song output of many species of male songbirds (Ball and Balthazart, 2010), and testosterone administration has been shown to promote song output of females in seasonal songbirds (Gahr, 2014). Many studies have shown testosterone-dependent brain plasticity (Dittrich et al., 2014; Hartog et al., 2009; Madison et al., 2015; Nottebohm, 1980; Sartor et al., 2005), and transcriptomic effects of testosterone in the song control system (Dittrich et al., 2014; Frankl-Vilches et al., 2015; Larson et al., 2015; Thompson et al., 2012). This experiment focused on early effects of testosterone in HVC and time-course study of molecular progression after testosterone treatment, both had not been studied in detail.

We examined the transcriptomes of the HVC after systemic testosterone implantation in adult female canaries. Strikingly, the plasma testosterone concentrations rose at least 17-fold after only one hour of treatment (Figure 16B). Such concentrations exceeded normal range of female canaries (Fusani et al., 2001; Madison et al., 2015) as well as male canaries during the breeding season (Leitner et al., 2001b). More than 2,600 genes (approximately 14% of all annotated canary genes) were differentially regulated compared to the controls (Figure 17A), while plasma testosterone concentration increased. Throughout the duration of our experiment, testosterone was constantly released into the bloodstream from the implants to produce elevated the plasma testosterone concentrations (Figure 16B), in which none of the implants were empty at the time of sacrifice.

Discussion

Testosterone can be converted to a major estrogen 17β-estradiol by aromatase (*CYP19A1*), to an active androgen 5α-dihydrotestosterone (5α-DHT) by 5α-reductase (*SRD5A2*), or to an inactive androgen 5β-dihydrotestosterone (5β-DHT) by 5β-reductase (*AKR1D1*). The expression and enzymatic activities of these three enzymes have been shown in songbird brains, including canaries (Fusani et al., 2001; Metzdorf et al., 1999; Soma et al., 2003b; Vockel et al., 1990a; Vockel et al., 1990b). Androgenic as well as estrogenic metabolites lead to anatomical changes in the brain (Sartor et al., 2005; Yamamura et al., 2011) potentially via androgen and estrogen receptors. These steroid receptors can exert genomic effects (transcription) by nuclear receptors or non-genomic effects by membrane-associated receptors (Diotel et al., 2018; Frankl-Vilches and Gahr, 2018; Gahr, 2001). The nuclear androgen receptor (*AR*) can bind with high affinity to androgens such as 5α-DHT and testosterone (Katoh et al., 2006) and act as a transcription factor. Similarly, two avian nuclear estrogen receptors (*ESR1* and *ESR2*) have been identified to bind with high affinity to estrogen such as estradiol (Kon et al., 1980).

After one hour of implantation, we observed increased expression of *AKR1D1* that encodes 5β-reductase (Figure 17C) that converts testosterone to a biologically inactive form of DHT (5β-DHT). In addition, up-regulation of *UGT1A1* and *UGT1A9* expression, each encoding an isoform of the UDP-glucuronosyltransferase 1A subfamily, is responsible for the glucuronidation of steroids. This process lowers the toxicity of lipophilic substances by conjugating hydrophilic glucuronides in various tissues, including brain tissue (Guillemette, 2003; Ouzzine et al., 2014). In humans, UGT1A1 is known to catalyze the glucuronidation of 17β-estradiol with high activity (Itäaho et al., 2008). Meanwhile UGT1A9 does not exhibit detectable activity to 17β-estradiol, but might work as a sequester of the substrate (Itäaho et al., 2008). Moreover, we observed the down-regulation of *HSD17B4* after testosterone-treatment, a gene that encodes the type 4 17β-hydroxysteroid dehydrogenase. Members of the 17β-hydroxysteroid dehydrogenase family can reversibly convert androstenedione to testosterone or convert estradiol to estrone depending on individual isoform and local factors like pH and the concentration of cofactors (Diotel et al., 2018; Greaves et al., 2014). The zebra finch HSD17B4 preferentially converts 17β-estradiol to estrone,

which is less potent than 17β-estradiol (London et al., 2010). In summary, the high levels of testosterone after one hour of implantation activated the gene transcription of multiple enzymes, which might accelerate the inactivation of testosterone and estradiol.

After eight hours of implantation the elevated plasma testosterone concentrations gradually decreased (Figure 16B), leading to a down-regulation of *AKR1D1* and an increased expression of *SRD5A2*, the gene that encodes 5α-reductase to convert testosterone to active androgen 5α-DHT. The up-regulation of *UGT1A1* was abolished at that time point and 17β-hydroxysteroid dehydrogenase genes *HSD17B4* as well as *HSD17B12* were down-regulated. The activity of HSD17B12 has not been studied in the avian species; the human HSD17B12 can convert estradiol to estrone in cell culture (Saloniemi et al., 2012). Up-regulation of *SRD5A2* and down-regulation of *HSD17B4* and *HSD17B12* suggest that in HVC the catabolism of bioactive androgen (5α-DHT) and estrogen (estradiol) at T8h was decreased leading to potential testosterone effect via nuclear androgen and estrogen receptors. As observed, *ESR1*, the gene encodes estrogen receptor α, were up-regulated at this time point (Figure 17C).

After three days of testosterone implantation, up-regulation of *SRD5A2* was abolished but the expression of *AKR1D1* and *HSD17B12* was up-regulated (Figure 17C). This indicated that the promotion of the biosynthesis of 5α-DHT was abolished; but the biosynthesis of the inactive 5β-DHT and the inactivation of estradiol were again promoted. Correspondingly, *ESR2* was down-regulated at T3d.

By two weeks of treatment, many genes encodes enzymes involved in steroidogenesis were up-regulated, including *HSD3B2*, *CYP17A1*, *SRD5A2*, *CYP19A1*, *ARK1D1* (except for the down-regulation of *HSD17B12*), the nuclear steroid receptors (*AR* and *ESR2*), as well as the membrane-associated androgen receptor *GPRC6A* (Pi et al., 2010). The differential gene expression of many steroidogenesis enzymes suggests potentially active biosynthesis of the intermediate steroidogenesis metabolites (Figure 17C). These steroids might subsequently influence gene expression; this might correspond to the high number of

differentially expressed genes at T14d (approximately 7,700 genes, equivalent to 42 % of all canary genes, Figure 17A). The HVC volume was significantly greater than that of the control animals at T14d.

II. 2. Testosterone-driven activity changes preceded morphological changes

The anatomy of HVC likely underwent continuous growth and only after two weeks does the volume reaches statistical differences. Three birds of T7d had comparable HVC volume with those of T14d animals (Figure 16C). While T7d failed to pass the statistical test for significance as reported by a previous study (Madison et al., 2015). The HVC volume of T14d birds was comparable to published data where the female canaries were implanted with testosterone for three weeks or longer (Fusani et al., 2003a; Hartog et al., 2009; Madison et al., 2015; Nottebohm, 1980; Rasika et al., 1994b). The regulated genes associated with the search-terms "dendrite," "axon," and "spine" were enriched after one hour of testosterone treatment. However, testosterone-treated birds started singing 3 days after testosterone implantation. These results suggest that initiation of singing might result from testosterone-dependent modulation of expression of genes associated with neuronal activity by, which might precede the gross anatomical changes. Accordingly, we observed enrichment of search-terms "serotonin" (up-regulated) and "glutamate" (down-regulated) at early time points (T3h and T8h) and enrichment for "GABA" (up-regulated) at T3d, when initial singing occurred (Figure 19B, Supplementary Table 2 and (Dittrich et al., 2014)). Testosterone treatment increased the number of gap junctions in female canary HVCs (Gahr and Garcia-Segura, 1996). Although in our dataset, genes associated with "gap junction" were not enriched at any time points (Figure 19B), *GJB1* (Gap junction beta-1 protein; also known as connexin 32, *Cx32*) was up-regulated at T1h and T3h, *PANX2* and *PANX3* (pannexin 2 and 3, respectively) were up-regulated at T8h (Supplementary Table 2). Moreover, 15 genes associated with gap junction were up-regulated at T14d (Supplementary Table 2) suggesting increase in number of gap junctions beyond this time point.

II. 3. Molecular choreography of testosterone-driven angiogenesis and neurogenesis

Testosterone-dependent brain plasticity in the HVC of adult female canary is associated with vascular expansion and neuron addition (Goldman and Nottebohm, 1983; Louissaint et al., 2002). It has been shown that the vascular expansion takes place in the HVC within one week after testosterone implantation (Louissaint et al., 2002) and this is induced by HVC's endothelial cell secretion of vascular endothelial growth factor (VEGF) after four days of implantation. In addition to VEGF, mRNA of its principle receptor KDR is up-regulated by testosterone or estradiol *in vivo* within four days (Louissaint et al., 2002). Inhibition of KDR prevents testosterone-induced angiogenesis as well as neuronal recruitment (Hartog et al., 2009; Louissaint et al., 2002). In this experiment, *KDR* and *FLT4* (the other principle receptors of VEGF) were expressed at higher levels than those of the controls onwards from one hour after testosterone treatment (Figure 17C). In addition, *FLT1* was up-regulated at T8h, while *VEGFA* and *VEGFC* were up-regulated at T3d (Figure 17C). Likewise, GO-term enrichment analysis showed that genes associated with circulatory system development (GO:0072359) were enriched at all time points (Figure 19A). The transcription of genes associated with "endothelial cell" was enriched at T3d (Figure 19B). Therefore, the results of our transcriptomic analyses were in accordance with previous finding that stated testosterone induce vascular expansion in the HVC. However, our result suggests that HVC responds to testosterone very rapidly and starts to transcribe genes relevant for angiogenesis shortly after one hour of treatment.

We explored the possibility that there might be a master regulator of testosterone-driven angiogenesis and neuron recruitment upstream of *VEGFs* by utilizing a gene co-expression network-based bioinformatics strategy. WGCNA assigned many relevant genes for the testosterone-driven changes that were already identified by previous experiments into a sub-network (turquoise module, Supplementary Table 3). The members of the turquoise module include *VEGFA, VEGFC* and VEGF receptors (*KDR, FLT1* and *FLT4*), *BDNF*, *IGF1*, matrix metallopeptidases (*MMP2* and *MMP9*) (Hartog et al., 2009; Kim et al., 2008; Louissaint et al., 2002; Rasika et al., 1999; Rasika et al., 1994a), sex steroid receptors (*AR*, *GPRC6A*, ESR1 and *ESR2*) and 20 steroidogenesis enzymes. The most well-connected gene in the WGCNA network was *SP8*, a zinc-finger transcription factor required for survival of pallial progenitor cells during corticogenesis

(Zembrzycki et al., 2007). Most importantly, we identified potential targets of SP8, which includes *VEGFA* and *ESR2*. We identified that *GATAs* might form a transcription complex with *SP8* and regulate the transcription of *VEGFA* and *ESR2*. Unfortunately, because the promoter sequences of canary *SP8* was incomplete, we could not examine the presence of the steroid receptor responding elements *in silico*. Future experiments are needed to verify whether *SP8* can form a transcription complex with *GATAs* and whether *SP8* can stimulate the expression of *VEGFA* and *ESR2* with and without *GATAs*.

Pharmacological inhibition of VEGF receptors blocks angiogenesis, neuronal recruitment as well as testosterone-induced song in adult female canaries (Hartog et al., 2009). However, brain derived neurotrophic factor (BDNF) can restore the effects of VEGF receptor inhibition and thus it is necessary for singing occurrences by canaries (Hartog et al., 2009). It was found that the endothelial cells can secrete BDNF upon stimulation of testosterone, 5α-DHT or estradiol *in vitro*; and that the BDNF mRNA was elevated after two weeks of testosterone treatment *in vivo* (Louissaint et al., 2002). Testosterone-dependent neuron recruitment into the HVC requires BDNF (Louissaint et al., 2002; Rasika et al., 1999). This neurotrophic factor can be released post-synaptically to the robust nucleus of the arcopallium (RA) and regulate neuron survival in RA (Johnson et al., 1997). In our study, *BDNF* was up-regulated at T7d, the time point when birds sing relatively stereotypical canary song, and at T14d, the time point when the HVC volume was significantly greater than that of the controls. The receptor for BDNF (*NTRK2*, also known as *TrkB*) was down-regulated before T3d and the down-regulation was abolished at later time points (Figure 17C). *NTRK2* does not seem to be hormonally regulated (Wissman and Brenowitz, 2009). This suggests that the elevation of *BDNF* followed singing, because most of the birds started singing by the end of four days. Even though earlier study found that singing *per se* could increase the production of BDNF in male canaries (Li et al., 2000), BDNF up-regulation may not be sufficient for female singing. Preliminary experiment injecting viral vector carrying *BDNF* did not induce singing in adult female canaries (Ko and Gahr, unpublished data). Local administration of testosterone to preoptic area motivated male canaries to sing,

and this treatment increased the rate of singing (Alward et al., 2013). Hence, in addition to BNDF, female singing behavior must be induced by other molecules or androgenic metabolites.

Increase of BDNF production driven by neuronal activity in avian species is possible. Numerous research on mammals concerning activity induced BDNF expression focus on how normal brain develops epilepsy (Grabenstatter et al., 2012). Prolonged seizure increases both BDNF mRNA and protein levels in in temporal lobe tissue of patients with epilepsy (Murray Karl et al., 2000). BDNF may regulate $GABA_A$ receptor subunits gene expression (Lund et al., 2008). It has been proposed that activity induced BDNF activates JAK/STAT signaling pathway and leads to increases in ICER, an isoform of CREM (cAMP responsive element modulator). ICER recognizes adenosine 3',5'-monophosphate response element (CRE) contained in *GABRA1*, and represses the transcription (Grabenstatter et al., 2012; Lund et al., 2008). Alteration of $GABA_A$ receptor subunits composition determines the intrinsic properties of each channel (Grabenstatter et al., 2012). In our dataset, *CREM* was the only gene connected to *BDNF* with adjacency value > 0.5 in the WGCNA network. From this dataset, up-regulation of both *BDNF* and *CREM* were apparent at T7d and T14d. Also $GABA_A$ receptor subunits (*GABRB1*, *GABRD*) were down-regulated at T7d, and four α subunit (*GABRA1*, *GABRA2*, *GABRA4* and *GABRA5*), two β subunit (*GABRB2* and *GABRB3*) and two γ subunit isoforms (*GABRG1* and *GABRG2*) of $GABA_A$ receptor were down-regulated at T14d (Supplementary Table 2). Deducing from another source to support our observation, Genomatix-MatInspector program (Cartharius et al., 2005) identified adenosine 3',5'-monophosphate response elements in the promoters of the above mentioned GABA receptors. Therefore, we are tempted to speculate that the initial singing of female canaries stimulated by elevated testosterone via preoptic pathway is activated by the BDNF production in the HVC, Increased levels of BDNF down-regulate expression of $GABA_A$ receptor subunits henceforth disinhibits neuronal activity in the HVC and eventually induces female song.

III. Testosterone doesn't make you male

III. 1. The canary is a suitable model for studying sex differences in singing and gene expression

The song control nucleus HVC exhibits anatomical sex differences in all songbird species that have been examined thus far (Brenowitz et al., 1985; Gahr et al., 2008; Gahr et al., 1998a; Gurney and Konishi, 1980; Hall et al., 2010; Lobato et al., 2015; MacDougall-Shackleton and Ball, 1999; Nottebohm and Arnold, 1976). In all cases, male songbirds possess a bigger HVC compared to female songbirds of the same species, despite the fact that in some species females sing regularly or even more frequently than males (Brenowitz et al., 1985; Gahr et al., 2008; Hall et al., 2010; Lobato et al., 2015). Therefore, the question about what constrains female songbirds arises. The canary is a suitable model for this question. In this species, females sing rarely (experiment I and (Pesch and Güttinger, 1985)), whereas males sing regularly but with seasonal variations (Leitner et al., 2001a; Leitner et al., 2001b; Voigt and Leitner, 2008). Singing of both males and females is inducible by testosterone (Fusani et al., 2003a; Hartley and Suthers, 1989; Heid et al., 1985; Herrick and Harris, 1957; Leonard, 1939; Madison et al., 2015; Nottebohm, 1980; Shoemaker, 1939). Thus, by studying female and male canaries with or without the singing phenotype under different breeding conditions, we identified that the sex identity and circulating testosterone levels were the major determinants for HVC gene expression patterns in canaries (Figure 21). The singing phenotypes might influence HVC gene expression patterns, however, the extent of its effect was smaller than those of sex and testosterone levels, judging from the PCA result (Figure 21). This might also imply that relatively fewer genes are required for song development.

III. 2. Possible causes for sex differences in gene expression

One of the major differences between males and females in sexually reproductive animals is the sex chromosome complement. In birds, the male genome contains autosomes and a pair of Z chromosomes,

while the female genome contains the same autosomes, a single copy of the Z chromosome and a single copy of the W chromosome. Because in avian species sex chromosome gene dosages are not completely compensated as in mammals, i.e., many Z chromosome genes are expressed in higher dosages than autosomal genes in males (Itoh et al., 2007; Mank, 2013; Nätt et al., 2014; Uebbing et al., 2015; Wolf and Bryk, 2011), because the Z chromosome on average harbors more than 700 protein-coding genes (Yates et al., 2016) and because the W chromosome harbors less than 45 protein-coding genes (Smeds et al., 2015), at least 600 protein-coding genes might be expressed in higher dosages in males than in females. The activity of many genes including transcription factors depends on gene dosage (Birchler et al., 2001; Doghman et al., 2013; Schulz, 2017). Thus, gene dosage differences of 600 protein-coding genes might result in profound sex differences in gene expression. Indeed, sex differences in gene expression have been reported in multiple tissues and animal species (Frésard et al., 2013; Itoh et al., 2007; Nätt et al., 2014; Uebbing et al., 2015; Wolf and Bryk, 2011; Yang et al., 2006).

Testosterone is circulated in higher concentrations in males than in females. Testosterone can be converted to 5α-dihydrotestosterone (5α-DHT) and 17β-estradiol, which activate androgen receptor (AR) and estrogen receptors (ERs), respectively. Both AR and ERs are transcription factors playing important roles in the transcription of numerous genes (Bourdeau et al., 2004; Pihlajamaa et al., 2015; Takayama et al., 2007; Wilson et al., 2016). Systemic administration of testosterone to adult canaries dramatically changed the behavioral phenotype (from non-singing to singing) within days and substantially influenced HVC transcriptomes (Experiment II). The transcriptional effect of testosterone was indeed the major determinant for HVC gene expression patterns in canaries (Figure 21). However, the HVC transcriptomes of the testosterone-treated birds were not similar to the non-treated birds of the same breeding condition of the same sex (Figure 22). In addition, testosterone did not masculinize the HVC transcriptomes of the non-breeding female, as the transcriptomes of testosterone-treated females were not similar to both un-treated male groups (Figure 21).

Discussion

Several studies have investigated the genetic basis of male traits such as male plumage, which often seem Z-linked (Irwin, 2018; Price, 2002; Pryke, 2010; Sæther et al., 2007). Singing in canaries is a male trait. Females are sexually attracted by high-frequency modulated male song phrases (Kreutzer et al., 1992; Leitner et al., 2001a; Vallet et al., 1996). Thus, it is not surprising that we identified the enrichment of Z-linked genes among male-specific differential genes (against non-singing phenotype, Supplementary Table 10). Our result provides a potential gene list for further investigation for male-beneficial alleles that increase the fitness of males (Irwin, 2018). Because the blood testosterone levels are higher in the spontaneous singing (non-breeding) females than in the non-singing non-breeding females (Experiment I), the activation of singing is likely testosterone-dependent in these females as in males (Hartley and Suthers, 1989; Heid et al., 1985; Leitner et al., 2001a; Nottebohm et al., 1987). Thus, the potential master regulator for inducing singing might be testosterone-sensitive and Z-linked. One of such candidates is *DMRT1* (doublesex and mab-3 related transcription factor 1), which was present in the sex-shared gene list. *DMRT1* is a Z-linked gene and is required for male sex-determination in birds and other animal species (Herpin and Schartl, 2015; Lambeth et al., 2014; Smith et al., 2009). Overexpression of *DMRT1* in female chicken embryos reduces aromatase expression in the gonads, and induces pathway that triggers development of the testis (Lambeth et al., 2014). Thus, higher gene dosage of *DMRT1* might indirectly regulate circulating testosterone concentration and induce singing in canaries. However, whether overexpression of *DMRT1* in canary HVC induces singing needs to be validated by future experiments.

Although singing female canaries shared relatively less genes with singing male canaries (Figure 22), the results of GO-term enrichment analysis suggested that the sex-specific genes might be functionally similar. For example, estrogen receptor α (*ESR1*) was male-specific, while estrogen receptor β (*ESR2*) was female-specific. The protein products of *ESR1* and *ESR2* are ERα and ERβ, respectively; both act as transcription factors by binding to the estrogen response elements (EREs). Empirical results showed that ERα and ERβ bind to the same ERE motif (Zhao et al., 2010a), thus might have functional overlap. However,

regulation of ERα and ERβ are often cell context-dependent, thus whether gene networks of ERα and ERβ are functionally overlapped should be further investigated in male and female cells.

IV. Singing female songbirds express female-specific gene networks in their song control systems.

IV. 1. Transcriptional sex differences were observed in HVC of three songbird species

In contrast to mammals, dosage compensation is incomplete in birds (Itoh et al., 2007; Mank, 2013; Naurin et al., 2011; Wolf and Bryk, 2011). The imbalanced Z-chromosomal gene expression could lead to "default" sex-specific gene expression. Indeed, we found that ~11% of gene expression in the sexually identical singing species, forest weaver, was sex specific (Figure 24; >1,300 genes), and that Z-chromosomal genes accounted for 22% of this sex difference. Approximately 100 Z-chromosomal genes were HVC-specific and shared by all males. Whether the default male-specific transcriptomes are consistent in different brain regions remains to be evaluated. A previous meta-analysis of the transcriptomic data of large brain regions did not identify sex as a major factor in differential gene expression (Drnevich et al., 2012), which would be expected if area-specific transcriptomes were diluted in homogenates of larger brain regions. In contrast, we identified many sex-biased autosomal genes by studying a defined brain nucleus, which is critical for singing and is anatomically sexually different in all songbird species (Brenowitz et al., 1985; Gahr et al., 2008; Gahr et al., 1998b; Hall et al., 2010; Lobato et al., 2015; Nottebohm and Arnold, 1976; Schwabl et al., 2015). Furthermore, we expect that the transcriptional sex differences would even increase if we obtained neuron type-specific transcriptomes of HVC. Thus, our comparative approach suggests the existence of a fundamental transcriptional sex difference in the brain regions of songbirds and that females of some species can functionally compensate at the level of neural networks for this fundamental transcriptional sex difference (e.g., the forest weaver HVC), while females of other species cannot. In the latter case, sex differences in singing behavior can be observed (e.g., the cordon bleu and

canary HVC). Although transcriptional differences do not necessarily translate into proteome differences (Uebbing et al., 2015), it is unlikely that post-transcriptional mechanisms could balance the vast number of sex-biased transcribed genes in the HVC.

IV. 2. Possible causes for species-specific transcriptional sex differences

The phylogenetic distribution of female singing suggests that the latter was lost and regained many times during songbird evolution (Brenowitz et al., 1985; Gahr et al., 2008; Odom et al., 2014; Schwabl et al., 2015). The forest weaver (family Passeridae) are more closely related to the cordon bleu (family Estrildidae) than to the canary (family Fringillidae), based on gene sequences and morphological features (Christidis, 1986; Jetz et al., 2012). The hierarchical clustering result of HVC_{spec} genes resembled this phylogeny (Figure 27A). If we assume that females of the common ancestor of our three songbird species sang, then the female cordon bleus must have regained singing, as females of early-branching estrildid species do not sing (Soma and Garamszegi, 2015). Since female singing was lost and regained repeatedly during the evolution of songbirds (Odom et al., 2014), it is conceivable that the different ways to compensate for the imbalanced Z-chromosomal gene expression led to family-, genus- or even species-specific molecular networks in the HVC. To support, we observed various levels of molecular species-specificity. First, the identities and the putative biological functions of sex-biased genes were largely species-specific (Figure 26D). Second, even among the HVC transcripts shared by both sexes, we found species- and sex-specific regulation (Figure 27E and 5F). Third, the chromosomal locations of female-biased genes were distinctly enriched across species, whereas male-biased genes were enriched on the Z-chromosome except for comparisons involving the spontaneously singing female canaries. Fourth, the singing female canaries but not the other two species (forest weaver and cordon bleu females) up-regulated many Z-chromosomal genes above the levels found in the homologous area of the male brain (Figure 26A). Imbalanced sex chromosomal gene expression might lead to sex-specific *cis*- or *trans*-regulation and result in sex-specific expression of many autosomal genes. In the HVC, we detected ~50 female-biased autosomal genes associated with epigenetic regulation in

spontaneously singing female canaries, ~40 in female cordon bleus, only seven in female forest weavers, and none among all male-biased genes (Supplementary Table 12 and Supplementary Table 13).

IV. 3. Female-specific transcriptomes reflected female-specific neural connectivity

Female-specific transcriptomes might reflect female-specific neuron types and/or connectivity. In *C. elegans*, the sex-shared neurons are strongly sexually different in their synaptic wiring patterns (Oren-Suissa et al., 2016). In humans, diffusion tensor imaging demonstrated that women and men have different connectivity patterns in the brain (Ingalhalikar et al., 2014). In our dataset, gene networks related to neuron differentiation and connectivity (dendritic, axonal and synaptic) were the most prominent functions of female-specific HVC genes (Figure 27C and Supplementary Table 19). Both the elaboration of neuronal projections and synapse formation are critical for neuronal connectivity. At least 40 Rho signalling-associated genes, which are crucial for axon and dendritic spine morphology by regulating cytoskeleton dynamics (Hall and Lalli, 2010), were expressed in higher levels in the spontaneously singing female canaries than in the non-singing male canaries of the same season (Supplementary Table 12). Unfortunately, the morphology of HVC neurons, including spines, dendrites and axons, has never been studied in females that sing regularly without testosterone treatment. In buff-breasted wrens, in which females and males duet with sex-specific contributions, small but significant anatomical sex differences were revealed in the RA neurons (a premotor nucleus of the song control system efferent of the HVC) (DeVoogd et al., 1988). More specialized neuron types (reflected in the more heterogeneous functional gene networks of females, Figure 27C and Supplementary Table 19) and/or increased connectivity might lead to female-specific circuits in the HVC and consequently to a female-specific motor pattern, since the basic function of the HVC concerns the temporal patterning of song (Hahnloser et al., 2002). Indeed, the motor movements of testosterone-treated female canaries were different from those of males when singing identical song syllables (Mendez et al., 2006). Alternatively, as suggested by the work in the stomatogastric nervous system, sex-specific neuron types and circuits might nevertheless result in sexually identical motor

patterns (Marder, 2011). Future anatomical and neurophysiological studies are required to resolve how female songbirds achieve entire sexually identical songs (in the case of forest weavers) or at least song parts (in the case of cordon bleus) with different HVC transcriptomes from males.

V. General discussion

V. 1. Testosterone regulation of gene expression

Since Nottebohm revealed that systemic testosterone treatment increases the volumes of song control nuclei in female canaries (Nottebohm, 1980), testosterone-driven brain plasticity of female canaries generates much scientific interest. Decades of studies (reviewed in (Balthazart and Ball, 2016; Brenowitz and Larson, 2015; Chen et al., 2013) conclude that several cellular mechanisms could underlie the testosterone-induced volumetric increase, including cell number, intercellular space, or the soma size of song nuclei cells (Tramontin and Brenowitz, 2000; Yamamura et al., 2011). Recruitment of new neurons is a possibility to regulate neuron numbers of the HVC in particular (Alvarez-Buylla et al., 1990; Goldman and Nottebohm, 1983; Vellema et al., 2010).

Circulating testosterone passes the blood-brain barrier entering the brain, and it is converted to estradiol by aromatase in the HVC or its' surrounding. Estradiol (but not 5α-DHT) triggers endothelial cells in the HVC to secrete vascular endothelial growth factor (VEGF), which activates the VEGF pathway and the process of angiogenesis and secretion of brain derived neurotrophic factor (BDNF) from endothelial cells (Louissaint et al., 2002). BDNF mediates the recruitment and survival of newborn neurons (Rasika et al., 1999) during a restricted time window 14-20 days after the new cells are born (Alvarez-Borda et al., 2004). The newborn neurons arrive at the HVC as early as three days after birth and transition to post-migratory neurons peaks at eight days after birth (Kirn et al., 1999). The new neurons project to RA earliest 15 days after birth (Kirn et al., 1999) and BDNF is released to postsynaptic cells in RA stabilizing the projection (Li et al., 2000). The activation of VEGF pathway is necessary for initiation of female singing in testosterone-treated female canaries, because pharmacological inhibition of VEGF receptor prevents singing; concurrent

over-expression of BDNF in the HVC of such birds restores the effect of VEGF receptor antagonist (Hartog et al., 2009). Therefore, BDNF plays crucial roles in female singing, however, over-expression of BDNF in RA, in the nidopallium outside HVC (Hartog et al., 2009), and in the HVC (own preliminary results) did not induce singing in the females. Therefore, BDNF might not be the only gene necessary for induction of singing; instead, it might belong to a gene network that is necessary for the process.

The current literature is lacking on molecular mechanisms taking place before the activation of VEGF pathway. In addition, although many seminal studies have disclosed molecular and cellular mechanisms of neuronal recruitment and survival, these work generally focused on the timeframe after 4 days to approximately one month, when testosterone-treated females start singing and their songs are crystallized, respectively. No studies have investigated molecular mechanisms before singing.

In the second experiment of this thesis, I aimed to bridge the gap in the literature by sampling the HVC transcriptomes of multiple time points from the onset of the testosterone implantation to two weeks after the implantation. I identified many testosterone-responsive genes in the HVC at each treatment period. By using WGCNA, a bioinformatics gene network approach that integrates transcriptomic, anatomical and behavioral data, I identified gene clusters that have prominent relevance of testosterone-driven changes, such as increased song length and HVC volume. Among the gene clusters relevant for testosterone-driven changes, the hub gene was *SP8*, whose mRNA expression was correlated to the highest number of other genes' expression levels. *SP8* is a zinc-finger transcription factor that belongs to SP/KLF family (Limame et al., 2014), and is crucial in angiogenesis (Safe and Kim, 2008), CNS development (Bell et al., 2003), as well as adult neurogenesis in mice (Waclaw et al., 2006). The SP family plays important roles in estradiol-dependent gene regulation in breast cancer cell lines (Safe and Kim, 2008). In addition, both ERα and ERβ have been shown to act in synergy with the SP family; of note, they might act in different manners depending on promoter and cell-context (Gustafsson, 2000; Safe and Kim, 2008).

By database research, I identified 625 potential targets of SP8, including *GATA2*, *LHX6*, *CUX2, VEGFA*, and *ESR2*. GATA2 is expressed in the nervous system and is necessary for angiogenesis in maintenance of multipotential progenitors and hematopoietic stem cells (Fujiwara et al., 2004). In zebra fish embryo, *GATA2* mRNA expression is dependent on *SP8* mRNA expression; the *SP8* knock-down morphant embryos have reduced neuron numbers in the spinal cord (Penberthy et al., 2004). *LHX6* and *CUX2 (Weiss and Nieto, 2018)* are transcription factors that have crucial roles in adult neurogenesis (Fogarty et al., 2007; Liodis et al., 2007). Thus, it is plausible that estradiol activates SP8, which in turn activates its downstream effectors required for testosterone-induced angiogenesis and neurogenesis in female canary HVC. However, future work should be conducted to test whether SP8 is activated by estradiol in female canaries and which interaction partners of SP8 are necessary for inducing singing (see VI. Summary and future directions).

Testosterone's transcriptional effects do not limited to female canaries. Transcriptional effects of testosterone in the brains have been reported for female European robins, male zebra finch, male Gambel's white-crowned sparrow, and female and male dark-eyed juncos (Dittrich et al., 2014; Frankl-Vilches et al., 2015; Larson et al., 2015; Peterson et al., 2013; Thompson et al., 2012). In the third experiment, I showed that testosterone implantation altered HVC gene expression in both female and male canaries (Figure 22). Approximately 42% of male or 65% of female testosterone-responsive genes overlapped between the two sexes, indicating testosterone-dependent gene regulation might be sex-specific. In the dark-eyed junco study (Peterson et al., 2013), testosterone-responsive gene were also sex-specific. Future studies included more songbird species could reveal whether sex-specific testosterone gene regulation is general for all songbird species.

V. 2. Testosterone regulation of singing behavior

Behavioral and brain plasticity of female canaries after testosterone manipulation provide a platform for studying molecular, cellular and behavioral regulation of testosterone. In this model, high dosage of testosterone reliably induces singing behavior and increases the size of the song control nuclei such as HVC

(Figure 16, Table 6, and (Fusani et al., 2003a; Madison et al., 2015; Nottebohm, 1980)). A similar scenario occurred in some females of untreated captive populations, as I reported that approximately 5% of spontaneous singing female canaries have nearly six times higher plasma testosterone concentrations than their non-singing cohort (Figure 14). The blood plasma testosterone concentrations of the spontaneous singers were higher than those of the non-singers (Figure 14). The elevated testosterone levels of the spontaneous singers might originate from the ovarian tissues, given that the oviduct weight of the singers was greater than that of the non-singer (Figure 14), and that the ovarian tissues is the primary source of testosterone in female bird (Johnson, 1986), although I did not quantify the ovarian testosterone levels of these females. In addition to singing behavior, an approximately 1.7 times volumetric increase in the HVC of the spontaneous singing females was observed (Figure 14). Their HVC volume is similar to that of 2-week-testosterone implanted female canaries (spontaneous singer: 0.18 ± 0.04 mm^3 (mean ± SD); 2-week-testosterone implanted bird: 0.25 ± 0.04 mm^3, Mann-Whitney U test, $U = 6$, $p = 0.06$, $n = 12$). Therefore, a certain concentration of blood plasma testosterone seems to be crucial for induction of singing and an increased HVC volume in both spontaneous singing and testosterone-treated female canaries.

Studies of male canaries showed that testosterone regulates several aspects of singing behavior at multiple sites (Alward et al., 2018). Specifically, local testosterone implantation solely in the medial preoptic nucleus (POM) motivates male canaries to sing, whereas birds implanted solely in HVC sing little or no songs (Alward et al., 2016b). Male canaries received local testosterone implantation in both POM and HVC sing stereotypic songs but at a lower amplitude, suggesting that song amplitude might be regulated elsewhere in the brain or the periphery (Alward et al., 2016b). In an attempt to study peripheral effects of AR signaling on song characteristics, Alward et al. implanted male canaries with bicalutamid, an androgen receptor antagonist that does not cross the blood-brain barrier, and found that songs of these birds have reduced song durations, syllable repetition rates and complexity (Alward et al., 2016a). An additional layer of testosterone regulation is that androgenic and estrogenic metabolites of testosterone govern separate aspects on singing. A series of experiments aimed to disentangle effects of androgenic regulation on song

features at different sites have been performed in male canaries. Bilaterally implantation of flutamide, a potent AR antagonist, in HVC of male canaries reduces variability of syllable-type usage and syllable sequences, while the same treatment in RA reduces song stability and trill bandwidth stereotypy (Alward et al., 2017). Similar experiments designed to examine effects of estrogens in the CNS (excluding peripheral systems) or locally in the HVC, the only song control nuclei expresses ERα, have not been done. Thus, testosterone regulates distinct characteristics of singing behavior in male canaries in both periphery and several brain regions in a non-redundant manner (Alward et al., 2017; Alward et al., 2016b). However, since these implants are close to the ventricular walls, diffusion of the hormones into other brain areas is a problem.

Stimulation of female singing in canaries required both androgenic and estrogenic metabolites of testosterone (DeVoogd and Nottebohm, 1981). Unfortunately, the local effects of testosterone and its metabolites at different sites in the brain or peripheries have not been studied in detail in female canaries. Therefore, I do not know whether testosterone regulates individual song features in the same way in female canaries as described above for males. Nevertheless, the singing female canaries (both spontaneous and testosterone-treated) have both elevated circulating testosterone levels and elevated syllable repetition rates, which has been shown being estrogen-sensitive in both female and canaries (Fusani et al., 2003a; Rybak and Gahr, 2004). More work is required to extend our understanding of testosterone regulation in female songs (see VI. Summary and future directions).

V. 3. Sex differences always exist

It is clear that testosterone is important for induction and maintenance of singing in male and some species of female songbirds (see Introduction, p.22). To investigate whether testosterone regulates singing by similar genetic mechanisms in both males and female canaries, I compared the HVC transcriptomes of seven groups of canaries, including breeding and non-breeding males and females, and non-breeding males

and females implanted with testosterone, and finally a group of spontaneously singing females (Figure 21). Among these seven groups, four groups sang (breeding males, testosterone-implanted males, and females and spontaneously singing females). Singing *per se* did not dominate the clustering of their transcriptomes. Instead, the testosterone-treated females and males were clustered on a separate branch from the other five untreated groups. On the branch of the untreated groups, the sex dominated the clustering: the spontaneous singing females were clustered with breeding and non-breeding females, whereas breeding and non-breeding males formed another cluster. Therefore, although testosterone triggered singing in both non-breeding females and males, its transcriptomic effect did not mimic natural singing birds. In fact, testosterone-treated birds expressed more differential genes compared to non-breeding birds (also non-singing) than natural singing birds.

Nevertheless, the common differential genes (relative to non-singing birds) of testosterone-treated and natural singing female and males might provide indications of singing-related genes. For example, c-fos and ZENK were up-regulated in both of testosterone-treated and natural singing females. c-fos and ZENK are immediate early genes that are activated by singing (Feenders et al., 2008; Jarvis and Nottebohm, 1997; Jarvis et al., 1997; Kimpo and Doupe, 1997; Mello and Ribeiro, 1998). Furthermore, I identified more than 1,500 differentially expressed genes that were male-specific, more than 1,900 genes were female-specific, and relative few were sex-shared (641). The canary has approximately 15,609 protein-coding genes (MPI_MOLGEN, 2014). Thus, approximately 4% protein-coding genes were sex-shared in the HVC of singing canaries. In contrast, female singing canaries expressed ~3% more protein-coding genes than males. Intriguingly, GO-term enrichment analysis of female- and male-specific genes showed that most GO-terms recurred in both lists. In addition, ER signaling regulates syllable repetition rate in both male and female canaries in similar manner (Fusani et al., 2003a; Rybak and Gahr, 2004). Thus, it is possible that although female canaries activate a different set of genes, these gene networks execute similar biological functions (e.g., singing) as the male-specific gene networks.

Discussion

Are sex differences always present in the HVC of songbirds? To answer this question, I additionaly compared the HVC transcriptomes of two other songbird species, forest weavers and blue-capped cordon bleus. In these two species both male and females sing naturally. Songs of male and female cordon bleus were sex-specific (Geberzahn and Gahr, 2011), whereas forest weaver mated-pairs sing identical songs (Wickler and Seibt, 1980). The comparison of HVC transcriptomes showed that sex differences existed in both species, although the number of sex-biased expressed genes in forest weavers was lower than in the cordon bleus. In addition, the numbers of sex-biased expressed genes in both species were lower than in breeding and non-breeding canaries. Are sex-biased genes sex chromosomal genes? I checked this by mapping the transcripts to the zebra finch genome, because cordon bleu and forest weaver genomes are not available and the canary genome has not been annotated to chromosome levels. I found that more than 78% of the sex-biased genes from the three species were autosomal, which is not an exception. Transcriptional studies on sex differences in multiple animal models including birds, insects and mammals, have reported similar findings (Mank, 2013; Mank et al., 2008; Wijchers and Festenstein, 2011). In addition to sex-specific gene sets, the expression profiles of the sex-shared genes showed sex-specific patterns, indicting the regulation or the relationship of genes in the sex-shared gene networks were different in each sex.

Several sex-specific gene regulatory mechanisms might explain sex differences in gene networks and expression pattern. First, sex-specific gene regulation by *cis*-regulatory elements is one possibility (Meiklejohn et al., 2014). For example, distinct sex hormone milieu in the HVC of males and females might influence the regulation of sex steroid receptors in a dose-dependent manner (Doghman et al., 2013; Schulz, 2017). Although the concentrations of androgens and estrogens of adult canaries and species other than zebra finches have not been measured locally in HVC (for instance by *in vivo* micro-dialysis) or from dissected tissues, using radioimmunoassay or mass spectroscopy (Schlinger, 2015; Shah et al., 2011; Taves et al., 2011), the aromatase activity is higher in presynaptic boutons of certain brain regions of male as compared to female zebra finches (Rohmann Kevin et al., 2006). Likewise, 3β-HSD activity (Introduction,

Figure 3) is higher in the telencephalon of male zebra finches relative to females (Soma et al., 2004). In addition, elevation of peripheral testosterone levels increases aromatase mRNA expression and activity in adult canaries (Fusani et al., 2001). Thus, a higher local concentration of estradiol is possible in the male brains leading to sexually differential regulation of hormone-sensitive transcription factors.

Second, the regulation of autosomal genes might be different between the sexes due to incomplete Z chromosome dosage compensation (Birchler et al., 2001; Doghman et al., 2013; Schulz, 2017). Because birds do not completely compensate dosages of the Z chromosomal genes and many genes are regulated dose-dependently (Birchler et al., 2001; Doghman et al., 2013; Schulz, 2017), the Z-linked gene dosages might result in a different transcription landscape in between females and males, and might affect both genes on sex chromosomes and autosomes.

Third, sex chromosome complement has been shown inducing sex-specific epigenetic difference. For example, in murine embryonic stem cells with an XX chromosome complement display global DNA hypomethylation compared with XY and X0 (Wijchers and Festenstein, 2011; Zvetkova et al., 2005). Relatively few studies on epigenetic regulation have been conducted in birds, however, there is indication of sex differences in DNA methylation patterns in chicken brains (Frésard et al., 2013; Nätt et al., 2014).

Fourth, sex-specific expression of particular splice variants and usage of exons have been associated with sexually differential gene expression (Jazin and Cahill, 2010; Wijchers and Festenstein, 2011). A genome-wide study detected widespread sex difference in gene expression and splicing in 12 CNS human brain regions (Trabzuni et al., 2013). Moreover, several isoforms of the *AR*, *ESR1* and *ESR2* are derived from alternative splicing and promoter usage (Arnal et al., 2017; Thomas and Gustafsson, 2011), and have been found in the brain of mammals including humans (Hu et al., 2014; Ishunina et al., 2013; Kundu et al., 2015). Splice variants can have different protein structure and function; for example, an ERα isoform is primarily localized in the plasma membrane and the cytoplasm and mediates membrane-initiative effects of estrogen signaling (Thomas and Gustafsson, 2011); an another truncated ERα isoform

exhibits hormone-independent activity (Arnal et al., 2017). In zebra finches, *ESR1* splice variants occurring in higher density in neurons distributed throughout the hypothalamus (Frankl-Vilches and Gahr, 2017). *AR* splice variants have not been analyzed in birds.

Finally, sex-linked alleles, such as single-nucleotide polymorphisms (SNPs), might result in nonsynonymous substitution of amino acids, alter protein properties and lead to sex-specific traits. For example, melanocortin 1 receptor gene (*MC1R*) is responsible for melanin-based color polymorphism in the barn owl (*Tyto alba*): the ancestral valine at position 126 encodes white coloration and the derived isoleucine allele encodes rufous coloration (Ducret et al., 2016). While each sex can express any phenotype, male barn owls are on average whiter than females. Furthermore, a study found that more than 22% male traits are Z-linked in birds, which is a rate higher than at random (Price, 2002). This suggests sexual selection might drive the evolution of Z chromosome (Irwin, 2018). For example, a single Z-linked locus is associated with two color morphs (red or black head color) of Gouldian Finches (*Erythrura gouldiae*) (Pryke, 2010). Both male and females prefer mates of the same color type as themselves, and the preference is also Z-linked (Irwin, 2018; Pryke, 2010). Mating of mixed morphs results low fitness offspring (Pryke, 2010). In *Ficedula* flycatchers, three species-specific male plumage traits are Z-linked (Sæther et al., 2007) and sexual preferences of females for those male traits are also Z-linked (Sæther et al., 2007).

In addition to coding sequence evolution, sexual selection appears to drive a rapid turnover in sex-biased gene expression primarily through evolution of male expression levels, and the degree of sexual selection predicts the proportion of male-biased genes (Harrison et al., 2015). This is reminiscent to our result, in which I observed higher sex-biased genes in the canaries. Female canary prefers male songs with higher syllable repetition rate (Kreutzer et al., 1992; Vallet and Kreutzer, 1995). Intriguingly, the frequency of being on Z chromosome of female-biased gene of the spontaneously singing female was higher than cordon bleu and forest weaver females. This might hint that canary “singing genes” are on the Z chromosome and the male gene dosage might be necessary for activate singing. Because circulating

testosterone concentrations were higher in the spontaneously singing females than the non-singing females, it is possible that the elevated testosterone up-regulates the "singing genes" on the Z chromosome and induces singing, an otherwise male trait, in 5% of the female canary population.

In summary, besides the W chromosome hosting ~40 genes and sublet differences in sex-linked allelic polymorphisms, males and females of the same species share most of their genome. Yet, multiple levels of regulation allow sex differences and at the same time compensation at every turn. Sex differences and compensation have been shown in epigenetics, gene expression, alternative splicing and functional gene and neural network in various species (De Vries, 2004; de Vries and Sodersten, 2009; McCarthy et al., 2009; Portman, 2017; Ratnu et al., 2017). These examples also demonstrate species-specific evolutionary routes of sexually antagonistic variation and the complex dynamics of male–female coevolution (Pennell et al., 2016).

VI. Summary and future directions

In the present thesis, I monitored more than one hundred female canaries, obtained a group of spontaneously singing female canaries and described their song characteristics. Because of their rare occurrences, their songs were not described in details. Moreover, I found that their circulating testosterone levels were higher than non-singing female canaries. Furthermore, I conducted testosterone-implantation experiment on female canaries and studied the testosterone effects on HVC transcriptomes in a timely manner. Using bioinformatics and integrative approaches, I speculated that a zinc-finger transcription factor is crucial in initiating testosterone-driven processes, such as angiogenesis. By comparing HVC transcriptome of testosterone-induced singing and spontaneously singing female canaries, I showed differences in HVC gene expression. Similarly, differences were found in HVC transcriptomes between testosterone-induced singing non-breeding males and singing breeding males. Clearly testosterone induces singing in both females and males, nevertheless, naturally singing birds perhaps regulate gene expression

in a more efficient and precise way by regulating only half as many genes than testosterone-treated birds. Finally, I demonstrated that transcriptional sex differences were present in the HVC of canaries of both breeding and non-breeding seasons, and two other songbird species. However, birds with similar singing performance showed reduced transcriptional sex differences, indicating potential mechanisms for behavioral compensation. However, several questions remained open and require further investigation.

VI. 1. What makes spontaneously singing females sing? Why and when do they sing?

In the first experiment, the spontaneously singing females were housed in social isolation, and, when they were not monitored for singing, they were housed in a single-sexed aviary. During the experiment (approximately 2 years), they were held under non-breeding light regime and did not encounter any male birds. Because this combination of factors (social isolation, long-term abnormal light schedule and single-sexed housing) was a reliable method to spot singing females, I observed that females were more likely to sing after being socially isolated for more than 4 days, but decreased singing after 2 weeks of isolation. However, these three factors also confound the true reason of why these females sang. Social isolation has been shown altering gene expression patterns in the zebra finch brain (Drnevich et al., 2012; Park and Clayton, 2002), a highly social species. Although canaries are much less social, social isolation *per se* might be ruled out as a reason to sing, because not all socially isolated females had elevated plasma testosterone levels and exhibited singing behavior. High level of female-female competition for mates has been associated with female song occurrences (Langmore, 1998). Thus, the lack of opportunity to find a male and constantly housed under short-day length (non-breeding condition) might drive these female birds to be "ready for mating" all the time. Indeed, the oviduct weight of singing females was heavier than that of the non-singing birds, indicting the birds were in breeding condition despite the housing condition. Circulating testosterone level is higher before egg laying in many bird species (Ketterson et al., 2005; Schwabl, 1996). High testosterone level in the yolk might be advantageous and adaptive for the offspring (Groothuis et al., 2005), i.e. female song development would be a collateral effect of increasing the fitness of

the offspring. Future study employing backpack microphones (Gill et al., 2016), which allows free-moving and social interaction between males and females in natural context and under natural light schedule, would provide answers to why and when female canaries sing. Additional experiments can be designed with various sex ratios in group-housing aviaries; this would answer whether higher female-female competition is related to occurrences of female singing in canary.

VI. 2. Is SP8 estradiol-sensitive and the master regulator of testosterone regulation?

Testosterone regulated a large number of genes in the HVC at each implantation period. By bioinformatics approach that integrated physiological (e.g., body weight, testosterone levels), behavioral (e.g., song length, syllable repetition rate) and anatomical (e.g., HVC volume), I focused on a hub gene of a sub-network that was highly associated with the testosterone-driven changes. This hub gene was *SP8*, an estradiol-sensitive zinc-finger transcription factor and crucial for angiogenesis (Safe and Kim, 2008), which might act in synergy with ERα and ERβ in cancer cell lines (Gustafsson, 2000). In mice, SP8 regulate the generation of interneurons in the olfactory bulb (Waclaw et al., 2006). However, several steps should be taken before I conclude that SP8 is the master regulator of testosterone-induced singing. First, it is important to test whether canary SP8 is also estradiol-sensitive. Pilot studies with primary forebrain cell culture from male and female canaries can be designed to study the responsiveness of SP8 to estradiol and androgens. Second, interaction partners of SP8 should be verified, for example, by mass spectrometry-based proteomics (Li et al., 2017). Moreover, the downstream DNA targets of SP8 should be verified by CHIP-sequence against SP8 antibody; multiple cell contexts should also be tested, namely, with and without estradiol and SP8's interaction partners. Lastly, if SP8 is the master regulator for inducing female singing, loss-of-function study should abolish testosterone-induced singing; experiment using siRNA or CRISPR/Cas9 knockout protocol against *SP8* would be informative.

VI. 3. How to disentangle androgen and estrogen effects in the HVC?

Discussion

The activation of VEGF receptor is estrogen-dependent, and is crucial for testosterone-induced angiogenesis and necessary for induction of female singing (Hartog et al., 2009; Louissaint et al., 2002). If SP8 is inducible by estrogen in female canary HVC, it might act as an up-stream regulator of the VEGF pathway, which up-regulates the expression of a ligand of the VEGF receptor (VEGFA), alone or act in synergy with other factors. However, estrogen alone is not sufficient for inducing female singing in canaries (DeVoogd and Nottebohm, 1981). Similarly, implantation of 5α-DHT without estradiol is not sufficient; a combination of estradiol and 5α-DHT is required for song induction and able to increase the volume and neuron density in HVC (DeVoogd and Nottebohm, 1981; Yamamura et al., 2011). Interestingly, such treatment (implantation of 5α-DHT without estradiol) to females that had been treated with testosterone half a year ago stimulates singing (Vellema et al., in submission). Thus, this suggests temporal dissociation of estrogens and androgens is tolerable for inducing female song, although how far apart can the two hormones act is still open. This also raises the probability that the androgens might motivate singing in females as in males, while the estrogens might be responsible for the organizational requirements for singing. What motivates the birds and what are these organizational requirements remained to be elucidated and tested.

Interestingly, potential androgen responsive elements (AREs) are present in the proximate promoter sequences of canary of estrogen receptors (*ESR1*, *ESR2, GPER1)*. Thus, it is possible that androgen acts through AR to motivate singing by activating transcription of the ERs. Subsequently, estradiol induces ERs and factors, such as SP8, that stimulate cascades promoting the formation of necessary anatomical structures, such as a vascular system in the HVC, which allows sufficient energy supply (Louissaint et al., 2002). Indeed, the testosterone-implanted birds started to sing plastic/subsongs after approximately 4 days. Their songs from this time were shorter than 3 seconds, which increased to approximately 7 seconds by the 14th day (Table 6). During the first 2 weeks, genes related to angiogenesis and neuronal recruitment were expressed (Figure 17), and the vascular system in HVC expands (Louissaint et al., 2002). Thus, these females had been motivated to sing before anatomical infrastructure was enhanced. In the case of the 5α-

DHT re-implanted female canaries (Vellema et al., in submission), the birds were first implanted with testosterone and songs developed. Their songs reached a stable level after about 6 months and stopped after testosterone implants were removed. However, after the birds received a second implantation with 5α-DHT, they started singing in the first 2 days and sang at a comparable level as what they achieved before testosterone was removed. The recurring vocal motor patterns of these female canaries provide a possible example for temporal dissociation of estrogen and androgen. Single implantation of estradiol or 5α-DHT did not stimulate singing nor increase the HVC volume in female canaries, but it did increase the cell density of fusiform doublecortin positive cells, an indicator for migrating neurons, in the HVC (Yamamura et al., 2011) and increase dendritic length in RA (DeVoogd and Nottebohm, 1981). Thus, estradiol alone can alter anatomical properties; nevertheless, it is not sufficient for inducing female singing. Whether these subtle anatomical changes fulfill organizational requirements should be tested. Experiments with recurrent 5α-DHT implantations with a temporal delay did not induce female singing (Vellema personal communication), whereas to best my knowledge, experiments that implant female canaries first with estradiol then with 5α-DHT with a temporal delay have yet to be conducted. Future work aiming to study temporal segregation of estradiol and 5α-DHT would be informative for revealing the minimum gene-expression requirements for female singing in canaries.

VI. 4. Are transcriptional sex differences confined in certain cell types?

In the HVC of songbirds, transcriptional sex differences were observed in every case: in three different species of songbirds, in both breeding and non-breeding canaries, and in testosterone-treated canaries. Moreover, transcriptional sex differences were also observed in the entopallium, the avian visual cortex, and in the cerebellum. Both brain regions do not show obvious sexually differential functions. Thus, it is likely that transcriptional sex differences are observed in every brain regions and organs in all sexually reproducing animals (Brawand et al., 2011; Harrison et al., 2015; Itoh et al., 2007; Mayne et al., 2016; Rotllant et al., 2017; Uebbing et al., 2013; Yang et al., 2006). In theory, because male and female somatic

cells have different sex chromosome complement, transcriptional sex differences might manifest in each cell. Thus, the question of whether all cell lineages is sexually differential, or some but not others would provide information about fundamental sex differences in single-cell level resolution. In addition, because neurons are the building blocks of neural network, this information would provide an indication about whether neural networks are fundamental sexually differential. Moreover, because GO-term enrichment analysis indicated that female-specific genes in the HVC are associated with dendrite and dendrite spines, if neurons are the primary sexually differential cell types, does the neuronal morphology of female neurons (RA projection, Area X projection or interneuron) differ from male neurons? Anatomical analysis using anterograde or retrograde labeling dyes or fluorescent-expressing viral vectors would be useful to answer these questions.

VI. 5. Technical constrains

It is undoubtedly that microarray transcriptomes provide abundant and invaluable information about how testosterone regulates gene expression in female HVCs and what genes are sexually different in multiple species of songbirds. However, several technical constrains have limited the interpretation of our data. First, as mentioned above, the bulk transcriptomes obtained by dissecting the whole HVC could not differentiate transcriptional changes among cell types. By using single-cell mRNA sequencing technique, one can investigate which cell types are under the most testosterone influences. Second, a transcriptome is a collection of mRNAs that are being transcribed at the time of sampling; thus, rapid non-genomic changes caused by hormone, such as electrical properties of neurons, could not be studied by this method. Instead, classical electrophysiological methods would be appropriate. Third, multiple post-transcriptional and post-translational regulatory mechanisms result in putative time delay between detected mRNA transcripts and functional proteins. The transcriptomes might provide a general direction of what is being and will be regulated; however, they do not provide a precise temporal resolution. Fourth, transcriptomic studies are correlational in nature. Causality of gene function needs to be verified by carefully designed experiments *in*

vitro in cell culture and *in vivo* in transgenic or viral transfected birds. However, causality of gene network remained difficult to tackle and verify. Lastly, the interpretation of transcriptomic studies is largely relying on literatures, which are heavily biased to rodents, humans, or cancer cell lines abnormal physiology. Although many homolog genes appear conserved in gene function, evidence also shows species-specific regulation and change of function depending on contexts. Thus, experiments specially designed to verify the gene in question in a particular species would be required before drawing conclusions.

VII. Conclusion

In light of recent development of genome editing tools, it is not impossible to efficiently manipulate an individual gene and to study the impact in the brain and on freely-behaving animals. With such tools in hand, transcriptomic data are necessary and essential for providing comprehensive background knowledge and revealing potential gene of interest that govern brain and behavioral plasticity. The present thesis laid the groundwork and open many questions to be answered in the upcoming future. It also provides rich datasets to be further explored by applying future advancement in data-mining strategies.

Materials and Methods

Animals

Adult canaries (*Serinus canaria*, at least one-year-old) used in all experiments were either purchased from local breeders during breeding seasons (June to August) or bred at the animal facility of Max Planck Institute for Ornithology in Seewiesen, Germany. Sex was confirmed by PCR using P2 and P8 primers for CHD genes (Griffiths et al., 1998) and visual inspection of reproduction system after sacrifice. These birds were housed in single-sexed aviaries in local light regimen. For non-breeding canaries, the light schedule was gradually (over six to eight weeks) switched to non-breeding condition 9/15 (light/dark) for at least 8 weeks before the experiments. Food and water were provided *ad libitum* at all times.

In the first two experiments, non-breeding female canaries were used, and their singing activity was monitored (see Song monitoring, p. 139). Six spontaneously singing female and nine non-singing female canaries were in the first experiment. The singing females were sacrificed within our hour after singing had been observed. Body weight was measured and the brain and other organs (syringes, spleens and oviducts) were dissected, weighed, snap frozen on dry ice and stored at -80°C for further analysis.

In the second experiment, 39 non-singing female canaries were used and implanted with testosterone, and sacrificed after the intended implantation periods (see Testosterone implantation, p. 146, and Table 5). Body weight was measured and the brain and other organs (syringes, spleens and oviducts) were dissected, weighed, snap frozen on dry ice and stored at -80°C for further analysis.

In the third and fourth experiment, male and female canaries were housed in single-sexed aviaries before song monitoring (see Song monitoring, p. 139). Birds in breeding condition were sacrificed between March and July, whereas birds in non-breeding condition were sacrificed between October and January (six

birds per group, Table 7). Six non-breeding male canaries were implanted with testosterone for two weeks before sacrifice. The T14d females used in the second experiment were included in this experiment. The HVC samples of spontaneously singing non-breeding female canaries used in the first experiment were included in the third experiment (referred as non-breeding singing females).

In the fourth experiment, two additional songbird species were used. Blue-capped cordon bleus (*Uraeginthus cyanocephalus*) were bred at our animal facilities and housed in pairs in breeding condition 10/14 (light/dark), whereas forest weaver (*Ploceus bicolor*) pairs were caught and sacrificed in their breeding territories in Eastern South Africa in accordance with permits issued by the local authorities (Chief Professional Officer for Research at the Natal Parks, Game and Fish Preservation Board, P. O. B. 662, Pietermaritzburg 3200). The brain was dissected and stored at -80°C for further analysis.

Song monitoring

For monitoring singing activity, canaries were held alone in sound-attenuated boxes and recorded continuously up to four weeks. A microphone in each box was connected to a PR8E amplifier (SM Pro Audio), feeding into an Edirol USB audio capture device (Edirol UA 1000) connected to a computer. Songs were recorded at a sampling rate of 44.1 kHz and 16-bit resolution using the software Sound Analysis Pro 2011(Tchernichovski et al., 2000). Amplitude filtering (>24 dB) functionality was used.

In the first experiment, female canaries with potential to develop song were identified by visual inspection of sound spectrograms (sonograms). Birds producing calls composed of more than five syllables were selected for further monitoring. When the recording period exceeded four weeks, they were switched back to same-sex aviaries in non-breeding condition for at least four more weeks before another song monitoring period started. Song monitoring was repeated three to five times from February 2012 to August 2015 to monitor song activity and song development of six singing female canaries. All non-singing females were monitored at least for four weeks to make sure they did not sing.

In the second experiment, all females were monitored at least for four weeks to make sure they did not sing before testosterone implantation. After implantation, testosterone-treated female canaries were monitored for singing activity. Singing activity of T7d and T14d birds was observed.

The canaries used in the third and fourth experiments as well as the cordon bleus used in the fourth experiment were held alone in sound-attenuated boxes, and recorded continuously up to four weeks. Songs of forest weaver pairs used in the fourth experiment were recorded to ensure that both mates were singing.

Song analysis

Song analysis was done using Multi_Channel_Analyser (MCA), a custom program with graphical-user-interface written in MATLAB (version R2016b, Mathworks). MCA loads song files and generates sound spectrograms using fast Fourier transformation with sliding time windows of 294 samples and 128 samples of overlap between adjoining sections. These settings lead to a spectrogram with a resolution of 150 Hz on the frequency axes and a resolution of 3.76 milliseconds on the time axis. We analyzed the recordings from six birds, nine hours of recordings per individual at least every four days (Figure 6a, Table 1). Due to background noise, syllable detection was done in three steps. First, sound segments (songs and calls) were manually selected by visual inspection. Second, syllables were automatically detected within the selected song segments using MCA. Third, song segments were selected if the segment was longer than three seconds, contained no inter-syllable pause longer than 1.5 seconds and contained less than three consecutive pauses longer than 0.4 seconds. These relatively long gaps occur because spontaneous female songs are generally loose in temporal organization of the syllables. Song segments not passing these criteria were therefore referred to as unstable songs (Figure 6a).

Since canary syllables can be very short and usually occur as phrases with a high syllable repetition rate, we selected syllables as follows. First, the audio signal is filtered with a high-pass filter at 2.0 kHz. Next, we detected at which time the values of the audio samples crossed a certain amplitude threshold, which

was called Threshold_wav_amplitude in MCA. Treshold_wave_amplitude is relative to the maximum intensity of the recorded audio signal and is scaled between -1 and 1. For this study, the Treshold_wave_amplitude was set at 0.001. Subsequently, we determined the interval between all subsequent crossing time points and calculate the frequency for each time the threshold was crossed. Finally, we set a threshold for this time series of frequencies, called Threshold_syllable_freq in MCA, and determined at which time points this threshold was crossed in upward and downward direction, representing the syllable start and its end respectively. We set the threshold_syllable_freq at 50 Hz. A second threshold represents the interval between syllable start and end, was set at 15 ms, i.e., all syllables had durations longer than 15 ms.

Subsequently, digital samples of detected syllables were exported and further used to calculate sound features including song-level parameters like song length, number of syllables in a song, repetition rate and the downward slope (in this paper called slope coefficient, α), as well as syllable-level parameters like syllable length, syllable interval, peak frequency and wiener entropy. Song length was calculated by subtraction of the timestamp at the end of the last syllable and the timestamp at the start of the first syllable of a song. Repetition rate was defined as the number of syllables in the song divided by the song length in seconds. Syllable length was calculated by subtraction of the timestamp at the end of the syllable and at the start of that syllable. Syllable interval was calculated by subtraction of the timestamp at the onset of the subsequent syllable and at the end of the syllable. All syllables were high-pass filtered at 500 Hz and normalized by dividing by their root mean square (RMS) value. The total amount of samples per syllable was normalized by adding 4096 zeros in front and adding zeros behind such that the total amount of samples was 44100. From these normalized syllables we calculated a spectrum, peak frequency and Wiener entropy. Peak frequency was the frequency at which the power was at maximum in the spectrogram of a syllable. Wiener entropy is a unit less measure of signal noise that provides a measure of the width and uniformity of the power spectrum. A noise of Wiener entropy 0 would be a pure tone noise (Tchernichovski et al., 2000).

To get a general impression of the song structure, we analyzed the low frequency components of the amplitude modulation of the song. Therefore, we calculated the power of each sample by means of a Hilbert transformation, applied a low pass filter at 441 Hz and down-sampled them to a rate of 441 samples per second. We then calculated the spectrum and analyzed the frequency range between 0.5 and 2 Hz. This range represents the amplitude modulation below the slowest syllable repetition rate (0.5 s) and above the shortest song length (2 s). The syllable repetition rate is represented in the spectrum as a peak in the frequency range between 2 and 30 Hz. The spectrum below 2 Hz represents the song organization at the level of phrases. In this range, the spectrum is not organized in peaks but as a downward slope. After taking the log transformation of the frequency and the amplitude axes of the power spectrum, the downward slope can be calculated by taking the linear regression. The slope coefficient α represents the level of predictability or surprise in sound structures by reflecting the change in amplitude or loudness of the subsequent phrases (Gardner, 1978; Gisiger, 2001; Voss and Clarke, 1975, 1978). For example, white noise has a power spectrum of f^{α}, where $\alpha = 0$. White noise is a random superposition of waves over a wide range of frequencies (Gisiger, 2001). Flicker noise (1/f-noise, or pink noise) is defined by $\alpha \in [-1.5, -0.5]$, sometimes loosely as α close to -1. Sounds with f^{α} ($\alpha = -1$) spectral density are considered pleasant and enable for stochastic composition. For example, the frequency fluctuations of music have a f^{α} ($\alpha = -1$) spectral density at frequencies down to the inverse of the length of the piece of music (Voss and Clarke, 1978). Brownian noise has a power spectrum of f^{α}, $\alpha = -2$. The Brownian noise signal at a particular time t can be reproduced by adding a random offset to each sample to obtain the next one, thus Brownian noise is strongly correlated to time (Gilden et al., 1995; Gisiger, 2001; Voss and Clarke, 1975). Blue noise is defined by $\alpha \in [0.5, 1.5]$. A blue noise signal has a power spectrum with amplitude proportional to its frequency. Therefore, bird song defined as blue noise means this song has highly repetitive units with short repetitive intervals. A song with white noise characteristics means syllables in this song are likely organized randomly, whereas a song with pink noise characteristics indicates a temporal organization of syllables in a

nonrandom way making it appear pleasant. Lastly, a song with brown noise characteristics has repetitive units with long repetitive intervals (in contrast to blue noise song).

Syllable sorting

In order to study the syntax in the first experiment (p. 50), we developed "SylSorter", a tool which semi-automatically categorizes syllables into clusters based on Structural Similarity Index, SSIM (Wang and Bovik, 2002, 2009) (see below). We compared up to 3000 syllables for each recording period per bird and classified these syllables into clusters by a custom algorithm written in MATLAB (available at https://github.com/maggieMCKO/SylSorter).

All syllables were high-pass filtered at 500 Hz and normalized by dividing them by their root mean square (RMS) value. At this point the syllables were resampled to a total amount of 44100 samples. Resampling was done by taking the Fourier transformation, zero-filling the frequency space to 44100 samples and then taking the inverse Fourier transform. From the resampled signal we calculated a spectrogram using a sliding window of 4410 samples and 2205 samples of overlap between adjoining sections. The number of DFT points was calculated as 11025000 divided by the number of samples in the syllable. This led to a correct scaling of the frequency. The resulting two-dimensional pictures had the same number of pixels for all syllables. The clustering algorithm included three parts: grouping similar syllables into a cluster, reassigning syllables within a cluster and merging similar clusters. In short, the sound spectrogram of all syllables was compared by Structural Similarity Index, SSIM (Wang and Bovik, 2002, 2009). SSIM index measures changes in structural information of two images, therefore it has been widely used for evaluating image processing, and has been applied for evaluating audio signal quality (Kandadai et al., 2008). SSIM score is computed locally within a sliding window that moves pixel-by-pixel across the image resulting in a SSIM map. The SSIM score of the entire image is then computed by averaging the SSIM values across the image. Three image properties are accessed by the SSIM index: the local patch luminance, the local patch contrast and the local patch structure (Wang and Bovik, 2009). These three elements reflect

sound amplitude, signal-to-noise ratio, and frequency modulation of song syllables. Calculations were done in MATLAB using the "ssim" function with an output between -1 and 1 ($-1 < SSIM \leq 1$). SSIM equals to 1 when the two images are identical. Clusters were initially formed by collapsing syllables with the maximum SSIM value into one cluster. This resulted in clusters with different sizes (the number of syllables in one cluster). Subsequently, the lowest 2% of syllables in a cluster were reassigned to other clusters if a higher SSIM value was found. Subsequently, clusters were merged if all SSIM values of the containing syllables (compared to the representative syllable of the other clusters) were higher than the threshold. Reassigning and merging was repeated until the number of clusters stopped changing. Lastly, a pair of clusters (cluster a and b) were further merged if the SSIM value between the average syllables of cluster a and of cluster b was higher than the threshold, and if the average syllable length of cluster a is within the range of one standard deviation from the mean syllable length of cluster b ($\mu_b - \sigma_b < \mu_a < \mu_b + \sigma_b$).

Radioimmunoassay of plasma testosterone

Blood (< 150 µl) was taken from the wing vein and collected into heparinized micropipettes for plasma testosterone analyses. Blood samples were centrifuged (2,500 rpm, 10 min) to separate the plasma from blood cells, and stored at -80°C until further analysis. Testosterone metabolites were measured with a radioimmunoassay using a commercial antiserum against testosterone (T3-125, Endocrine Sciences, Tarzana, USA) as previously described (Goymann et al., 2002). Standard curves and sample concentrations were calculated with Immunofit 3.0 (Beckman Inc. Fullerton, CA) using a four-parameter logistic curve fit and corrected for individual recoveries. Because the testosterone antibody used showed significant cross-reactions with 5α-dihydrotestosterone (44%), our measurement may include a fraction of 5α-DHT.

In the first experiment, the blood of all birds including singing and non-singing birds were sampled within four days after they were housed in sound-attenuated recording boxes. For the female singers, the blood was sampled no longer than one hour after their last songs before sacrifice. All blood samples were taken between 8 and 11 am and were taken within 3 min to avoid the effect of handling (Wingfield et al.,

1982). Testosterone concentrations were assayed in duplicates, in three separate assays. The mean extraction efficiency for plasma testosterone was 85.2 ± 3.42% (mean ± SD, N = 30). All samples were above the lower detection limit of the testosterone (0.35, 0.36 and 0.38 pg per tube). The intra-assay coefficients of variation of a chicken plasma pool were 3.4%, 12.8%, and 1.9%. The inter-assay coefficient of variation as determined by the variation of the chicken plasma pool between all assays was 10.6%.

In the second experiment, all birds were blood-sampled four days after they were housed in sound-attenuated recording boxes, and at least 4 days before testosterone implantation (see below) to ensure on the day of implantation the birds were not stressed from previous handling. Blood was sampled again when the birds were sacrificed. All blood samples were taken between 8 and 11 am and were taken within 3 min to avoid the effect of handling (Wingfield et al., 1982). Testosterone concentrations were assayed in duplicates, in 4 separate assays. The mean extraction efficiency for plasma testosterone was 84.0 ± 6.8% (mean ± SD, N = 120). The lower detection limits of the testosterone assays were 0.35, 0.34, 0.34 and 0.38 pg per tube, all samples were above the detection limit. The intra-assay coefficients of variation of a chicken plasma pool were 4.4%, 8.7%, 4.1%, and 1.9%. The inter-assay coefficient of variation as determined by the variation of the chicken plasma pool between all assays was 5.9%.

In the third experiment, blood samples of breeding female and male canaries, non-breeding female and male canaries and testosterone-implanted non-breeding males were taken before sacrifice. All samples were above the lower detection limit 0.38 pg per tube of the testosterone. The intra-assay coefficient of variation was 6.5 ± 0.14 %. The inter-assay coefficient of variation as determined by the variation of the chicken plasma pool between all assays was 0.6%. The plasma testosterone concentrations of non-breeding singing females and of testosterone-treated non-breeding females were taken from the first experiment and the second experiment (T14d), respectively.

Testosterone implantation

Silastic™ tube (Dow Corning; 1.47 mm inner diameter, 1.96 mm outer diameter, 0.23 mm thickness) was cut 7 mm in length, and was loaded with testosterone (86500, Fluka) as dense as possible. The two ends of the Silastic™ tube were sealed by silicone elastomer (3140, Dow Corning). After closure, the implants were cleaned with 100% ethanol to remove testosterone particles and then were immersed in ethanol overnight in the hood to ensure no leakage at both ends. Implants with apparent damp were discarded. One day before the implantation the implants were incubated in 0.1M phosphate buffered saline (PBS) overnight enabling the immediate release of testosterone upon implantation (Rasika et al., 1994b). We started implantation right after the light was turned on in the morning, approximately at 8:30 am with 20 minutes interval between each bird, considering the scarification time. A small incision was made on the back of the bird over the pectoral musculature, and one testosterone implant was placed subcutaneously. The skin was closed by application of tissue glue. In the second experiment (p. 65), we implanted six groups of female canaries for the intended period of implantation (1h, 3h, 8h, 3d, 7d, 14d, see Table 5). For the controls, we implanted the animals with empty 7-mm silastic tube also sealed by silicone elastomer. In the third experiment (p. 81), the female and male non-breeding canaries were implanted with testosterone implants for two weeks (Table 7). After the intended period of implantation, the birds were sacrificed by an overdose of isoflurane, the body weights were recorded, the brains and oviducts were dissected, weighed, snap frozen on dry ice and stored at -80□ until further use. Testosterone implants were checked, and they were all in place, and were not empty at the end of the experiments.

HVC volume measurement

Brains were sagittally sectioned in either 40 µm × 4 + 20 µm × 2 or 50 µm × 4 + 14 µm × 3 with a cryostat (Jung CM3000 Leica). The thick sections (40 or 50 µm) were mounted on glass slides for subsequent tissue micro-dissection for microarray analysis, whereas the thin sections (20 or 14 µm) were mounted on RNase-free Superfrost slides for Nissl staining or RNAscope® *in situ* hybridization assay (Wang et al., 2012).

All sections were stored at -80°C until further processing. One set of thin serial sections (20 or 14 µm) was sequentially hydrated (100%, 90%, 70%, 20% ethanol and distilled water, each solution for 50-60 seconds), stained with 0.1% thionin solution for 5-8 seconds, and serially dehydrated (distilled water, 20%, 70%, 90% and 100% ethanol, each for 30 seconds). Finally, the slides were immersed in xylene and cover-slipped with Roti-Histokitt II mounting medium (Roth). The HVC areas were measured based on microphotographs containing HVC taken with a Leica DM6000 B microscope using ImageJ2 (Rueden et al., 2017) (Fiji distribution (Schindelin et al., 2012)). All brains were coded so that the observers were blind towards any additional information about the sections they measured during the delineations. Volumes were derived from the summed area measurements multiplied by the section thickness and the inter-section distance.

RNAScope® *in situ* hybridization assay

In situ hybridization for mRNA expression of *ALDH1A1*, *AR*, *HSD17B12*, *SRD5A2*, and *UTS2B* was performed on paraffin-embedded HVC sections (20 or 14 µm in thickness) using the RNAScope®2.0 HD Detection Kit (Advanced Cell Diagnostics), following protocols provided by the manufacturer. Hematoxylin counterstaining was performed. The stained tissue sections were imaged using a Leica DM6000 B microscope (Leica). Quantification was done with ImageJ2 (Rueden et al., 2017) (Fiji distribution (Schindelin et al., 2012)). Three birds were used for a given probe at a given time period (3 slices for each bird). We measured the density of accumulated chromogenic particles in the stained HVC sections for all probes. Then, briefly delineated the HVC and measured the area. Subsequently, we inverse selected and filled the area outside of the HVC with white background color using custom macro file, "Macro_quantify_density_singleProbes_cut.ijm". We used "Color Threshold" function (Color space: RGB; red channel: 0 to 47; green channel: 0 to 165; blue channel: 0 to 160) to select accumulated chromogenic particles, and used "Analyze Particles" function (minimum size = 8 μm^2) to count the number of accumulated chromogenic particles (macro file: "Macro_quantify_density_singleProbes_quantify.ijm"). The

density of chromogenic particles was calculated for each section and the mean density was calculated for each bird. For *AR* and *UTS2B*, we additionally measure the area by each chromogenic particle, and subsequently calculated the sum and the percentage of occupied area versus the HVC area in the same sections for at given time point. The custom macro files are available at https://github.com/maggieMCKO/RNAscopeQuantification.

Microarray procedures and annotation

For total RNA extraction, the song control nucleus HVC (experiment II, III, and IV) and the visual area entopallium (ENT, experiment IV) was dissected from the abovementioned thick sections (40 or 50 µm) under a stereomicroscope and transferred into an Eppendorf containing 340 µl of RLT buffer mixture (containing DTT, Qiagen). Our dissection procedure using rather thin sections reduces the contamination of HVC tissue with surrounding tissues. In this regard, we used separate sets of dissection tools dissecting different tissues. Then, RNA was extracted using the RNeasy® Micro Kit (Qiagen). RNA quality was assessed using the Agilent Model 2100 Bioanalyzer (Agilent Technologies). RNA concentrations were assessed using a Nanodrop 1000 spectrometer (Thermo Fisher Scientific). The RNA of all samples was of good quality with high and comparable RNA integrity numbers (RIN > 7). The purified total RNA samples (at least 100 ng per sample) were subsequently processed and hybridized using the Ambion WT Expression Kit and the Affymetrix WT Terminal Labeling and Controls Kit. The resulting cDNA was hybridized to the Custom Affymetrix Gene Chip® MPIO-ZF1s520811 Exon Array, which has been used and validated in successful cross-species (zebra finches and canaries) hybridization studies (Dittrich et al., 2014; Frankl-Vilches et al., 2015). In the second experiment, we also verified the microarray results by RNAscope® *in situ* hybridization assay. The 5.7 million male zebra finch-specific probes correspond to approximately 4,711,133 probe sets and to 25,816 transcripts published in public databases (NCBI and Ensembl). Our custom array lacks probes for the W chromosome genes due to the absence of W chromosome annotation in the zebra finch genome assembly (Warren et al., 2010). We annotated more

than 90% of the transcripts to 12,729 human orthologous genes using several public databases (Ensembl, GenBank, UniProt, and DAVID (Benson et al., 2005; Consortium, 2015; Flicek et al., 2014; Huang et al., 2008, 2009; Yates et al., 2016)) and commercial databases (El Dorado, Genomatix GmbH (Genomatix), RRID:SCR_008036). Hybridization was performed for 16 hours at 45°C and 60 rpm in the GeneChip Hybridization Oven 640. The arrays were washed, stained and scanned using the Affymetrix GeneChip Fluidics Station 450 and Affymetrix GeneChip scanner 3000 7G. The CEL files were generated by the Affymetrix® GeneChip® Command Console® Software (AGCC), and quality control for evaluating the success of individual hybridizations was assessed by the Affymetrix® Expression Console™ software. The average percentage of present probes was > 90% for all samples. The microarray data discussed in the second experiment have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002), and are accessible through the GEO Series accession number GSE118522 ("GEO: GSE118522"). Likewise, the microarray data discussed in the third and fourth experiments are accessible through the GEO Series accession number GSE83674 ("GEO: GSE83674").

Normalization of microarray expression data

We calculated the normalized expression of the CEL files of using the "justRMA" function in the affy R package (Gautier et al., 2004). We collapsed values on probe-set-level to transcript-level, and then to gene-level by using the "collapseRows" function of the WGCNA R package (v1.51) (Langfelder and Horvath, 2008, 2012; Miller et al., 2011). In the second experiment, the normalized expression of 39 female canaries was used in a weighted gene co-expression network analysis (WGCNA, see p. 153), while the normalized expression of 42 canaries was used in hierarchical clustering (p. 151) and principal component analyses (p. 152) in the third experiment.

Differential gene expression analysis

In the second experiment, differential gene expression was calculated by comparing testosterone-implanted female canaries with control female canaries (implanted with an empty Silastic™ tube). In the third experiment, differential expression was calculated by comparing singing birds with non-singing birds. Specifically, non-breeding female canaries implanted with testosterone and non-breeding singing female canaries were compared with non-breeding female canaries separately. Likewise, breeding male canaries and non-breeding male canaries implanted with testosterone were compared with non-breeding male canaries. In the forth experiment, the hybridization efficiency was significantly different between canaries and forest weavers (FW) and between canaries and cordon bleus (CB) (Kruskal-Wallis test followed by Dunn's Kruskal-Wallis test for multiple comparisons, chi-squared = 29.73, degrees of freedom = 3, adjusted P = 0.04 (Can-FW); 0.002 (Can-CB)); 3×10^{-5} (Can-ZF (zebra finches)); 0.02 (CB-ZF); 0.003 (FW-ZF). Considering possible cross-species bias in hybridization, differential expression was calculated using two groups within the same species 1) male to female (Figure 24, Figure 25, and Figure 26) or 2) HVC to ENT (Figure 27).

Differential expression was calculated by the ChipInspector software (v21, El Dorado Database vE28R1306, (Genomatix), RRID:SCR_008036). ChipInspector is a single probe-based analysis tool for microarray data that can increase sensitivity compared to conventional probe-set based analyses, such as robust multi-array analysis. ChipInspector consists of four steps: single probe-transcript annotation (ensuring up-to-date annotation), total intensity normalization, SAM analysis (significance analysis of microarrays, adapted to single probe handling), and transcript identification based on significantly changed probes (Cohen et al., 2008). SAM was described in detail in (Tusher et al., 2001). Briefly, SAM computes a statistic d(i) for each probe, which is a score based on the change in gene expression between two groups of samples relative to the standard deviation of repeated measurement for that probe (Tusher et al., 2001). To identify potential significantly changed probes, SAM creates artificial background data by randomly

permuting the array results. For each permutation, SAM computes $d_p(i)$ and calculates the expected $d_E(i)$ of each probe, which is defined as the average $d_p(i)$ over all permutations. A scatter plot of d(i) vs $d_E(i)$ is created, and probes with d(i) higher than a certain threshold (Delta) are deemed significant. The Delta value is a distance from the d(i) = $d_E(i)$ line on the scatter plot. Then, SAM estimates the percentage of probes identified by chance (the percentage of the average number of falsely significant genes over all permutations), i.e., the false discovery rate (Tusher et al., 2001). A false discovery rate can be estimated for each Delta threshold; a smaller Delta can increase the number of significant genes but at the cost of increasing the estimated false discovery rate. We therefore chose a Delta threshold that gives a 0% false discovery rate for all our analyses. We used the group-wise exhaustive comparison tool in ChipInspector to analyse differential expression; the minimum coverage for each transcript was set to 10 significant probes. The significantly differentially expressed transcripts obtained were annotated to human orthologous genes. For transcripts belonging to the same genes, the average expression was calculated if all transcripts were regulated in the same way (e.g., all up-regulated or all down-regulated). If the direction of regulation of all significant probes of a given transcript were not consistent, the direction and the average were determined by the majority probes (> 60% of all significant probes). Transcripts without human orthologous gene annotation were removed before subsequent analyses.

Hierarchical clustering analysis

The correlation between gene expression profiles of experimental groups was calculated using Spearman's ρ based on 1) the normalized HVC gene expression (p. 149) in the third experiment, or 2) the differential gene expression profiles (HVC with respect to ENT) in the fourth experiment. From these values (Spearman's ρ), we calculated a Euclidean distance matrix, which was used for hierarchical clustering analysis (average method). This computation was performed in R (RCoreTeam, 2015), using the package "ggplot2" for visualization (Wickham, 2009).

Principal component analysis (PCA)

In the first experiment, PCA was applied to either the median and interquartile range (IQR) of song-level parameters (song length, number of syllables in a song, repetition rate and slope coefficient α)(Figure 8), or to song-level parameters (song length, number of syllables in a song, repetition rate and slope coefficient α) (Figure 9). The computation was carried out using the "pca" function of the R package "pcaMethods" (v1.60.0, method = svd (Stacklies et al., 2007)). Data were centered and scaled by unit variance. Subsequently, the calculated score values (projections on to each principal component (PC)) were used to calculate center of mass of each PC for each bird. Euclidean distances were calculated from the score values of recording periods to the center of mass of a given PC, and then an average Euclidean distance over the first six PCs were calculated. The average Euclidean distances provide a proximate estimation for song change over time and allow between-individual comparison.

PCA has been successfully applied to genome-wide expression studies and can provide potential insights into variable selection (Ringner, 2008). In the third experiment, PCA was applied to the normalized HVC gene expression data of 42 canaries, including breeding females and males, non-breeding females and males, testosterone-treated non-breeding females and males and spontaneously singing non-breeding females (Table 7), to identify variables contributed most variation in the dataset. In the fourth experiment, PCA was applied to the normalized HVC gene expression data of 18 males and 16 females of cordon bleus, forest weavers and canaries. (However, the values of one female forest weaver and one female cordon bleu differed so strongly from the others that they formed their own components and are not shown here). The gene expression levels were used as variables and the birds' identities as observations. The computation was carried out using the "pca" function of the R package "pcaMethods" (v1.60.0 (Stacklies et al., 2007), method = svd). The data were centered but not scaled because the expression data had already been normalized. The calculated scores and loadings were used to plot Figure 21 and Figure 27.

Weighted gene co-expression network analysis (WGCNA)

WGCNA R package (v1.51) (Langfelder and Horvath, 2008, 2012) was used to identify functional units of gene co-expression network that are relevant for the testosterone-induced morphological and behavioral changes (traits). A total of 32 traits were included in the second experiment (p. 75), for example, plasma testosterone concentrations, HVC volumes and song parameters (Figure 20A and Table 6). In co-expression networks, genes are seen as "nodes" and "edges" (or connection strengths) between nodes are determined by the pairwise correlations between gene expression profiles. WGCNA is an unsupervised analysis method that clusters genes based on their expression profiles. This method uses soft thresholding based on the Pearson correlation matrix to determine the connection strengths between two genes. Network construction method preserves gene co-expression information and highly robust, weighted co-expression networks (Fuller and Horvath, 2012).

Before network construction, we calculated the normalized expression of the CEL files using the "justRMA" function in the affy R package (Gautier et al., 2004). We followed authors of the WGCNA R package's suggestion of using the "collapseRows" function in the WGCNA R package to aggregate gene expression data to gene level (Miller et al., 2011). We constructed a signed hybrid network composed from all HVC samples (n = 39, Table 5) using the "adjacency" function (type: "signed hybrid"). Judging from the fitted curves calculated by the "pickSoftThreshold" function, the soft thresholding power for construction of adjacency matrix was set at 6. The adjacency value has a range from zero to one. The calculated adjacency matrix was fed into the "TOMsimilarity" function to calculate topological overlap matrix. Then, we used the "cutreeDynamic" function to cluster genes into modules (sub-networks within the overall network) based on topological overlap matrix (the "TOMsimilarity" function). We used a minimum module size of 100 genes and deepSplit was set equal to four. We merged similar modules using the "mergeCloseModules" function (cutHeight set equal to 20%). Module Eigengenes is the first principal component of a module's gene expression profile, a method of summarizing an entire module in one vector (Burkett et al., 2018;

Langfelder and Horvath, 2008, 2012). We calculated Module Eigengenes using the "moduleEigengenes" function and calculated correlation matrix between the Module Eigengenes and the traits using the "cor" function (Pearson) in R (RCoreTeam, 2015). Gene significance is the Pearson correlation between a gene's expression profile and the traits (Burkett et al., 2018; Langfelder and Horvath, 2008, 2012). The overall network connectivity as well as intramodular and intermodular connectivity were calculated using the "intramodularConnectivity" function in the WGCNA package. Extracting direct neighbors of *SP8* was done by exporting adjacency matrix using the "exportNetworkToCytoscape" function with a cutoff of 0.5. The interactions between *SP8* and its direct neighbors were evaluated using Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING, v10.5 (Szklarczyk et al., 2015)). The interactions with moderate confidence were retained (combined score greater than 0.4) and visualized using Cytoscape (environment v3.5.1 (Bindea et al., 2009; Shannon et al., 2003)).

Gene Ontology (GO)-term enrichment analysis

We used ClueGO (v2.5.0, RRID:SCR_005748), an application built under the Cytoscape (environment v3.5.1, RRID:SCR_003032, (Bindea et al., 2009; Shannon et al., 2003)), to predict the putative biological functions of the genes of interest. This software performs GO-term enrichment hierarchical analyses and fuses GO terms with similar functions. The enrichment was determined by the right-sided test and corrected by the Bonferroni step-down method considering multiple comparisons. The results were plotted using ggplot2 R package (Wickham, 2009).

Overrepresented transcription factor binding sites or pairs analysis

In the second experiment (p. 75), we extracted promoter sequences from the direct neighbors of *SP8* in the WGCNA adjacency matrix found in previously sequenced canary genome (Frankl-Vilches et al., 2015). The sequences are available for public use at http://public-genomes-ngs.molgen.mpg.de/cgi-bin/hgGateway?db=serCan1 (GEO: GSE50070). We defined promoter sequence as 1,000 bp genomic

sequence upstream from putative transcription start site of a given gene. We used the overrepresented transcription factor binding sites toolbox of the Genematix software suite (Genomatix-overrepresented TFBS tool) (Cartharius et al., 2005; Genomatix; Quandt et al., 1995; Sui et al., 2005) to search transcription factor binding sites (TFBS) and determine whether single TFBSs (or TFBS pairs) are overrepresented within the input sequences against annotated zebra finch promoters because the option for canary was unavailable in the Genomatix software suite (Genomatix). Considering the high probability that the promoter sequences of canaries and zebra finches are not conserved (Frankl-Vilches et al., 2015), we repeated the calculation using the canary promoter sequences of a randomly sampled genes set in the turquoise module of the WGCNA network as the user-defined background. The size of the random samples matched the number of direct neighbors of *SP8* (n = 1,151). The two repeats gave the same result: the binding sites of SP8 (V$SP1F) were overrepresented in the promoter sequences of the 1,151 direct neighbors of *SP8*.

To evaluate the probability that SP8 forms transcription factor complex with GATA (the TF binding sites within 10 to 50 bp distance (middle to middle) from each other), we used the module search function of the Genomatix-overrepresented TFBS tool. Because the options of using user-defined background (the matching size random sample described above) and using canary promoters were not possible, this analysis was done using zebra finch annotated promoters as background.

Enrichment analysis for cell types, neuronal processes and neurotransmitters

In the second experiment (p. 72), we searched 33 terms associated with cell types, neuronal protrusion types and biosynthesis of neurotransmitters and neurotransmitter receptors. Downloaded gene lists associated with the 33 terms (Supplementary Table 2) by using the “Genes and gene products” tool of the AmiGO 2 (v2.4.26, release date 2017-07-05, (Ashburner et al., 2000; Consortium, 2017)). Subsequently, we used the Fisher’s exact test to determine whether a certain time point is enriched for the search-term in question (using the “fisher.test” in R (RCoreTeam, 2015), the alternative hypothesis set to “greater”)

against canary genome background. The p values were adjusted by the Bonferroni's method using the "p.adjust" function in R (RCoreTeam, 2015). More specifically, we calculated an expectation value (E) for a given time point by multiplying the number of differential genes and the number of genes associated with a given search-term and then dividing by the total number of canary genes (n = 17797 genes, https://www.ncbi.nlm.nih.gov/genome/?term=canary), i.e.,

$$E = \frac{\#_{time\ point} \times \#_{searched\ term}}{\#_{canary\ genes}}$$

The enrichment score was defined as percentage change between the number of observed genes and the expectation value (E) for a given time point and a given search-term, i.e.,

$$Enrichment\ score\ (\%) = \frac{\#_{observed} - E}{E}$$

The results were plotted using ggplot2 R package (Wickham, 2009).

Enrichment analysis chromosomal location

We annotated information of carrier chromosomes to gene lists of interest based on a zebra finch genome (El Dorado, Genomatix GmbH (Genomatix)), because such information is not available for the canary. We acknowledged potential bias would have been caused by this approach. Subsequently, we used the Fisher's exact test to determine whether a given gene list is enriched for a chromosome in question (using the "fisher.test" in R (RCoreTeam, 2015), the alternative hypothesis was set equal to "greater") against canary genome background (15,609 protein-coding genes, https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Serinus_canaria/101/). The p values were adjusted by the Bonferroni's method using the "p.adjust" function in R (RCoreTeam, 2015).

Experimental design and statistical analysis

In the first experiment, 15 adult female canaries were used (six were spontaneously singing and nine were non-singing) for song monitoring, HVC volume measurement, and weight measurements (body, brain, syringes, and oviduct). Shapiro-Wilk normality test was used to test data distribution. To evaluate effects from different song recording periods on song parameters (song length and repetition rate), we either used Mann-Whitney Test for birds with two recording periods, or Kruskal-Wallis rank sum test (by using the "kruskal.test" function in R (RCoreTeam, 2015)) followed by Bonferroni-Dunn post hoc test (by using the "posthoc.kruskal.dunn.test" function in the PMCMR R package, (Pohlert, 2014)) for birds with three recording periods. Plasma testosterone level differences between singers and non-singers were tested by two-sided Mann-Whitney Test ("wilcox.test" function in R) (RCoreTeam, 2015). Pearson's correlation coefficients were calculated by the "cor" function in R. P values were calculated by the "cor.test" function, and further corrected for multiple comparisons using the "p.adjust" function (FDR) in R.

In the second experiment, 39 adult female canaries were used (Table 5). All animals (n = 5 or 6 birds per group) were used for microarray analysis, HVC volume measurement and weight measurements (body, brain and oviduct). For HVC volume measurement, one-set of thin brain slices were used, typically 8-10 slices. Statistical significance of HVC and weight measurements was determined by Kruskal-Wallis rank sum test (the "kruskal.test" function in R (RCoreTeam, 2015)) followed by Bonferroni-Dunn post hoc test (the "posthoc.kruskal.dunn.test" function in the PMCMR R package, (Pohlert, 2014)). For RNAScope® *in situ* hybridization, three birds were used for a given probe at a given time point (3 slices for each bird). Statistical significance was determined by Mann-Whitney U Test (the "wilcox.text" function in R, the alternative hypothesis was set to "two-sided"). Birds of T7d and T14d were used for song analyses. Mann-Whitney U Test (with same parameters listed above) was used to determine statistical significance. All thick brain slices, typically 24-32 slices, (see above Microarray procedures and annotation) containing the HVC were used for microarray analysis. Differential gene expression were analyzed by comparing treated

groups against the control group using the ChipInspector software (v21, El Dorado Database vE28R1306 (Genomatix), see Differential gene expression analysis, p. 150). Fisher's exact test was used to test whether a given gene list is enriched for a search-term in question (see Enrichment analysis for cell types, neuronal processes and neurotransmitters, p. 155).

In the third experiment, 42 adult canaries were used (Table 7). All animals (n = 6 birds per group) were used for microarray analysis and HVC volume measurement. For HVC volume measurement, one-set of thin brain slices were used, typically 8-10 slices. For microarray analysis, all thick brain slices (see above Microarray procedures and annotation) containing the HVC used, typically 24-32 slices for HVC. Differential gene expression were analyzed by comparing singing canary groups against the non-singing canary groups using the ChipInspector software (v21, El Dorado Database vE28R1306 (Genomatix), see Differential gene expression analysis, p. 150). Fisher's exact test was used to determine whether a given gene list is enriched for a chromosome in question (see Enrichment analysis chromosomal location, p. 156).

In the fourth experiment, a total of 60 birds were used (Table 8). All animals (n = 6 birds per group) were used for microarray analysis, HVC volume measurement, and song syllable counting. For microarray analysis, all thick brain slices containing the HVC and ENT were used, typically 24-32 slices for HVC and 16-20 slices for ENT. For HVC volume measurement, one-set of thin brain slices were used, typically 8-10 slices. Differential gene expression was analyzed by comparing males with females of a species (sex-biased genes), or by comparing HVC with ENT of a sex of a species (HVC-specific genes, see Differential gene expression analysis, p. 150). Fisher's exact test was used to test whether sex-biased genes were enriched on a chromosome (see Enrichment analysis chromosomal location, p. 156). Differences between autosomal and Z-chromosomal female-to-male ratios (F:M) were assessed with a non-parametric Mann-Whitney test, using "wilcox.test" (two-sided) in R (RCoreTeam, 2015).

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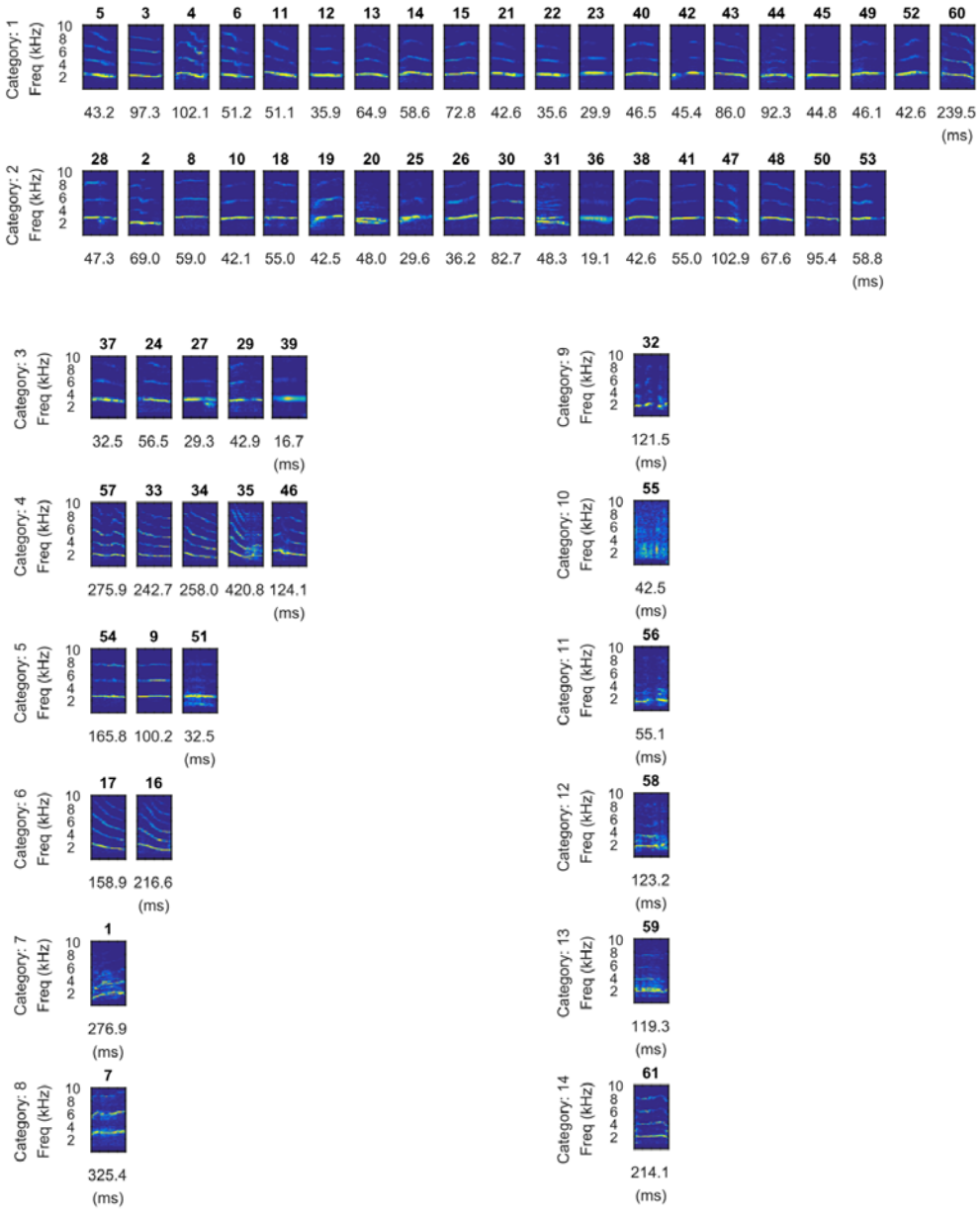
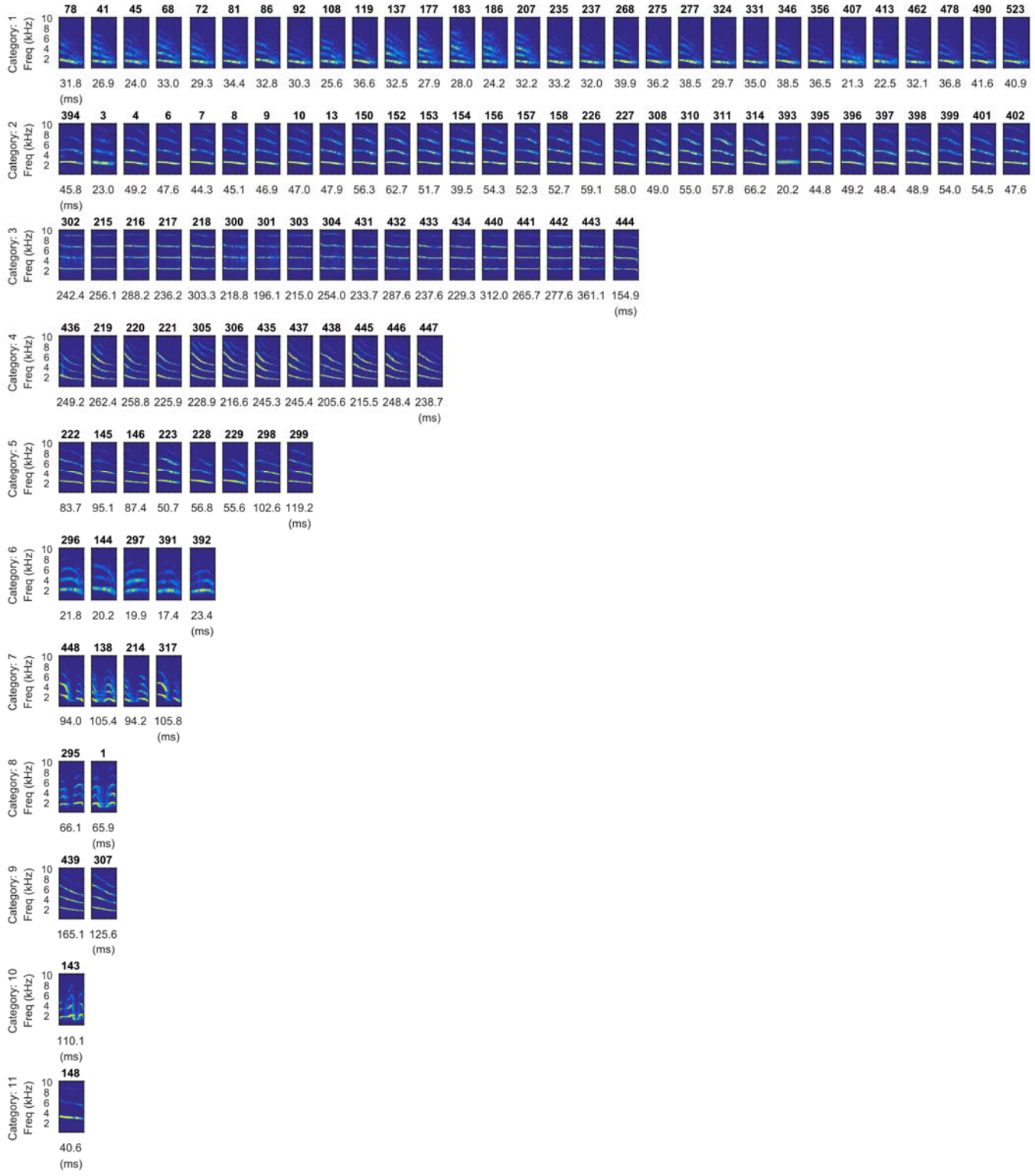
Appendix

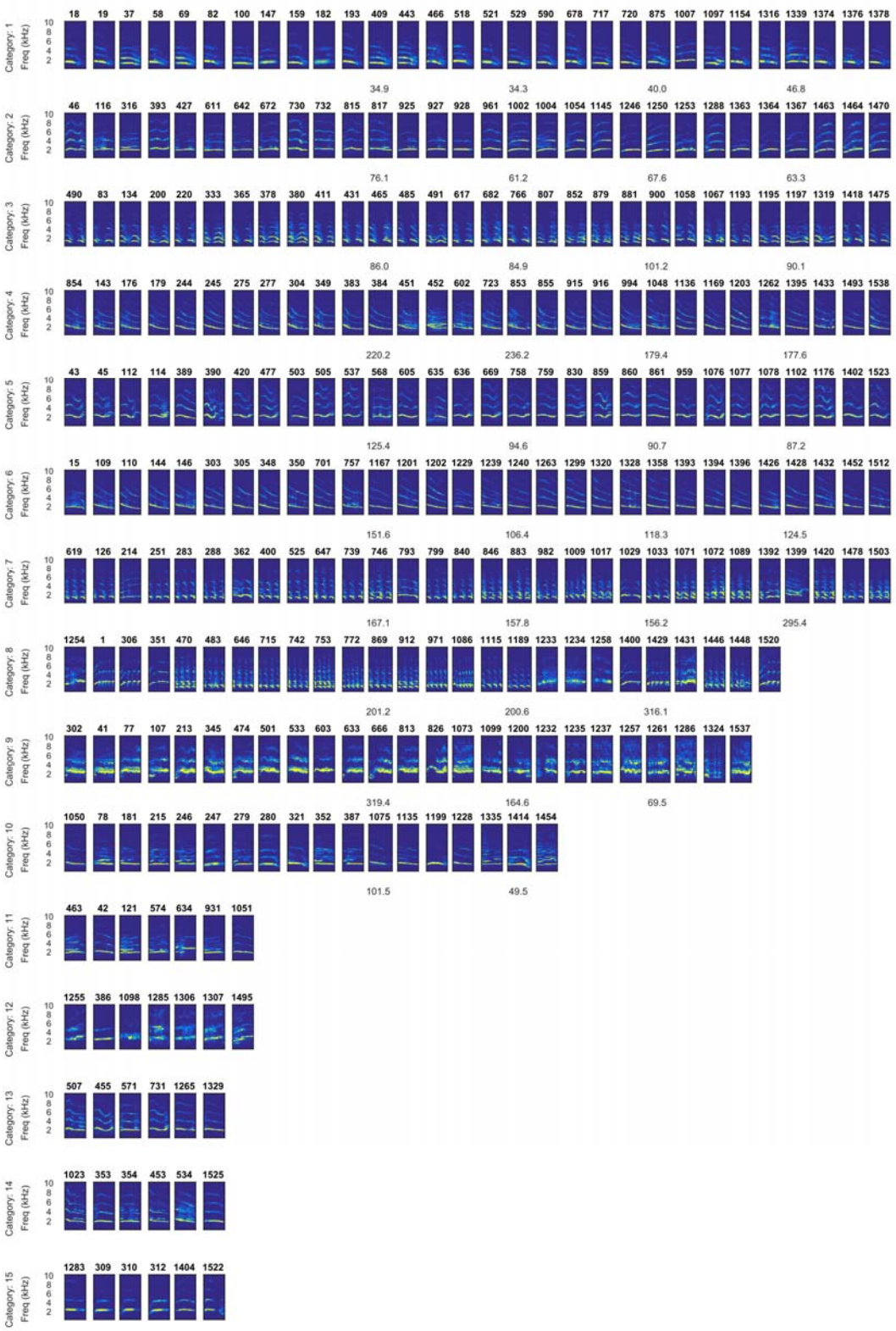
I. Supplementary figures and legends



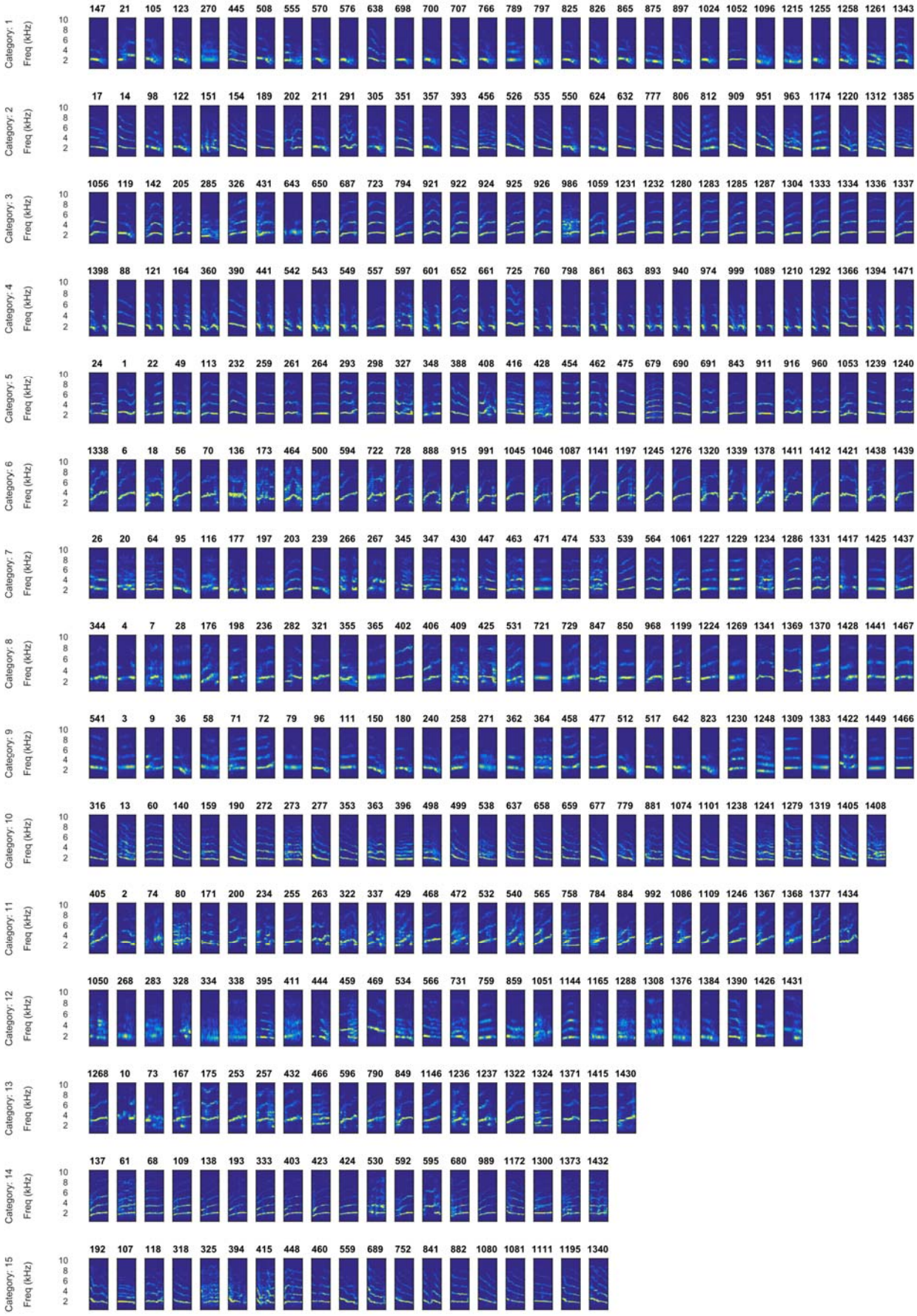
Supplementary Figure 1. Demonstration of clustering performance of SylSorter on syllables from songs of period 1 of bird 3.



Supplementary Figure 2. Demonstration of clustering performance of SylSorter on syllables from songs of period 2 of bird 3.



Supplementary Figure 3. Demonstration of clustering performance of SylSorter on syllables from songs of period 1 of bird 2. The 15 most frequently used syllables are shown.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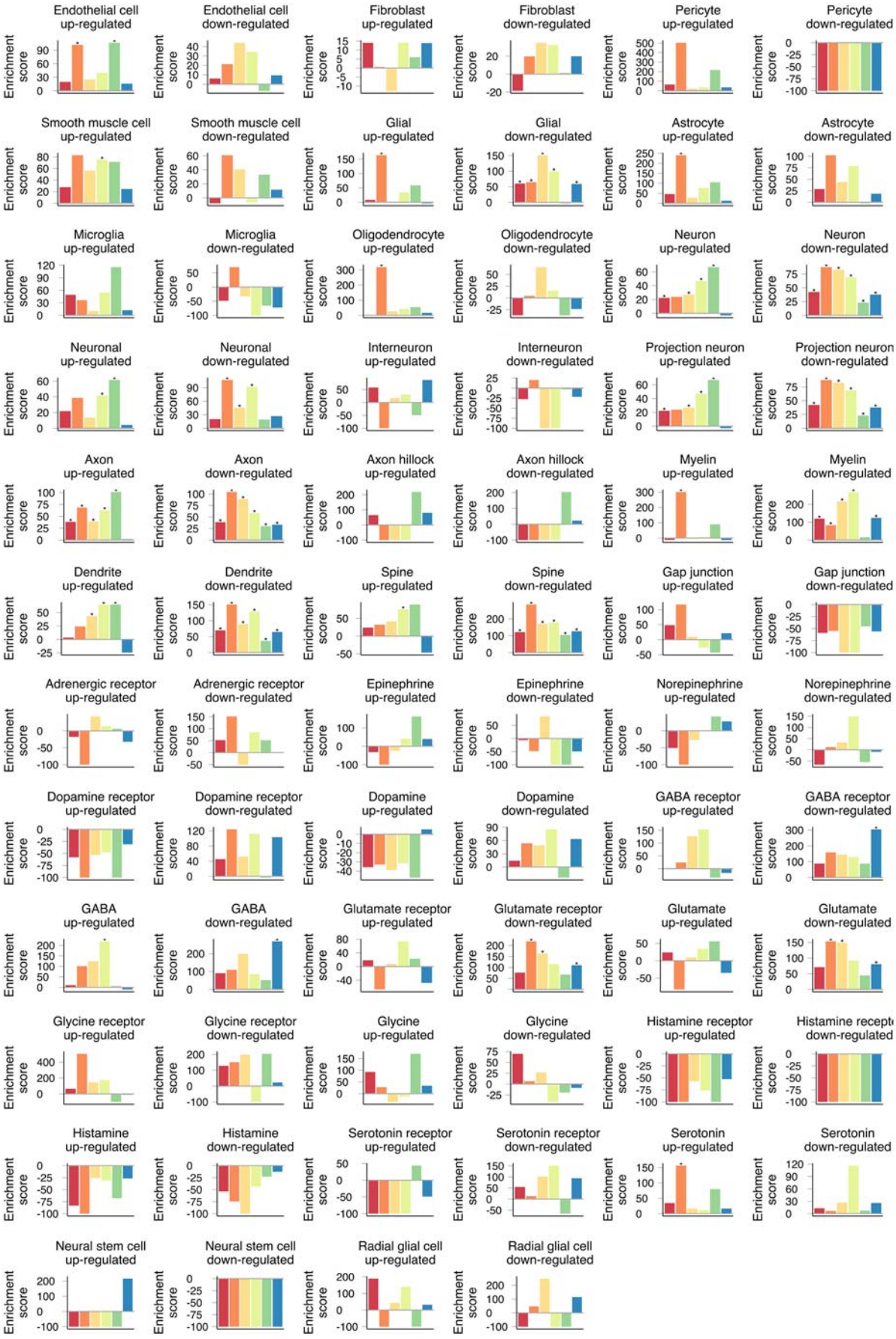
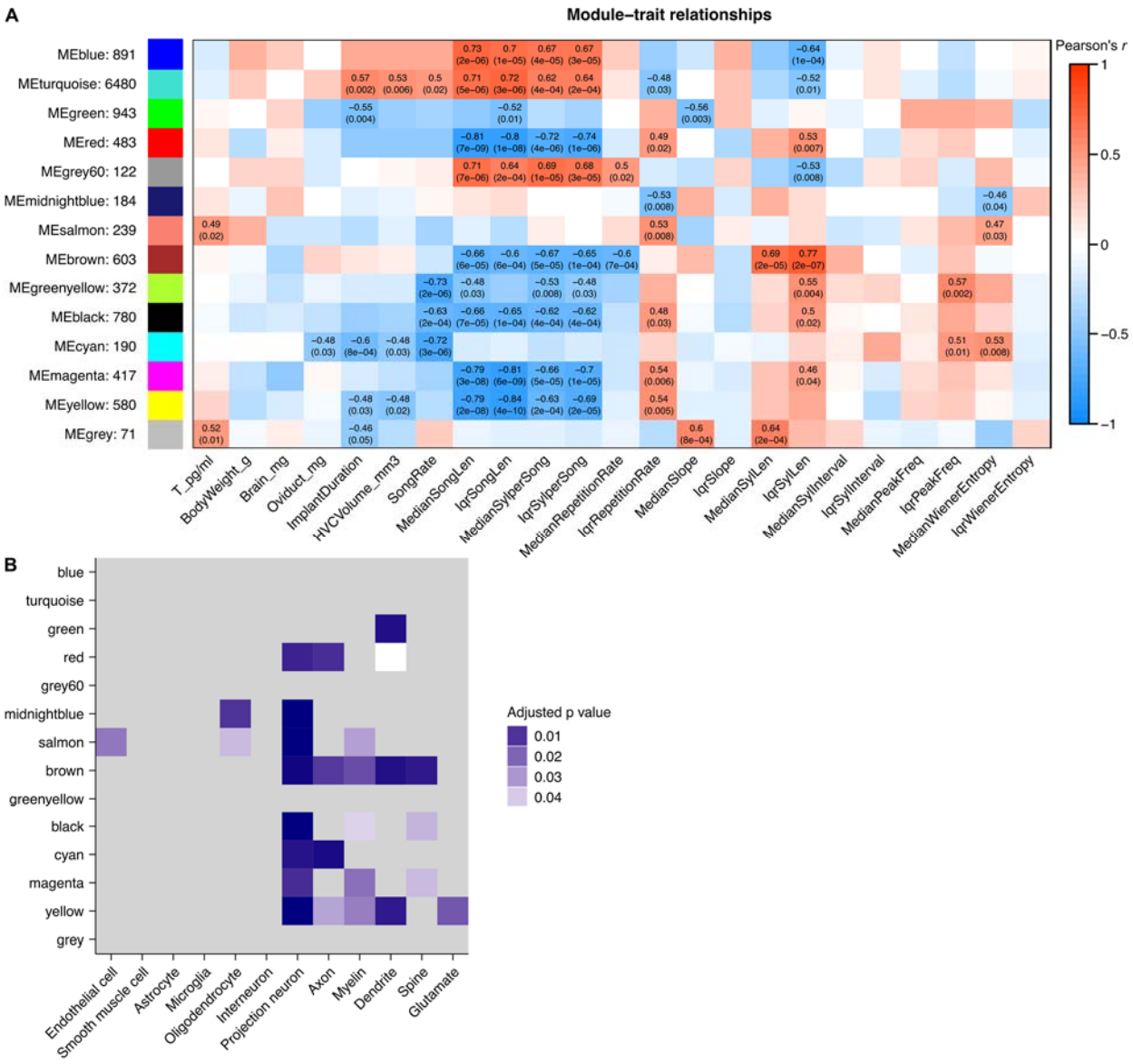
Freq (kHz)
147 21 105 123 270 445 508 555 570 576 638 698 700 707 766 789 797 825 826 865 875 897 1024 1052 1096 1215 1255 1258 1261 1343
Category: 2
Freq (kHz)
17 14 98 122 151 154 189 202 211 291 305 351 357 393 456 526 535 550 624 632 777 806 812 909 951 963 1174 1220 1312 1385
Category: 3
Freq (kHz)
1056 119 142 205 285 326 431 643 650 687 723 794 921 922 924 925 926 986 1059 1231 1232 1280 1283 1285 1287 1304 1333 1334 1336 1337
Category: 4
Freq (kHz)
1398 88 121 164 360 390 441 542 543 549 557 597 601 652 661 725 760 798 861 863 893 940 974 999 1089 1210 1292 1366 1394 1471
Category: 5
Freq (kHz)
24 1 22 49 113 232 259 261 264 293 298 327 348 388 408 416 428 454 462 475 679 690 691 843 911 916 960 1053 1239 1240
Category: 6
Freq (kHz)
1338 6 18 56 70 136 173 464 500 594 722 728 888 915 991 1045 1046 1087 1141 1197 1245 1276 1320 1339 1378 1411 1412 1421 1438 1439
Category: 7
Freq (kHz)
26 20 64 95 116 177 197 203 239 266 267 345 347 430 447 463 471 474 533 539 564 1061 1227 1229 1234 1286 1331 1417 1425 1437
Category: 8
Freq (kHz)
344 4 7 28 176 198 236 282 321 355 365 402 406 409 425 531 721 729 847 850 968 1199 1224 1269 1341 1369 1370 1428 1441 1467
Category: 9
Freq (kHz)
541 3 9 36 58 71 72 79 96 111 150 180 240 258 271 362 364 458 477 512 517 642 823 1230 1248 1309 1383 1422 1449 1466
Category: 10
Freq (kHz)
316 13 60 140 159 190 272 273 277 353 363 396 498 499 538 637 658 659 677 779 881 1074 1101 1238 1241 1279 1319 1405 1408
Category: 11
Freq (kHz)
405 2 74 80 171 200 234 255 263 322 337 429 468 472 532 540 565 758 784 884 992 1086 1109 1246 1367 1368 1377 1434
Category: 12
Freq (kHz)
1050 268 283 328 334 338 395 411 444 459 469 534 566 731 759 859 1051 1144 1165 1288 1308 1376 1384 1390 1426 1431
Category: 13
Freq (kHz)
1268 10 73 167 175 253 257 432 466 596 790 849 1146 1236 1237 1322 1324 1371 1415 1430
Category: 14
Freq (kHz)
137 61 68 109 138 193 333 403 423 424 530 592 595 680 989 1172 1300 1373 1432
Category: 15
Freq (kHz)
192 107 118 318 325 394 415 448 460 559 689 752 841 882 1080 1081 1111 1195 1340

Supplementary Figure 4. Demonstration of clustering performance of SylSorter on syllables from songs of period 2 of bird 2. The 15 most frequently used syllables are shown.


Endothelial cell up-regulated
Endothelial cell down-regulated
Fibroblast up-regulated
Fibroblast down-regulated
Pericyte up-regulated
Pericyte down-regulated
Smooth muscle cell up-regulated
Smooth muscle cell down-regulated
Glial up-regulated
Glial down-regulated
Astrocyte up-regulated
Astrocyte down-regulated
Microglia up-regulated
Microglia down-regulated
Oligodendrocyte up-regulated
Oligodendrocyte down-regulated
Neuron up-regulated
Neuron down-regulated
Neuronal up-regulated
Neuronal down-regulated
Interneuron up-regulated
Interneuron down-regulated
Projection neuron up-regulated
Projection neuron down-regulated
Axon up-regulated
Axon down-regulated
Axon hillock up-regulated
Axon hillock down-regulated
Myelin up-regulated
Myelin down-regulated
Dendrite up-regulated
Dendrite down-regulated
Spine up-regulated
Spine down-regulated
Gap junction up-regulated
Gap junction down-regulated
Adrenergic receptor up-regulated
Adrenergic receptor down-regulated
Epinephrine up-regulated
Epinephrine down-regulated
Norepinephrine up-regulated
Norepinephrine down-regulated
Dopamine receptor up-regulated
Dopamine receptor down-regulated
Dopamine up-regulated
Dopamine down-regulated
GABA receptor up-regulated
GABA receptor down-regulated
GABA up-regulated
GABA down-regulated
Glutamate receptor up-regulated
Glutamate receptor down-regulated
Glutamate up-regulated
Glutamate down-regulated
Glycine receptor up-regulated
Glycine receptor down-regulated
Glycine up-regulated
Glycine down-regulated
Histamine receptor up-regulated
Histamine receptor down-regulated
Histamine up-regulated
Histamine down-regulated
Serotonin receptor up-regulated
Serotonin receptor down-regulated
Serotonin up-regulated
Serotonin down-regulated
Neural stem cell up-regulated
Neural stem cell down-regulated
Radial glial cell up-regulated
Radial glial cell down-regulated
Enrichment score

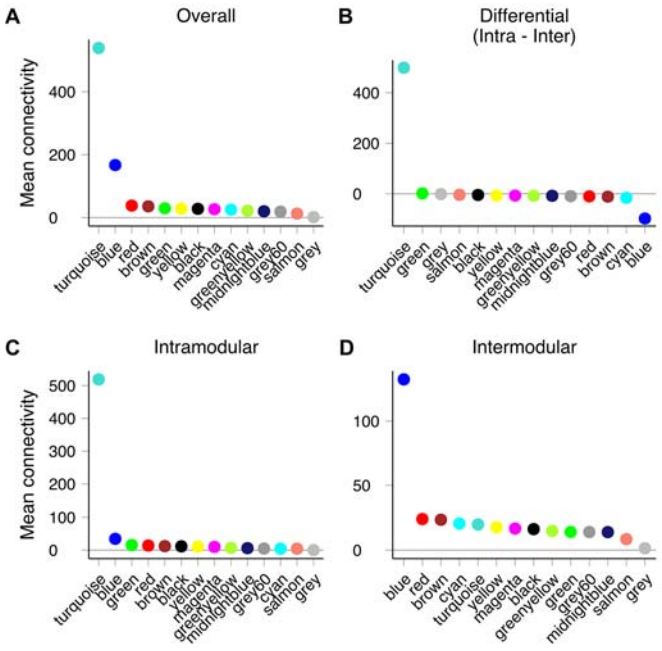
Supplementary Figure 5. Search term enrichment analyses focused on the genes associated with the major cell types, neuronal protrusion types and biosynthesis of neurotransmitters and neurotransmitter receptors in the HVC.

The enrichment score was defined as a percentage increase of decrease of the number of observed genes than the expectation value (E) for a given time point and a given search term (see methods). A positive enrichment score means the time point is likely enriched for a given search term. Significance was determined by Fisher's exact test followed by the Bonferroni multiple correction. *: adjusted p value < 0.05.



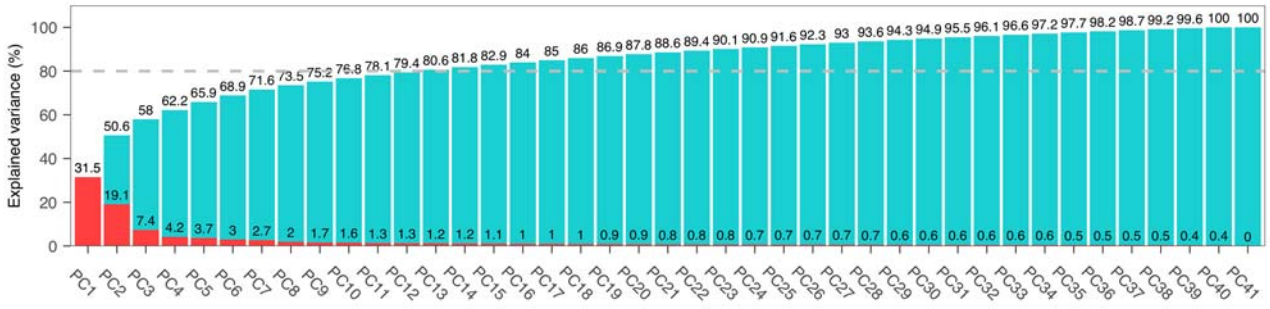
Supplementary Figure 6. Genes belong to the same modules have similar Gene significance (GS) patterns.

A, WGCNA, classified genes into 14 modules (sub-network within the overall network) and associated the modules (using Module Eigengenes, MEs) with physiological traits (including plasma testosterone concentrations (T), and weights of body, brain and oviduct), the HVC volume and song measurements. Iqr: inter-quartile range. B, Search term enrichment analyses of the 14 modules showing 8 modules are associated with neuronal properties.



Supplementary Figure 7. The ranked connectivity of WGCNA modules. A) overall B) differential C) intramodular and D) intermodular connectivity.

A Module with high differential connectivity indicating the genes are more densely connected within module than with the genes from other modules.



Supplementary Figure 8. A PCA scree plot shows percentage of explained variance by each principal component.

II. Supplementary Tables and Legends

Supplementary Table 1. Pearson's correlation analysis of song features and physiological measurements.

Supplementary Table 2. Expression levels of genes associated with particular cell type.

Supplementary Table 3. Intramodular connectivity of genes assigned to the turquoise module.

Supplementary Table 4. List of genes (625) contains SP8 binding sites in proximal promoter sequences.

Supplementary Table 5. List of genes (169) contains SP8 and GATA binding sites in proximal promoter sequences.

Supplementary Table 6. Pearson's correlation analysis of variables and principal components.

Supplementary Table 7. Female-specific genes.

Supplementary Table 8. Male-specific genes.

Supplementary Table 9. Sex-shared genes.

Supplementary Table 10. Fisher's exact test of female-specific, male-specific and sex-shared genes.

Supplementary Table 11. GO-term enrichment analysis of female-specific, male-specific and sex-shared genes.

Supplementary Table 12. Female-biased HVC genes.

Female-biased genes, i.e., genes with negative values of average log2-fold change (LFC.ave < 0), identified in this study are listed. The columns of regulatory function (regulatory.function) and matrix family of transcription factors (matrix.family) were retrieved from El Dorado databases (Genomatix) and indicate the potential regulatory roles of the genes.

Supplementary Table 13. Male-biased HVC genes.

Male-biased genes, i.e., genes with positive values of the average log2-fold change (LFC.ave > 0), identified in this study are listed. The columns of regulatory function (regulatory.function) and matrix family of transcription factors (matrix.family) were retrieved from El Dorado databases (Genomatix) and indicate the potential regulatory roles of the genes.

Supplementary Table 14. GO-term enrichment analysis of sex-biased genes.

GO-term enrichment analyses of the six groups (FWm, CBm, Cm, FWf, CBf, and CfS) were performed separately and summarized in this table.

Supplementary Table 15. Female-specific HVCspec genes across singing groups.

The HVC_{spec} genes that were expressed in the females of all singing species are listed. The columns of regulatory function (regulatory.function) and matrix family of transcription factors (matrix.family) were retrieved from El Dorado databases (Genomatix) and indicate the potential regulatory roles of the genes.

Supplementary Table 16. Male-specific HVCspec genes across singing groups.

The HVC_{spec} genes that were expressed in the males of all singing species are listed. The columns of regulatory function (regulatory.function) and matrix family of transcription factors (matrix.family) were retrieved from El Dorado databases (Genomatix) and indicate the potential regulatory roles of the genes.

Supplementary Table 17. Commonly expressed HVCspec genes across singing groups.

The HVC_{spec} genes that were expressed in both females and males of all singing species are listed. The columns of regulatory function (regulatory.function) and matrix family of transcription factors (matrix.family) were retrieved from El Dorado databases (Genomatix) and indicate the potential regulatory roles of the genes.

Supplementary Table 18. GO-term enrichment analysis of the commonly expressed HVCspec genes.

Supplementary Table 19. GO-term enrichment analysis of the female-specific HVCspec genes.

Supplementary Table 20. GO-term enrichment analysis of the male-specific HVCspec genes.

Author contributions

This thesis was completed by Meng-Ching Ko (M-CK), and with cooperation with many scientists and research technicians.

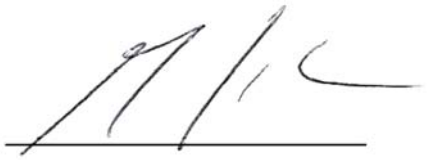
Experiment I: M-CK screened and recorded female canaries. M-CK collected female canary blood samples, measured weights of organs and body, sectioned the brain, measured the HVC volume. Vincent Van Meir (VVM) developed the Multi_Channel_Analyser (MCA) program. M-CK and VVM developed the SylSorter tool. M-CK and VVM analysed the data. M-CK visualized the data. M-CK, VVM and Manfred Gahr (MG) wrote the manuscript.

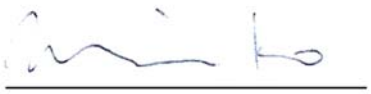
Experiment II: M-CK, Carolina Frankl-Vilches (CF-V), and Manfred Gahr (MG) designed the experiment. M-CK implanted and recorded female canaries. M-CK collected female canary blood samples, measured weights of organs and body, sectioned the brain, measured the HVC volume. M-CK and MG performed the tissue isolation for microarray. M-CK and Antje Bakker (AB) performed the RNA extractions and microarray hybridizations. Nina Sohnius-Wilhelmi (NS-W) performed RNAscope® *in situ* hybridizations. M-CK analysed, visualized the transcriptomic data, and was a major contributor in writing the manuscript. CF-V and MG provided supervision throughout the project. MG conceived of the study and financial support.

Experiment III: M-CK, Carolina Frankl-Vilches (CF-V) and Manfred Gahr (MG) wrote the manuscript. M-CK implanted female canaries. M-CK and MG collected canary blood samples. M-CK and MG measured weights of organs and body, sectioned the brain, measured the HVC volume. M-CK and MG performed the tissue isolation for microarray. M-CK and Antje Bakker (AB) performed the RNA extractions and microarray hybridizations. M-CK analysed, visualized the transcriptomic data, and was a major contributor in writing the manuscript. MG conceived of the study and financial support.

Author contributions

Experiment IV: M-CK, Carolina Frankl-Vilches (CF-V) and Manfred Gahr (MG). M-CK implanted female canaries. M-CK and MG collected canary blood samples. MG collected forest weavers and blue-capped cordon bleus. M-CK and MG measured weights of organs and body, sectioned the brain, measured the HVC volume. M-CK and MG performed the tissue isolation for microarray. M-CK and Antje Bakker (AB) performed the RNA extractions and microarray hybridizations. M-CK analysed, visualized the transcriptomic data, and was a major contributor in writing the manuscript. MG conceived of the study and financial support.





Manfred Gahr

Doktorvater

Meng-Ching Ko

Doktorandin

Acknowledgments

I am deeply grateful to Prof. Dr. Manfred Gahr for giving me the opportunity to start my PhD in his department in Germany, and to enrich my experiences in academic research and in the world. I thank him for providing the maximum support, while giving the maximum freedom to pursue my research interests. I also want to thank him for his kind mentorship, his patience, and his human empathy with my family and me.

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to Seewiesen. I thank Anja for helping me implant and sacrifice canaries in early mornings, and for sexing many canaries for me. Small talk with you has always brightened my day. I thank Christina and Judith for showing me Cryostat tricks, which helped speed things up and reduced my back pain. I also thank Judith for your caring for my well being, and for always sharing your fantastic holidays with me.

I thank the people who taking care of birds and maintaining the facilities. Many thanks to Stefan Leitner for providing canaries and sharing his knowledge on canary behaviors. I want to thank all animal caretakers, especially Roswitha Brighton and David Witkowski. I thank Roswitha for all the small talks we talked about and her kindness. I thank Lisa Trost for dealing with authorities. I also want to thank Willi for maintaining sound boxes, keeping an eye on the clocks of sound boxes, and for digitizing audiotapes that were recorded before I was born to this world.

I would like to thank Wolfgang Goymann and Monika for helping with the radioimmunoassay. I would also like to thank Monika for her hospitality, her generosity, and her abundant knowledge in gardening. Her asian cooking is by far the best and made me feel like at home. I also want to thank her for shuttle me between Weiheim and Seewiesen and for showing me the secret swimming spot.

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I would like to thank Diana Werner for the English lessons and for many weeks' devotion of conditional sentences. If it hadn't been for her lessons, my English would have been worse (hope I got it right). And thank to my native English speaker friends Glenn, Jessie, Lisa Gill, Maude, and Rosalie for editing my thesis and manuscripts.

I thank Markus for teaching me driving, for elevating my terrible manual-car-driving skills to a normal level, and for stopping me from terrifying my passengers. By doing so, he gave me a pair of real legs that enables true mobility during my PhD time. I would also like to thank him for driving me around - back-and-forth to work, food shopping, Stammtisch at Tutzinger hof, and more - for months, and for inviting people to his place (drinking) so that I ended up with Vincent.

I enjoy sharing office with Shouwen, Pietro, Nico, and Amanda. I thank Shouwen, who unexpectedly showed up in my garden on my first day in Germany, made me a welcome dinner (chicken wings), and instantly made me feel like at home. I appreciate his company for many years, the daily walk to get coffee, and yearly winter walks on the icy lake. I thank Pietro for being the first Italian who cooked real pasta for me, and his daily dosage of sarcasm, which had nurtured my mental health until his departure to Alaska. Since then I've been living in agony and frequently bothered by headaches. I thank Nico for the aquarium initiative, for bring life into our office but sadly not on my desk, and for being the most upbeat person in our office. I also thank Nico and Lu for sharing their lunch, because they can't bear with my food. I want to thank Amanda for all her endless sharing of cakes, cookies, and food, her tolerant and sweet personality, and her creativeness for baking, handcraft, and more. I also thank her for dragging me to social events, thus breaking me out from social isolation.

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I want to thank my dog for being cute and lighting up my mood whenever I see him, and my sister for playing my role in the family. Most importantly, I want to thank my parents for their support and their love.

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Awards

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Publications

- Ko, M.-C., Frankl-Vilches, C., Bakker, A., and Gahr, M. (2018). Singing female songbirds express female-specific gene networks in their song control systems. (in preparation)
- Ko, M.-C., Van Meir, V., Vellema, M., and Gahr, M. (2018). Spontaneous female canary songs. (submitted)
- Ko, M.-C., Frankl-Vilches, C., Bakker, A., Sohnius-Wilhelmi, N., and Gahr, M. (2018). Time-lapse transcriptomic profiling of testosterone-induced singing behaviour in adult female canaries. (in preparation)
- Dittrich, F., Ramenda, C., Grillitsch, D., Frankl-Vilches, C., Ko, M.-C., Hertel, M., Goymann, W., Ter Maat, A., and Gahr, M. (2014). Regulatory mechanisms of testosterone-stimulated song in the sensorimotor nucleus HVC of female songbirds. BMC Neurosci 15, 128.
- Vellema, M., Ko, M.-C., Frankl-Vilches, C., and Gahr, M. (2014). What Makes a Marker a Good Marker? Brain, Behavior and Evolution 84, 5-7.
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