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Inter- and Intrasexual Dimorphism in the Song and Song Control System of Duetting White-Browed Sparrow Weavers

Cuvillier Verlag Göttingen

INTER- AND INTRASEXUAL DIMORPHISM IN THE SONG AND SONG CONTROL SYSTEM OF DUETTING WHITE-BROWED SPARROW WEAVERS

Dissertation

der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

vorgelegt von

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Januar 2005

durchgeführt am

Max-Planck-Institut für Verhaltensphysiologie

in Seewiesen

Bibliografische Information Der Deutschen Bibliothek
Die Deutsche Bibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über http://dnb.ddb.de abrufbar.
1. Aufl. - Göttingen : Cuvillier, 2005
Zugl.: München, Univ., Diss., 2005
ISBN 3-86537-501-4

1. Gutachter: Prof. Dr. Benedikt Grothe
2. Gutachter: PD Dr. York Winter
Tag der Abgabe: 12. Januar 2005
Tag der mündlichen Prüfung: 23. Mai 2005

Nonnenstieg 8, 37075 Göttingen
Telefon: 0551-54724-0
Telefax: 0551-54724-21
www.cuvillier.de

1. Auflage, 2005
Gedruckt auf säurefreiem Papier

ISBN 3-86537-501-4

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1. GENERAL INTRODUCTION

1.1. Sexually dimorphic behaviour and brain

Since a long time it has been known that males and females differ in their behaviours. Such sex differences, named sexual dimorphisms, occur in two forms. Sex-specific behaviours are those exclusively shown by one sex and include female life-birth and male intromission. More often behaviours are sex-typical, which means the pattern is more frequently shown by one sex but can be produced under certain conditions by the other sex as well. Examples are clasping in frogs, singing and copulation-solicitation display in songbirds and mounting in many mammalian species (Beach 1961; Kelley 1988; Gahr 1994).

Sexually dimorphic behaviour patterns have been well studied in many groups of animals since the beginning of the last century, especially in the context of reproduction. At the same time, it was recognised that sex differences in circulating androgens and oestrogens account for sex differences in behaviour patterns (Beach 1961). By the 1960s, it was known that steroid hormones exert their effects on behaviour by acting on specific brain regions and distinct areas were identified that accumulate steroid hormones (Hutchison 1967; Pfaff 1968; Stumpf 1968).

However, it was not until the 1970s that the first sex differences in brain structure of vertebrates had been discovered (Raisman & Field 1971; Raisman & Field 1973). These studies focused on reproductive behaviour in rats and revealed a sex difference in the type of synaptic input to the preoptic area of the rat brain, an important region for the control of male and female sexual behaviour (Pfaff 1980). These studies were followed by the discovery of the sexually dimorphic nucleus of the preoptic area (SDN-POA), which is about eight times larger in males than in females (Gorski et al. 1978; Gorski et al. 1980). These sex differences were so prominent that one could detect them without a microscope. Soon after the initial report of sexually dimorphic neural structures in mammals, Nottebohm & Arnold (1976) described sex differences in the size of the song control nuclei in songbirds. They found in zebra finches (*Taeniopygia guttata*) and canaries (*Serinus canaria*) that several telencephalic song nuclei were many times larger in the male than in the female brain and it is only the male that normally sings in these species. Later on in other birds and mammals and in all other classes of vertebrates neural sexual dimorphisms were reported, e.g. in the goldfish (*Carassius auratus*) (Rao et al. 1996), in the African clawed frog (*Xenopus laevis*) (Kelley & Dennison 1990), in a species of whiptail lizards (*Cnemidophorus inornatus*) (Crews et al. 1990), in Japanese

quail (*Coturnix japonica*) (Adkins-Regan & Watson 1990), in the guinea pig (Hines et al. 1985) and in humans (Swaab & Fliers 1985).

The traditional view about the development of these sex differences in brain structure is that genetic sex determines gonadal sex and gonadal sex determines phenotypic sex (for review, see Arnold 2002). Early experiments on mammals confirmed that manipulations of steroid hormone levels at certain periods during ontogeny permanently affect structural sex differences in the brain and let Phoenix et al. (1959) to propose that steroid hormones act in two fundamentally different modes: organisational and activational. Sex steroids act early in life to *organise* neural structures and these effects are permanent. In adulthood, sex steroids *activate* differentiated brain structures to mediate behaviours. Therefore, female guinea pigs prenatally exposed to androgens will show less feminine behaviour in adulthood because androgen had organised their brains in a masculine manner (Phoenix et al. 1959). Conversely, males deprived of androgen during ontogeny will develop a feminine brain (Young et al. 1964). Many of the examples of neural sex differences, which were discovered in the 1970s, were conform to the organisational hypothesis. For example, exposure of newborn female rats to testosterone masculinises the size of the SDN-POA whereas castration of newborn males results in a feminine appearance of this region (Gorski et al. 1978). However, the sexual differentiation of brain and behaviour in the zebra finch fits only partly the hypothesis. There, exposure of juvenile females to steroid hormones, especially oestrogen, masculinises the song control system and song behaviour, but depriving young males of steroids does not prevent masculine development (Arnold 1997). In birds, there is now increasing evidence that sexual differentiation is controlled not only by gonadal steroid hormones but also by brain-intrinsic, genetically determined mechanisms (Wade & Arnold 1996; Agate et al. 2003; Gahr 2003).

1.2. Polymorphic behaviour and brain

From earlier studies on alternative reproductive strategies, e.g. in fish and amphibians, it was recognised that the adult phenotype not only comes in two forms, male and female, but also differs between individuals of the same sex (Howard 1978; Gross 1982). Such polymorphism is found for example in the plainfin midshipman (*Porichthys notatus*), a sound-producing fish with two distinct male phenotypes, which differ in reproductive and vocal behaviour. Whereas type I males engage in nest-building and egg-guarding and generate a mate song during the breeding season, type II males are non-territorial, sneak spawn and do not vocalise (Bass 1992). Furthermore, the differences in vocal

behaviour are paralleled by differences in the cellular structure of the motor neurons innervating the sonic muscles, which are responsible for sound generation (Bass & Andersen 1991). Among humans, homosexuality and transsexuality represent well-studied examples of behavioural polymorphism. There, similar to reports from fish, the phenotypic within-sex differences were found to be reflected in differences in brain structure (Swaab & Hofman 1995; Zhou et al. 1995). For instance, the volume of the suprachiasmatic nucleus of the hypothalamus is about twice as large in homosexual then in heterosexual men (Swaab & Hofman 1990).

With respect to the organisational-activational concept (see 1.1.), which predicts the differentiation of sexually dimorphic behaviour and brain structure, the existence of polymorphic phenotypes raises the question about the general applicability of this hypothesis. According to it, an ‘organising’ effect would mean that the adult phenotype is predetermined during a critical period of development and remains fixed throughout life, whereas an ‘activational’ effect would induce the different phenotype in adulthood and should be reversible. A first attempt to solve this problem has been made by Moore (1991), who proposed the ‘relative plasticity hypothesis’. He distinguished between two different forms of polymorphism, a system with *plastic* phenotypes, in which adult individuals change from one type to another, and a system with *fixed* phenotypes, in which individuals attain one phenotype before sexual maturity and do not change it in adulthood. From this, the hypothesis states that differences between individuals of the fixed phenotype should be organised by early steroid hormone action whereas differences between individuals of the plastic phenotype are due to the activational action of steroid hormones. Data obtained from studies on mating systems and endocrine profiles in several vertebrate taxa were in support of the hypothesis (reviewed in Moore 1991). For example, in tree lizards (*Urosaurus ornatus*), which exhibit the fixed alternative phenotype regarding throat colour, castration or testosterone treatment of male hatchlings results in significant alterations of adult phenotype, which is not obtained when applying the similar treatment to adult individuals (Hews et al. 1994; Hews & Moore 1995). On the other hand, cooperatively breeding bird species such as the pied kingfisher (*Ceryle rudis*), the Florida scrub jay (*Aphelocoma coerulescens*) or the white-browed sparrow weaver (*Plocepasser mahali*) were regarded as having plastic alternative phenotypes because helpers may eventually become breeders. There, the hormone profiles of adults differed in respect to phenotype supporting an activational effect of steroid hormones. However, not all available data could be fit to the predictions of the hypothesis (Moore 1991) and studies exploring the neural basis underlying behavioural polymorphism are so far mainly conducted in a few species of fish and in

humans. Nevertheless, extensive studies on one of these fish species, the African cichlid *Haplochromis burtoni*, revealed the remarkable result that social behaviour exerts profound effects on the reproductive axis and on the brain structures controlling reproduction (Fernald 2002). This pattern is not restricted to maturation but also occurs in adult fish. Males of ascending social status experience an increase in gonad size and an increase in the size of gonadotropin-releasing hormone containing neurons of the preoptic area. These changes revert when the dominant position is lost. Such a system allows the fish to respond quickly to changes in social environment and mating opportunities (Fernald 2002).

1.3. The study of birdsong

For studying the neural mechanisms of behaviour, birdsong represents an attractive model, which enables an integrative approach of behavioural and neurobiological studies because of the following reasons: 1) the brain pathways controlling song learning and song production are well characterised, 2) the circuitry is sensitive to steroid hormones both during development and in adulthood, 3) in most species the song control system is sexually dimorphic, 4) the behaviour can be clearly measured and quantified and 5) birdsong is a sexually dimorphic, learned behavioural pattern.

Birds use their song for inter- and intra-sexual communication. The song of a male may signal to a female the presence of a potential mate whereas to another male, song may convey information about his location and territory ownership (Catchpole & Slater 1995). The hormonal basis of birdsong was inferred early from observations on the relationship between seasonal occurrence of song and reproduction (Armstrong 1963).

Songbirds (*Passeriformes: Oscines*), which account for about half of all bird species, acquire the stereotyped song pattern typical of adult birds by vocal learning. Song learning occurs in two steps during ontogeny, in a sensory phase and in a sensorimotor phase. During the sensory phase, birds build up an auditory template of songs they hear during this period, thereby showing innate preference for conspecific song. Later on during the sensorimotor phase, birds match their vocal output to the acquired template, a process requiring auditory feedback. In some species these two periods overlap, such as in the zebra finch and in the canary, while in others they can be separated by several months, during which the bird needs not to rehear the song, e.g. in the swamp sparrow (*Melospiza georgiana*). When vocal learning is restricted to a critical period at the juvenile stage, these species are referred to as “age-limited” learners, such as the chaffinch (*Fringilla coelebs*) and the white-crowned sparrow (*Zonotrichia*

leucophrys). In contrast, others, like the canary, the starling (*Sturnus vulgaris*) and the red-winged blackbird (*Agelaius phoeniceus*) can modify and expand their song repertoires in adulthood and are called “open-ended” learners (Konishi 1985) (Nottebohm 1999). However, the ability to acquire new auditory memories persists in adult birds independently of a sensitive period and exists even in females that never produce any song. This allows birds to show preference to songs of their mate or to discriminate between songs of territorial neighbours (Nottebohm 1999).

Song behaviour in temperate zone birds is a seasonal trait, highly correlated with breeding and is mainly confined to males though there are species where song is produced in the non-breeding season (Schwabl & Kriner 1991; Rost 1992; Leitner et al. 2001a) and where females occasionally sing (Gerber 1955; Pesch & Guettinger 1985; Kriner & Schwabl 1991). In sharp contrast is the pattern found in the tropics (23.5° N - 23.5° S), where about 80 % of all passerine species live. Due to less variability in day length, climate and food availability compared with the temperate zone, breeding seasons are prolonged and many species are residents and year-round territorial. Furthermore, song is a common characteristic of both males and females and occurs intensively in the context of territory defence (Stutchbury & Morton 2001). Duetting, a primarily tropical phenomenon, represents a special form of singing, where both members of a mated pair vocalise in precise temporal coordination (Farabaugh 1982). This type of song production has evolved independently in distinct taxonomic groups leading to a great diversity among duetting species regarding song characteristics.

Several years after the initially described sex differences in the song control system of canaries and zebra finches, in which females do rarely sing or not at all (Nottebohm & Arnold 1976), duetting songbirds became an interesting subject in this field of research because they represent the other extreme in terms of song behaviour (Brenowitz et al. 1985). Comparative studies on three neotropical duetting wrens of the genus *Thryothorus*, the bay wren (*T. nigricapillus*), the rufous and white wren (*T. rufalbus*) and the buff-breasted wren (*T. leucotis*) and on the African duetter, the white-browed robin chat (*Cossypha heuglini*), which all differ in the degree of sexually dimorphic song production, confirmed previous results showing that the sex differences in song repertoire size correlated well with the sex differences in the volume of song nuclei in the brain (Brenowitz et al. 1985; Brenowitz & Arnold 1986; Brenowitz 1997). However, in a recent study by Gahr et al. (1998) this view was challenged. Their study species, the slate-coloured boubou shrike (*Laniarius funebris*) exhibits no sex difference in duet repertoire size despite a pronounced sex difference in song nuclei volume.

1.4. The neural control of birdsong production and learning

The song control system consists of a network of interconnected nuclei of the fore-, mid- and hindbrain that control song learning and song production. The brain nuclei of these two main pathways have been identified by means of lesion and tract-tracing studies (Nottebohm et al. 1976; Nottebohm et al. 1982). Within the descending motor pathway that controls song production, HVC (nucleus (n.) hyperstriatalis ventrale, pars caudale or high vocal center) of the neostriatum projects to RA (n. robustus archistriatalis), which sends projections to the midbrain nucleus DM (n. dorsomedialis of n. intercollicularis) and to the brainstem motor nucleus nXIIts (n. hypoglossus, pars tracheosyringealis) that innervates the muscles of the sound-producing organ, the syrinx (Fig. 1.1, blue arrows)[1]. When the motor neurons of the syrinx are stimulated, the syringeal muscles contract and the expiratory air stream leads to vibrations of the medial and lateral labia, which results in sound production (Goller & Larsen 1997). Lesions of any of the nuclei within this pathway prevent the bird from singing. Nucleus RA also projects to brainstem nuclei (Ram, rVRG), which send their projections to regions of the spinal cord, which in turn innervate respiratory muscles (Wild 1997). Electrophysiological studies revealed a hierarchical organisation of the nuclei within the motor pathway (McCasland 1987; Vu et al. 1994; Yu & Margoliash 1996).

Within the pathway that controls song learning, also known as anterior forebrain pathway (AFP, Fig. 1.1, green arrows), HVC projects rostrally to Area X, which projects via the thalamic nucleus DLM (n. dorsolateralis thalami, pars medialis) to lMAN (lateral n. magnocellularis of the anterior neostriatum) and finally to RA. Neurons of lMAN also send projections to Area X, providing the potential for feedback. Lesions of any of the AFP nuclei do not interrupt adult song production but interfere with vocal learning in juveniles. Nucleus HVC appears to be a major site for the integration of information because it is connected with both pathways and receives auditory (from field L), visual and somatosensory input (from Uva, n. uvaeformis, and from Nif, n. interfacialis, for review, see Margoliash 1997).

Several nuclei of the song control system have been shown to exhibit steroid hormone sensitivity, i.e. express steroid hormone receptors (for review, see Gahr 2001). Androgen receptors (AR) can be found in all nuclei of the descending motor pathway as well as in some nuclei of the AFP (lMAN, mMAN, DLM; Fig. 1.1, red areas). Oestrogen receptors (ER) are restricted to nucleus HVC (Fig. 1.1, orange area), which is therefore the only nucleus that contains both AR and ER. However, cells that do not express the receptor could

[1] see Appendix for revised nomenclature.

still be ‘sensitive’ because steroids cannot only act via genomic but also via non-genomic mechanisms (McEwen 1994). The spatio-temporal expression pattern of steroid hormone receptors is probably genetically controlled. For example, the AR expression in HVC of zebra finches is already sexually dimorphic at posthatching day 9 when the receptors first appear (Gahr & Metzdorf 1999). After the initial induction of the receptors during development, the expression might be retained throughout life as in the case of most songbirds studied so far. Alternatively, the expression is restricted to a short period as in the female zebra finch and is lost afterwards due to the pronounced neuronal death, which subsequently leads to the many times smaller song nuclei compared to males.

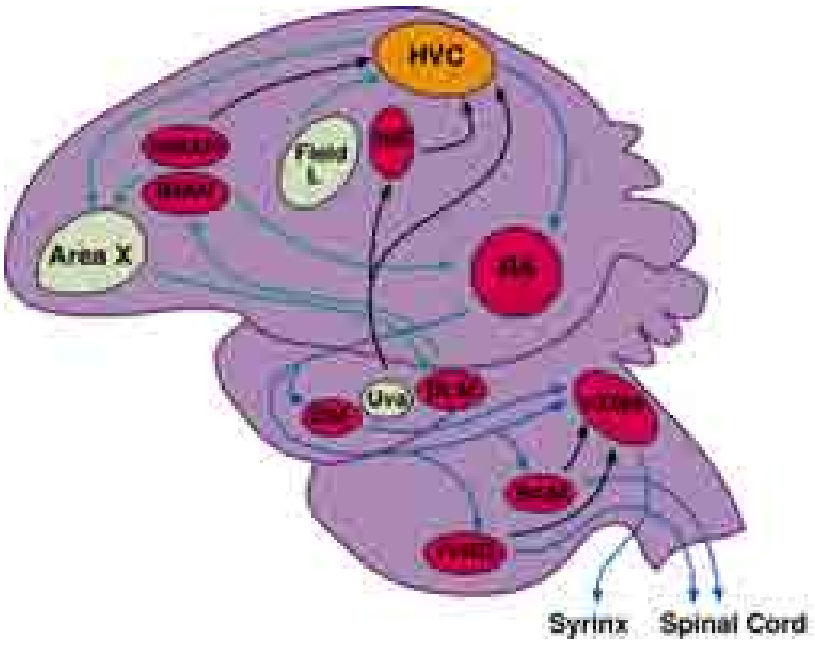


Fig. 1.1: Schematic parasagittal view of the vocal control system of songbirds showing the song nuclei and their projections (arrows). Song production is controlled by the descending motor pathway (blue arrows), which includes the nuclei HVC, RA, DM, nXIIts, Ram and rVRG. Vocal learning involves the nuclei of the anterior forebrain pathway (green arrows), consisting of HVC, Area X, DLM, lMAN and RA. HVC receives input from field L, an auditory region, as well as from nuclei Uva, Nif and mMAN. Vocal areas that express androgen receptors are in red, those that express both, androgen and oestrogen receptors (HVC only) are in orange and those that express neither are in grey. *Abbreviations*: DLM, nucleus (n.) dorsolateralis thalami, pars medialis; DM, n. dorsomedialis of n. intercollicularis; HVC, n. hyperstriatalis ventrale, pars caudale; lMAN, lateral n. magnocellularis of the anterior neostriatum; mMAN, medial n.magnocellularis of the anterior neostriatum; Nif, n. interfacialis; nXIIts, n. hypoglossus, pars tracheosyringealis; RA, n. robustus archistriatalis; Ram, n. retroambigualis; rVRG, rostroventral respiratory group; Uva, n. uvaeformis. (After Gil & Gahr 2002).

1.5. Aim of the study

In the current view of brain-behaviour differentiation of songbirds it is thought that the degree of sex differences in song nuclei size correlates with the degree of sex differences in the complexity of song behaviour, expressed in repertoire size (for review, see Ball & MacDougall-Shackleton 2001); Schlinger & Brenowitz 2002). However, there are a number of studies showing that female song nuclei differ from those of males in far more features beyond brain area size, e.g. neuron density, neuron size, synaptic properties, steroid hormone target cells (reviewed by Balthazart & Adkins-Regan 2002). Moreover, studies on several species, in which females sing, such as white-crowned sparrows (*Zonotrichia leucophrys*) (Baker et al. 1984; Baptista et al. 1993), white-throated sparrows (*Zonotrichia albicollis*) (DeVoogd et al. 1995) and slate-coloured boubou shrikes (Gahr et al. 1998) together with results obtained from testosterone-induced singing in female domesticated canaries (Nottebohm 1980; Appeltants et al. 2003) do not support such a relationship between song nuclei size and song complexity. For example, two white-throated sparrow morphs show the same degree of sex difference in HVC size although in one morph both males and females sing and have repertoires of similar size whereas in the other morph only the male sings (DeVoogd et al. 1995). Rather these data show that female song control is accomplished in a different way than it is in males. So far, functional interpretations about the observed sex differences within the song system are rare (Ball & MacDougall-Shackleton 2001). Clearly, studies are needed investigating brain-behaviour relationships at a more detailed structural level. Duetting species are interesting subjects in this regard due to their great diversity in song behaviour (Farabaugh 1982).

The aim of the present study was to investigate the neural basis of male and female song behaviour in duetting white-browed sparrow weavers (*Plocepasser mahali*). This species was chosen because it exhibits behavioural polymorphism in terms of song production, which allows conducting a comparative study. White-browed sparrow weavers are cooperatively breeding birds of eastern and southern Africa that live in groups of 2 to 10 individuals, with a dominant breeding pair and male and female subordinates (Collias & Collias 1978; Lewis 1982). Song production can be divided into two major types, duet song (when produced by more than two individuals called chorus song) and solo song. Whereas duet and chorus songs are common among all group members and are performed throughout the year, the solo song is restricted to the dominant male and only produced during the breeding season (Ferguson 1988a; Wingfield & Lewis 1993). The existence of two adult male phenotypes, dominants and subordinates, which differ in the production of solo

song, enables fruitful inter- and intrasexual comparisons of brain structure and behaviour. The focus for analysing the structure of the song control system, particularly of nucleus HVC was to go beyond brain area size by using a cytochemical approach in addition to the commonly used characterisation by cytoarchitecture. Such an approach allows identifying differences within functionally defined neuron subpopulations.

The thesis is divided into five experimental chapters. At the beginning, I give a detailed description of the song behaviour of males and females (chapter 4). Thereby I focus on the song output of dominant pairs recorded in Zimbabwe and these data are completed by studies on captive birds. The following chapter characterises the song nucleus HVC in both sexes by means of cytoarchitecture, e.g. volume, total number of cells and cell density of the nucleus (chapter 5). In the next chapter (chapter 6.1), I use the cytochemical approach and focus on the steroid hormone sensitivity of nucleus HVC by analysing the expression pattern of androgen and oestrogen receptors. In chapter 6.2, I introduce synaptic proteins as a new group of neurochemical markers for the characterisation of the song system. The basic expression pattern of these proteins and their steroid hormone sensitivity was characterised in the zebra finch because these birds are less precious than white-browed sparrow weavers and represent a model species for the study of birdsong. In chapter 6.3, I apply this new type of marker to the song system of male and female white-browed sparrow weavers. In chapter 7, I investigate the gonadal activity of males and females by measuring circulating levels of androgens and oestrogens and explore their influence on the steroid hormone sensitivity of the song system and on the expression of song. Chapter 8 covers a preliminary experiment studying the influence of exogenous testosterone on the song behaviour in females, which should reveal whether sex differences in adult song production could be attributed to ‘organisational’ or ‘activational’ effects of steroid hormones. At last, the results from all chapters are integrated and discussed (chapter 9).

2. ANIMALS AND STUDY AREA

2.1. The white-browed sparrow weaver

The white-browed sparrow weaver (*Plocepasser mahali*) belongs to the sub-family of sparrow weavers (Plocepasserinae) within the family of Ploceidae (weaverbirds). Its distribution ranges from Sudan and Ethiopia in northeastern Africa to the southern parts of Africa. Five subspecies are recognized that are geographically separated. *P. mahali melanorhynchus* and *P. mahali pectoralis* occur in the northern regions, whereas *P. mahali mahali*, *P. mahali stentor* and *P. mahali terricolor* occur in southern Africa (Clancey 1980). In my study, I investigated the subspecies *P. mahali terricolor* that is a common resident bird in southern Zimbabwe and in South Africa (Fig. 2.1). White-browed sparrow weavers can be distinguished by a bold white eyebrow, a brown head and a blackish brown face and crown. Its rump and underparts, as well as the tail coverts are white. In the northern subspecies, the breast is marked with brown spots and the sexes are alike. In the southern subspecies, sexes can be distinguished by their different bill colours. Females have a horn-coloured, and males have a black bill (Earle 1983b). The body length is 16-18 cm and body weight ranges from 40-54 g (Maclean 1993).

Fig. 2.1: Pair of white-browed sparrow weavers. The male is on the left, the female on the right side.

The habitat of sparrow weavers is *Acacia* savannah, bush and open grassland, usually below 1500 m. Birds live in pairs or groups (colonies) of up to 10 individuals (Collias & Collias 1978; Lewis 1982; Ferguson 1988b). Colonies consist of a dominant breeding pair and subordinate males and females. The species is year-round territorial and all colony members engage in territory

defence. Territory size shows large variation between different populations in different habitats and ranges from about 4000 m^2 in Kenya (Collias & Collias 1978) to 121000 m^2 in a South African population (Ferguson 1988b). White-browed sparrow weavers are cooperatively breeding birds, i.e. subordinates help raising offspring of the dominant pair (Collias & Collias 1978; Lewis 1982). Birds build their roosting nests into a single prominent tree or in several adjacent trees within the territory. Nests consist of long grass stems and resemble a bunch of straw. Roosting nests have two entrances and breeding nests only one. Nest building occurs year round. Birds usually forage on the ground on seeds and insects within the territory and prefer open, disturbed areas near human settlements that have animal husbandry facilities. The breeding season is usually restricted to the rainy season (Earle 1983a).

Fig. 2.2: Roosting nests of a colony of white-browed sparrow weavers in an *Acacia* tree. Nests are usually built in a single large tree or several adjacent smaller trees. Each colony member has several roosting nests.

2.2. Study area

The study was conducted in Zimbabwe, which is a land-locked country in south central Africa (15° 30' - 22° 20' S, 25° - 33° E). It is bordered by Botswana in the west, Zambia in the north, Mozambique in the east, and South Africa in the south (Fig. 2.5). It covers an area of 390500 km^2. The capital and largest city is Harare with 2.3 million inhabitants (2004 estimation), another large city is Bulawayo with 865000 inhabitants. The terrain consists of a plateau with four regions. The high veld, above 1200 m, crosses the country from southwest to northeast. On each side lies the middle veld, 900 - 1200 m high, and beyond is the low veld, at elevations below 900 m. The fourth region, the Eastern

Highlands, is a mountainous belt along the border of Mozambique, with the highest point in Zimbabwe, Mt. Inyangani (2,592 m). Annual rainfall varies from about 1780 mm in the Highlands to less than 640 mm in the south, and occurs mostly between October and April (Fig. 2.3).

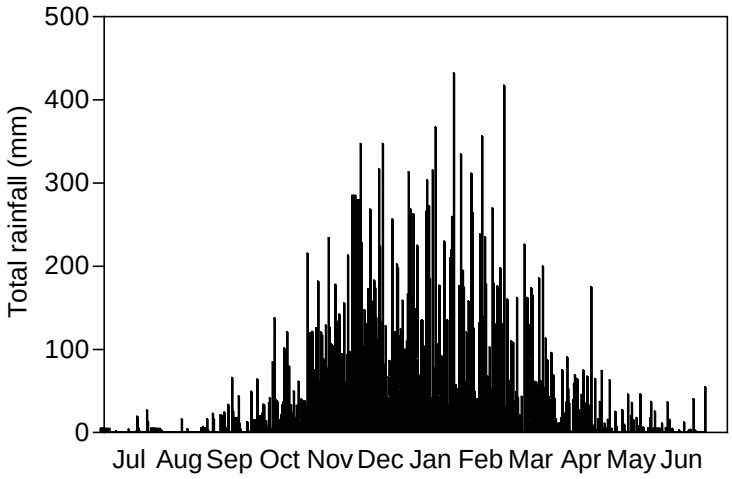


Fig. 2.3: Total monthly rainfall in the years 1949-2001 at Falcon College near Esigodini / Zimbabwe (20° 14'S, 28° 58'E). Rainfall occurs mainly between October and April, with peak values from December to February. That period is also characterised by considerable variation between years.

Although located in the tropics, Zimbabwe has a mild climate due to its height. In locations above 1000 m, mean temperatures range from 13°C in July (winter) to 23°C in January (summer) (Sträßer 1998). Nevertheless, minimum temperatures can drop below the freezing point in winter and can rise well above 30°C in summer.

2.3. Study site

After evaluating sparrow weaver habitats, I found a suitable study site in the south western part of Zimbabwe, about 60 km southeast of Bulawayo near the village of Esigodini between 20°08'S - 20°14'S and 28°56' - 29°01'E (Fig. 2.5). This area mainly consists of commercial farmland at altitudes ranging from 1120 – 1235 m in the transition zone between middle veld and high veld. The landscape is characterised by acacia savannah, bushy hillsides, and open grassland. Data were collected in two consecutive years during the rainy season: from January 25 to March 19 in 2000, and from January 30 to March 1 in 2001, after a final selection of two suitable study areas during an explorative journey

in January/February 1999. During the periods of data collection, we hit the peak of the rainy seasons, which was January/February in 2000 and February in 2001 (Fig. 2.4).

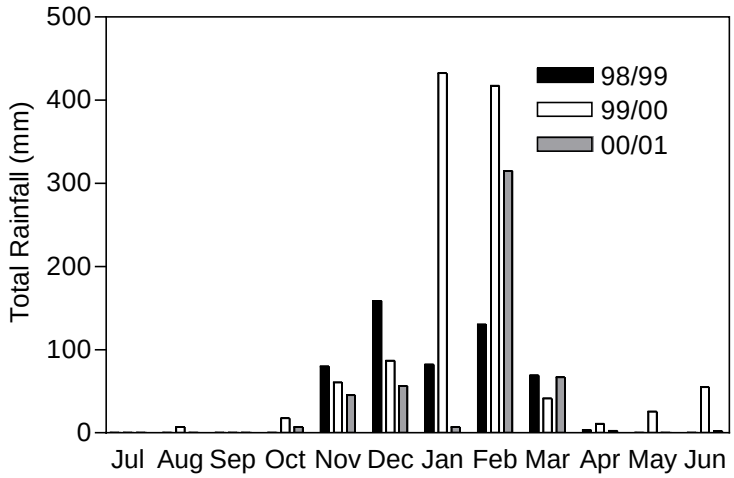
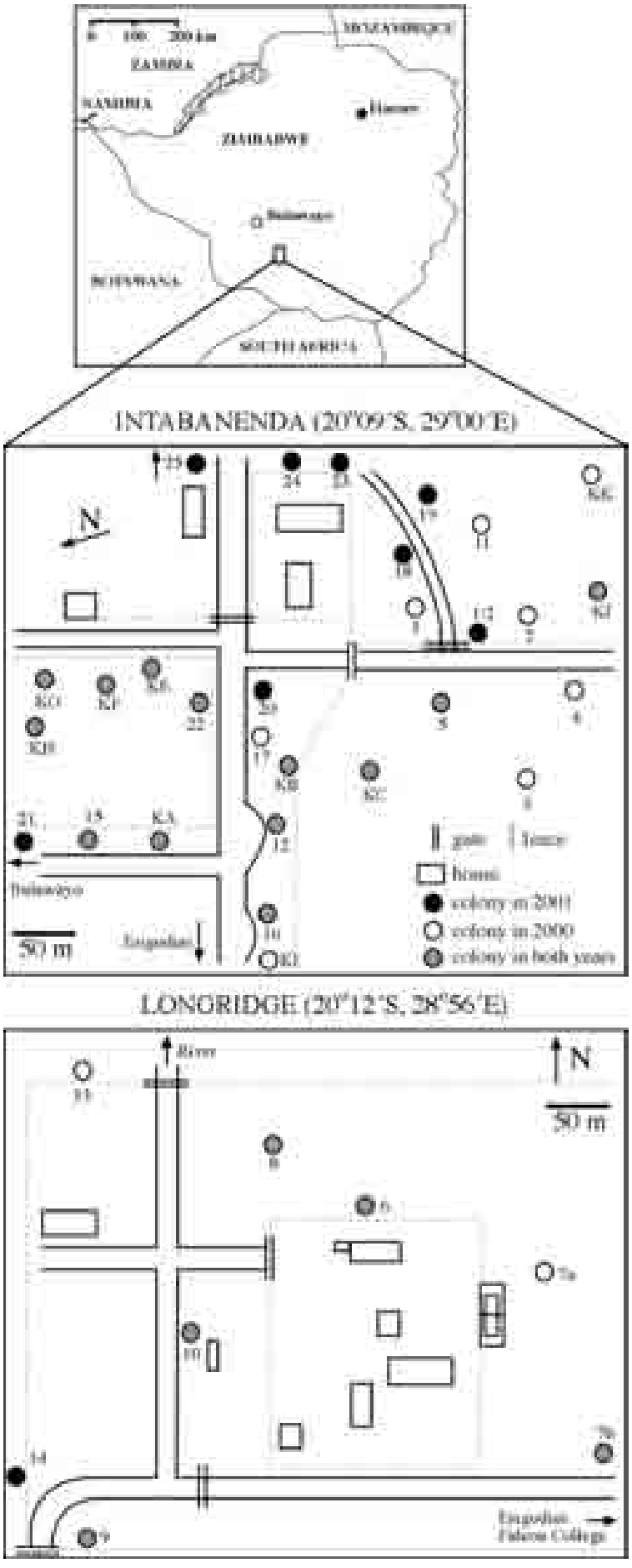


Fig. 2.4: Monthly rainfall from July 1998 to June 2001 at Falcon College near Esigodini / Zimbabwe (20° 14'S, 28° 58'E). The peak rainfall varied from year to year, but occurred mainly between December and February. January and February 2000 were characterised by a huge amount of rain that comprised 74 % of the rainfall of the period 99/00.

I conducted my study on two commercial farms, which were 10 km apart from each other (Fig. 2.5). I identified 28 colonies in 2000 and 24 colonies in 2001 on these study sites. Neighbouring colonies were at least 40 m apart. Territory sizes ranged from 2500-10000 m² and were slightly smaller than reported previously from another study in northern Zimbabwe (Vernon 1983). Large colonies persisted throughout both study years, whereas small colonies were often abandoned after one breeding season.

Fig. 2.5: (next page): The two study sites in southern Zimbabwe, southeast of Bulawayo on commercial farmland. The circles indicate sparrow weaver colonies in different years. The majority of colonies persisted longer than one year. As the figure shows, colonies can frequently be found in the vicinity of buildings, roads and fences.


ZAMBIA
ZIMBABWE
BOTSWANA
INTABANENDA (20°09'S, 29°00'E)
N
50 m
home
colony in 2011
colony in 2000
colony in both years
LONGRIDGE (20°12'S, 28°56'E)
River
N
50 m

3. GENERAL METHODS

3.1. Field methods

3.1.1. Capture, measurement and blood sampling of birds

Birds were captured with mist nets during the day and with a special trap at night to catch them inside their roosting nests. This trap consisted of an adjustable pole mounted to a net. Immediately upon capture, a blood sample was taken by puncture of the wing vein. Blood was collected in heparinised capillary tubes and immediately centrifuged with a mini-centrifuge (Bayer diagnostics) at 2500 rpm for 10 minutes. Plasma samples were stored at -20°C until analysis. All birds received a unique combination of a numbered aluminium ring and two coloured plastic rings. Birds were sexed according to the colouration of their beak (Earle 1983b). In the subspecies *Plocepasser mahali terricolor* that I was studying, males have a black beak and females have a horn-coloured beak (see also chapter 2.1). Birds were considered immature when bill colour was not uniform. From these birds, the sex could not be identified unequivocal. Several morphological measurements were taken: Body weight using a "Pesola" precision scale with an accuracy of 0.5 g, wing length using a wing measuring device (ruler) with an accuracy of 0.05 cm, bill length and tarsus length using a calliper with an accuracy of 0.01 cm. Fat and muscle scores were estimated by means of a scale ranging from 0-8. The measurements followed the instructions for bird banding of the "Vogelwarte Radolfzell/Germany" and are shown in Table 3.1.

Table 3.1: Morphological measurements of male and female white-browed sparrow weavers (mean ± SE).

	Males (N = 42)	Females (N = 47)
Body weight (g)	47.07 ± 0.39	44.42 ± 0.38
Wing length (cm)	10.33 ± 0.03	10.03 ± 0.03
Tarsus length (cm)	2.52 ± 0.01	2.50 ± 0.01
Bill length (cm)	1.50 ± 0.01	1.46 ± 0.01
Fat score	3.07 ± 0.14	3.51 ± 0.18
Muscle score	1.90 ± 0.05	1.93 ± 0.06

3.1.2. Song recording

All vocalisations were recorded with a Sony TCD-5M portable cassette recorder (Sony Corp., Tokyo, Japan) equipped with a Sennheiser ME-88 directional microphone (Sennheiser electronic, Wedemark, Germany).

3.1.3. Behavioural observations

At each colony of interest, behavioural observations were conducted to identify the social status of each bird. A male was considered dominant, when it was singing repeatedly the solo song at dawn and when it was the last bird of the colony entering the roosting nest at night. Additional males within the colony, which were fully mature but did not show the behaviour of the dominant male, were considered subordinate. The dominant female was identified by its breeding activity (e.g. copulation solicitation display, incubating eggs), by its frequent song output together with the dominant male and by chasing other females of the colony.

3.1.4. Perfusion of birds

For neuroanatomical analyses, only ringed birds were selected from which song recordings had been made. Birds were recaptured at night, between 7 and 10 pm, in their roosting nests, transferred to the field station and kept overnight singly in cages, supplied with food and water. On the following day, birds were killed with an overdose of chloroform and immediately perfused transcardially, first with 0.9 % saline, followed by 4 % phosphate-buffered formaldehyde (FPBS). Brains were removed and postfixed in FPBS at 4°C until analysis. The brains of fourteen dominant females, fourteen dominant males and eight subordinate males were collected for analysis. The gonads and syrinx of each bird were removed and stored in FPBS at 4°C.

3.2. Laboratory methods

3.2.1. Tissue preparation

Brains were removed from FPBS and immersed in 15 % RNase-free phosphate-buffered sucrose, followed by 30 % phosphate-buffered sucrose for several days at 4°C. Brains were then placed onto a freezing-microtome (Leica, Bensheim, Germany) and were cut into 30 µm parasagittal sections. The sections were transferred to sterile microwell plates (Nunc, Wiesbaden, Germany) filled with 1x phosphate-buffered saline (PBS). With a sterile brush sections were mounted onto Superfrost-Plus microscope slides (Menzel Gläser, Braunschweig, Germany) using a petri dish filled with 1x PBS and were then air-dried for 3 days. To prevent contamination of the tissue with ribonucleases (RNases), buffers and double-distilled water (ddH_2O) were treated with 0.1 % diethylpyrocarbonate (DEPC), which inhibits RNases. Details about the treatment of the brains are given in the respective chapters.

3.2.2. Nissl staining

The Nissl stain thionine labels those parts of the cell, which are highly basophilic, e.g. free ribosomes and ribosomes bound to the rough endoplasmatic reticulum (Raine 1994). Because these organelles are important in the process of protein synthesis, the intensity of the staining serves as indicator of overall cellular activity. Based on this method several brain areas belonging to the song control system can be identified.

Air-dried sections were hydrated in descending concentrations of ethanol (100 %, 90 %, 70 %, and 20 %) and ddH_2O each for 1 min and then immersed in 0.1 % thionine acetate (Serva, Heidelberg, Germany) for 20-30 sec. The slides were rinsed in ddH_2O and dehydrated in ascending concentrations of ethanol, each for 1 min. After immersing them for 1 min in xylene (Roth, Karlsruhe, Germany), slides were coverslipped with Roti-Histokitt (Roth) and dried in horizontal position for 2 days. For counterstaining of slides processed with in situ hybridisation, 0.005 % thionine acetate was used to give a very light staining of the sections.

3.2.3. In situ hybridisation

3.2.3.1. Cloning of cDNA probes

From the following proteins cDNA probes were generated to use in in situ hybridisation studies: androgen receptor (AR), oestrogen receptor (ER), synaptosomal-associated protein 25 kDa (SNAP-25), synaptoporin (SPO) and syntaxin 1B (STX).

The cloning of a partial zebra finch AR cDNA (759 bp) and ER cDNA (926 bp) was done in our laboratory and has been described previously (Gahr & Metzdorf 1997). The AR sequence revealed by reverse transcriptase polymerase chain reaction (RT-PCR) is identical to a partial zebra finch AR cDNA (1.4 kb) recently isolated from a phage cDNA library (Perlman et al. 2003). It extends from the DNA-binding domain into the steroid-binding domain. The ER fragment is 100 % homologous to nucleotides 751-1676 of the zebra finch ER cDNA cloned by Jacobs et al. (1996). The probe used for in situ hybridisation encompasses about 60% of the DNA-binding domain and most of the oestrogen-binding domain of the ER.

Based on sequence information available from other species PCR was used to amplify fragments of SPO, SNAP-25 and STX from the zebra finch. The mRNA was prepared from brain tissue by using the Dynabeads mRNA DIRECT kit (Deutsche Dynal GmbH, Hamburg). The synthesis of first-strand cDNA was done with SUPERSCRIPT II Reverse Transcriptase (Life Technologies) and oligo (dT)-primer. The resulting RNA-DNA hybrids were subsequently used in PCR's to generate pieces of the appropriate SPO and SNAP-25 genes. For SPO the forward primer was 5'-TTYGCMTTYGCMACNTGYGG-3' and the reverse primer was 5'-GTYTCYTTRAACACRAAYCA-3'. For SNAP-25 degenerate primers were designed to sequences of exon 2 (forward primer 5'-TGGCNGARGAYGCNGAYATG-3') and exon 7 (reverse primer 5'-CTRTCDATYTGNCGRTTYTG-3', respectively). For STX the forward primer was 5'-TTYGAGCARGTNGARGARAT-3' and the reverse primer was 5'-GCCATRTCCAYRAACATRTC-3'.

PCR was carried out for 40 cycles by using the following parameters: 94°C for 1 minute, 48°C (SPO) or 55°C (SNAP-25) for 2 minutes, 72°C for 2 minutes. Amplified fragments were purified, blunt-ended and cloned into the Sma I site of the plasmid vector pGEM7ZF (Promega, Mannheim, Germany). Resultant clones were sequenced to verify the authenticity and fidelity of the amplification. The cloned SNAP-25 sequence of the zebra finch is around 530 bp in length (GenBank no. AY 531112) and 96 % identical to its chicken counterpart. Two alternatively used exon 5 sequences, 5a and 5b, each 118 bp in

length and around 75 % identical to each other were described in chicken (Bark 1993). In the SNAP-25 sequence, exon 5b is incorporated. Because the sequences of the other exons are in common to both isoforms (SNAP-25a and SNAP-25b), riboprobes prepared from the SNAP-25 sequence should detect both isoforms. The cloned partial SPO sequence of the zebra finch is 560 bp in length (GenBank no. AY 531113) and is 91 % identical to the chicken SPO, also called synaptophysin II (Bixby 1992). The cloned STX sequence is 560 bp in length and is 87 % identical to its human counterpart (Genbank accession no. AY028792).

3.2.3.2. Preparation of RNA probes

For transcription of the antisense or sense RNA probes, the plasmids containing the cDNA sequence were linearized with restriction enzymes and transcribed from the SP6 or T7 promoter, respectively. For the synthesis and labelling of the probes, the linearized template DNA was mixed with 4 µl 5x transcription buffer (Promega), 2 µl 0.1 M dithiothreitol (DTT), 0.5 µl RNasin (Promega), 4 µl of each 2.5 mM ATP, GTP, UTP, 2.4 µl 0.1 mM unlabeled CTP, 37.5 µCi 5'[α-^{35}S] CTP (1250 Ci/mmol, Perkin Elmer Life Sciences GmbH), 0.5 µl SP6 or T7 RNA polymerase. After incubation for 90 min at 37°C the DNA template was removed by digestion with 1 µl DNase I (Promega) for 15 min at 37°C. To remove unincorporated nucleotides, 1 µl yeast tRNA, 7.5 µl 10 M ammonium acetate and 70 µl 100% ethanol were added, the solution was cooled for 30 min at -20°C and centrifuged for 15 min at 13000 rpm. The supernatant was discarded, 900 µl 70 % ethanol was added and centrifuged for 5 min at 13000 rpm. The supernatant was discarded, the RNA pellet was air-dried for 30 min and resuspended in 20 µl 0.1 mM DTT. To estimate the amount of incorporated ^{35}S-CTP, 1 µl of RNA solution was transferred to a szintillation vial, mixed with szintillation fluid and counted in a beta-counter. The RNA solution is then stored at -20°C until use. The sense RNA probes were used as controls in the in situ hybridisation studies.

3.2.3.3. In situ hybridisation

Our protocol followed the procedure described by (Whitfield et al. 1990) with modifications. Air-dried sections were fixed in 4 % FPBS, washed twice in phosphate buffer (PBS), then in 0.1 M triethanolamine-HCL and then in 0.25 % acetic anhydride in 0.1 M triethanolamine-HCL. Sections are then washed twice in 2x sodium chloride-sodium citrate (SSC) and dehydrated in ascending concentrations of ethanol. The ^{35}S-labeled antisense or sense RNA probe (8 x

10^6 cpm/ml) was heat-denaturated in the hybridisation buffer (50 % formamide, 600 mM NaCl, 10 mM Tris-HCL pH 7.5, 0.02 % Ficoll, 0.02 % bovine serum albumin (BSA), 0.02 % polyvinylpyrrolidone, 1 mM EDTA, 0.01 % salmon testicular DNA, 0.05 % total yeast RNA, 0.005 % yeast transfer RNA, 10 % dextran sulphate, 0.1 % sodium dodecyl sulphate, 0.1 % sodium thiosulphate, and 100 mM DTT. On each slide 50 µl (400,000 cpm) of hybridisation fluid were pipetted, covered with a glass coverslip and hybridised for 15 h at 55°C. After hybridisation, the slides were immersed in 2x SSC for 15 min to float off the coverslips. The slides were then treated with RNase A (20 µg/ml) in RNase buffer (0.5 M NaCl, 10 mM Tris-HCL pH 8.0, 1mM EDTA) for 30 min at 37°C and incubated in the same buffer for 30 min at 37°C. The slides were washed in 2x SSC for 30 min at 50°C, in 0.2x SSC for 30 min at 55°C and in 0.2x SSC for 30 min at 60°C, dehydrated in ascending concentrations of ethanol and air-dried.

3.2.3.4. Autoradiography

To detect autoradiographic silver grains, the slides were dipped into Kodak NTB-2 nuclear track emulsion diluted 1:1 with 0.1 % aerosol 22 (Sigma, Taufkirchen, Germany) at 42°C and then exposed at room temperature for 3-14 days. The exposure time varied depending on the RNA probe used and the half-life of ^{35}S (87 days) and had been optimised to give specific labelling with low background labelling. Slides hybridised with the probes for the synaptic proteins SNAP-25 and SPO required an exposure time of 3-5 days, whereas the probes for the oestrogen receptor (ER) were exposed for about 14 days. The slides were developed in Kodak D19 for 2 min, rinsed in water for 30 sec and fixed in Kodak fixer for 5 min at 16°C. After washing the slides in water for 45 min, they were air-dried overnight. The slides were then counterstained with the Nissl stain thionine and examined under the microscope using darkfield illumination.

3.2.4. Radioimmunoassay of steroid hormone levels

The androgens 5α-dihydrotestosterone (DHT) and testosterone (T) and the oestrogen 17ß-oestradiol (E2) were measured by radioimmunoassay (RIA) after extraction and partial purification on diatomaceous earth (celite) micro-columns using a modification of the methods described by (Wingfield & Farner 1975).

3.2.4.1. Reagents

Antisera were obtained from Endocrine Sciences (Tarzana, USA): DT3-351 (DHT), T3-125 (T), E17-94 (E2). Standard steroids were purchased from Sigma

(USA) and tritiated steroids from Perkin Elmer Life Sciences. The assay buffer for androgens and oestradiol was 1.0 M phosphate-buffered saline with 1 % gelatine and 1 % sodium azide, pH 7.0 (PBSG).

3.2.4.2. Extraction of steroids from plasma

Plasma samples (30-100 µl) were transferred to glass extraction tubes and incubated overnight at 4 °C with 1500 dpm each of tritiated DHT, T and E2 in PBSG, to determine extraction efficiency (recovery). Samples were extracted twice with distilled dichloromethane for 2 h at RT. After centrifugation, the organic phase was separated from the aqueous phase by plunging the extraction tube into an ethanol-dry ice bath; the aqueous phase freezes within a few seconds and the organic phase can be decanted into a clean glass tube. The organic phase was then dried under a nitrogen stream in a 40 °C water bath prior to chromatography. The dried extracts were re-dissolved in 0.5 ml of 10 % ethylacetate (EA) in isooctane.

3.2.4.3. Chromatography on celite micro-columns

Columns were prepared by packing 5 ml serological pipettes with 0.5 ml of a celite:water mixture (3:1; weight:volume [w:v]) and 0.5 ml of a celite:propandiol:ethylenglycol mixture (4:1:1, w:v:v). A glass bead was inserted at the bottom of the pipette to prevent leaking of the celite from the columns. The columns were packed with celite:water mixture (“water trap”) first and then with the celite:glycols mixture by using a glass rod. The water trap prevents the exit of the glycols from the columns when high concentrations of polar solvent are used. Columns were mounted on a holder and exposed to a nitrogen stream with constant pressure and washed twice with 2 ml isooctane. The re-suspended samples were loaded on the columns and washed again with 4 ml isooctane. Then steroid hormones were separated on the basis of their polarity by eluting the columns with increasing concentrations of EA in isooctane. DHT was eluted with 10 % EA, T with 20 % EA and E2 with 40 % EA. The fractions were dried under nitrogen, re-dissolved in 300 µl PBSG and kept overnight at 4 °C for equilibration. Aliquots (80 µl) were then transferred to scintillation tubes, mixed with scintillation fluid (Ready Safe, Beckman, USA) and counted to an accuracy of 2-3 % to estimate the recoveries. The residuals were stored at –20 °C until radioimmunoassays were conducted.

3.2.4.4. Radioimmunoassay

With this technique, unknown amounts of plasma steroids compete with known amounts of tritiated steroids for the binding of a known amount of antibody. Concentrations of steroids in plasma samples can be calculated by comparison with a standard curve.

A standard curve was set up by serial dilution of a stock standard solution. Aliquots of the corresponding fractions were transferred in duplicates (2x 100 µl) to glass assay tubes. The antiserum was added to the assay tubes, followed after 30 min by 5000 dpm of labelled hormone (13500 dpm for T). Samples were then incubated for 20 h at 4 °C (25 °C for DHT). Free steroids were separated from the bound fraction by addition of dextran-coated charcoal and centrifugation. The aqueous phase was decanted in scintillation vials, mixed with scintillation fluid and counted to an accuracy of 2 %.

3.2.4.5. Data calculation and quality controls

Standard curves and sample concentrations were calculated with Immunofit 3.0 software (Beckman Inc. Fullerton, USA), using a four parameter logistic curve fit. The lower detection limit of the standard curves was determined as the first value outside the 95 % confidence intervals for the zero-standard (B_{Max}). A summary of the assay parameters is shown in Table 3.2. Lower detection limits for androgens and oestrogens ranged from 0.40 to 1.90 pg / tube. For statistical analyses, non-detectable values were assumed equivalent to these minimum detectable values therefore giving a conservative estimate of hormone levels. Hormone levels (pg/ml) for each sample were adjusted for individual recoveries and the amount of plasma per sample. The average recoveries were between 64 and 81 %. Samples with plasma volume < 30 µl were removed from the data analysis.

Table 3.2: Assay parameters for DHT, T and E2 radioimmunoassays.

Steroid hormone	No. of assays	Mean recovery (%)	Lower detection limit (pg / tube)	Intra-assay CV (%)	Inter-assay CV (%)
DHT	3	80.7	1.90	2.5	4.9
T	3	75.1	1.25	1.7	6.0
E2	3	64.8	0.40	1.5	4.1

4. DUET AND SOLO SONG OF WHITE-BROWED SPARROW WEAVERS

4.1. Introduction

In the northern temperate zone, singing of songbirds is mostly confined to males. It is known that male song functions in territory defence and mate attraction (Catchpole & Slater 1995). In species where females do sing, their song is often shorter and less complex than male song and usually only produced at restricted times of the year (Hoelzel 1986; Baptista et al. 1993). However, there is evidence that female song in these species is not just a by-product of temporarily high levels of testosterone but serves functions as in males (for review, see Langmore 1998).

Among tropical songbirds, female song is commonly found. Whereas only little is known about female solo song, much attention has been paid to the phenomenon of duet singing, which refers to the acoustic interaction between both members of a pair, often occurring with precise temporal coordination (Farabaugh 1982). Duetting is not restricted to oscine songbirds. So far, it has been described for over 200 bird species from 44 families, and oscines account for 55 % of all duetting species (Farabaugh 1982). Duets can consist of simple patterns, such as the exchange of call notes, e.g. in parrots (Wright & Dorin 2001) or can be precisely synchronised, giving the impression that the vocalisation is produced by a single bird (Wickler & Seibt 1980). When male and female contributions occur in a strictly alternated fashion, duets are sung ‘antiphonally’; when both birds produce exactly the same pattern of syllables at the same time duets are sung in unison (Thorpe 1975).

Although duetting is described for a wide variety of taxonomic groups, three characteristics have been found to correlate with duetting: year-round territoriality, prolonged monogamous pair bonds and occurrence in the tropics (Farabaugh 1982). Over the last decades, many different hypotheses have been generated to understand why some bird species coordinate their songs to form duets while the majority of species does not. For many of the hypotheses, evidence from descriptive and experimental data is insufficient. In a recent review (Hall 2004) identified the most promising hypotheses to explain the functions of duets, which are mate guarding by both sexes, joint resource defence and signalling commitment.

Duetting styles can vary tremendously from one species to another. There are four major characteristics, which could be used to describe and classify duets of most species: 1) relative participation of the male and female in duet singing, 2) temporal pattern of sounds, i.e. are songs completely or partially overlapping

or antiphonal, 3) precision of timing, i.e. variability of the interval between mates' syllables, 4) complexity of duet organisation (Farabaugh 1982; Farabaugh 1983). Concerning the last characteristic, (Helversen 1980) described three levels of complexity. At the lowest level, mates exchange simple call notes as in D'Arnaud's barbet (*Thrachyphonus d'arnaudii*) (Wickler & Uhrig 1969), the spectacled weaver (*Ploceus ocularis*) (Skead 1953) or the Aldabra white-throated rail (*Dryolimnas cuvieri aldabranus*) (Huxley & Wilkinson 1979). At the second level, one bird has a repertoire of songs, which is combined with a single sound of the mate as in the gonolek (*Laniarius barbarus*) (Grimes 1965), the black-headed gonolek (*Laniarius erythrogaster)* (Hooker & Hooker 1969) or the white-browed robin chat (*Cossypha heuglini*) (Wickler 1974; Todt et al. 1982). At the highest level, both partners have large repertoires of songs and combine them to complex duets as in the Slate-coloured bou-bou shrike (*Laniarius funebris*) (Wickler 1972), the African Drongo (*Dicrurus adsimilis*) (Helversen & Wickler 1971), the Spotted Morning Warbler (*Cichladusa guttata*) (Todt & Fiebelkorn 1980), the Buff-breasted wren (*Thryothorus leucotis*) (Farabaugh 1983) or the Plain wren (*Thryothorus modestus*) (Mann et al. 2003).

The white-browed sparrow weaver (*Plocepasser mahali*) is a member of the family Ploceidae (Fry & Keith 2004) that is primarily composed of non-duetting species. So far, in this family duetting has been described only for two other species belonging to the genus *Ploceus*, the African forest weaver (*Ploceus bicolor*) (Wickler & Seibt 1980; Seibt et al. 2002) and the Spectacled weaver (*Ploceus ocularis*) (Skead 1953; Kunkel 1974). Song behaviour of the white-browed sparrow weaver is characterised by the complex solo song of the dominant male of a colony and by duet songs produced mainly by the dominant pair. Additionally, all colony members engage in chorus singing, which in structure resembles duet songs. Vocalisations of this species have first been described by Ferguson (1988a). He distinguished nine different call types, including the duet song (= territorial call) and the male solo song (= mating song) and recognised those as complex songs in contrast to the other call types. Wingfield & Lewis (1993) observed the male solo song and the 'highly synchronised and complex' chorus vocalisations when they studied hormonal and behavioural responses to simulated territorial intrusions. Both studies describe the male solo song as occurring seasonally in the context of breeding whereas duet and chorus songs occurred throughout the year and were associated with territorial aggression. From their observations, the authors conclude further that each group member could produce the entire duet or chorus song alone, and when birds were singing together, different members of the group were singing different sections of the song.

The aim of the present study is to investigate the neural basis of song production in males and females. Thus, the focus of this chapter is on the type of song produced by both sexes and their repertoire organisation. I collected repertoires of duet song and male solo song from dominant individuals of colonies in Zimbabwe. An important aspect thereby was to obtain song repertoires from the same birds that were later on used for the characterisation of the song control system in order to establish a relationship between those parameters. However, song recordings from the field did not provide sufficient information about the exact repertoire sizes of males and females. To this end, white-browed sparrow weavers were studied in captivity at our institute in Seewiesen. From investigations on six captive males, I was able to determine total repertoire sizes of the solo song and to compare these data to those obtained in Zimbabwe. In addition, I conducted a more detailed study of the structure and organisation of duet songs on three captive pairs focusing on the relative participation of both sexes in duets, on the temporal pattern and on the repertoire organisation. From these data, I could assess total repertoire sizes of males and females recorded in Zimbabwe and furthermore, I could make predictions about the repertoire size of subordinate males.

4.2. Methods

4.2.1. Song recordings from Zimbabwe

All vocalisations were recorded with a Sony TCD-5M portable cassette recorder (Sony Corp., Tokyo, Japan) equipped with a Sennheiser ME-88 directional microphone (Sennheiser electronic, Wedemark, Germany). All birds used for song analyses were previously captured and colour-ringed and their social status was known. For details on capture and identification of social status, see chapter 3.1. Recordings were obtained within a distance of two to ten meters from the bird.

4.2.1.1. Recordings of the solo song of dominant males

Solo songs of eight dominant males were recorded between February 6 and March 10, 2000. These males belonged to seven colonies; from one colony (No. 10), two dominant males were sampled. In this case, during the observation period, two different males of that colony were repeatedly observed singing the solo song on different days. The colony consisted only of two males and one female and it could be that there was a conflict about who holds the dominant position.

Each male was recorded once in the morning between 5:00 and 5:45. In previous observations, I determined the approximate starting time of the solo song, which was generally coincident with or slightly before first light. After three to ten days following song recording, these males were recaptured and sacrificed for neuroanatomical analysis. In addition, recordings of solo songs were obtained from eight dominant males during the field seasons 1999 to 2001 which were not used for neuroanatomical analysis.

4.2.1.2. Recordings of duet songs of dominant pairs

Duet songs were recorded from dominant pairs of eight different colonies between February 6 and March 10, 2000. These birds were sacrificed for neuroanatomical analysis. However, from one colony the dominant female could not be obtained. Therefore, one female was collected during the following field season, in February 2001, and duet songs from this colony were recorded. All duet recordings were made between 5:45 and 19:00. From most colonies, recordings of 50 to 60 duet songs could be obtained. From two colonies, only 10 and 34 duet songs respectively were available. The difference in number of duet songs sampled was due to birds being observed for a variable number of time and due to differences in song rates between colonies and between different days.

4.2.1.3. Song analysis

Sonagraphic analysis was done on a Macintosh Power Book G4 (Apple Computer Inc.) equipped with the sound analysis software Canary 1.2.1 (Cornell Laboratory of Ornithology, Ithaca, NY, USA). Sonagraphic printouts were generated with a laser printer Tektronix Phaser 840. For sound acquisition, songs were digitised with a sample rate of 22 kHz and a sample size of 16 bits. For frequency and time resolution of spectrograms, filter bandwidth was set at 342 Hz, with a frame length of 256 points and a grid resolution of 5.8 ms.

Song quantification was performed on spectrograms by visual inspection. Spectrograms of syllables used by each bird were sorted according to its structure by two independent observers. Each syllable in a bird's repertoire was assigned a type number by comparing it to a cumulative library of syllable types. Discriminating syllables of the solo song was not difficult because variation between different types always exceeded intra-type variability. Discrimination between syllable types of duet song was slightly more difficult because different types sometimes graded into others. Nonetheless, inter-observer reliability was high.

For duet songs recorded in the field, it was not possible to distinguish between vocalisations of the male and the female. Consequently, I could not assign syllable repertoires to individual birds. Therefore, the duet syllable repertoire constitutes the number of different syllable types sung by the dominant pair of a given colony. For each duet, song length and total number of syllables were determined and the number of syllables per second was calculated.

4.2.2. Song recordings from birds in captivity

In March 2000, ten male and ten female white-browed sparrow weavers were captured near our study site in Zimbabwe and imported to Germany. During the following years, the birds were kept pairwise in aviaries at the Max-Planck-Institute in Seewiesen. All pairs built roosting and breeding nests in the outdoor compartment of the aviaries. From 2001 to 2004, pairs reproduced successfully. Offspring were separated from their parents when six to eight months old. They were then kept singly in indoor aviaries with visual and acoustic contact.

4.2.2.1. Recordings of the solo song of dominant males

In 2001 and 2004, solo songs of in total six captive males were recorded. Between April and September, depending on the pairs' breeding activities, males were singing at dawn. Recordings were made close to the outdoor aviaries within five meters from the roosting nest using a Sony TCD-5M portable cassette recorder (Sony Corp., Tokyo, Japan) equipped with a Sennheiser ME-88 directional microphone (Sennheiser electronic, Wedemark, Germany). For each male, the solo song was recorded three times. Recordings were made in August 2001 from one male and in May/June 2004 from five males.

4.2.2.2. Recordings of duet songs from pairs

For detailed analysis of duet singing, three pairs were recorded in cages (84 x 40 x 54 cm) in a soundproof room using a Sony TCD-5M portable cassette recorder equipped with two Sennheiser ME-67 directional microphones. Males and females of each pair were housed in separate cages with visual contact and in front of each cage a microphone was installed that was surrounded by foamed material to further reduce reverberation. Both microphones were two meters apart. The female track was recorded into the right channel and the male track into the left channel of the cassette recorder. Recording levels were kept identical for both channels. Recordings were made on different days between 10:00 and 18:00 over a period of four to six weeks. To stimulate singing,

another pair was kept in cages in the same room but without visual contact to the pair, which was recorded. The different parameters of the three pairs from which recordings were obtained, are summarised in Table 4.1.

Table 4.1: Parameters of the pairs used for detailed analysis of duet song in captivity.

	Pair No. 1	Pair No. 2	Pair No. 3
Birth	♀: 12.08.2001	♀: 14.04.2002	caught as adult
	♂: 08.06.2001	♂: 03.07.2001	caught as adult
Mating experience prior to recording	no	♀: no ♂: yes	yes
In the recording room since	23.04.2002	23.03.2003	15.03.2003
Recording session	21.05. – 10.07. 2002	09.05. – 18.06. 2003	01.04. – 08.05. 2003
Pair singing in the background	no	yes	yes

4.2.2.3. Song analysis

Sonagraphic analysis was done on a Macintosh Power Book G4 (Apple Computer Inc.) equipped with the sound analysis software Canary 1.2.1 (Cornell Laboratory of Ornithology, Ithaca, NY, USA). Sonagraphic printouts were generated with a laser printer Tektronix Phaser 840. For sound acquisition, songs were digitised with a sample rate of 22 kHz and a sample size of 16 bits. For the frequency and time resolution of sonograms, filter bandwidth was set at 342 Hz, with a frame length of 256 points and a grid resolution of 5.8 ms.

For the solo song, quantification of syllable types was performed as described in section 4.2.1.3. For each recording session, a separate library of syllable types was constructed. Additionally, syllable types were compared to a cumulative library constructed from all three recording sessions to determine the increase in repertoire size between consecutive recordings and to estimate total repertoire size. Furthermore, song length was measured from each recording.
For duet songs, male and female recordings were analysed as separate tracks in sonograms and spectrograms. Printouts of spectrograms were generated displaying the male track in the upper pane and the female track in the lower pane. For *Pair No. 1,* discriminations in sorting male and female syllables were not difficult because both birds, although vocalising simultaneously, sang different syllable types and no syllables in unison. From these recordings, I

generated a cumulative library of syllable types for each bird and I determined the proportion of syllable types shared. For *Pairs No. 2 + 3*, discriminations between male and female syllable types were more difficult because these pairs were vocalising in duet bouts and some syllables were sung in unison. Although male and female vocalisations were recorded with different microphones into two separate channels, it was not possible to prevent that a signal recorded in one track always occurred, at lower amplitude, in the second track.

To clearly distinguish between syllables sung by one individual and those sung in unison, I measured the difference in average intensity (dB) between the selected syllable on track 1 and the same syllable appearing on track 2. The outline of a syllable was selected with cursors and intensity measurements were performed with the built-in function of the software (Fig. 4.1). In harmonic syllables, I measured the intensity at the fundamental frequency only. Syllables sung by a single bird on track 1 appeared on track 2 at half the intensity of track 1. Syllables with similar intensity values on both tracks were clearly sung by both birds in unison. When the difference in intensity for a selected syllable was less than two-fold between both tracks, I inspected the composition of the syllable in more detail. Intensity measurements and stereo playback at different speeds then revealed that parts of the syllable were sung in unison and other parts produced by a single bird. In these cases, I considered the syllable as sung by both birds. The following parameters of the duet songs were measured: total number of different syllables observed, number of different syllables sung by each sex constituting the bird's duet repertoire size, number of syllable types shared. Per individual duet bout, I determined the bout length, the total number of syllables, the proportion of syllables sung by each sex and the proportion of syllables sung in unison.

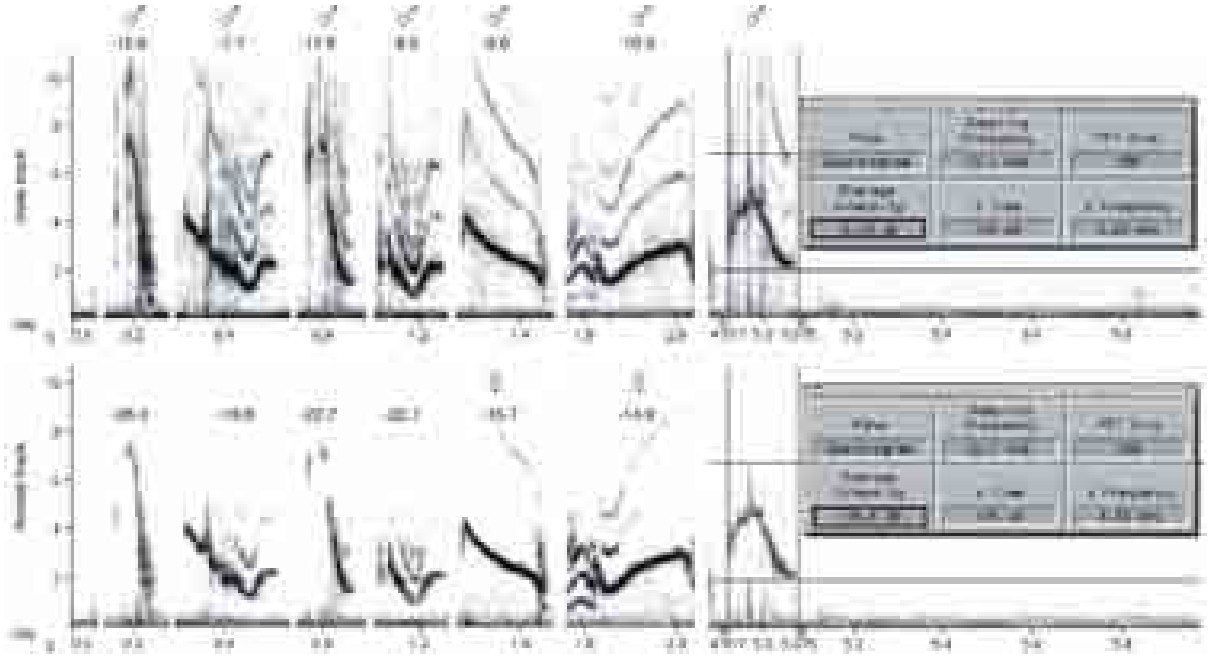


Fig. 4.1: Measurement of the average intensity of a selected syllable to identify if it was sung by a single bird or in unison. These measurements are shown for seven different syllables. For the last syllable, the procedure done with the software is outlined. In this example, the male sings all syllables and the female participates in two syllables (see text for details).

4.2.3. Statistical analysis

Statistical analyses were done with Systat 10.2. and Prism 3.0 (GraphPad Inc). All data were analysed by non-parametric statistics. Song length and repertoire size of the solo song was compared between males from Zimbabwe and those recorded in Seewiesen with the Mann-Whitney U test. Relationships between song length and repertoire size were analysed with Spearman correlation. The Friedman analysis of variance was used to analyse the differences in repertoire size of solo and duet song respectively between different recording sessions. Posthoc comparisons were performed as described by (Siegel & Castellan 1988). All tests were two-tailed and the significance level was fixed at $\alpha = 0.05$.

4.3. Results

4.3.1. General description of vocal behaviour

Vocalisations of white-browed sparrow weavers can be classified into solo songs, duets, chorus songs and calls. In this study, I focus on solo and duet songs. Data on these behaviours are quantified in detail in the sections described below (4.3.2, 4.3.3).

From field observations it was derived that solo songs were performed exclusively by the dominant male in the colony, duet songs were mainly performed by the dominant pair, whereas all colony members engaged in chorus singing and emitted calls. Solo songs were only sung once a day at dawn. Duet songs were performed throughout the day. Duets could be initiated by either sex and were not strictly antiphonally sung but some syllables were sung in unison. The structure of chorus songs resembled that of duets except that more than two birds participated. This type of song was frequently heard during aggressive encounters with neighbouring colonies. On a diurnal basis during the breeding season, song behaviour of a colony had the following pattern: At dawn, the dominant male started singing the solo song while still in the roosting nest. This song continued for up to 20 minutes with several pauses of various lengths. During singing, the male mostly left the nest and continued to sing from different trees within the territory. After the dominant female had left the nest, the pair would begin to duet. The frequency of duet singing seemed to be highest during the first hour in the morning. During the day, bouts of duet and chorus singing alternated with foraging and resting periods. At dusk, the dominant pair sang a series of duets while the other colony members already entered the roosting nests. The dominant male was the last bird entering the roosting nest, after 5 to 10 minutes of continuous calling.

4.3.2. Solo songs of dominant males

4.3.2.1. Terminology of the solo song

The solo song consists of a series of consecutive syllables either sung as single syllables or in phrases, interrupted by pauses, which can last up to one minute. Song structure is shown in spectrograms (Fig. 4.2), which plot the frequency (kHz) on the vertical axis against time (s) on the horizontal (the amplitude of a given signal is represented by a greyscale value between white and black). The smallest unit in a spectrogram is the song element. Syllables can consist of one or more elements. Song phrases were defined as combinations of same or

different syllable types where at least one type is repeated several times and contains usually no pauses longer than 200 ms. The length of the solo song is the time interval between the first syllable sung by the male and the last syllable before it started duetting with the female. The frequency range of the solo song comprises about 10 kHz, ranging from 0.5 to 10.5 kHz. A sequence of 90 seconds from a typical solo song is shown in Fig. 4.5.

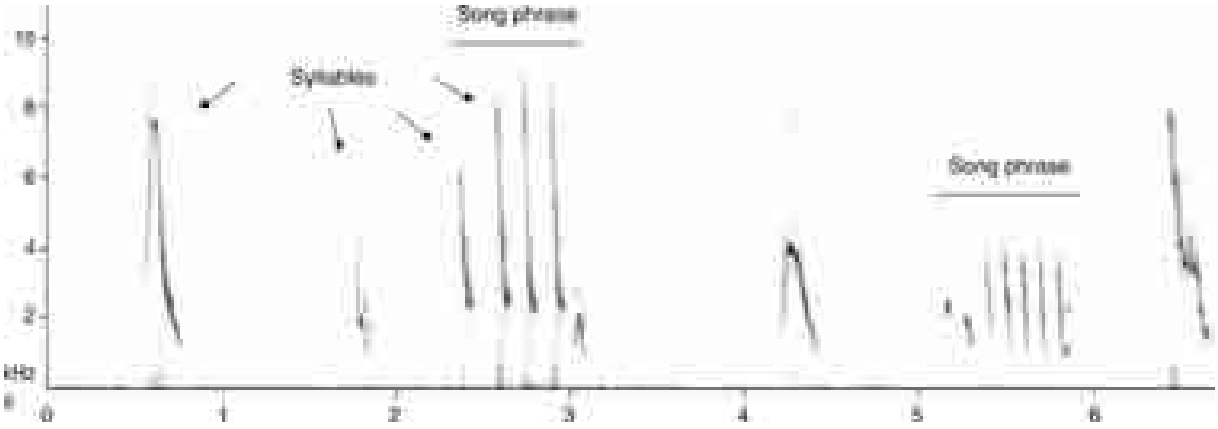


Fig. 4.2: Spectrogram showing a section out of the male solo song. A spectrogram represents the frequency composition of a signal over the time. The solo song consists of a series of syllables either sung as single syllables or combined in phrases. Arrows indicate four different syllable types.

4.3.2.2. Repertoire size of males from Zimbabwe

Solo songs from in total 16 dominant males were obtained during the field seasons 1999 to 2001. Song lengths and repertoire sizes are shown in Table 4.2. For each male, the data represent the performance of the song on a single morning. The eight males used for neuroanatomical analysis of the song control system were all sampled in 2000 and their observed repertoire sizes are shown in the upper part of Table 4.2. For male 77 the song sample (200 seconds) obtained was incomplete, therefore its repertoire size might be underestimated. Mean repertoire size across all males was 66.6 ± 3.7 different syllable types. There was large variation between males, ranging from 48 to 92 syllable types. Also, the length of the song differed greatly between males. Mean song length was 666.9 ± 68.6 seconds. However, there was no correlation between song length and repertoire size (r_s = 0.29, p = 0.28, N = 16). Males singing longer solo songs did not have larger repertoires. As the measurements of song length include all pauses a male makes between phrases, it could be that males that sang longer songs also made pauses of longer duration.

Table 4.2: Observed repertoire size from a single performance of the solo song of dominant males in Zimbabwe. The upper part contains the males used for neuroanatomical analysis.

male ID	Song length (s)	No. of different syllable types
28	370	52
29	940	58
40	1130	72
64	620	77
67	1050	92
69	810	48
77	200	60
78	800	92
1	530	67
3	1010	84
9	410	51
36	540	54
89	810	49
C	510	58
MRS1	520	78
MRS2	420	73

Curves of the number of new syllable types accumulated over time (in 10 second intervals) for each male are depicted in Fig. 4.3. For clearness, the data were split into two figures, showing the eight males sampled in 2000 on the left side and all other males on the right side.

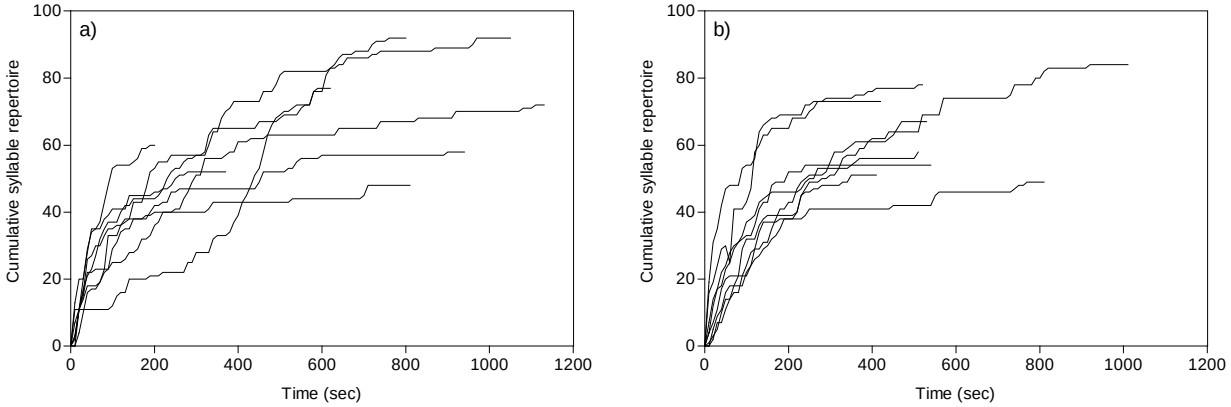


Fig. 4.3: Syllable type accumulation curves for the solo song of 16 males from Zimbabwe. In a) the data for the eight males which were used for analysis of the song system are plotted. Data of all other males are shown in b). One male made large pauses in the beginning of the song, which gave a less steep increase in the first part of the curve. For one bird, the sample obtained was incomplete (male 77). In this case, the curve did not become asymptotic. For all other birds, the asymptotic shape suggests that the male sings the majority of its syllable repertoire in a single performance.

The curves reach an asymptotic shape when per unit of time no or only few new syllable types are sung. For all males, (except for male 77) curves become eventually asymptotic suggesting that a single performance of the solo song contains the majority of the birds' syllable repertoire. The shape of the curve for one bird was exceptional (Fig. 4.3a). In this case, the male made large pauses in the beginning of its song.

4.3.2.3. Repertoire size of males in captivity

More accurate estimates of absolute repertoire sizes of the solo song could be obtained from six captive males, which were each sampled on three different days (Table 4.3). Mean length of the solo song per individual performance averaged over all males was 1669.4 ± 234.0 seconds. Compared to the males recorded in Zimbabwe, captive males sang significantly longer solo songs (U = 0.0, p = 0.0005). However, this was not reflected in repertoire size. Mean number of different syllable types sung by captive males per individual performance was 58.28 ± 3.71, which was not significantly different from males sampled in the field (U = 63.0, p = 0.269). Also, similar to males in the field, repertoire size was not significantly associated with song length in captive males (r_s = -0.029, p = 1.00). For the six males, the cumulative syllable repertoire of three consecutive recordings and the number of different syllables sung in each recording session are shown in Table 4.3. By using the Friedman test the cumulative repertoire size was compared between recording sessions 1 to 3. The Friedman test revealed a significant effect of the recording session on repertoire size (F = 11.47, p = 0.0001). Multiple comparisons showed that there was a significant increase in repertoire size between the first and the second recording session but not between the second and third session (Fig. 4.4).

Table 4.3: Syllable repertoire of the solo song from six captive males obtained on three consecutive days.

Male ID	Cumulative syllable repertoire			Syllable repertoire per individual performance			Syllable repertoire per individual performance in % of total repertoire			Mean (%)
	day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3	
1	82	88	88	82	74	67	93.2	84.1	76.1	84.5
2	60	65	65	60	57	56	92.3	87.7	86.2	88.7
3	61	66	67	61	56	61	91.0	83.6	91.1	88.6
4	52	54	54	52	45	42	96.3	83.3	77.8	85.8
5	57	65	65	57	54	58	87.7	83.1	89.2	86.7
6	56	58	58	56	54	57	96.6	93.1	98.3	96.0

Therefore, minimal two recording sessions reveal the complete repertoire of a male's solo song. To find out the proportion of the total repertoire the males sings per single performance, I calculated the percentage of the repertoire obtained on each day with respect to the total repertoire (cumulative syllable repertoire on day 3, Table 4.3). In a single performance of the solo song, a male sings 88.4 ± 4.1 % of its total syllable repertoire.

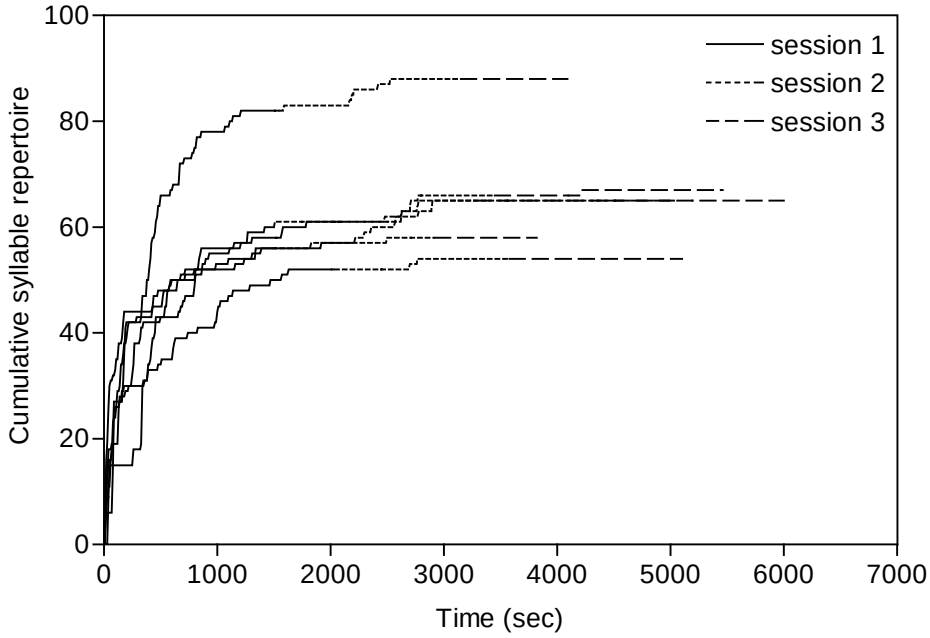
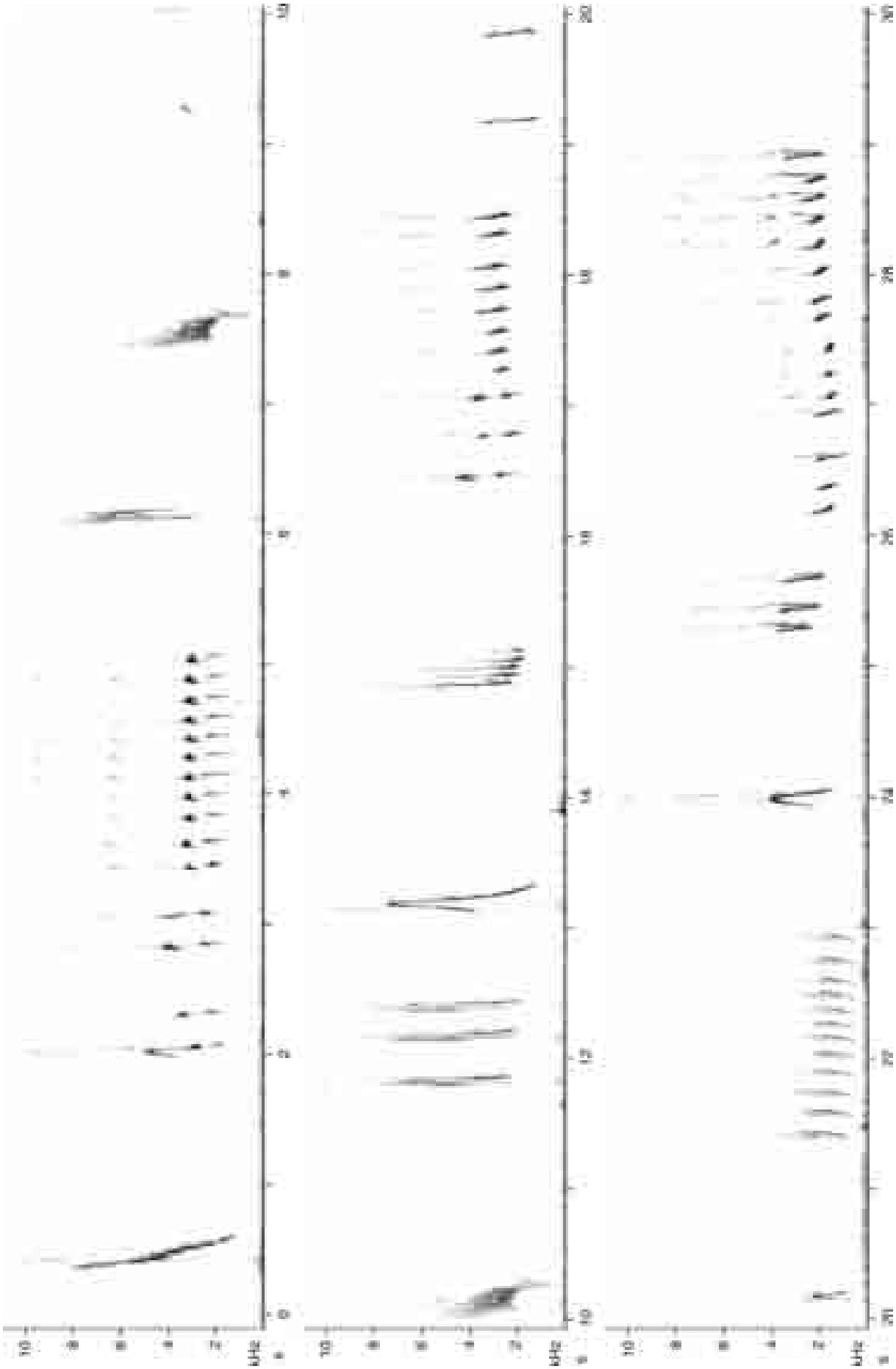
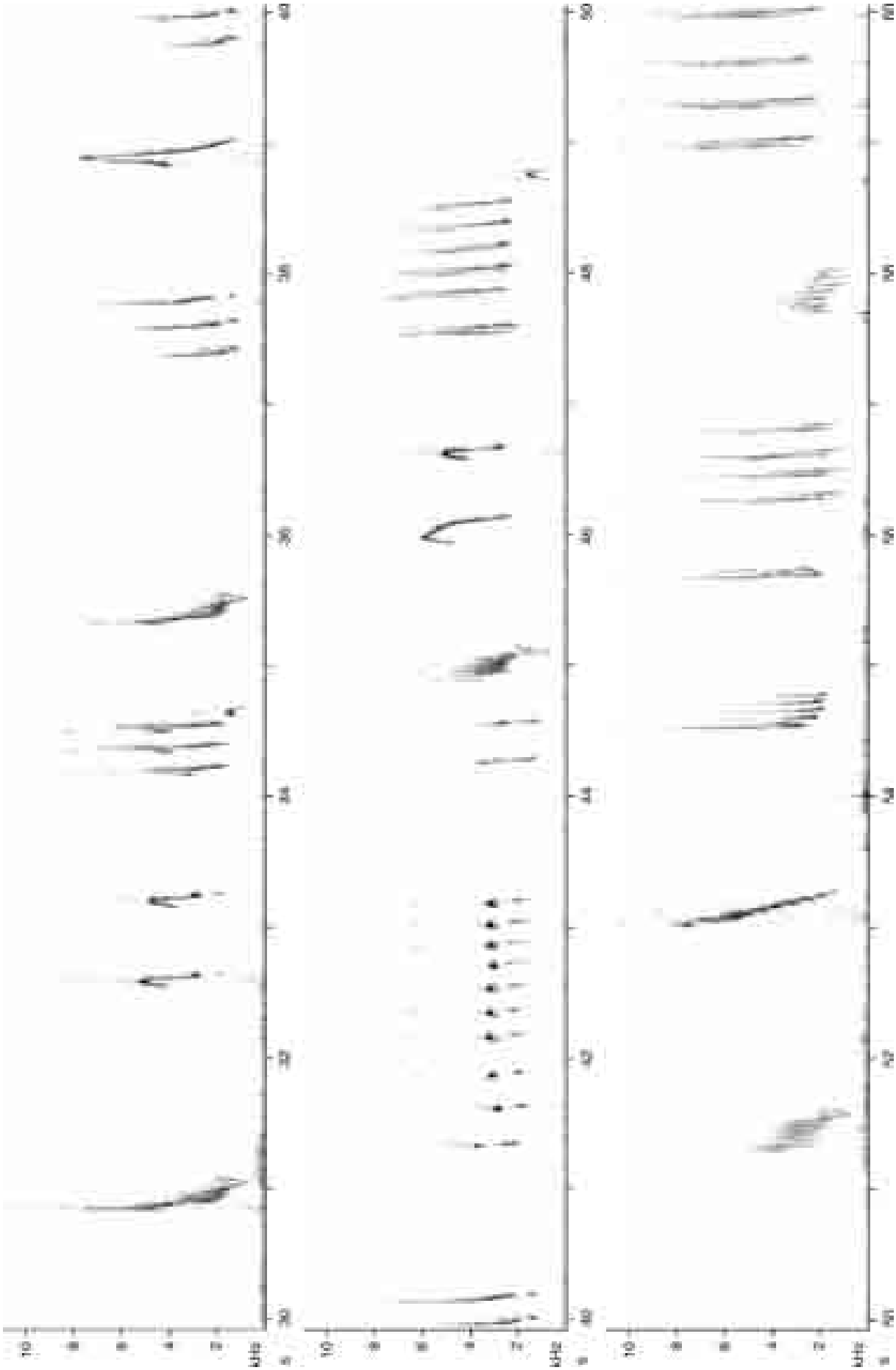
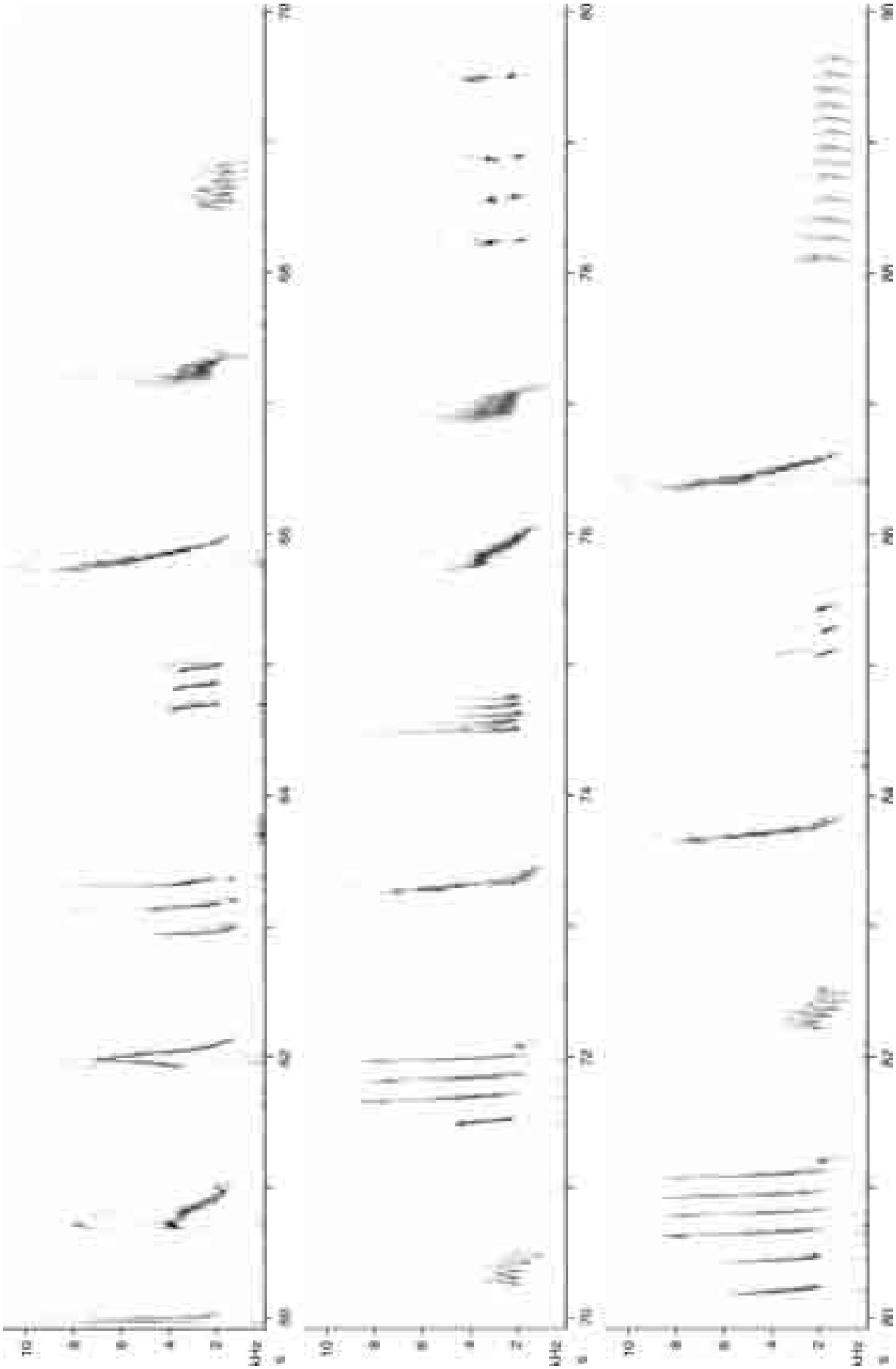


Fig. 4.4: Cumulative syllable repertoires of the solo song from six captive males recorded on three consecutive days. The different patterns indicate the different recording sessions. Repertoire sizes significantly increased from session 1 to session 2 but not from session 2 to session 3 (ANOVA followed by Tukey's HSD test). Therefore, on average, two recording sessions are necessary to reveal the male's solo song repertoire.

Fig. 4.5: (next pages): A sequence of 90 seconds out of the solo song from a dominant male recorded in Zimbabwe. The song consists of single syllables and syllables occurring in repetitions and is interrupted by pauses of various lengths. Mean song length of males in Zimbabwe was 666.9 ± 68.6 seconds.







4.3.3. Duet songs of dominant pairs

4.3.3.1. Terminology of the duet song

Duets are defined as overlapping bouts of sounds produced by both members of a pair (Farabaugh 1982). A duet bout of a pair consists of a series of very rapidly sung syllables in a continuous, precisely timed fashion and lasts usually 2 to 4 seconds (Fig. 4.6). Syllables are produced antiphonally or in unison. Often a duet starts with a harsh buzz (type 1, Fig. 4.6a), performed by either sex. Alternatively, duets can start with 1 to 3 introductory syllables (plain numbers, Fig. 4.6b). During the duet, usually two high frequency-modulated syllable types (indicated by numbers in squares, Fig. 4.6a, b) are alternated with a low frequency-modulated whistle-like syllable type, which is often of harmonic structure (indicated by numbers in circles, Fig. 4.6a, b) and mostly sung by the female (personal observation). This pattern is repeated several times within a duet bout. A single bird was never observed to produce a complete duet alone. The duet song comprises a frequency range of 0.5 to 9.5 kHz.

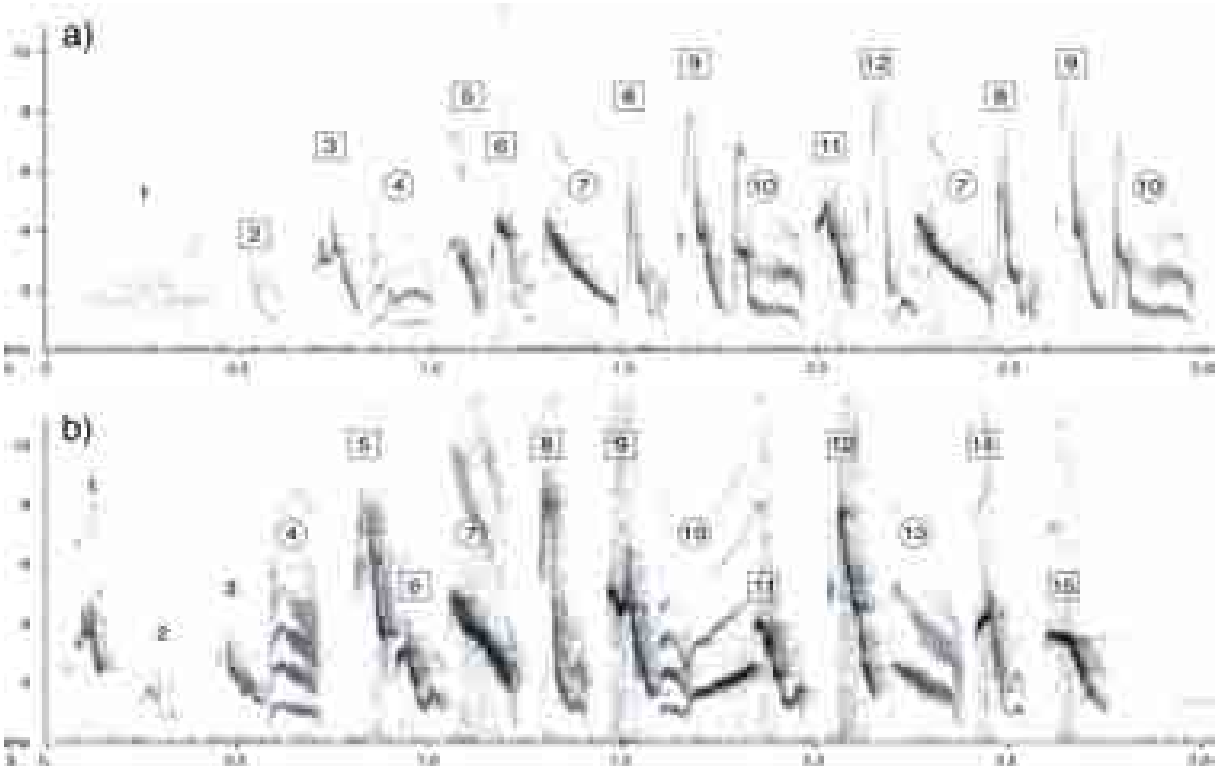


Fig. 4.6: Spectrogram showing two duet bouts of a dominant pair from Zimbabwe. The different syllable types are numbered. Duets are initiated by either sex. Typically, after a harsh buzz (type 1 in **a**) or 1 to 3 introductory syllables (types 1 to 3 in **b**), two frequency-modulated syllable types (numbers in squares) are alternately produced with a whistle-like syllable type (circled numbers) and this pattern is repeated several times. In **a**, types 7 to 10 are repeated once, in **b**, all types are different.

4.3.3.2. Repertoire size of pairs from Zimbabwe

Duet songs from dominant pairs of nine colonies were obtained. This sample covers all male and female individuals, which were used for neuroanatomical analysis. From most colonies, 50 to 60 duet bouts could be recorded, except for colonies 15 and 19 (Table 4.4). Mean song bout length was 2.85 ± 0.28 seconds and birds sang 5.00 ± 0.28 syllables per second. Mean duet repertoire size of dominant pairs was 51.2 ± 2.0 different syllable types, ranging from 45 to 61 syllable types. The curves of the cumulative number of new syllable types versus the number of duet bouts observed are depicted in Fig. 4.7. All curves (except for colony 19) reach the horizontal, indicating that sample sizes were sufficient to approach the probable limit of a colony's duet repertoire.

Table 4.4: Observed repertoire size and song parameters of duets from dominant pairs of nine colonies.

Colony no.	No. of duet bouts recorded	Observed repertoire size	Mean bout length (s)	No. of syllables per bout	No. of syllables per second
1	56	61	3.40	17.0	5.01
2	58	45	2.45	11.9	4.71
9	50	52	2.79	14.6	5.33
10	50	47	2.93	14.9	5.06
12	50	53	2.58	12.1	4.72
15	34	47	2.84	15.6	5.44
16	63	60	2.69	13.0	4.71
17	60	50	3.07	14.6	4.82
19	10	46	2.88	14.9	5.22

The Friedman test was used to investigate the increase in cumulative repertoire size as function of the number of duet bouts recorded. I compared the repertoire sizes at five sampling points, i.e. when 10, 20, 30, 40 and 50 duet bouts were recorded. Data from the two colonies with small sample sizes (10 and 34 duet bouts recorded) were not included in the analysis. Fifty duets was the maximum sample size, which could be obtained from most pairs. From four pairs more than 50 duets were recorded. In one of these, repertoire size increased further by a single syllable type; in three others, it remained constant (Fig. 4.7). The Friedman test revealed a significant influence of the number of duet bouts recorded on repertoire size ($F = 27.27$; $p < 0.0001$). Posthoc comparisons showed that repertoire sizes obtained after 10 and 20 song recordings were significantly smaller than repertoire sizes obtained after 50 recordings ($p < 0.05$). Repertoire sizes obtained after 30 and 40 song recordings were not significantly different from those at sampling point 50 ($p > 0.05$). This shows

that a minimum of 30 duet bouts reveals the total repertoire of a pair. I conclude that the observed repertoire size (Table 4.4, except for colony 19) represents the total repertoire size of these pairs.

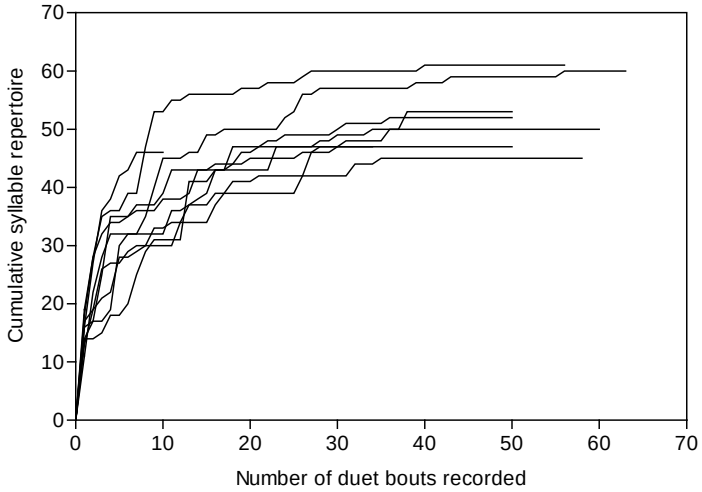


Fig. 4.7: Syllable type accumulation curves for duet song of nine pairs from Zimbabwe. For all but one colony (No. 19, see Table 4.4), the curves become asymptotic showing that the total repertoire size is approached.

For each colony, the library of syllable types of the male's solo song was compared with that of the duet song. None of the syllable types were found to match in structure. This indicates that males possess two distinct syllable repertoires. A sample of 20 syllable types from each repertoire of a male is depicted in Fig. 4.8.

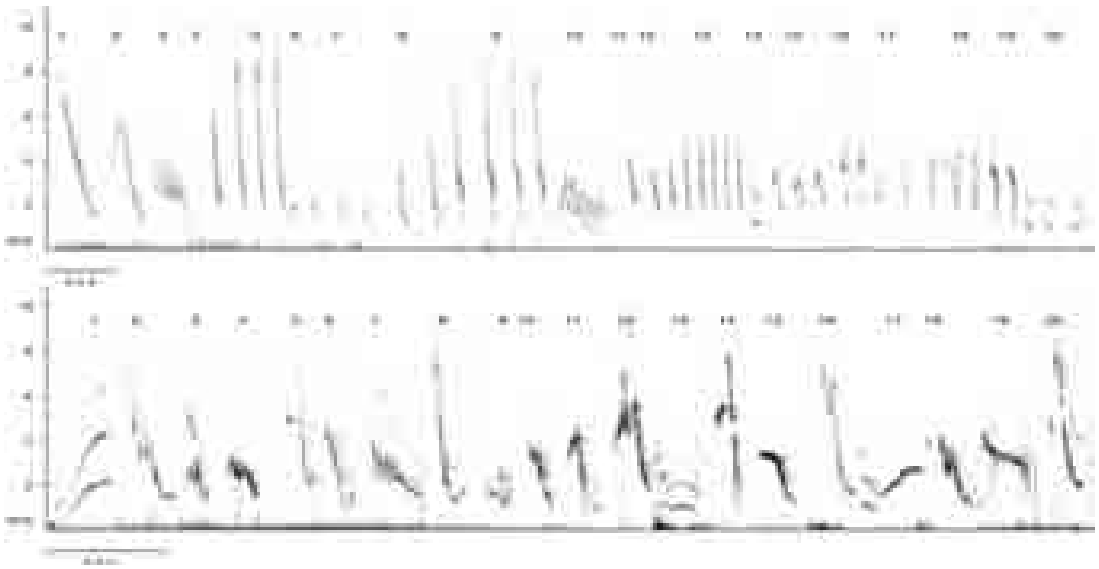


Fig. 4.8: A sample of 20 syllable types from a male's solo and duet song repertoire. Different types are numbered. Syllable types from both repertoires are all different; none did match in structure. Note, most syllables of solo song occur in repetitions whereas duets consist of single syllables. Time scales are different.

4.3.3.3. Duet repertoire size of individuals estimated from three pairs in captivity

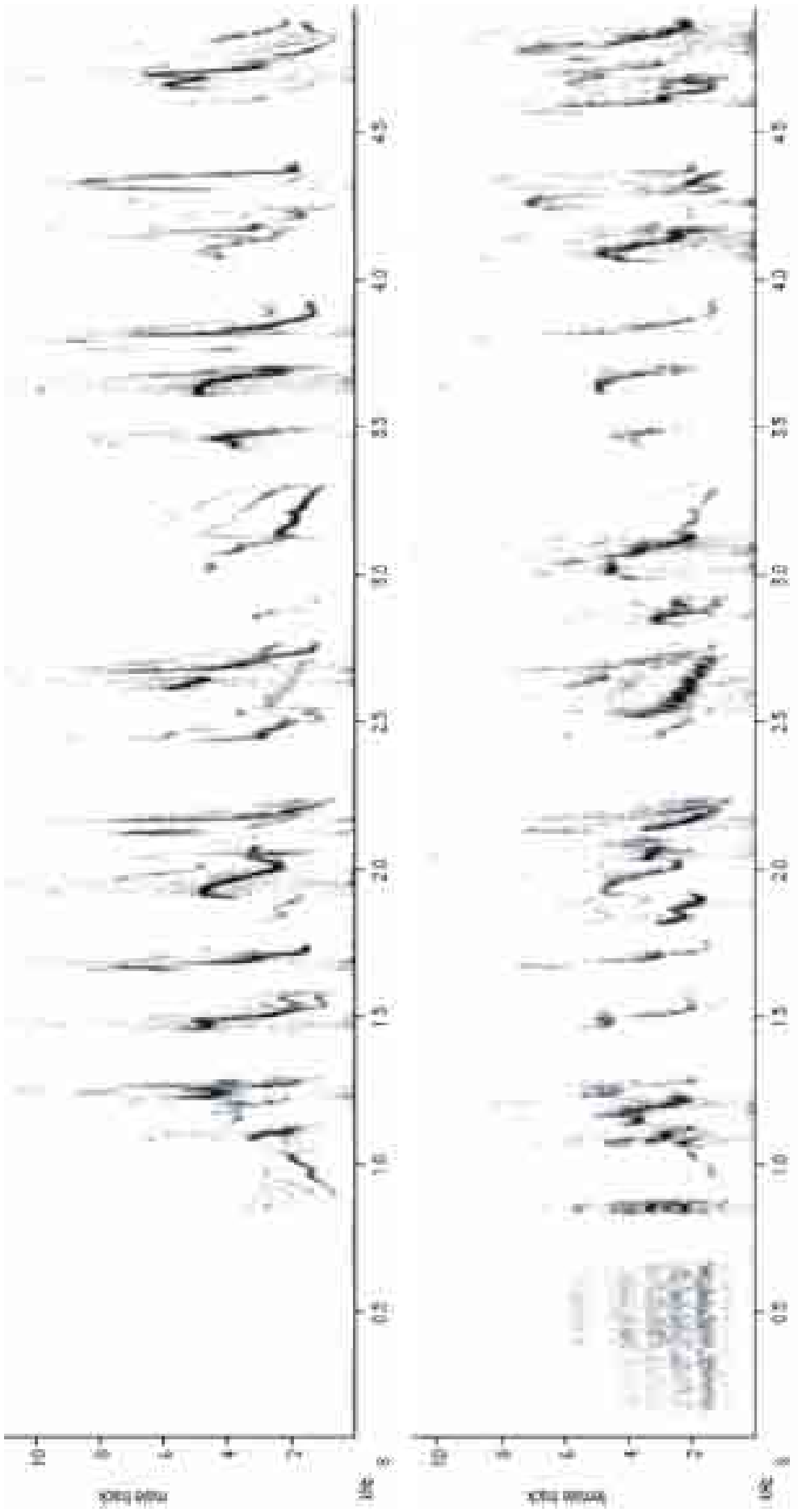
In the previous section, I have presented data on total duet repertoire size of dominant pairs, because from field recordings it was impossible to distinguish between syllables sung by the male and those sung by the female. The whistle-like syllable types (Fig. 4.6) were an exception. Based on observations, I could assign these syllables to the female. The focus of my studies with captive birds therefore was to estimate duet repertoire size of individual birds and to clarify how each sex contributes to duet singing. To this end, I studied song behaviour of three pairs in more detail. The following data are descriptive only because sample size was not sufficient for statistical testing.

Pair No. 1 consisted of individuals, which were raised in captivity. They were separated from their parents when seven (♀), respectively nine (♂) months old and then kept for two months in acoustic and visual contact with other males and females until recording sessions started (see Table 4.1 for details on recording set-up). At beginning of the recording, the male was 11 and the female 9 months old. During recordings, both sexes were continuously and simultaneously vocalising but they did not sing in precisely timed duet bouts as described for pairs from Zimbabwe (section 4.3.3.1.) and no syllables were sung in unison. Therefore, it was not difficult to discriminate between male and female syllables (Fig. 4.9.). The number of different syllable types categorised as duet syllables was found to be nearly identical in both sexes and surprisingly was almost two times as high as the mean number of duet syllables found in colonies in Zimbabwe (Table 4.5). Furthermore, I found that 72 syllables were shared between both birds, representing 75 % of the male's and 83 % of the female's duet repertoire (Table 4.5).

Table 4.5: Song parameters analysed in a pair of white-browed sparrow weavers in captivity.

Song parameters	Male	Female
Seconds of song analysed	4230	4230
Repertoire size of duet syllables	96	87
Shared repertoire size of duet syllables	72	72
Percent of the bird's duet repertoire shared	75	83

Fig. 4.9: (next page): Sequence from song recordings of *Pair no. 1*. Duet bouts were not coordinated or precisely timed and lacked the structure described for pairs from Zimbabwe (section 4.3.3.1.). Both sexes sang no syllables in unison, which facilitated sorting male and female syllable types.



Pair No. 2 consisted of individuals which were raised in captivity and separated from their parents when six (♀), respectively eight (♂) months old. They were then kept in acoustic and visual contact with other males and females until recording sessions started (Table 4.1). During this time, the male was paired for two months with a female of the same age. However, this pair did not persist because of the death of the female and the male was then kept singly for the rest of the time. At the beginning of the recording period, the male was transferred together with a new female into the recording room. At this time, the male was 20 and the female was 11 months old. During recording, the pair was vocalising in duet bouts with syllables sung antiphonally and some syllables sung in unison. However, most of the duets were not exactly timed and were often incomplete in structure, i.e. the whistle-like syllable types, which usually occur several times within a duet and are mostly sung by females, were missing (Fig. 4.10). Song characteristics measured in these recordings are summarised in Table 4.6. In total, 78 different syllable types of duet song were identified, which is about 15 % less than observed in *Pair No. 1*. Both sexes had equally sized syllable repertoires, comprising each about 95 % of the total duet repertoire. Further, per duet male and female contributed equal numbers of syllables and 58 % of the syllables were sung in unison.

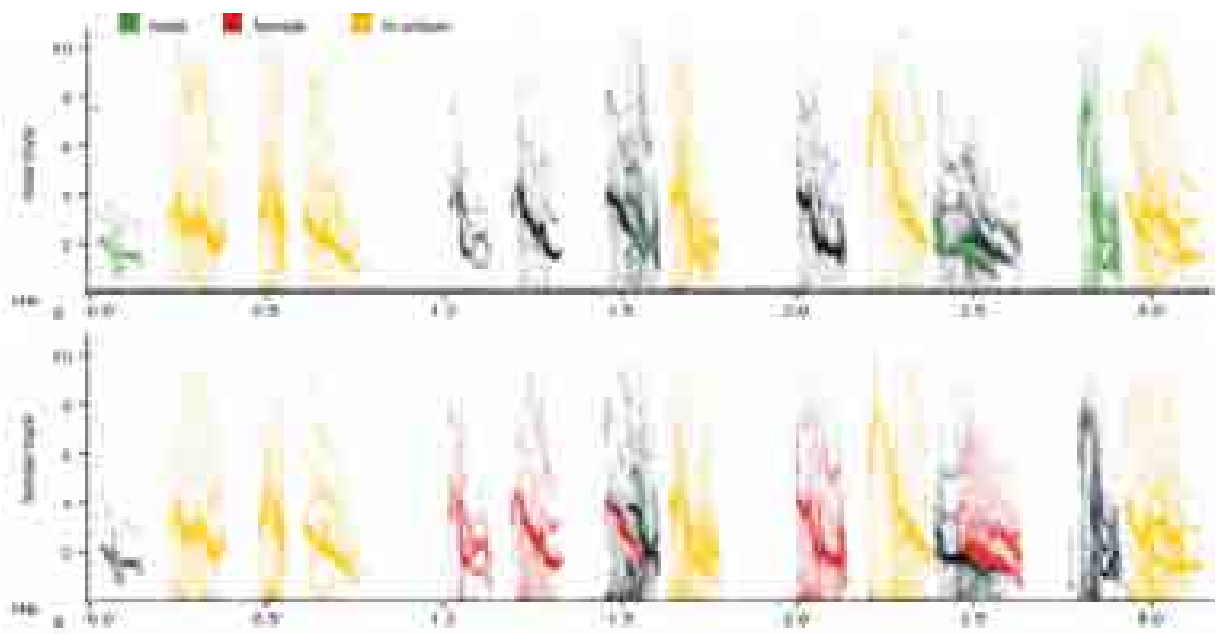


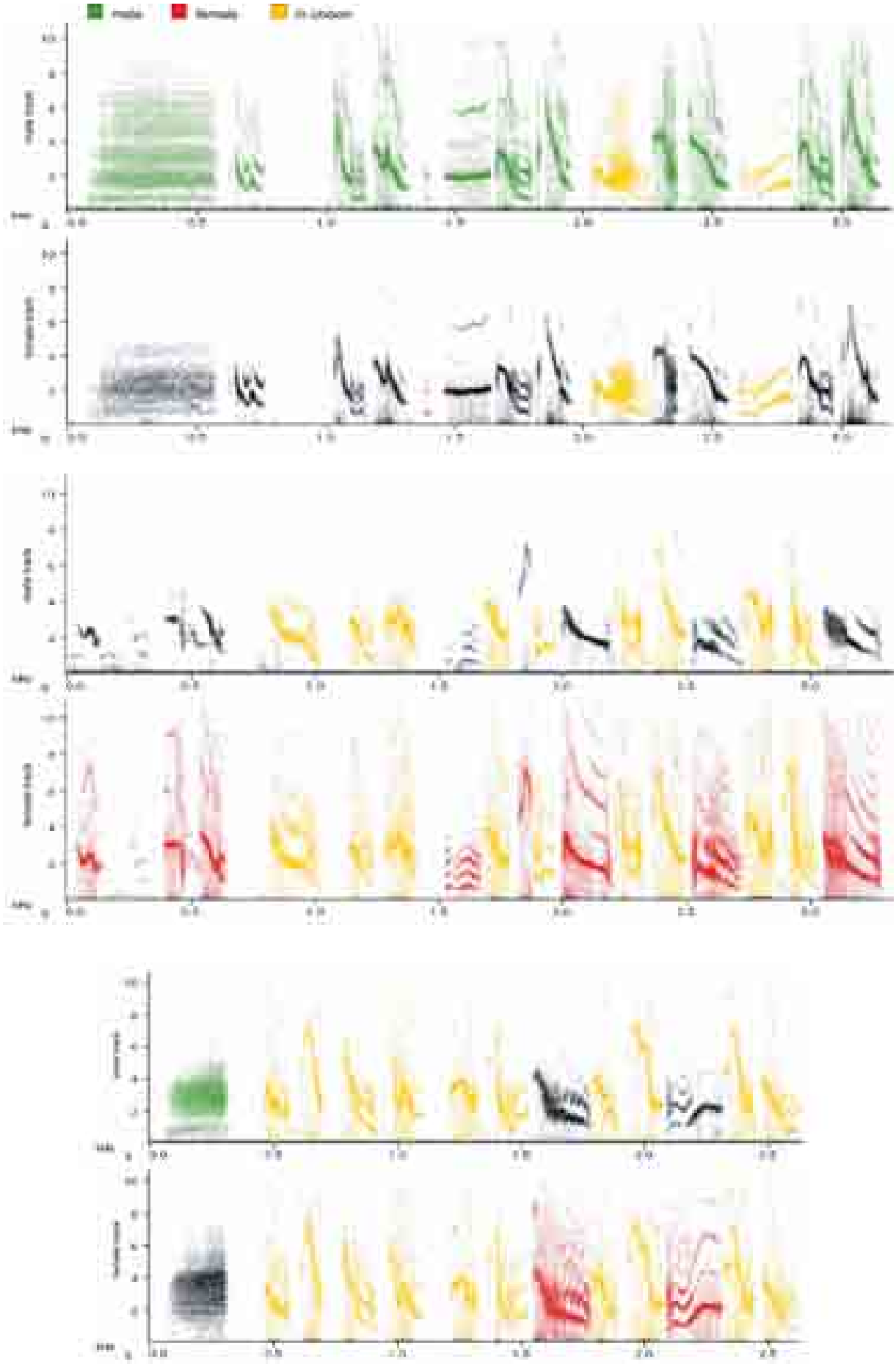
Fig. 4.10: Duet bout from recordings of *Pair No. 2*. Syllables sung by each sex and those sung in unison are colour-marked. Duets are not precisely timed. On two occasions, male and female were singing different syllables (time point 1.5 and 2.5). Whistle-like syllable types, which are mainly produced by the female, were mostly missing.

Table 4.6: Parameters of duet song analysed in two pairs of white-browed sparrow weavers in captivity.

Song parameters	Pair No. 2 (N=38 duets)	Pair No. 3 (N=27 duets)
Duet length (s)	3.36 ± 1.34	2.68 ± 0.97
No. of syllables per duet	14.95 ± 5.96	13.37 ± 4.79
No. of syllables per second	4.49 ± 0.61	5.00 ± 0.69
Percent of syllables sung by male per duet	78.84 ± 24.32	78.63 ± 18.62
Percent of syllables sung by female per duet	79.62 ± 14.29	83.13 ± 22.88
Percent of syllables sung in unison per duet	58.76 ± 27.36	62.03 ± 23.19
Total number of different duet syllables	78	59
Female syllable repertoire size	74	58
Male syllable repertoire size	75	55
Shared repertoire size	71	54

Pair No. 3 consisted of individuals, which were caught in February 2000 as adults in Zimbabwe. Since that time, they were kept as a pair in a large aviary with acoustic contact to other wild-caught pairs. These pairs behaved similar to those observed in Zimbabwe. Duetting was frequent throughout the day. However, song rate decreased when the birds were transferred to the recording cages. Without acoustic stimulation by another pair, birds would hardly sing. Therefore, sample size (27 duets) is rather low. Song characteristics are summarized in Table 4.6. Pairs were singing completely structured and precisely timed duet songs (Fig. 4.11.) as I have described for pairs from Zimbabwe (section 4.3.3.1). Examples of three duet bouts are given in Fig. 4.11. The total number of different syllables sung was 59, which is 25 % less than in the previous pair and about 40 % less than in *Pair No. 1*. Similar to both pairs, male and female had nearly identical duet repertoire sizes. Further, per duet song, male and female contributed equal numbers of syllables and they sang 62 % in unison. When comparing with *Pair No. 2*, it is noticed that in *Pair No. 3*, the number of syllables sung per second is higher and duet songs are shorter. These data are an indication for precisely timed duets with shorter pauses between syllables, which could not be accomplished by the inexperienced *Pair No. 2*.

Fig. 4.11: (next page): Three duet bouts from *Pair No. 3* showing the contribution of each sex to the song. Syllables sung by either sex are colour-marked in the respective tracks and syllables sung in unison are marked in both tracks. Both sexes can sing large parts of the duet alone (compare first and second duet bout). However, the female participates in almost all whistle-like syllable types. The third duet occurred four times and therefore, most frequently in the recordings. Almost all syllables were sung in unison.


male
female
in chorus

Detailed analysis of recordings from this pair revealed further insights into the structural composition of duets. Two (and sometimes three) frequency-modulated syllable types (Fig. 4.6, numbers in squares) repeatedly occurred in the same combination, which I defined as phrases (Fig. 4.12). A single syllable type could be part of different phrases. In total, I identified 26 phrase types and 9 whistle-like syllable types. Different phrase types together with whistle-like syllable types are combined to constitute a duet bout. Some phrase types occur in a fixed pattern with others, thereby building motifs, whereas others may occur in various combinations. Further, I found that certain whistle-like syllable types preceded certain phrase types. Summarized for all recordings, the female produced 53 % of these syllables alone whereas the male sang only 4 % of them alone. In unison, the pair produced 44 % of such syllables. This confirms my field observations and suggests a leading part of the female within duets. Further recordings from adult pairs in captivity are necessary to confirm the duet structure determined from this pair.

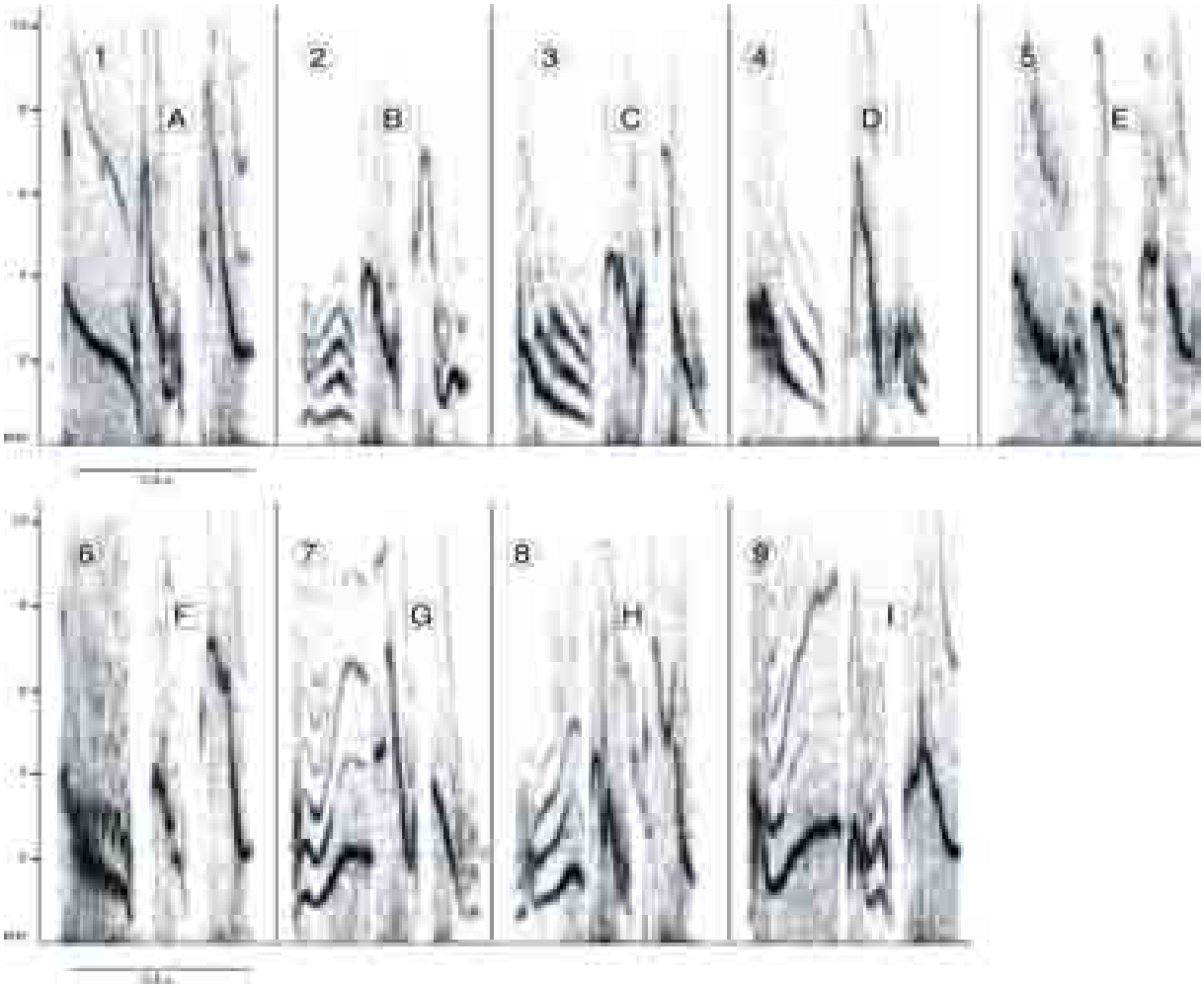


Fig. 4.12: Examples of nine different phrase types (indicated by letters), which are preceded by different whistle-like syllable types (indicated by numbers) and are repeatedly produced in this combination.

In conclusion, the data obtained from three captive pairs show that duet songs have a complex structure. Young, inexperienced pairs are not able to sing precisely coordinated duets, i.e. the whistle-like syllable type is often missing, and different syllable types are produced at the same time. Duet songs of experienced birds in captivity are similar in structure and time to those described for pairs in Zimbabwe. Both pair members contribute equally to duet singing and about 60 % of syllables in each duet are sung in unison. Further, mates have duet repertoires of similar size and share about 95 % of syllable types. Duet repertoires can comprise as many as 96 different syllables. However, as experience in duet singing increases, pairs reduce their overt repertoire by 25 to 40 %. In this process, most sex-specific syllables are lost. Duet syllables are organised in phrase types, which occur in combination with certain whistle-like syllable types.

4.3.4. Estimates of total repertoire size of individual birds recorded in Zimbabwe inferred from studies on captive birds

4.3.4.1. Repertoire size of solo song

For males and females used in neuroanatomical analyses, I used the data obtained from captive birds to estimate repertoire sizes of individual birds. Analysis of solo song from six males in captivity revealed that a single recording session was not sufficient to determine the bird's repertoire. However, after two recording sessions, there was no further significant increase in repertoire size. Per single performance, a male sang on average 88.4 % of its total repertoire. Based on this result, I estimated total repertoire size of the solo song for each male (Table 4.7.). Mean estimated repertoire size of the solo song was 78.1 ± 7.0 syllable types.

4.3.4.2. Repertoire size of duet song

Results from studies on three captive pairs have revealed that both members of the pair have similar sized duet syllable repertoires. In the two pairs, which were singing in duet bouts each sex sang 93 to 98 % of the total number of duet syllable types found. Therefore, I conclude that the repertoire sizes determined from dominant pairs of colonies in Zimbabwe can be fully assigned to each member of those pairs (Table 4.7.). Total repertoire size constitutes in males the sum of duet and solo song repertoire and in females the duet song repertoire (Table 4.7).

Mean total repertoire size of males is 128.3 ± 7.2 and that of females 50.1 ± 1.9 syllables. This difference is highly significant (U = 0.0, p = 0.0002, N = 8).

Table 4.7: Estimated individual repertoire size of duet and solo song inferred from studies in captive birds.

Sex / ID	Colony No.	Repertoire size of solo song		Repertoire size of duet song	Total repertoire size
		observed	estimated	observed = estimated	
♂ 28	10	52	59	47	106
♂ 29	10	58	66	47	113
♂ 40	2	72	81	45	126
♂ 64	9	77	87	52	139
♂ 67	12	92	105	53	158
♂ 69	17	48	54	50	104
♂ 77	16	60	68	60	128
♂ 78	15	92	105	47	152
♀ 26	9	-		52	52
♀ 27	10	-		47	47
♀ 31	1	-		61	61
♀ 38	2	-		45	45
♀ 53	17	-		50	50
♀ 54	12	-		53	53
♀ 79	15	-		47	47
♀ 146	19	-		46	46

4.3.4.3. Implications for the repertoire size of subordinate males

For this group of males no quantitative data are available. Nonetheless, there is evidence that these males have similar sized duet repertoires as dominant individuals. First, in Zimbabwe, I frequently observed all colony members singing chorus songs at their territory boundaries and these songs resemble in structure those of duet songs. Second, young males in captivity are able to sing up to 96 different duet syllables (see 4.3.3.3), which they later on might adjust to their partner's repertoire. Regarding the solo song repertoire, males in captivity perform courtship display and sing sequences of solo song already when eight months old. In song recordings of *Pair no. 2*, the male sang in addition to duet syllables 29 different syllables, which resemble types of the solo song. This suggests that a solo song repertoire already exists in young males. Further experiments are needed to determine the exact repertoire size of subordinate males.

4.4. Discussion

In the present chapter, I have described the song behaviour of male and female white-browed sparrow weavers. **A)** Solo songs were recorded from dominant males of colonies in Zimbabwe and were recorded from captive males kept in our institute. 1) Although captive males sang longer songs than males in the field, repertoires of solo song per single performance were of similar size and ranged from 48 to 92 syllable types. 2) A male sings on average 88 % of its total solo song repertoire in a single performance. 3) The syllables of the solo song repertoire can be clearly distinguished from those of the duet syllable repertoire. **B)** Duet songs were recorded from dominant pairs of colonies in Zimbabwe. 1) Recordings of 30 duet bouts were sufficient to obtain the total repertoire of a dominant pair. 2) Duet repertoires ranged from 45 to 61 different syllable types. **C)** Detailed duet structure and organisation was studied in three captive pairs, which differed in respect to age and duetting experience. 1) Pair members have duet repertoires of similar size, share about 95 % of syllables and contribute equally to individual duets. 2) Within duets, 60 % of the syllables are sung in unison. 3) Syllables are arranged in phrases and the whistle-like syllable type, mostly sung by the female, seems to determine the following phrase type. 4) In pairs with duetting experience, overt repertoire size is lower and duets are more precisely timed than in inexperienced pairs.

4.4.1. The solo song of dominant males

4.4.1.1. Song pattern

The solo song of male white-browed sparrow weavers consists of a series of repeated and non-repeated syllables occurring in phrases or as single syllables and separated by pauses of various lengths. The male starts at dawn and songs can last up to 20 minutes. Such a performance was not heard at other times of the day. Importantly, syllable types used in solo singing were different from those used for duetting. The structure and temporal pattern of the song did not allow any further subdivision. Although phrases could occur repeatedly in the same combination of syllable types no fixed patterns of phrase types, i.e. motifs or song types were identified. Therefore, the quantification of the syllable repertoire was the most reliable measurement of song versatility for this type of song. The singing style, i.e. with immediate variety, resembles that of the nightingale (*Luscinia megarhynchos*) or the blackbird (*Turdus merula*), and contrasts with species such as the great tit (*Parus major*) or the chaffinch (*Fringilla coelebs*) that both possess a repetitive singing style, i.e. sing with

eventual variety (Todt & Naguib 2000). Among duetting species, male solo song occurs frequently, e.g. in the southern bou-bou shrike (*Laniarius ferrugineus*) (Harcus 1977), in the white-browed robin chat (Todt et al. 1982), in the spotted morning warbler (Todt & Fiebelkorn 1980) or in *Thryothorus* wrens (Farabaugh 1983; Levin 1988). However, in all of the species studied so far, solo singing is referred to as when males or females sing parts of their duet contributions alone. The pattern found in white-browed sparrow weavers, where males have two distinct repertoires can therefore be regarded as unique. Preliminary observations of anteater chats (*Myrmecocichla aethiops*), a group living and duetting turdid species of East Africa, however, suggests that this species has a similar pattern of song behaviour, with males singing solo songs only before dawn (Helversen 1980). Unfortunately, no further data on repertoire composition are available.

4.4.1.2. Repertoire size

Single recordings of solo song from males in Zimbabwe revealed repertoires ranging from 48 to 92 different syllable types, representing almost 100 % difference between males. Such differences are also known from other species with large repertoires, e.g. the starling (Eens et al. 1992), the sedge warbler (Catchpole 2000) or the northern mockingbird (Derrickson 1988). The variation found by Eens et al. (1992) was attributable to the fact that yearling males had significantly smaller repertoires than older males. An age related increase in repertoire size could account for the variation found in white-browed sparrow weavers. Currently, no data are available whether these birds can acquire new syllable types as adults. If this is not the case, total repertoire must have been learned early in life and then not being performed until reaching a dominant position, which could be one or two years later. Subordinate males could be even older than dominant males (J. Wingfield, personal communication). It seems more reasonable to suggest a difference in repertoire size between new and already established territory holders especially when considering that costs might be involved in maintaining large repertoires (Gil & Gahr 2002). The question whether larger repertoires require more brain space or other morphological adaptations of the song control system will be explored in a later chapter.

Recordings from captive males showed that a single recording session did not cover a male's total repertoire. At least two recording sessions from each male in Zimbabwe would have been necessary. The solo song of males is a complete song sequence, which can only be heard once a day. Therefore, one would probably expect that males include as much information as possible in

such a single performance. However, this might depend on whether the solo song is mainly used in intra- or inter-sexual communication (see below). Single performances of captive males covered 84 to 96 % of their total repertoire. The measure of syllable repertoire size obtained from males in Zimbabwe is therefore a good estimate of total repertoire size and enables to make comparisons among males. The finding that captive males sang significantly longer solo songs than males in the wild is certainly related to conditions associated with captivity, e.g. reduced foraging time due to high food abundance, fewer interactions with neighbouring colonies.

4.4.1.3. Song length

High variation among white-browed sparrow weavers occurred in the length of the song performance. Because larger repertoires were not related to singing longer songs, such variation might represent different singing strategies. In nightingales, three forms of temporal performance roles have been identified, namely birds being inserters, overlappers or autonomous singers, which are thought to convey different types of information between territorial neighbours (Hultsch & Todt 1982). Neighbouring colonies of white-browed sparrow weavers in Zimbabwe were about 50 meters apart providing the opportunity for vocal interaction among solo singing males. Counter singing of males during solo vocalisations has been observed for example in the tropical anteater chat (Helversen 1980).

4.4.1.4. Possible functions of the solo song

Previous studies on white-browed sparrow weavers have indicated that the male solo song is associated with the breeding season and that it was not affected by simulated territorial intrusions (Ferguson 1988a; Wingfield & Lewis 1993). My own observations point in the same direction that this type of behaviour functions not primarily in territorial defence. In Zimbabwe, certain colonies started dawn vocalisations throughout the study period with duet singing although males of neighbouring colonies performed solo songs. Moreover, territorial challenges during the day were accompanied by intense duet and chorus vocalisations but never by solo singing. Similar patterns were observed in captivity. Not all males performed solo song exactly at the same time of the year. Rearrangements of pairs in neighbouring aviaries usually released high rates of duet vocalisations upon neighbours, exceeding the daily song pattern several times and could last up to three days. However, there is also evidence, that males from pairs, which were not in acoustic contact with conspecifics

would hardly sing solo song. Together, these observations suggest a function of solo song in both inter- and intra-sexual communication.

The behavioural pattern of the solo song closely resembles the dawn chorus of territorial species from the northern temperate zone and some of the functional explanations proposed for this type of behaviour might be applicable to the solo singing of white-browed sparrow weavers. Dawn chorus describes the phenomenon that territory owners vocalise during the breeding season at high rates usually 30 to 60 minutes before dawn, whereas singing rates decrease dramatically after sunrise and seem to be less intense during daytime singing (Catchpole & Slater 1995). A striking feature of the dawn chorus is the use of different song patterns than those used for singing during the day. Several hypotheses to explain the functions of dawn chorus have been proposed, which can be categorised into three classes, serving 1) functions intrinsic to the singer, 2) social functions and 3) functions related to environmental pressures (Staicer et al. 1996). Among the first category, the 'self-stimulation-hypothesis' seems a good explanation, which states that dawn singing may stimulate hormone production in the singers and prepare them for social interactions such as mating. Because the time window of favourable breeding conditions is much larger in the tropics compared to temperate zones, this could be a way in regulating gonadal growth and steroid hormone production. Dominant males have significantly larger testes than subordinates (Wingfield et al. 1991, this study) and testosterone levels were found to peak in dominants in the mid-breeding season (Wingfield et al. 1991, not found in this study). Social functions include mate attraction, mate stimulation, mate guarding, social dynamics among neighbours and the handicap hypothesis, stating that dawn singing is a reliable signal of male quality if this type of song is costly. It seems reasonable that the solo song functions in stimulating reproductive development of the mate, ensuring reproductive synchrony between mates. Intra-sexually, the solo song might be used to assess and establish social relationships between neighbouring males, because it is a long, simultaneous display. Further, there is indirect evidence that the solo song is costly, because captive males spent significantly longer times singing than males in the wild. The third category contains hypotheses explaining dawn singing because of low predation risk, enhanced acoustic transmission or inefficient foraging. Taken together, the solo song of white-browed sparrow weavers most likely fulfils functions in both, inter- and intra-sexual communication and is certainly closer associated with breeding activity than with territoriality. Further experiments are needed to test particular hypotheses.

4.4.2. The duet song of dominant pairs

4.4.2.1. Song pattern and repertoire size

The duet songs of white-browed sparrow weavers are complex vocalisations, consisting of parts produced either alternately or in unison and comprise a large number of different syllable types. In each duet bout, syllables are mostly arranged in phrases, containing a whistle-like syllable and two frequency-modulated syllables. Such phrases are combined in various ways to form motifs, also called song types. Some of these song types were repeatedly found in its complete structure, indicating that duets are not random sequences of syllables. Nonetheless, due to the high number of phrase type combinations possible, I measured the syllable repertoire rather than the song type repertoire.

In terms of complexity, the duet song resembles that described for the African Drongo most closely (Helversen & Wickler 1971). There, single duets, lasting 2 to 4 seconds, can comprise as many as 40 syllables. Because birds sing strictly alternating, each bird contributes up to 20 syllables. From studying the duets of a single pair more detailed, the authors could assign 24 different syllable types to bird A and 41 types to bird B. The sexes could not be identified. However, there is evidence, that this sample does not comprise the birds' entire repertoire and both partners could even possess equally sized repertoires (W. Wickler, personal communication). Further, the occurrence of a particular syllable within the duet depends on the preceding syllable of the partner and on that of the bird's own song. Similarly, in duets of white-browed sparrow weavers the occurrence of a particular combination of frequency-modulated syllables constituting a phrase depends on the preceding whistle-like syllable type that is mostly sung by the female (Fig. 4.12.). Among the neotropical wrens of the genus *Thryothorus* that comprises at least eight duetting species (Farabaugh 1982), the highest complexity is found in the Plain wren (*Thryothorus modestus*) (Mann et al. 2003). Duets start with an introductory phrase produced by the male, followed by an A-phrase of the female, which is then sung in an alternate fashion with a B-phrase of the male, producing 2 to 4 "AB" cycles. Repertoires of each phrase type comprise 15 to 25 different phrases, males having therefore twice as large repertoires as females. However, per individual duet, the same "AB" phrase types were mostly cycled repeatedly. Switching of phrase types was influenced by the phrase type selection of the partner, which is similar to the African drongo and the white-browed sparrow weaver.

Duet repertoire sizes of dominant pairs in Zimbabwe ranged from 45 to 61 different syllable types. Studies on two captive pairs confirmed that each

partner could sing almost the entire repertoire (mean: 95.5 %) and that both pair members therefore had similar repertoire sizes and contributed equally to individual duets. In terms of the level of complexity as defined by (Helversen 1980), the white-browed sparrow weaver clearly belongs to the third category, comprising the highest level of complexity. Concerning repertoire size, no duetting species with larger syllable repertoires have been described so far. Further, the white-browed sparrow weaver is special regarding the temporal pattern of duets. Most species possess a distinct temporal pattern, ranging from antiphony, which is often based on sex-specific repertoires, e.g. in buff-breasted wrens, bay wrens or several shrikes of the genus *Laniarius*, to synchrony e.g. in the African forest weaver. In contrast, captive white-browed sparrow weavers produced on average 60 % of the syllables within duets in complete synchrony whereas the remaining syllables were uttered by either sex. Such song pattern can only arise when both partners share large parts of their repertoire. Although both pair members were capable of producing all syllables within duets alone, this was never observed. It cannot be excluded that conditions related to captivity had an influence on the pattern of singing, i.e. the degree of syllables produced in synchrony. Birds were kept singly in cages throughout the study period and during recordings, they were prevented from visual contact due to the recording set-up used. There is evidence from one study of captive tropical bou-bou shrikes (*Laniarius aethiopicus*), that on occasions the normally antiphonal singing pattern can be switched for a short time to complete synchrony, e.g. when pair members were reunited after a period of separation (Thorpe 1975).

4.4.2.2. Development of duet singing

The study of captive birds allowed drawing conclusions about the development of duet singing in pairs because individuals of two pairs were young and were not paired to their partner before. Surprisingly, repertoire sizes of the three pairs gradually declined from the inexperienced to the most experienced pair but were always identical in pair members. Also, the temporal pattern was shaped, the first pair vocalising continuously whereas the second pair already sang in duet bouts but with less precision and producing fewer syllables per second than the third, well established pair. Such a pattern has so far not been described for other duetting species. However, Todt et al. (1982) mentioned that in white-browed robin chats both sexes vocalise equally during sub-song and crystallisation but afterwards young females reduce the utterance of song pattern. Young slate-coloured bou-bou shrikes acquire their complete repertoire when 6 to 8 months old and there was no evidence that it had ever been changed after mating or remating later in life (Wickler & Sonnenschein 1989). African forest weavers,

however, which acquire their full repertoire usually when 5 to 6 months old, are able to adjust their song type repertoire to conspecifics up to an age of two years (Seibt et al. 2002). These patterns are compatible with the model of action-based learning proposed by Marler & Peters (1982) and Marler (1997) for song learning in males. The model proposes a developmental overproduction and a selective attrition of unused song types during crystallisation. The process of selection is mediated by social interactions and its timing varies considerably. Whereas in some species it is finished once the birds reach sexual maturity, it can in others be extended into the first breeding season (Marler 1997). Under the last aspect, the pattern observed in white-browed sparrow weavers seems to fit the model. The pairs recorded in captivity were already sexually mature and produced duet syllables similar in structure to older pairs (Figs. 4.9, 4.10). During pair formation, both members might build up a pair-specific duet repertoire, where they combine a certain number of syllables to fixed phrases and duet motifs. Other syllables in an individual's repertoire may remain unused and are discarded. Social interaction, i.e. pair formation could therefore trigger the selective attrition of syllable types. It remains to be seen whether duet repertoire composition of pair members changes later in life after experimentally induced remating with different partners. There is evidence from a study of buff breasted wrens (*Thryothorus leucotis*) that newly established pairs build up a larger number of duet types from each individual's song type repertoire whereas old pairs minimise the number of duet combinations (Farabaugh 1983).

4.4.2.3. Possible functions of the duet song

Ornithologists have known the phenomenon of 'duet singing' already since the end of the nineteenth century. Thorpe (1963) initiated the first detailed studies, when he investigated the temporal pattern of this behaviour in three different taxonomic groups in Uganda and his results stimulated others to conduct similar studies later on (Grimes 1965; Payne 1970). From extensive studies on several duetting species Thorpe (1972) derived the conclusion that duetting functions primarily in mate recognition and maintaining contact thereby replacing visual displays in species living in dense tropical vegetation. Since then, this topic has attracted a large number of studies and many hypotheses have been proposed about its functions. Harcus (1977) studied duet singing in the southern bou-bou shrike (*Laniarius ferrugineus*), the bokmakierie shrike (*Telophorus zeylonus*) and in the bar-throated apalis (*Apalis thoracica*). Because duetting was maintained in all three species throughout the year with an increase just before breeding, he concluded that duets function in maintaining pair bond and to achieve reproductive synchronisation between mates. By using playback, he

could also establish a function in territory defence. The latter function was also confirmed by Seibt & Wickler (1977) when measuring sound intensity from duet vocalisations of two species of barbets (*Trachyphonus usambiro, T. d'arnaudii*) and the slate-coloured bou-bou shrike. They found that pairs of both species vocalised with much higher intensity than would have been necessary because partners were perched next to each other. Further, the sound could be heard at least 50 meters away. Vocalisations of low intensity were given in the context of courtship. The authors therefore conclude that loud duet vocalisations were directed towards territory neighbours. This hypothesis is clearly applicable to white-browed sparrow weavers. Pairs usually perform loud duets when in close proximity and frequently engage in counter-duetting or chorusing with neighbours at their territory boundaries. In situations of instability, e.g. after invasions or after rearrangement of neighbours in captive pairs the intensity of duet song was even increased and remained high for several hours (personal observation). Further, simulated territorial intrusions elicited a significant increase in the frequency of chorus singing performed by all colony members (Wingfield & Lewis 1993). Chorus songs resemble duet songs in terms of structure and temporal pattern. In contrast, both pair members uttered very soft sounds during the long precopulatory display (Ferguson 1988a), personal observation). These data strongly support the hypothesis that territory defence is one function of duets in this species. Todt et al. (1982) derived similar results for duet singing in white-browed robin chats.

Later on the study of the highly synchronised duet songs of the African forest weaver, lead Wickler (1980) to propose the coyness hypothesis. The author states that duet songs originated from territorial displays but that the evolution of highly complex and pair-specific duets could not be explained by this function. Instead, elaborate and pair-specific duets require investment of time and energy from partners in learning such songs thereby signalling commitment to the partner and reducing the risk of desertion. A change of mates would involve new investment. In white-browed sparrow weavers, the existence of long-term partnerships (Lewis 1982) as well as the period of learning duet songs, so far only shown for young pairs in captivity, would support this hypothesis. It remains to be seen, whether duet song in this species is indeed pair-specific, because syllable types are shared between neighbouring colonies and whether learning is involved after mate change in older pairs. Interestingly, songs of African forest weavers do not change later in life. Experimentally established pairs in captivity with different original song type repertoires reproduced successfully without making effort to sing duets in unison (Seibt et al. 2002).

In conclusion, avian duetting is likely to serve multiple functions, which are not necessarily mutually exclusive. In a recent review, Hall (2004) discusses the most prominent hypotheses and points out those likely to be broadly applicable. In the following, I will shortly outline each hypothesis and discuss its application to white-browed sparrow weavers. 1) Maintaining contact between partners. This hypothesis was originally proposed for pairs living in dense vegetation, where visibility is limited. It can be rejected because birds live in open habitat and often only duet when perched close to each other. Duetting rates decline when out of sight. 2) Ensuring reproductive synchrony. This hypothesis predicts that duetting peaks just before breeding. It can be rejected, because duetting occurs throughout the year. Instead, I have proposed that male solo song functions in this regard because of its association with the breeding season (see section 4.3.1.3.). 3) Mate guarding. An individual engages in duets to advertise the mated status of its partner and to repel same-sex rivals attracted to its partner's solo song. Songs should be loud and reveal sex and location of the singer. This could apply to white-browed sparrow weavers, though there is no evidence that unmated males engage in solo singing. Also, breeding females are mostly replaced by females from within the group (Lewis 1982). 4) Guarding paternity. Males engage in duets to repel rivals that are attracted to female song seeking extra-pair copulations. This is unlikely to apply because of the complex precopulatory display and the occurrence of duet singing at times when the female is not fertile. 5) Preventing a partner being usurped. Here, the bird advertises the mated status of its partner to solitary intruders of the opposite sex. Long-term partnerships in white-browed sparrow weavers (Lewis 1982) speak against this hypothesis. 6) Joined resource defence. Duets are cooperative displays to advertise and defend territories and access to resources. Duets should be loud, easy to locate, both sexes participate and outsiders are approached together. In my opinion, this is the most likely hypothesis to explain duet singing in white-browed sparrow weavers. It has been discussed in more detail above. 7) Signalling quality. Engaging in precisely coordinated duets should be an indicator of individual quality. No data are available to accept or reject this hypothesis. 8) Signalling commitment. Originally proposed by Wickler (1980), partners signal their willingness to invest in several aspects of the partnership. The complex duet of white-browed sparrow weavers is likely to fulfil such a function. This hypothesis has been already discussed above. Taken together, the data available so far support most strongly the 'Joint resource defence' hypothesis but make the 'Signalling commitment' and the 'Mate guarding' hypotheses likely to apply. Experimental studies in the field and on pairs in captivity would provide the opportunity to test particular hypotheses.

5. INTER- AND INTRASEXUAL DIMORPHISM IN THE SONG CONTROL SYSTEM REVEALED BY CYTOARCHITECTURE

5.1. Introduction

Soon after the initial report of sex differences in brain structures in mammals, Nottebohm & Arnold (1976) discovered the first sexually dimorphic brain areas in birds in the song control system of zebra finches and domesticated canaries. This circuit consists of a group of interconnected distinct nuclei of the forebrain, midbrain and hindbrain, and is in its specialisations unique to oscine songbirds (Kroodsma & Konishi 1991; Gahr et al. 1993; Ball 1994; Gahr 2000). Studies, involving lesions of song control areas and recordings of neural activity have demonstrated that these brain structures are causally linked to song behaviour (Nottebohm et al. 1976; McCasland 1987). Among the motor nuclei required for song production are the forebrain nuclei HVC (nucleus hyperstriatalis ventrale pars caudale or high vocal center) and RA (nucleus robustus archistriatalis, Fig.1.1.). Both nuclei are directly connected by a projection from HVC to RA and indirectly via the anterior forebrain pathway. RA, in turn, projects directly to the brainstem nucleus nXIIts (nucleus hypoglossus pars tracheosyringealis), which innervates the musculature of the syrinx (Nottebohm et al. 1976; Nottebohm et al. 1982). HVC appears to be the integrative centre of the song system, it receives auditory, visual and somatosensory input and its neurons show selective auditory responses to the bird's own song (Katz & Gurney 1981; Margoliash 1986; Wild 1994). Further, neurons of the motor nuclei appear to be hierarchically organised, with larger motor units such as song syllables coded in HVC neurons and smaller motor units such as song elements in RA neurons (Vu et al. 1994; Yu & Margoliash 1996).

The sex differences found in brain structure of zebra finches and canaries were thought to correlate well with the sex differences in song behaviour. In zebra finches only the male sings and related to this, the volumes of HVC and RA are about five times larger in males than in females. In contrast to female zebra finches, female canaries can produce male-like songs (Nottebohm 1980; Vallet et al. 1996). In this species, HVC and RA volumes are only 2.5 to three times larger in males compared to females (Nottebohm & Arnold 1976). Subsequently, in several other songbird species sex differences in the volume of song nuclei and in song behaviour were reported (Baker et al. 1984; Gahr & Guettinger 1985; Bernard et al. 1993; DeVoogd et al. 1995; Hauber et al. 1999; Nealen & Perkel 2000; Leitner et al. 2002). In most of these studies, the sexual dimorphism in brain and behaviour is strongly male-biased. Together with comparative studies, (Brenowitz & Arnold 1986; DeVoogd et al. 1993;

MacDougall-Shackleton & Ball 1999) they support the initial idea that the degree of sex differences in the brain correlates with the degree of sex differences in song behaviour. Investigations of sexual differences in the brain have not been restricted to variation in nuclear volumes. Males and females were found to differ also in other cytoarchitectural features, e.g. neuron number, neuron density, soma size, dendritic morphology (Gurney 1981; DeVoogd & Nottebohm 1981b; Tramontin et al. 1998; Nealen & Perkel 2000) as well as in cytochemical properties, e.g. steroid hormone accumulating cells, distribution of steroid hormone receptors and neurotransmitter systems (Arnold 1980; Arnold et al. 1986; Grisham & Arnold 1994; Gahr & Metzdorf 1999; Appeltants et al. 2001).

More insight into the complex relationship between brain and behaviour came from work on duetting species (Brenowitz & Arnold 1985; Brenowitz & Arnold 1986; Brenowitz et al. 1985; Gahr et al. 1998). Although duetting patterns and repertoire sizes vary markedly, a common feature among duetting species is the high degree of temporal coordination between male and female vocalisations. Further, the degree of sexual dimorphism in song production varies between different duetting species, which gives rise to comparative analyses. Brenowitz & Arnold (1986) investigated two species of closely related tropical wrens, the rufous-and-white wren (*Thryothorus rufalbus*) and the bay wren (*T. nigricapillus*), which differ in their sexually dimorphic song production. Whereas in bay wrens males and females have similar repertoires and similar volumes of song nuclei, female rufous-and-white wrens exhibit significantly smaller volumes of HVC and RA and considerable smaller song repertoires than males. This study and others, concentrating also on cytoarchitectural and cytochemical properties of song control areas in *Thryothorus*-wrens (Brenowitz & Arnold 1985; Brenowitz & Arnold 1989) strongly supported the authors' view that sex differences in brain structure and song behaviour have coevolved.

However, in a recent study (Gahr et al. 1998) this notion was challenged. These authors investigated sex differences in the song system of the slate-coloured boubou shrike (*Laniarius funebris*), a duetting species with similar song complexity. Unlike as would be predicted from the studies mentioned above, properties of the song system, e.g. volumes and neuron number of HVC and RA were significantly larger in males compared to females. Obviously, in this species vocal complexity in females is achieved by using different structural properties than males. This study raises also the question about the functional significance of the neural sex differences.

To further clarify this question, I studied the song control system of white-browed sparrow weavers (*Plocepasser mahali*), a duetting species with

complex song behaviour of males and females (see chapter 4). My studies focus on cytoarchitectural and cytochemical properties of neural structures, because both properties might develop and change independently from each other and combined, these approaches are able to identify sex differences in functionally different neuron subpopulations (Gahr 1997; Gahr 2001). The cytoarchitecture of the song system will be the focus of this chapter, whereas cytochemical aspects will be analysed in chapter 6. For this study, I chose dominant males and dominant females with known song output. Moreover, I extended my investigations to subordinate males (for definition of the social status see General methods). Subordinate and dominant males differ in the production of solo song. Therefore, they provide an ideal model to examine the neural correlates of such intra-sexual dimorphism in more detail.

5.2. Materials and Methods

5.2.1. Animals

Birds were collected in March 2000 and February 2001 at our study site in Zimbabwe. All birds had previously been captured for ringing, measuring and blood sampling and their social status was known (see section 3.1. for details). The brains of fourteen dominant females, fourteen dominant males and eight subordinate males were analysed. From six dominant males and six dominant females, only the right hemisphere of the brain was used. The left hemisphere was stored for an intended study on synapse structure using Golgi impregnation (see section 3.2. for details).

5.2.2. Tissue processing

Details are described in General methods, section 3.2. Serial sections were collected for processing with Nissl stain. From both hemispheres the first series of sections was used for Nissl staining.

5.2.3. Morphometric analysis

Nissl-stained sections were examined using a microscope (Leitz Aristoplan) under brightfield illumination (63x magnification). Cells could be identified by their dark blue staining. Morphometric analysis was done using an image analysis system (Metamorph 4.6, Visitron, Germany) connected to the microscope. For volume measurement, the perimeter of the region of interest in

each section was drawn on digitized images (1.156 µm/pixel) and the area was calculated by a built-in function of the software. The volume of a nucleus was calculated as the sum of these measurements multiplied with the section thickness and the interval between sections (120 µm). Telencephalon volume was estimated by measuring every eighth section throughout the extent of the brain using a binocular (Leitz, Bensheim, Germany) attached the image analysis system (1.196 µm/pixel). In some birds, only the right hemisphere of the brain was analysed. In these cases the volumes of song nuclei and the telencephalon were calculated unilaterally. For the birds, from which both hemispheres were processed, volumes were calculated bilaterally and presented either separately for each hemisphere or as the total volume, which was the sum of the measurements from the right and left side of the brain.

The density of cells in HVC and in the surrounding tissue was estimated in Nissl-stained sections under a 100x oil-immersion objective via the image analysis system. Although I did not use specific markers to distinguish between neurons and glia cells, the histological staining used is likely to stain predominantly neurons (Ball & MacDougall-Shackleton 2001). Cell counts were performed in the right brain hemisphere at the lateral, central and medial level of the nucleus HVC. *Central* was defined as the section N/2 according to the number of sections (N) where HVC was visible. This section divided the HVC in a lateral and medial portion. The *lateral* level was section $N_l/2$ and the *medial* level was section $N_m/2$ ($N_{l,m}$ = number of sections in the lateral and medial portion, respectively). At each level, a counting frame of 10000 μm^2 was analysed within the borders of HVC and in the neostriatum adjacent to HVC using the digitised images. We counted all profiles that contained one or two nucleoli, throughout the entire depth (30 µm) of the section that fell within the boundaries of the counting frame. In a preliminary analysis in nucleus HVC of males and females, I found no regional differences in cell density (Repeated-Measures-ANOVA, all tests $p > 0.17$), therefore in the main analysis I used only the measurements from the *central* level of HVC and the adjacent neostriatum. Density measurements are presented as 10^4 cells/mm^3. The total number of cells in HVC was derived from multiplying the cell density by the volume of the nucleus HVC.

5.2.4. Statistical analysis

Statistical analyses were done with Systat 10.2. General Linear Models (GLM) were used whenever possible. If necessary, data were transformed to meet the assumptions for the use of GLM. Data are presented as means ± standard error. All tests were two-tailed and the significance level was fixed at $\alpha = 0.05$. The

differences between dominant males and females and between dominant males and subordinate males were analysed with t-tests. Correlations between HVC and RA volume were done separately for each sex using a Pearson correlation. Relative HVC and RA volumes were compared between sexes by means of a One-Way-ANOVA with sex as factor and telencephalon size as covariate. Two-Way-Repeated-Measures-ANOVA was used to analyse cell density, with sex (dominant male, dominant female) or status (dominant male, subordinate male) as between-subjects factor and region (HVC, neostriatum) as within-subjects factor. If the ANOVA revealed a significant effect of the between-subjects factor, the groups were compared at each level of the within-subjects factor with t-tests. If there was a significant effect of the within-subjects factor or a significant interaction, multiple comparisons were made with the Tukey's HSD test. Body measurements of dominant and subordinate males were compared with t-tests and because of multiple testing on a single hypothesis the standard Bonferroni technique was applied (Sokal & Rohlf 1996). Therefore, the significance level α was adjusted for the number of tests (k) carried out, which gives a significance level of $\alpha' = \alpha / k$. Because data of the body and brain measurements of dominant males were used in two comparisons, first with dominant females, second with subordinate males, the standard Bonferroni technique was applied ($\alpha' = 0.05/2$). On these data sets the significance level α' was fixed at 0.025 and is indicated in the respective sections of the results.

5.3. Results

5.3.1. Size of song control nuclei HVC and RA revealed from the right brain hemisphere

In fourteen dominant males and dominant females, the size of song nuclei HVC and RA was calculated from measurements of the right hemisphere of the brain. Males had significantly larger volumes of HVC than females (Table 5.1, Fig. 5.1) The volume of the right telencephalon did not differ between the sexes. Males were on average heavier than females however, no sex difference was found in the weight of the syrinx (Table 5.1). There was a positive correlation between the size of HVC and RA in males ($r = 0.618$, $p = 0.018$, $N = 14$) but not in females ($r = 0.419$, $p = 0.136$, $N = 14$).

Table 5.1: Body- and brain measurements (right hemisphere) of dominant males and dominant females.

	Males (N = 14)	Females (N = 14)	t	P
Body weight (g)	47.50 ± 0.67	44.82 ± 0.79	2.58	p = 0.016
Syrinx weight (g)	0.121 ± 0.003	0.112 ± 0.006	1.28	p = 0.211
HVC volume (mm^3)	1.726 ± 0.097	0.663 ± 0.043	10.05	p = 0.0001
RA volume (mm^3)	0.481 ± 0.032	0.271 ± 0.013	6.03	p = 0.0001
Telencephalon volume (mm^3)	390.47 ± 12.09	374.30 ± 14.46	0.85	p = 0.399

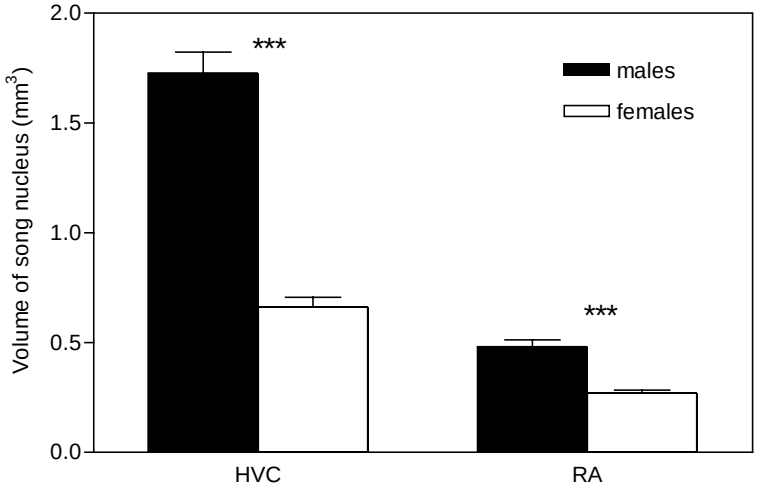


Fig. 5.1: Volumes of song nuclei HVC and RA revealed from the right brain hemisphere of dominant males (N = 14) and dominant females (N = 14). Both nuclei were significantly larger in males than in females (t-tests; *** = $p < 0.001$).

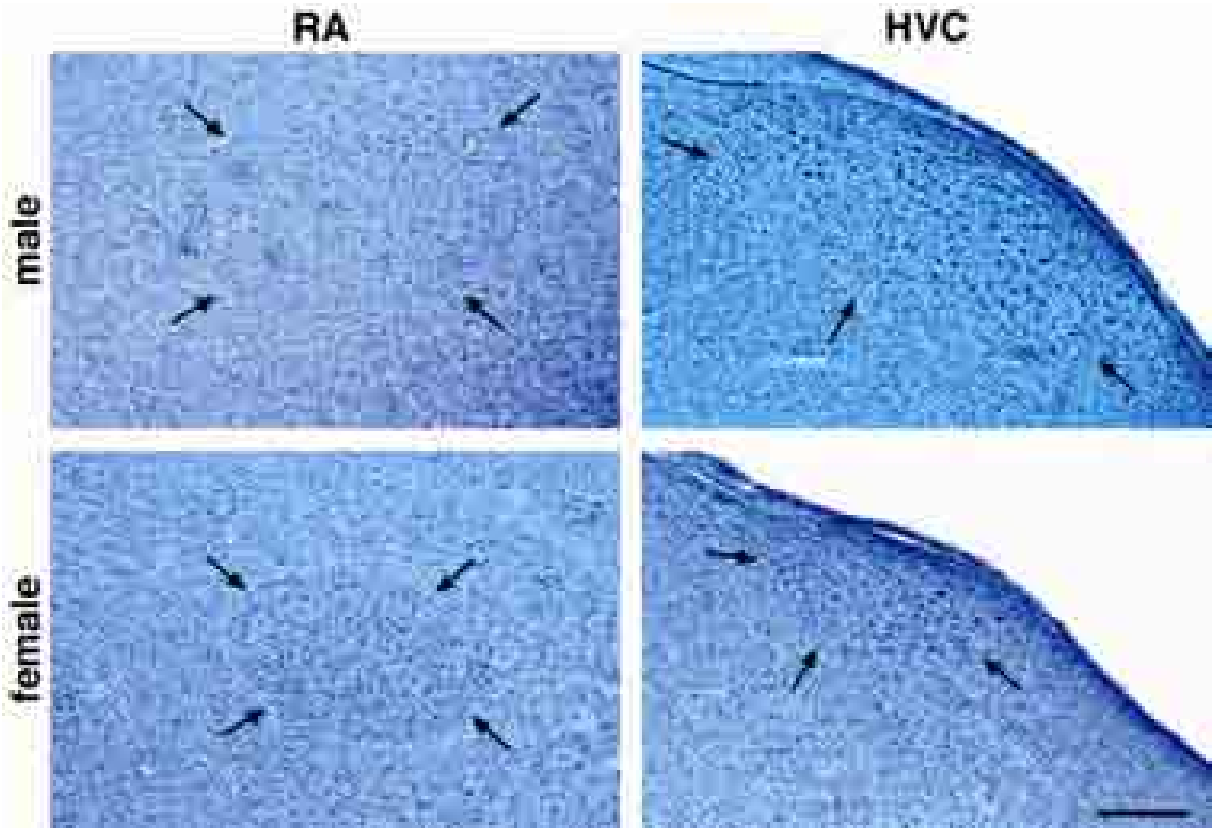


Fig. 5.2. Brightfield photomicrographs showing song control nuclei HVC and RA in Nissl-stained sections of males and females. The nuclei can be distinguished from surrounding tissue by larger, clustered and more darkly stained cells. Arrows indicate boundaries. Dorsal is to the top and caudal to the right in these sagittal sections. Scale bar = 300 µm.

5.3.2. Size of song control nuclei HVC and RA revealed from both brain hemispheres

The total volume of song nuclei HVC and RA was calculated for eight dominant males and females where measurements from both hemispheres were available. Total HVC volume was about three times larger in males than in females. Total RA volume was about twice as large in males than in females (Table 5.2). In both sexes, there were no significant hemispheric differences in the size of HVC (males: $t = 0.62$, $df = 7$, $p = 0.56$; females: $t = 1.55$, $df = 7$, $p = 0.17$) and RA (males: $t = 0.31$, $df = 7$, $p = 0.77$; females: $t = 0.65$, $df = 7$, $p = 0.54$).

Table 5.2: Brain measurements (both hemispheres) of dominant males and dominant females ($\alpha' = 0.025$).

	Males (N = 8)	Females (N = 8)	t	P
Total HVC volume (mm^3)	3.539 ± 0.201	1.195 ± 0.065	11.08	p = 0.0001
Total RA volume (mm^3)	1.039 ± 0.073	0.528 ± 0.037	6.20	p = 0.0001
Total telencephalon volume (mm^3)	815.52 ± 21.39	759.12 ± 15.19	2.15	p = 0.05

5.3.3. Telencephalon volume

Total telencephalon volume was numerically smaller in females than in males (Table 5.2). This difference only approached significance. When comparing the telencephalon volume of the left and the right hemisphere separately, no significant differences were found between sexes (left hemisphere: $t = 0.59$, $df = 14$, $p = 0.57$; right hemisphere: $t = 1.49$, $df = 14$, $p = 0.16$).

In studies on sexual dimorphism of brain regions, sex differences in overall body size may give rise to sex differences in brain size and consequently, could affect measurements of the brain region of interest. Still, there is not enough evidence to support such a relationship (Peters 1991; Sherry et al. 1993; Nealen & Perkel 2000). Body measurements (section 3.1.1) and telencephalon volumes in white-browed sparrow weavers do not clearly indicate, that males and females differ in the size of the body and the brain. However, when I analysed total HVC and RA volume separately in a One-Way-ANOVA including telencephalon volume as a covariate, males still had larger relative volumes of HVC (effect of sex: $F_{(1,13)} = 84.51$, $p = 0.0001$, effect of telencephalon: $F_{(1,13)} = 1.64$, $p = 0.22$) and RA (effect of sex: $F_{(1,13)} = 23.74$, $p = 0.0001$, effect of telencephalon: $F_{(1,13)} = 2.34$, $p = 0.15$) compared to females. These differences were highly significant.

Considering the non-significant differences of the left and right telencephali between sexes and the non-significant result from the larger sample size ($N = 14$, see 5.3.1) let me to conclude that there is not enough evidence to claim that males and females differ in telencephalon volume. Therefore, I decided to apply no correction for overall body size in the following analyses of brain structure of males and females (chapter 5 and 6).

5.3.4. Cell density and total cell number in HVC

Analysis of cell density in HVC and adjacent neostriatum revealed neither a significant main effect of sex ($F_{(1,14)} = 1.19$, $p = 0.29$, $\alpha' = 0.025$), nor of brain region ($F_{(1,14)} = 0$, $p = 0.99$, $\alpha' = 0.025$) on cell density, but a highly significant interaction between both factors ($F_{(1,14)} = 9.60$, $p = 0.008$, $\alpha' = 0.025$; Fig. 5.3a). Graphical inspection showed that density measurements of HVC and neostriatum differed between males and females. Posthoc comparisons revealed that, whereas both sexes had equal densities of cells in the neostriatum, females had a higher cell density within the nucleus HVC than males. Within each sex, cell density was not significantly different between HVC and neostriatum (Tukey's HSD test; Table 5.3). However, in respect to total cell number, females had significantly fewer cells in HVC than males ($t = 6.90$, $df = 14$, $p = 0.0001$,

α' = 0.025; Table 5.3, Fig. 5.3b). There was no correlation between HVC volume and cell density in HVC in either sex (males: r = -0.08, p = 0.854; females: r = 0.331, p = 0.424).

Table 5.3: Cell density and cell number in brain areas of dominant males and dominant females

	Males (N = 8)	Females (N = 8)
Cell density in HVC (x10^4 cells/mm^3)	24.04 ± 1.54	29.42 ± 0.90
Cell density in neostriatum (x10^4 cells/mm^3)	27.33 ± 1.89	26.08 ± 1.64
Total cell number in HVC (x10^4 cells)	42.00 ± 3.49	16.89 ± 1.05

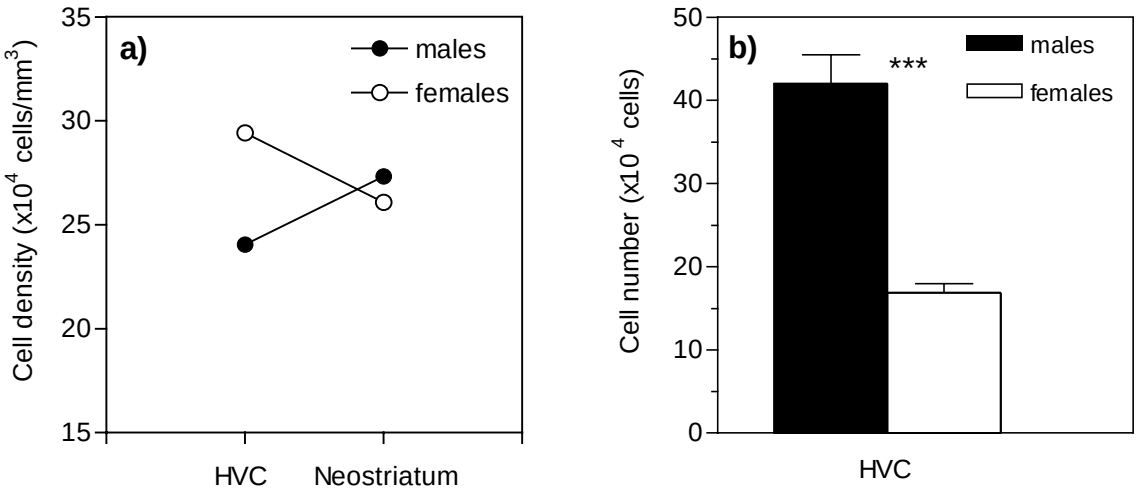


Fig 5.3: Interaction plot of cell density (a) in HVC and neostriatum of males and females and total cell number (b) in HVC. a: Cell density in HVC was significantly higher in females compared to males, whereas no sex differences were found in the neostriatum (Tukey's HSD test). b: Males had significantly more cells in HVC than females, t-test, *** = $p < 0.001$, α' = 0.025).

5.3.5. Does the size of the song control nuclei relate to social status?

To investigate whether there exists a relationship between the size of the song nuclei and social status, I analysed the brains of eight subordinate males and compared them with the brains of eight dominant males. In respect to body size and body condition, subordinate males did not differ from dominant males. Also, syrinx weight was identical between groups. However, gonads of subordinate males were smaller compared to dominant males (Table 5.4, Fig. 5.4a).

Table 5.4: Body measurements of males with different social status (Bonferroni adjusted: α' = 0.0063)

	Dominant males (N = 8)	Subordinate males (N = 8)	t	P
Body weight (g)	46.88 ± 1.06	45.63 ± 0.57	1.04	p = 0.314
Wing length (cm)	10.15 ± 0.10	10.33 ± 0.07	1.51	p = 0.153
Tarsus length (cm)	2.53 ± 0.02	2.47 ± 0.02	2.46	p = 0.027
Bill length (cm)	1.49 ± 0.01	1.48 ± 0.02	0.13	p = 0.900
Fat score	3.88 ± 0.48	3.38 ± 0.26	0.91	p = 0.376
Muscle score	1.88 ± 0.13	1.88 ± 0.13	0	p = 1.000
Testes weight (g)	0.196 ± 0.026	0.072 ± 0.018	3.88	p = 0.002
Syrinx weight (g)	0.117 ± 0.005	0.111 ± 0.004	1.03	P = 0.321

Total volumes of HVC and RA were about 1.4 times larger in dominant males compared to subordinate males (Table 5.5, Fig. 5.4b, 5.5). The total telencephalon volume was not significantly different between groups (Table 5.5).

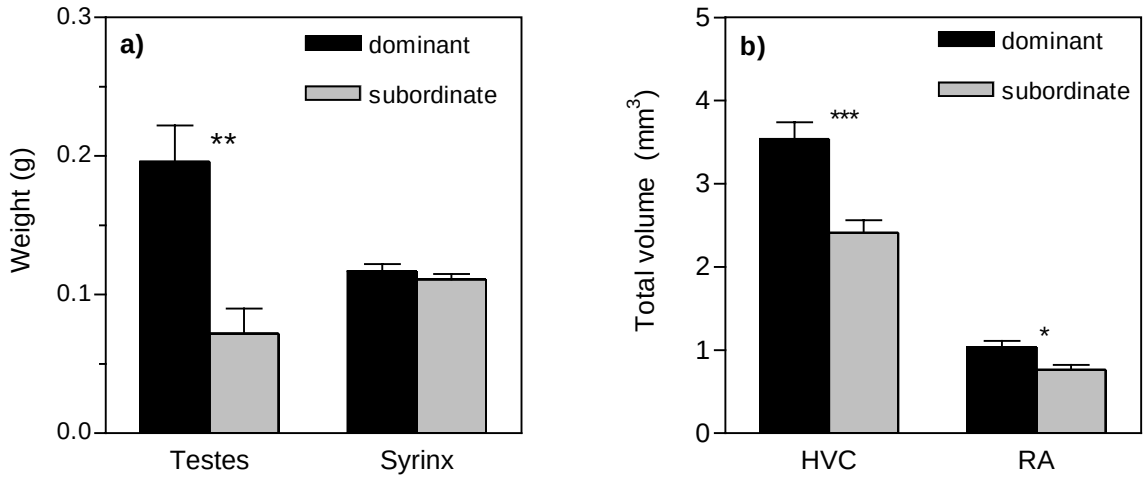


Fig. 5.4: a: Dominant males had significantly larger testes than subordinates (t-test, p = 0.002), but there was no difference in syrinx weight (t-test, p = 0.321, α' = 0.025). b: Dominant males had significantly larger volumes of HVC (t-test, *** = p < 0.001, α' = 0.025) and RA (t-test, * = p < 0.025, α' = 0.025) than subordinates.

Table 5.5: Comparison of brain characteristics of dominant and subordinate males (α' = 0.025)

	Dominant males (N = 8)	Subordinate males (N = 8)	t	P
Total HVC volume (mm^3)	3.539 ± 0.201	2.413 ± 0.147	4.53	p = 0.0001
Total RA volume (mm^3)	1.039 ± 0.073	0.763 ± 0.059	2.94	p = 0.011
Total telencephalon volume (mm^3)	815.52 ± 21.39	775.09 ± 11.68	1.66	p = 0.119

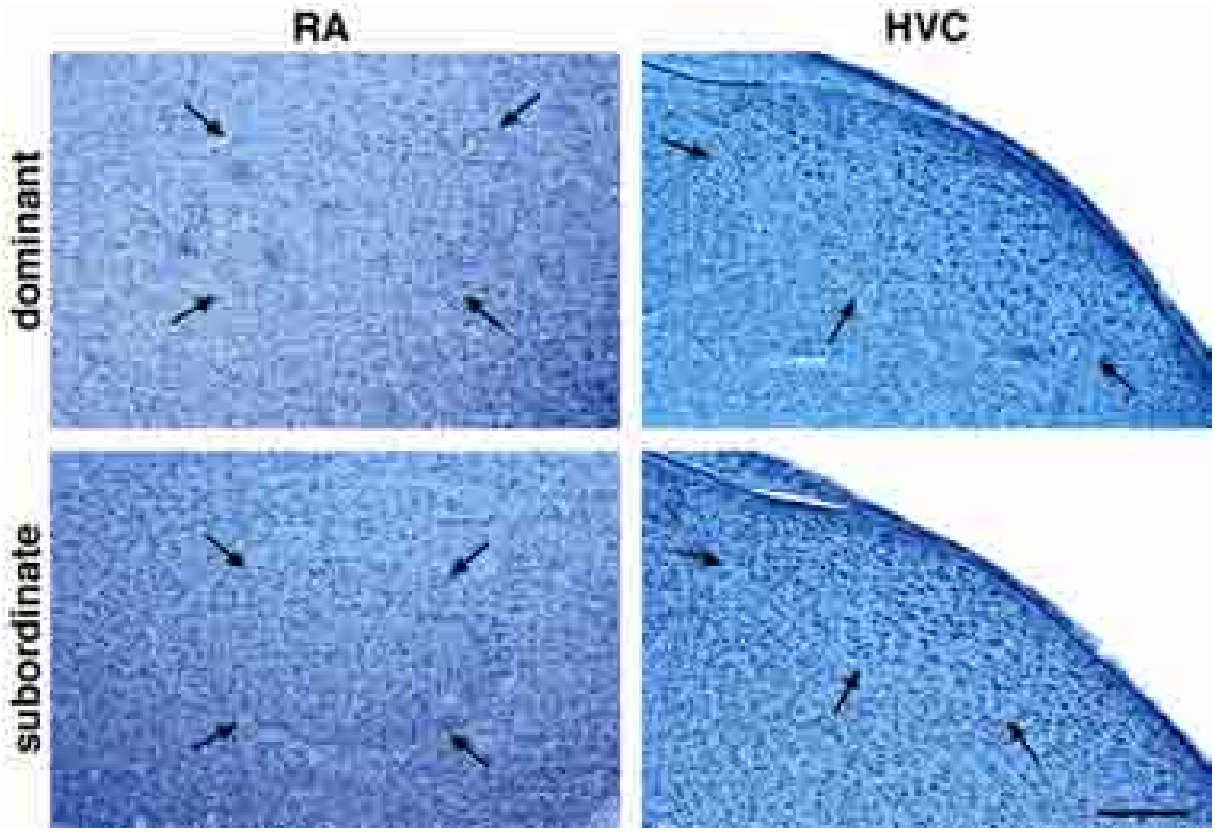


Fig. 5.5: Bright field photomicrographs showing song nuclei HVC and RA in Nissl-stained sections of dominant and subordinate males. Arrows indicate boundaries. Both brain areas were about 1.4 times larger in dominant males than in subordinates. Dorsal is to the top and caudal is to the right in these sagittal sections. Scale bar = 300 µm.

Analysis of cell density in HVC and adjacent neostriatum revealed neither a significant main effect of status ($F_{(1,14)}$ = 1.42, p = 0.25, α' = 0.025) nor of brain region ($F_{(1,14)}$ = 0.28, p = 0.60, α' = 0.025) on cell density, but a significant interaction between both factors ($F_{(1,14)}$ = 8.04, p = 0.013, α' = 0.025; Fig. 5.6a). Posthoc comparisons showed that cell density in HVC was equal in both groups of males, whereas subordinate males had a lower cell density in the adjacent neostriatum (Tukey's HSD test; Table 5.6). Total cell number was about 30% lower in subordinates compared to dominant males (t = 3.11, df = 14, p = 0.008, α' = 0.025; Fig. 5.6b). There was no significant correlation between HVC volume and cell density in HVC in either group of males (dominant: r = -0.08, p = 0.854; subordinate: r = -0.336, p = 0.415).

Table 5.6: Cell density and cell number in brain areas of dominant and subordinate males

	Dominant males (N = 8)	Subordinate males (N = 8)
Cell density in HVC (x10^4 cells/mm^3)	24.04 ± 1.54	24.38 ± 1.68
Cell density in neostriatum (x10^4 cells/mm^3)	27.33 ± 1.89	22.13 ± 1.22
Total cell number in HVC (x10^4 cells)	42.00 ± 3.49	29.21 ± 2.17

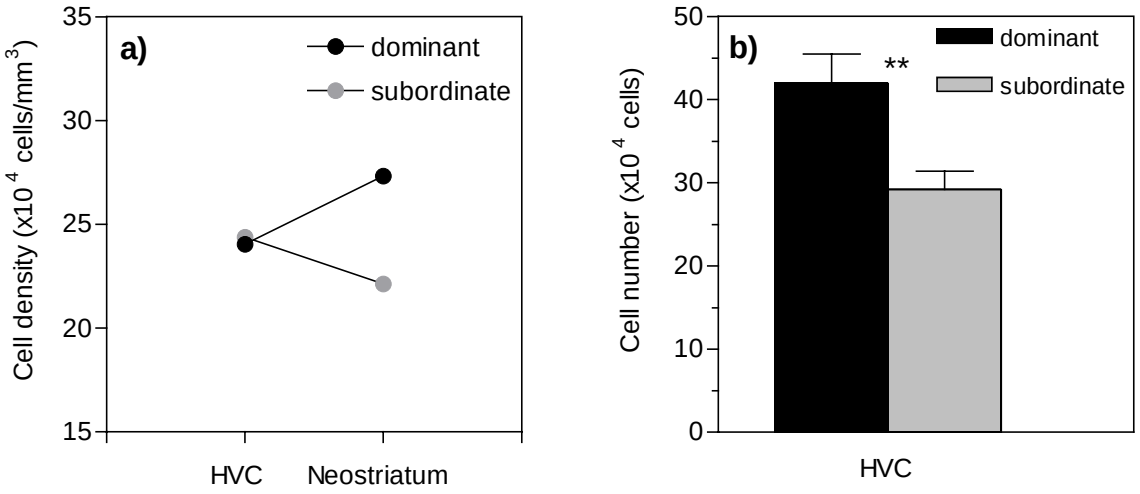


Fig 5.6: Interaction plot of cell density (a) in HVC and neostriatum of dominant and subordinate males and total cell number (b) in HVC. a: Cell density in HVC was not significantly different between both groups of males. In the neostriatum, cell density was significantly lower in subordinates than in dominant males (ANOVA, followed by Tukey's HSD test). b: Total cell number was significantly lower in HVC of subordinates compared to dominant males (t-test, ** = $p < 0.01$, $\alpha' = 0.025$).

5.4. Discussion

In this study, I investigated inter- and intrasexual differences in several cytoarchitectural properties of the song control system. 1) Dominant males and females differed from each other in respect to volumes of song control nuclei HVC and RA, cell density in HVC and the total number of HVC cells. Except for HVC cell density, all properties were larger in males. 2) No sex differences were found in the weight of the syrinx and in the volume of the telencephalon. 3) Males of different social status differed from each other in respect to testes size, volumes of song nuclei HVC and RA, the total number of HVC cells and cell density in neostriatum. All of these properties were smaller in subordinates than in dominant males. 4) No differences between both groups of males were found in overall body size, syrinx weight, cell density in HVC and the volume of the telencephalon.

5.4.1. Sexually dimorphic volumes of song nuclei HVC and RA

Sexually dimorphic behaviours of vertebrates associated with courtship and reproduction are well described (for review, see Kelley 1988) and it is known in many of these systems that the behavioural sex differences are paralleled by substantial anatomical sex differences in the brain (Gorski et al. 1978; Adkins-

Regan & Watson 1990; Crews et al. 1990). The song control system of songbirds represents a prominent example, in which the mechanisms mediating sex differences in behaviour are well investigated (Nottebohm & Arnold 1976; Arnold et al. 1986). In zebra finches, where only the male sings, the song nuclei HVC, RA, MAN and AreaX are up to five times larger in males than in females. Duetting songbirds, i.e. the bay wren (*Thryothorus nigricapillus*) with similar song behaviour of males and females constitute the other extreme and have song nuclei that are about 1.3 times larger in males than in females and therefore are much less sexually dimorphic (Brenowitz et al. 1985). Sex differences in volumes of brain areas are also reflected in sex differences in the cellular composition of these regions. For example, neurons in RA are larger, more numerous and more widely spaced in male zebra finches compared to females (Gurney 1981), whereas in two species of duetting *Thryothorus*-wrens no sex differences in RA neuron size and density exist (Brenowitz et al. 1985). However, inter-specific comparisons of sex differences do not reveal a consistent pattern, which could describe a relationship between brain area size or its cytoarchitectural features and the behaviour (Gahr et al. 1998), but see (Brenowitz & Arnold 1986; Ball et al. 1994). In the present study on duetting white-browed sparrow weavers, dominant males have about 2.6 times larger total song repertoires than females (section 4.3.4) and volumes of the song nuclei HVC and RA are 3.0, respectively 2.0 times larger in dominant males than in females. These sex differences are in agreement with the general pattern found in songbirds and they lie well between the two extreme species described above.

When extending the comparison to subordinate males, two interesting features emerge. First, subordinate males differ from dominant males in having 1.4 times smaller volumes of HVC and RA and these size differences are the same for both nuclei. Second, females differ from subordinate males in having 2.0, respectively 1.4 times smaller volumes of nuclei HVC and RA. The first observation shows that the song system of adult males can still increase in overall size later in life due to the action of epigenetic factors, probably related to the bird's social status. This finding will be discussed later in more detail. The fact that females have smaller song nuclei than subordinate males despite their similar vocal behaviour (section 4.3.4.3) could indicate that subordinate males have already learned their entire song repertoire early in life and therefore, the size of the brain areas is larger. Alternatively, the differences could reflect a genetically determined sex difference in the size of the song nuclei, which is not related to the behaviour. Experiments, involving the manipulation of song learning and adult song behaviour, i.e. rearing juveniles in acoustic isolation and

treating subordinate males with testosterone would help to clarify the underlying mechanisms.

The finding that the size differences between the sexes are smaller for RA than for HVC probably suggests that the female RA requires a relatively large size, i.e. large number of neurons or large neuron size, to accomplish the singing of highly complex duets and that its development depends partly on the development of the brain stem-syrinx pathway. White-browed sparrow weavers are monomorphic in respect to syrinx weight, whereas in species, where only the male sings, e.g. the zebra finch, the entire syrinx-control pathway (HVC, RA, nXIIts, syrinx) is highly sexually dimorphic (Nottebohm & Arnold 1976). Nucleus RA has, among others, one projection to the motor neurons of the nXIIts, which innervate the ventral and dorsal muscles of the syrinx. The dorsal muscles regulate the temporal fine structure of song and the precise timing of elements by gating the expiratory airflow (for review, see Margoliash 1997; Suthers 1999). It has been shown in zebra finches, that the syringeal muscles are important for the size of at least those RA neurons that project to nXIIts. Denervation of juvenile birds, by cutting the XIIth cranial nerve, not only reduced the volume and neuron number of the motor nucleus nXIIts but also reduced the size of one neuron type in the premotor nucleus RA (Lohmann & Gahr 2000).

5.4.2. Cellular properties in HVC differ between males and females

The size of neural structures is the outcome of several different cellular features, e.g. cell size, cell number, spacing of cells, synaptic organisation, internal connectivity and projections. In my study, I have focused on cell number and cell density in HVC. In the sexually dimorphic zebra finch, experimental studies on females have indicated that the development of the size differences and other properties in the song nuclei depends widely on the action of steroid hormones (Gurney & Konishi 1980; Gurney 1981). However, it has become apparent, that besides the action of epigenetic factors, such as gonadal steroid hormones, genetically determined factors are involved in the development of the sexually dimorphic song system (Arnold 1997; Gahr & Metzdorf 1999). Further, the mechanism, which regulates adult size, differs between song nuclei (Arnold et al. 1986). HVC of male zebra finches grows by continuous addition of new neurons until adulthood, whereas in females the number of neurons remains constant from day 20 on. In contrast to HVC, volume of RA increases in males mainly by an increase in soma size and neuronal spacing. In females, after day 25, there is a substantial loss of RA neurons due to neuronal death or migration (Bottjer et al. 1986). The sexually dimorphic development leads to the observed

sex difference in the volume of the song nuclei in adult birds and one consequence is that males have far more neurons in these nuclei than females. Sex differences in adult neuron number are also observed in the present study and in a study on another duetting species, the slate-coloured boubou shrike (Gahr et al. 1998).

Interestingly, cell density in HVC was higher in female white-browed sparrow weavers compared to males, whereas no sex difference was found in the neostriatum, which surrounds HVC. A similar pattern has been described for nucleus RA in zebra finches (Johnson & Sellix 2000) and in duetting white-browed robin chats (Brenowitz et al. 1985). From the data on mean RA and HVC volume and neuron number presented by (Gahr et al. 1998) it can be inferred that the same pattern exists in the slate-coloured boubou shrike. My study differed in respect to the method of cell counting, which could potentially have influenced my results. In Nissl-stained sections, I did not distinguish between neurons and glia cells and I used a nonstereological counting method. However, Nissl stain seems to work best in identifying neurons and not glia cells (Ball & MacDougall-Shackleton 2001). Further, both cell-counting techniques, nonstereological and stereological such as the optical dissector, have been proved to yield the same results (Tramontin et al. 1998). Therefore, it is unlikely that my cell counts were biased by these factors. Instead, maintaining higher cell densities within the song nuclei could be a general feature of females of duetting species to compensate for fewer or smaller neurons or smaller dendritic fields. That this feature is specific to the song system is supported by the fact that no sex difference in cell density occurred in the surrounding tissue. Higher cell densities in females have also been described in otherwise male-biased sexually dimorphic brain areas, the medial preoptic area and the SDN-POA of the rat (Madeira et al. 1999).

5.4.3. Which mechanisms could drive restructuring of brain areas in subordinate males?

Subordinate male white-browed sparrow weavers did not differ from dominant males in respect to body size and body condition. Also, their bill was completely black coloured (see section 3.1), which confirms that they were adult individuals. Testis size was the only variable, which could predict their social status. Subordinate males had significantly smaller testes than dominant birds. However, there was some overlap between groups and further, there was no correlation between testes size and plasma testosterone levels in either group. Both groups had similar levels of blood testosterone (see chapter 7). Regarding testis size these findings agree with results from an earlier study on white-

browed sparrow weavers, conducted on a different subspecies in Zambia (Wingfield et al. 1991). The authors suggested that subordinates are "psychologically castrated", which means, dominant birds suppress the reproductive maturity of subordinate birds (Brown 1978). Furthermore, Wingfield et al. (1991) found that subordinates were not stressed by their social rank, because body condition and plasma corticosterone levels were not different from dominant birds.

Considering these characteristics, the finding of differences in the gross morphology of the song control system between both groups of males was unexpected. No group differences were found regarding telencephalon size and syrinx weight, which is not surprising when keeping in mind that there exist no sex differences in these parameters despite large sex differences in the size of the song nuclei. The similar syrinx weight relates probably to the fact that subordinates engage as frequently as other colony members in duet and chorus song. Subordinate males had on average 30% smaller volumes of HVC and RA than dominant males. Because cell density in HVC was similar in both groups of males, the smaller HVC volume reflects the fact that subordinates have about 30% less cells in this nucleus. Therefore, the question arises, how the growth of HVC and RA is accomplished and what are the mechanisms leading to this increase.

My data indicate that there exists a general sex difference in the cellular composition of the song nuclei, at least in HVC, because both groups of males differ from females in having lower cell densities, which is probably linked to larger dendritic fields. This pattern also reveals that HVC in subordinates grows mainly by neuron addition rather than by increasing cell size and spacing between cells. Alternatively, it could be possible that in subordinates the final number of neurons was already present as immature cells, and therefore was missed in our cell counts, and by increasing their metabolic activity at later stages, those cells might grow and become visible with histological techniques (Gahr 1990a; Gahr 1997). On the other hand, the difference in total cell number between both groups of males could reflect a less developed glia and vascular system in subordinates. It has been suggested that in contrast to neurons, glia and endothelial cells can proliferate within HVC under the influence of testosterone (Goldman & Nottebohm 1983).

Neurogenesis and neuronal recruitment is a well-described phenomenon in adult songbirds and is studied mainly in relation to learned behaviours like singing (Goldman & Nottebohm 1983; Alvarez-Buylla et al. 1988; Kirn & Nottebohm 1993) and food-stooring (Barnea & Nottebohm 1994; Patel et al. 1997). Neuronal precursors are born in the ventricular zone of the lateral ventricle and migrate subsequently into the adjacent forebrain tissue. There, they

become eventually recruited into specific brain areas, where they differentiate (for reviews, see Goldman 1998; Gahr et al. 2002). Within the song control system, the recruitment of new neurons is restricted to AreaX and HVC and in the latter it affects only RA-projecting neurons and local interneurons (Paton et al. 1985; Alvarez-Buylla et al. 1988).

The functional relevance of neurogenesis and neuronal replacement has mainly been seen in the light of learning and the acquisition of long-term memory (reviewed by Nottebohm 2002, but see Gahr et al. 2002). However, during development, captive male white-browed sparrow weavers go through a period of subsong when 60 to 120 days old. At later stages, young males engage in singing duet and chorus songs with their siblings and parents but do not sing the solo song until separated and kept solitary or paired to a female (personal observation). Therefore, I believe that males acquire their final song repertoire already during early development but do not use part of it until entering a dominant position within a group. If this view is correct, the addition of new neurons in terms of learning or updating memory seems unlikely.

Rather, it is reasonable to suggest, that factors relating to the birds social status could affect the recruitment and/or survival of new neurons in HVC. One of such factors could be singing activity. It has been suggested for other species that song rate plays a role in promoting neuron recruitment or prevent new neurons from cell death (Li et al. 2000; Lipkind et al. 2002; Absil et al. 2003). At first sight, this seems unlikely for white-browed sparrow weavers, because all birds engage in duet and chorus singing year-round. However, only dominant males sing the long and complex solo song (see chapter 4). Increasing singing activity, when becoming dominant, could possibly drive the structural changes within the song nuclei by inducing the recruitment of new neurons into HVC. There is evidence that HVC can grow in size within seven days by about 60% due to addition of 50,000 neurons (Tramontin et al. 2000). Another factor influencing neuronal recruitment could be the steroid hormone sensitivity of brain areas possibly expressed at the level of the steroid hormone receptor (chapter 6.1) and which could be different in birds of different social status.

However, neuronal recruitment does not explain the growth pattern of RA, because the process has not been observed in this nucleus. In zebra finches, growth of RA during development is accomplished by increase in cell size and spacing, not by neuron addition (Bottjer et al. 1986). This mechanism could apply to the white-browed sparrow weavers as well; the outgrowth of axons from newly recruited RA-projecting HVC neurons could stimulate the restructuring in RA.

Because seasonal plasticity of the song system has been described in males of several songbird species (Tramontin & Brenowitz 2000), one could

argue, that the differences in HVC and RA volume between dominant and subordinate white-browed sparrow weavers reflect seasonal changes occurring in the brain and in song behaviour in this species, because the solo song of dominant males is thought to be restricted to the breeding season (Ferguson 1988a). This appears rather unlikely for two reasons. First, white-browed sparrow weavers in Zimbabwe are year-round territorial and have extended breeding seasons, which can last up to seven months and that are linked to the rainy season. Second, in captivity, white-browed sparrow weavers can breed any time of the year (personal observation). These facts do not suggest that pronounced seasonality has evolved in this species.

Experience-dependent restructuring of brain areas represents an alternative explanation for the observed anatomical differences in the song system of dominant and subordinate male white-browed sparrow weavers. According to this, the initial experience of performing a particular behaviour, in this case the singing of the solo song due to a change of the social status could drive the morphological changes in corresponding brain areas. Support for this hypothesis comes from a study on food-storing behaviour in birds and from studies on fish. Exposing hand-raised marsh tits (*Parus palustris*) for the first time to the experience of food-storing did induce growth of the hippocampal region (Clayton & Krebs 1994). The increase in volume and neuron number was not related to the amount of food-storing experience, which indicates a threshold effect. In an African cichlid fish (*Haplochromis burtoni*), which exhibits two different male phenotypes, experimentally induced changes in social status lead to alteration of neuron size in the preoptic area of the hypothalamus (Fernald 1995). Further studies on white-browed sparrow weavers, involving experimental manipulation of the social status, would certainly give more insight into the mechanism responsible for alterations of brain areas in adult birds.

6. CHARACTERISATION OF THE SONG SYSTEM BY CYTOCHEMISTRY

6.1. Sexually dimorphic and status-dependent expression of AR and ER mRNA in the song control nucleus HVC

6.1.1. Introduction

The sexual differentiation of brain and behaviour is not only determined by the action of gonadal steroid hormones (MacLusky & Naftolin 1981; Adkins-Regan 1987) but also by genetically determined mechanisms (Arnold 2002; Agate et al. 2003; Gahr 2003). It is thought that the temporal and area-specific expression of steroid hormone receptors in the avian brain develops under the control of brain-intrinsic factors (Gahr & Balaban 1996; Gahr & Metzdorf 1999). These receptors mediate the epigenetic, steroid hormone-dependent differentiation of brain areas (for review, see Gahr 1994).

In adult songbirds, sex steroids are responsible for the activation of singing (Leonard 1939; Nottebohm 1980; Vallet et al. 1996) and other reproductive behaviours, such as courtship display and copulatory behaviour (Pröve 1974; Walters & Harding 1988). Moreover, they cause pronounced morphological changes in the brain, thereby affecting volume and cellular composition of brain areas (Nottebohm 1980; Johnson & Bottjer 1993; Tramontin et al. 2003) as well as synapse anatomy (DeVoogd & Nottebohm 1981a; DeVoogd et al. 1985; Gahr & Garcia-Segura 1996).

Steroid hormones can act on target tissue via genomic and non-genomic mechanisms (McEwen 1994). The genomic action involves binding of the hormone to intracellular receptors, which function as ligand-activated transcription factors and modify gene expression. Steroids can also act directly and rapidly by alteration of electrophysiological properties of neurons or second messenger pathways. The dynamics of changes in song behaviour both during development and in adulthood in response to steroid hormones are characteristic of the genomic action via steroid hormone receptors (Gahr & Metzdorf 1997). However, non-genomic actions cannot be excluded. I focus in this chapter on the genomic action of steroid hormones by investigating the distribution and expression of androgen receptors (AR) and oestrogen receptors (ER) within the song system.

The presence of steroid binding sites within the song system has been confirmed by steroid binding studies (Arnold et al. 1976), immunocytochemical localisation of receptor proteins (Gahr et al. 1987; Balthazart et al. 1992; Gahr et al. 1993) and in situ hybridisation of the receptor mRNA (Nastiuk & Clayton

1995; Gahr & Metzdorf 1997). In the adult brain, AR has been found in several song nuclei, e.g. HVC, RA, MAN, Nif, nXIIts, suggesting that androgens exert their effects in various parts of the song system. In contrast, ER is only found in the nucleus HVC, indicating a restricted action of oestrogens. The expression of AR and ER in the song system is dynamic and both receptors are autoregulated by positive and negative feedback mechanisms (Nastiuk & Clayton 1995; Fusani et al. 2000; Leitner et al. 2001b, Fusani et al. 2003).

Studies on sex differences in steroid hormone accumulating cells and in the expression of its receptors were so far only conducted in zebra finches, canaries and two species of duetting *Thryothorus*-wrens (reviewed by Schlinger & Brenowitz 2002). Qualitatively, no sex differences were found in these species, i.e. the general distribution of steroid accumulation and expression of AR and ER is similar in both sexes. However, pronounced variation exists when making quantitative comparisons. The greatest sex differences in the proportion and absolute number of androgen-target cells in HVC were found in the zebra finch, where only the male sings, whereas in species with singing females these sex differences were less pronounced (canary, rufous and white wren) or not existing (bay wren). The comparative analysis suggests that a minimum level of androgen-target cells in HVC is required to learn and/or produce song (Brenowitz et al. 1996). Sexual dimorphism in the absolute number of steroid hormone target cells results from anatomical sex differences, i.e. volume and total number of neurons of brain areas.

In the previous chapter, I have described substantial sex differences in the gross anatomy of song nuclei in duetting white-browed sparrow weavers as well as intra-sexual differences in these parameters in males, which relate to their social status. This cytoarchitectural approach, which uses Nissl-stained material, provides only a limited amount of information, because Nissl stain is an indicator of overall protein synthesis within the cell and relates to overall cell activity. Therefore, sex differences in Nissl-defined brain areas could just reflect differential activation of cells, i.e. fewer cells in the female may be involved in overall protein synthesis, but in other features these cell populations might be similar. This view is supported by a study of Gahr (1990a), who demonstrated that the Nissl-defined HVC volume changes seasonally, whereas the volume defined by ER expressing cells remains constant throughout the year.

In the present study, I used the mRNA expression of AR and ER as markers to investigate sex differences in HVC cell subpopulations, namely AR- and ER expressing cells in the brain of white-browed sparrow weavers. This approach gives insights into the sex-specific regulation of these cell types and into the potential effects of local androgens and oestrogens on the morphology and function of nucleus HVC in adult males and females. Furthermore, it might

be able to clarify the observed intra-sexual variation in the size of the song nuclei.

6.1.2. Materials and Methods

6.1.2.1. Animals

The brains of eight dominant females, eight dominant males and eight subordinate males were analysed. These birds are the same as those used in chapter 5. For details, see section 5.2.1.

6.1.2.2. Tissue processing

In situ hybridisation of androgen receptor (AR) and oestrogen receptor (ER) mRNA was carried out as described in General Methods, section 3.2.3. For processing of AR mRNA the second series from the right hemisphere was used and for ER mRNA, the second series from the left hemisphere was used.

6.1.2.3. Morphometric analysis

Sections were examined using a microscope (Leitz Aristoplan) under darkfield illumination (63x magnification), connected to an image analysis system (Metamorph 4.6, Visitron, Germany). The volume of HVC was estimated on the basis of the distribution of AR expressing cells. The AR distribution allows the unambiguous delineation of the forebrain vocal control areas (Gahr 1997; Gahr & Metzdorf 1997). Further, AR allows a functional definition of the nucleus, because AR-expressing HVC neurons send projections in the descending motor pathway, which controls vocal output (Bottjer & Johnson 1997). For the measurement of volume, the perimeter of the region of interest in each section was drawn on digitised images (1.156 µm/pixel) and the area was calculated by a built-in function of the software. The volume of a nucleus was calculated as the sum of these measurements multiplied with the section thickness and the interval between sections (120 µm).

The length of HVC in its lateral to medial extension was derived from the number of sections where HVC was visible multiplied with the section thickness and the interval between sections (120 µm). The same procedure was applied to the Nissl-defined HVC. For estimation of the difference in HVC area between AR-defined and Nissl-defined HVC, every eighth section was sampled throughout the nucleus. At each sampling point, in adjacent sections from AR- and Nissl-HVC the area of the nucleus was measured and the area of AR-HVC

was expressed as percent of the area of Nissl-HVC. Sampling point 0 represents the first section where HVC was visible. Towards the medial border of the nucleus, HVC length varied between individuals, therefore, sampling points were eliminated when data from less than four individuals were available.

Oestrogen receptors were only expressed in the medial part of HVC and in the paraHVC (Johnson & Bottjer 1995), and therefore, its distribution was not a suitable marker to delimit HVC boundaries. The paraHVC constitutes AreaX-projecting HVC neurons, which extend beyond the medial limit of the Nissl-defined HVC. It was initially defined according to its high density of oestrogen-concentrating cells and the lack of androgen-concentrating cells in the brain of canaries (Johnson & Bottjer 1995). However, in white-browed sparrow weavers AR-expressing cells were frequently found in this area. It was not considered for volume estimation of the AR-HVC.

6.1.2.4. Estimation of mRNA expression level in HVC

Labelled areas were identified due to the elevated number of silver grains per visually inspected brain region and were compared with adjacent sections processed with Nissl stain. The expression level of such selected areas was than estimated with a 0-3 scale (- = no staining, 2-6 silver grains/cell; + = low intensity, 7-13 silver grains/cell; ++ = moderate intensity, 14-19 silver grains/cell; +++ = high intensity, 20-29 silver grains/cell). The same scale was used to estimate the expression level of synaptic proteins, described in the following chapters (chapters 6.2, 6.3). Therefore, the highest intensity of AR and ER mRNA expression did not necessarily get the highest score.

Quantitatively, AR mRNA expression in HVC was measured at the lateral, central and medial level of the nucleus. These levels were estimated according to the Nissl-defined boundaries of HVC. I counted the number of sections where HVC was visible. *Central* was the section N/2. This section divided the HVC in a lateral and medial portion. The *lateral* level was section $N_l/2$ and the *medial* level was section $N_m/2$ ($N_{l,m}$ = number of sections in the lateral and medial part, respectively). At each level, the mean of four measured areas (13100 μm^2 each) across HVC was taken (Fig. 6.1.1). To quantify the level of mRNA expression in a selected area, the image was converted to a greyscale image. A threshold level was then adjusted to separate the silver grains from the background. The above thresholded area was calculated by a built-in function of the software and was expressed as fractional area covered by silver grains. This measurement was named mRNA expression level.

The same measurements were done in the adjacent neostriatum, in an area immediately outside HVC at its ventral border. To correct for different amounts

of background labelling due to different sets of in situ hybridisations I measured the area covered by silver grains in a region of the same section lacking specific labelling. I chose the Tractus septomesencephalicus (TSM) as region for background labelling. Correction was done by subtracting the value for background labelling from the values of HVC and neostriatum, respectively. ER mRNA expression was only measured in HVC at the medial level and in the paraHVC (Fig. 6.1.1), according to the procedure described above.

6.1.2.5. Statistical analysis

Statistical analyses were done with Systat 10.2. General Linear Models (GLM) were used whenever possible. If necessary, data were transformed to meet the assumptions for the use of GLM. Data are presented as means ± standard error. All tests were two-tailed and the significance level was fixed at $\alpha = 0.05$. Data were analysed with Two-Way-Repeated Measures ANOVA with between-subjects factor sex (dominant male, dominant female) or status (dominant male, subordinate male) and within-subjects factor marker (Nissl, AR) or region (lateral, central, medial). For analysis of AR expression level, cell density was included as covariate. If there was no significant influence of the covariate, it was not included in further analyses. If the ANOVA revealed a significant effect of the between-subjects factor, the groups were compared at each level of the within-subjects factor with t-tests. If there was a significant effect of the within-subjects factor or a significant interaction, multiple comparisons were made with the Tukey's HSD test. Differences in the lateral-medial extent of AR-HVC compared to Nissl-HVC were analysed with paired t-tests, separately for each group of birds. Differences in the area size of HVC between the two labels (AR mRNA, Nissl) were analysed with one-sample t-tests and standard Bonferroni correction was applied ($\alpha' = \alpha / n$; n = number of comparisons). This was done separately for dominant males, dominant females and subordinate males. The adjusted significance levels α' were indicated in the text. Relationships between AR and ER expression levels, cell density, cell number and HVC volume, respectively, were analysed with a Pearson correlation. Because some data sets of dominant males were used in two comparisons, first with dominant females, second with subordinate males, the standard Bonferroni technique was applied ($\alpha' = 0.05/2$). On these data sets the significance level α' was fixed at 0.025 and this was indicated in the respective sections of the results.

6.1.3. Results

6.1.3.1. Brain distribution of AR and ER mRNA

Expression of AR- and ER mRNA was found in several brain areas of the white-browed sparrow weaver. No specific labelling was visible using the sense probes. The overall distribution of AR- and ER mRNA was similar in dominant males and females and in subordinate males (Table 6.1). AR mRNA was expressed in low to moderate intensity in most forebrain vocal control areas, i.e. HVC, RA, lMAN, mMAN, and in the brain stem nuclei nXIIts and Ram. AR expression in nucleus RA was very weak and occurred rarely in dominant birds. In all groups of birds, moderate AR expression was found in HVC. The expression extended beyond the medial border of HVC into a narrow band along the dorso-ventral border of the caudo-medial neostriatum. This region has been named “paraHVC” and was originally defined as an area of high density of ER accumulating cells, which lacks AR concentrating cells (Johnson & Bottjer 1995). However, AR was expressed in this region in all females and in most of the males analysed. The dorso-lateral extension of the AR expression showed high interindividual variability and the intensity appeared to be lower than in HVC. Besides the song system, AR mRNA was expressed in the diencephalon, i.e. the thalamic and the preoptic-hypothalamic region and in the mesencephalic nucleus intercollicularis (ICo).

In contrast to AR, the expression of ER was restricted to few forebrain areas. Within the song system, HVC and paraHVC were the only ER containing regions. In all birds, the lateral and central portion of HVC was devoid of ER expression. In females and subordinate birds, the medial HVC frequently contained ER mRNA, although at low levels. In few individuals, this pattern was restricted to the rostral part of the medial HVC. The paraHVC was the region as described above for the AR expression and contained ER expressing cells in all birds. Outside the song system, ER was only found in the diencephalon. The ER mRNA distribution within the hypothalamic-preoptic region was similar in all groups, with the highest expression level found in the ventromedial hypothalamus. The intensity of expression in these diencephalic regions appeared to be highest in females. The presence of ER in these brain regions is not songbird-specific, but represents a general avian pattern, as confirmed by studying 26 species from six avian orders (Gahr et al. 1993).

Table 6.1.1: Distribution of AR and ER mRNA in different brain areas of the white-browed sparrow weaver [1]

Brain areas	AR			ER		
Telencephalon	dom. male	dom. female	subor. male	dom. male	dom. female	subor. male
HVC (n. hyperstriatalis ventrale pars caudale)	++	++	++	+[b,d]	+[d]	+[d]
paraHVC [a]	+	+	+	+	+	+
RA (n. robustus archistriatalis)	+[b]	+[c]	+	-	-	-
lMAN (lateral n. magnocellularis)	+	+	+	-	-	-
mMAN (medial n. magnocellularis)	++	+	+	-	-	-
Area X	-	-	-	-	-	-
Nif (n. interfacialis) / field L	-	-	-	-	-	-
AHP (area parahippocampalis) dorsale / ventrale	-	-	-	-	-	-
Diencephalon						
DLM (dorsolateral n. of medial anterior thalamus)	-	-	-	-	-	-
Thalamus	+[c]	+[c]	+	-	-	-
POA (preoptic area)	+	+	+[f]	+[f]	++	+
HTH (hypothalamus)	+	+	+[f]	++[f]	++	++
Mesencephalon						
ICo (n. intercollicularis)	+	+	+	-	-	-
Rhombencephalon						
nXIIts (n. hypoglossus pars tracheosyringealis)	+[e]	++[e]	n.a.	-	-	-
Ram (n. retroambigualis)	+[e]	n.a.	n.a.	-	-	-
Cerebellum						
Purkinje cells	-	-	-	-	-	-

[a] region according to definition by (Johnson & Bottjer 1995)

[b] found in one individual

[c] found in few individuals

[d] only in the medial part of HVC

[e] data available only from one individual

[f] data available only from few individuals

[1] Intensity of hybridisation signals were estimated by visual inspection and classified on a 0-3 scale: - = no staining, + = low intensity, ++ = moderate intensity, +++ = high intensity, n.a. = data not available. The same scale was used to estimate the expression level of synaptic proteins, described in chapters 6.2, 6.3. Therefore, the highest intensity of AR and ER mRNA expression did not necessarily get the highest score.

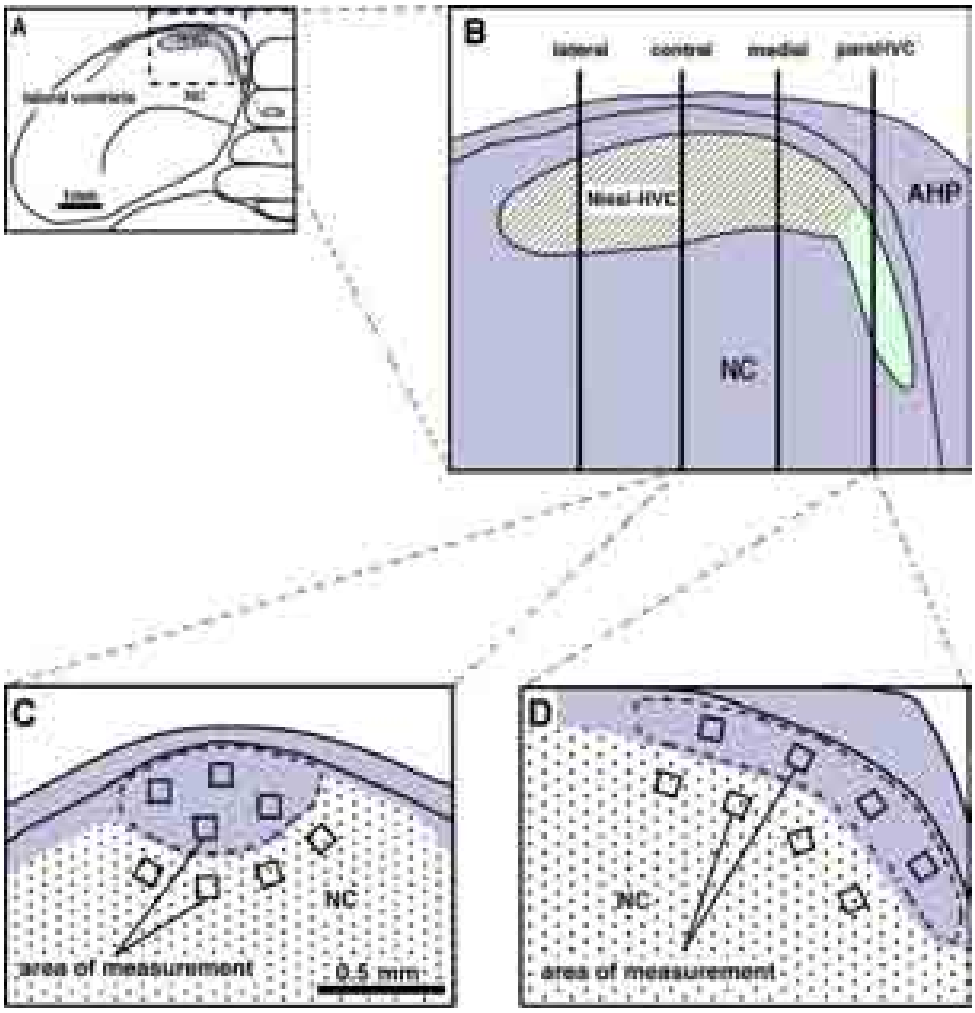
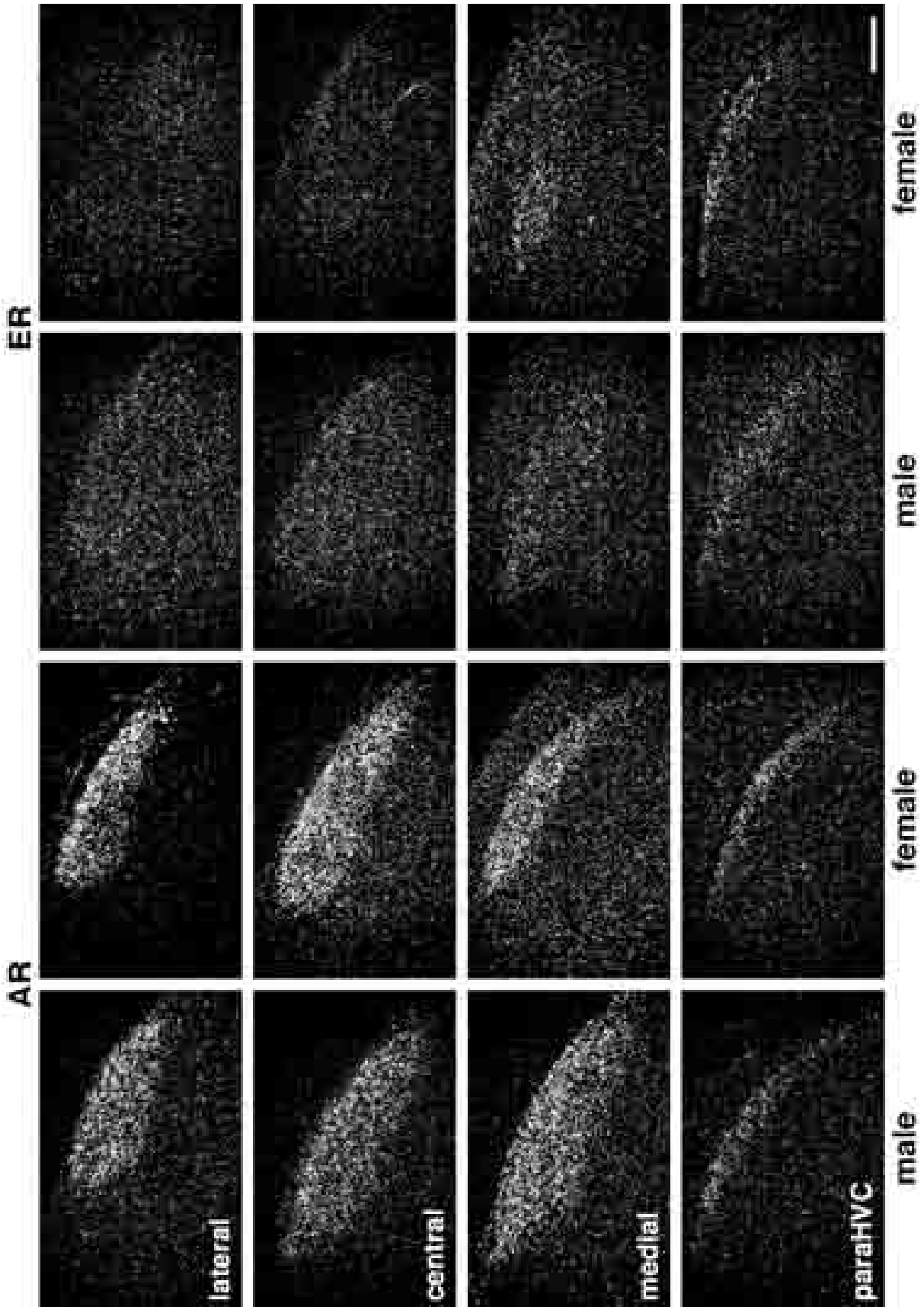


Fig. 6.1.1: The schematic drawings illustrate the procedure for measurement of mRNA expression levels of androgen receptor (AR) and oestrogen receptor (ER). (A) Coronal section of the caudal neostriatum. (B) Enlargement of the dashed square in (A). Four parasagittal regions (lateral, central, medial, paraHVC) were defined according to the lateral and medial limits of the Nissl-HVC (see section 6.1.2.4). (C) Central level of HVC showing the areas of measurement within the nucleus and in the adjacent neostriatum. Similar measurements were done at the lateral and medial level of HVC. (D) Parasagittal view of the paraHVC showing the areas of measurement within and outside of the nucleus. AHP, area parahippocampalis; Cb, cerebellum; NC, neostriatum caudale. Modified after Fusani et al. (2000).

Fig. 6.1.2: (next page): Darkfield photomicrographs showing the expression of androgen receptor (AR) and oestrogen receptor (ER) in nucleus HVC of dominant male and female white-browed sparrow weavers. Each row corresponds to one of the four parasagittal levels indicated in Fig. 6.1.1. In both sexes, AR was expressed throughout the nucleus and it could be used to delineate the boundaries of HVC. Further, AR-expressing cells were found in paraHVC in both sexes. ER expression was not detected at the lateral and central level of HVC. Low intensity of ER expression was found at the medial level in both sexes, in females sometimes restricted to the rostral part of HVC. In all birds, ER was expressed in paraHVC. Dorsal is to the top and caudal is to the right. Scale bar = 300 µm.


AR
ER
male
female
male
female
lateral
central
medial
paraHVC

6.1.3.2. AR mRNA expression in dominant males and dominant females

The AR mRNA was expressed throughout HVC and was elevated compared to the surrounding tissue. Therefore, it was possible to unambiguously delineate the nucleus with this criterion. The volume of HVC, delimited by AR mRNA distribution was compared between the sexes and with the volume of the Nissl-defined HVC (Table 6.1.2). The ANOVA revealed a highly significant effect of sex but not of marker on HVC volume (Table 6.1.3). The volume of HVC was significantly larger in dominant males than in females, irrespectively of the marker used. Moreover, there was a significant interaction between both factors. Posthoc comparisons showed that, whereas in dominant males AR-HVC was smaller than Nissl-HVC, there were no size differences between AR- and Nissl-HVC in females (Tukey's HSD test, Fig. 6.1.3).

Table 6.1.2: Comparison of AR- and Nissl-HVC volume (right hemisphere) of dominant males and females

	Males (N = 8)	Females (N = 8)
Volume of AR-HVC (mm^3)	1.575 ± 0.083	0.599 ± 0.034
Volume of Nissl-HVC (mm^3)	1.700 ± 0.092	0.573 ± 0.027

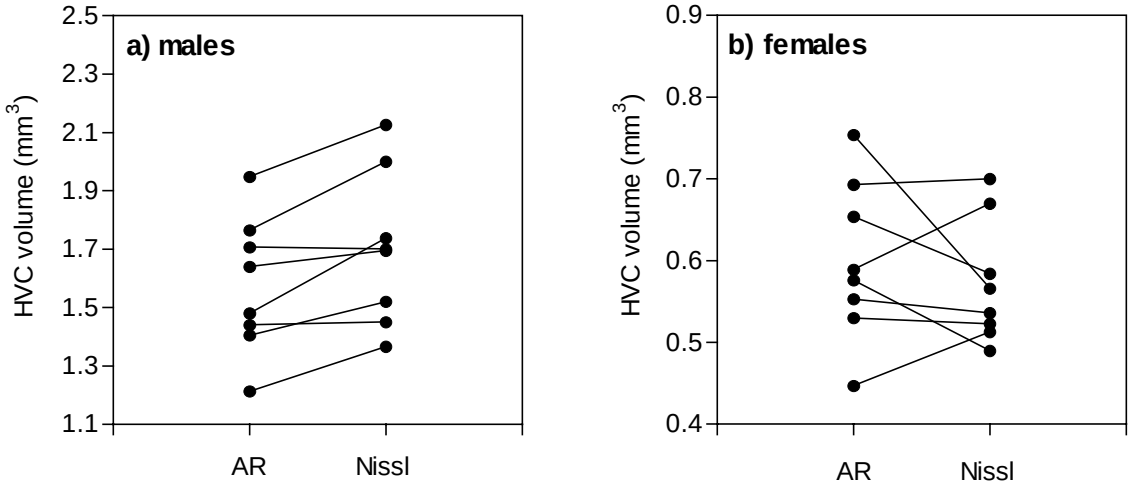


Fig. 6.1.3: Comparison of AR- and Nissl-defined HVC volumes in dominant males (a) and females (b). In males, the AR-HVC was significantly smaller than the Nissl-HVC, whereas no size difference was found in females (ANOVA, followed by Tukey's HSD test). Female HVC was about three times smaller than male HVC. Note that the scales at the graphs are different.

To further investigate the outline of HVC with the two different techniques, I measured the latero-medial extent of the nucleus and I calculated the differences in area size between Nissl- and AR-HVC throughout its extent. In dominant males, the latero-medial extent of HVC was not significantly different between the two labels ($t = 0.66$, $df = 7$, $p = 0.528$). In females, however, HVC extension was significantly larger when labelled with AR mRNA than with Nissl stain ($t = 4.00$, $df = 7$, $p = 0.005$). Concerning the differences in HVC area size, males and females showed different patterns. As indicated from the volumetric measurements, AR-HVC of males was smaller than Nissl-HVC at almost all parts of the nucleus. This size difference was significant at the central to medial level of HVC (Fig. 6.1.4 a; 1950 µm: $p = 0.0001$, 2190 µm: $p = 0.001$, $\alpha' = 0.0036$). The area size of the female AR-HVC was not significantly different from the area of Nissl-HVC throughout the nucleus (Fig. 6.1.4 b; $p > 0.10$ for all tests, $\alpha' = 0.0050$). In both sexes, variation between the two labels was largest in the lateral and medial part of HVC.

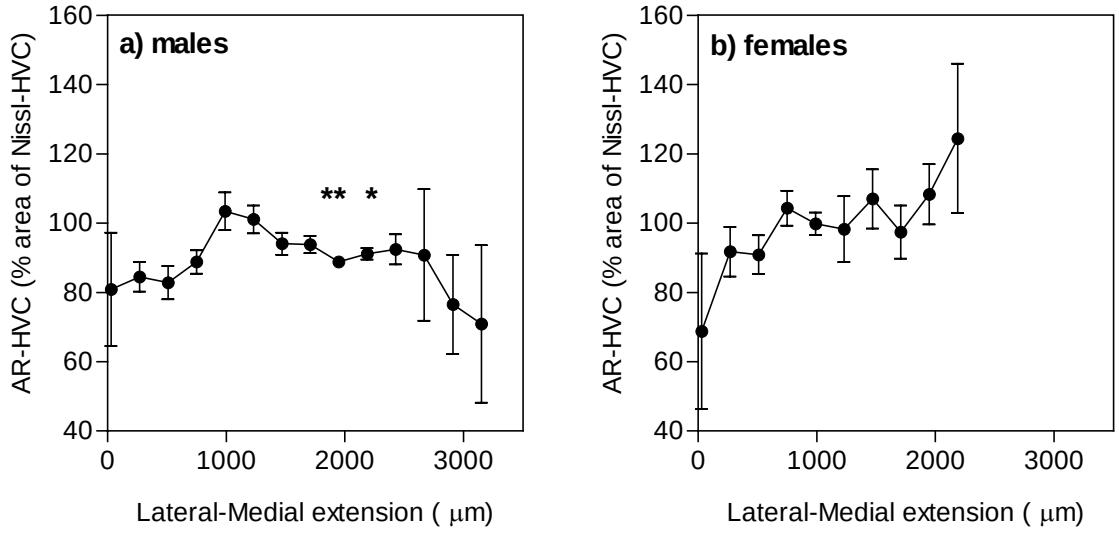


Fig. 6.1.4: Change in AR-HVC area size, expressed as percent of Nissl-HVC, throughout the lateromedial extent of the nucleus. In both sexes, variation between the two labels was greatest in the lateral and medial part of HVC. In males, the area of AR-HVC was significantly smaller than the area of Nissl-HVC towards the medial part of the nucleus (one-sample t-tests with applied Bonferroni-correction, * = $p < 0.002$, ** = $p < 0.0002$, $\alpha' = 0.0036$). In females, the area of HVC did not differ significantly between the two labels, $p > 0.10$, $\alpha' = 0.0050$).

The expression level of AR mRNA in HVC was measured at the lateral, central and medial level of the nucleus. Because cell density could potentially influence the density of AR expression, I included this variable as covariate in the analysis. The ANOVA revealed neither a significant effect of sex nor of cell

density and region on the AR expression level and no significant interactions (Table 6.1.3). Dominant males and females did not differ in the expression level of AR mRNA in HVC (Fig. 6.1.5). However, in the medial part of HVC, AR expression was numerically higher in females than in males (Fig. 6.1.5.a), but the ANOVA did not reveal a significant effect. When comparing AR expression at this level with a t-test, the difference between the sexes was significant ($t = 2.98$, $p = 0.01$, $\alpha' = 0.025$). Further, there was no significant correlation between the mean AR expression level in HVC and cell density in HVC in either sex (males: $r = -0.481$, $p = 0.229$; females: $r = -0.341$, $p = 0.408$). Also, mean AR expression did not correlate with the volume of the AR-HVC (males: $r = -0.304$, $p = 0.465$; females: $r = -0.545$, $p = 0.163$).

In the neostriatum, the ANOVA revealed neither a significant effect of sex, nor of cell density and region on the AR expression level and no significant interactions (Table 6.1.3). The AR expression level in the neostriatum did not differ between males and females (Fig. 6.1.5.b). There was no correlation between cell density and the mean AR expression level in the neostriatum (males: $r = 0.473$, $p = 0.237$; females: $r = 0.090$, $p = 0.833$).

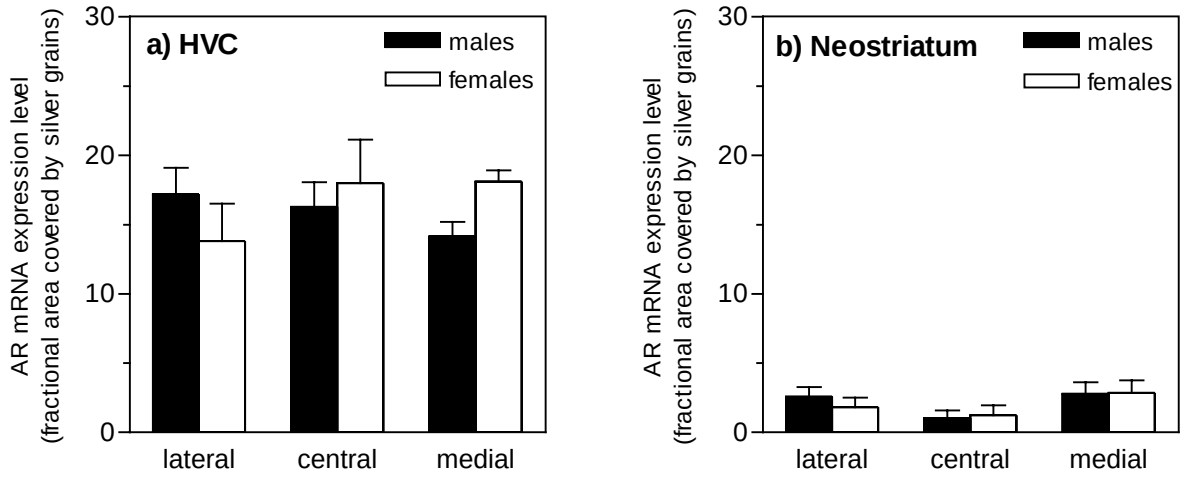


Fig. 6.1.5: Expression level of AR mRNA in HVC (a) and in the adjacent neostriatum (b) at the lateral, central and medial level of the nucleus. The ANOVA revealed no significant effect of sex or region on the AR expression level. In HVC, the lack of a significant difference at the medial level is possibly due to rather low sample size. When compared separately with a t-test, females had a higher AR expression level in this part of the nucleus, compared to males.

6.1.3.3. ER mRNA expression in dominant males and dominant females

The level of expression of ER mRNA was measured in the medial part of HVC and in the paraHVC (Fig. 6.1.1, 6.1.2). Due to a problem with the in situ hybridisation, data were only available from six males and six females. The ANOVA revealed a significant effect of sex and of region on ER expression level (Table 6.1.3). There was no significant interaction. Posthoc comparisons showed that in medial HVC the expression of ER was significantly lower in males than in females. Further, in males ER was expressed at higher intensity in the paraHVC compared to the medial HVC (Tukey's HSD test, Fig. 6.1.6).

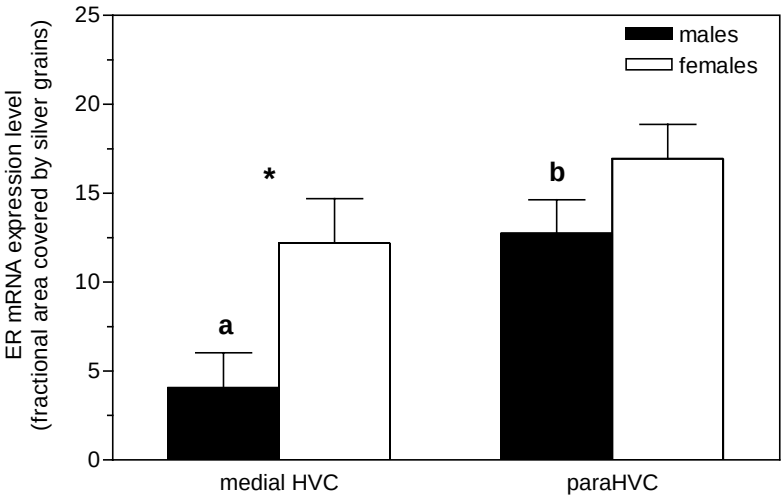


Fig. 6.1.6: Expression level of ER mRNA in the medial part of HVC and in the paraHVC. There were sex differences in the expression level in medial HVC with females having higher levels than males (Tukey's HSD test, * = $p < 0.05$. Further, males had higher ER expression in paraHVC compared to medial HVC (indicated by different letters).

Table 6.1.3: Two-Way Repeated Measures ANOVA of AR- and ER mRNA expression in dominant males and females ($\alpha' = 0.025$).

Factor	df	F	P
AR-HVC volume			
Sex (A)	1,14	192.15	0.0001
Marker (B)	1,14	1.76	NS
AxB	1,14	7.49	0.016
AR mRNA expression level in HVC			
Sex (A)	1,13	1.22	NS
Cell density (B)	1,13	1.64	NS
Region (C)	2,26	1.31	NS
AxC	2,26	0.27	NS
BxC	2,26	1.39	NS
AR mRNA expression level in neostriatum			
Sex (A)	1,13	0.00	NS
Cell density (B)	1,13	0.64	NS
Region (C)	2,26	1.52	NS
AxC	2,26	0.41	NS
BxC	2,26	0.82	NS
ER mRNA expression level			
Sex (A)	1,10	8.62	0.015
Region (B)	1,10	11.00	0.008
AxB	1,10	3.08	NS

6.1.3.4. AR mRNA expression in males in relation to social status

The HVC volume delimited by the distribution of AR mRNA was compared between dominant and subordinate males and with the Nissl-defined HVC volume (Table 6.1.4). The ANOVA revealed a significant effect of status and of marker on HVC volume (Table 6.1.5). The AR-HVC was significantly larger in dominant than in subordinate males, irrespectively of the marker used. In both groups, the AR-HVC was smaller than the Nissl-defined HVC (Tukey's HSD test, Fig. 6.1.7a, b).

Table 6.1.4: Comparison of AR- and Nissl-HVC volume (right hemisphere) of dominant males and subordinates

	Dominant males (N = 8)	Subordinate males (N = 8)
Volume of AR-HVC (mm^3)	1.575 ± 0.083	1.056 ± 0.089
Volume of Nissl-HVC (mm^3)	1.700 ± 0.092	1.211 ± 0.076

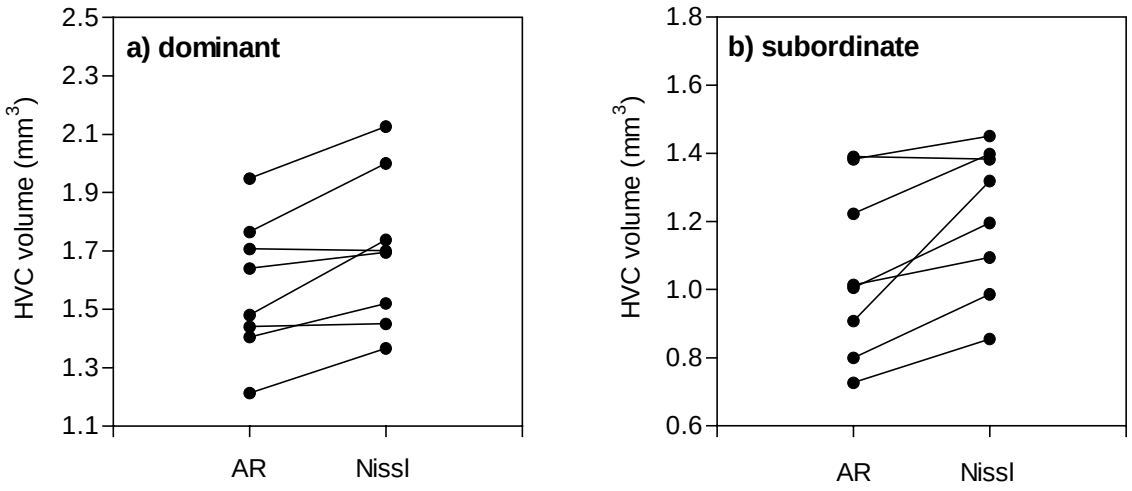


Fig. 6.1.7: Comparison of AR- and Nissl-defined HVC volumes in dominant males (a) and subordinate males (b). In both groups of males, the AR-HVC was smaller than the Nissl-HVC (ANOVA, followed by Tukey's HSD test). Subordinates had about 1.5 times smaller HVC volumes than dominant birds. Note that the scales of the graphs are different.

In subordinate and dominant males, the latero-medial extent of HVC did not differ significantly between the two labels (dominant males: $t = 0.66$, $df = 7$, $p = 0.528$; subordinate males: $t = 1.36$, $df = 7$, $p = 0.214$). Concerning the area size of AR- and Nissl-HVC, both groups of males showed similar patterns. The area of AR-HVC was smaller than Nissl-HVC throughout most parts of its latero-medial extent. These differences were significant towards the medial part and in

subordinates additionally in the lateral part of nucleus HVC (dominant males: Fig. 6.1.8 a; 1950 µm: p = 0.0001, 2190 µm: p = 0.001, α' = 0.0036; subordinate males: Fig. 6.1.8 b; 510 µm: p = 0.0007, 2190 µm: p = 0.0028, α' = 0.0042).

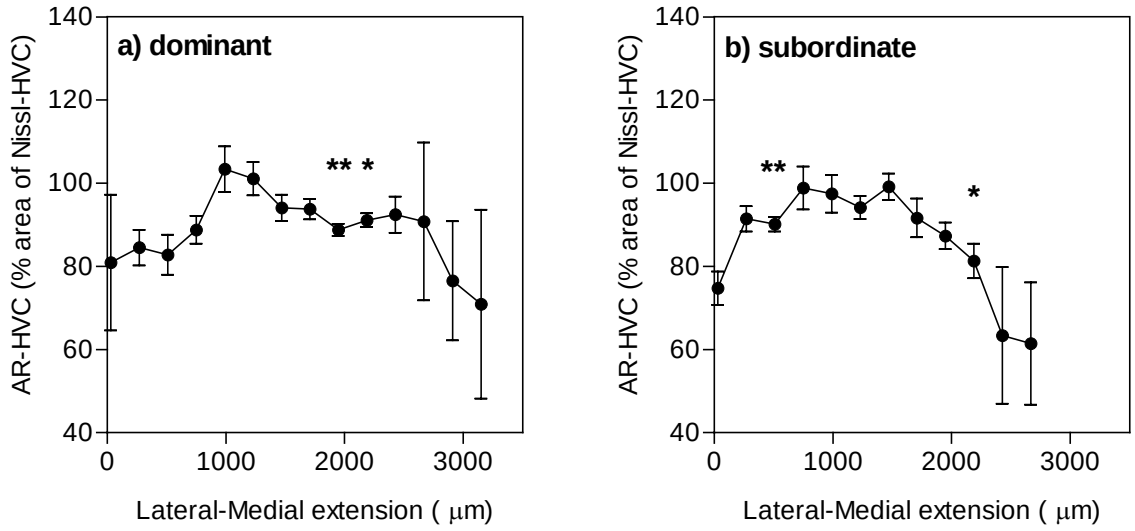


Fig. 6.1.8: Change in AR-HVC area size, expressed as percent of Nissl-HVC, throughout the lateromedial extent of the nucleus. In both groups of males, the area of AR-HVC was significantly smaller than the area of Nissl-HVC towards the medial part of the nucleus and in subordinates also in the lateral HVC (one-sample t-tests with applied Bonferroni-correction, dominant males: * = $p < 0.002$, ** = $p < 0.0002$, α' = 0.0036; subordinate males: * = $p < 0.003$, ** = $p < 0.0008$, α' = 0.0042).

The expression level of AR mRNA was measured at the lateral, central and medial level of HVC. Cell density was included in the analysis as covariate. The ANOVA revealed a significant effect of status and of cell density, but not of region on the AR expression level in HVC (Table 6.1.5). Therefore, I compared the expression level between both groups of males separately for each region with a One-Way ANOVA and included cell density as covariate. At the lateral and central level the AR expression level did not differ between males (effect of status: $F_{(1,13)} = 0.89$, NS; $F_{(1,13)} = 0.18$, NS, respectively, α' = 0.025). At the medial level AR expression was significantly higher in subordinates than in dominant males ($F_{(1,13)} = 9.26$, p = 0.009, α' = 0.025, Fig. 6.1.9.a). Cell density significantly affected AR expression only in the central region of HVC ($F_{(1,13)} = 7.60$, p = 0.016, α' = 0.025). There was a negative correlation between cell density and the mean AR expression level in HVC, which approached significance in subordinates (r = -0.718, p = 0.046) but not in dominant males (r = -0.481, p = 0.228). Furthermore, the volume of AR-HVC was positively

correlated with the mean AR expression level in HVC in subordinate males (r = 0.890, p = 0.003), but not in dominant males (r = -0.304, p = 0.465).

In the adjacent neostriatum outside of HVC, the ANOVA revealed a highly significant effect of status, but not of cell density and of region on the AR expression level and no significant interactions (Table 6.1.5). Therefore, cell density was not included in the further analysis. Posthoc comparisons showed that in subordinates, compared to dominant males, AR expression in the neostriatum was increased at the central (t = 3.26, p = 0.006) and medial level (t = 2.31, p = 0.037), whereas males did not differ at the lateral level of HVC (t = 0.49, p = 0.63, Fig. 6.1.9.b). Cell density did not correlate with the mean AR expression level in the neostriatum in either group of males (dominant: r = 0.473, p = 0.238; subordinate: r = 0.080, p = 0.850).

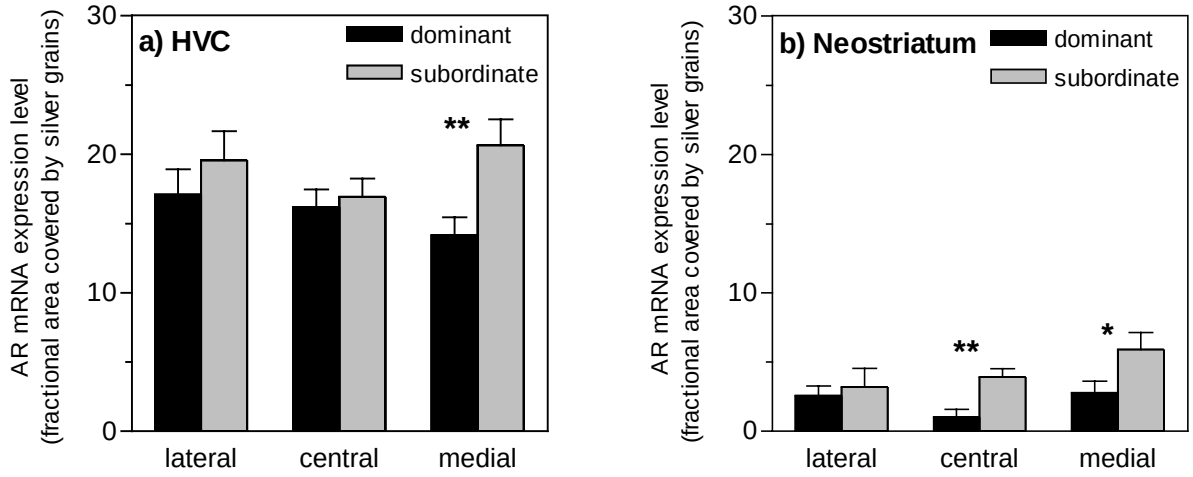


Fig. 6.1.9: Expression level of AR mRNA in HVC (a) and in the adjacent neostriatum (b) at the lateral, central and medial level of the nucleus. In HVC, subordinate males had a higher AR expression level in the medial part of the nucleus, compared to dominant males (ANOVA, ** = $p < 0.01$). In the neostriatum, the AR expression level in subordinates was significantly higher at the central and medial level of HVC (ANOVA, followed by t-test, * = $p < 0.05$, ** = $p < 0.01$).

6.1.3.5. ER mRNA expression in males in relation to social status

The level of expression of ER mRNA was measured in the medial part of HVC and in the paraHVC (Fig. 6.1.1, 6.1.2). The ANOVA revealed a significant effect of region but not of status on ER expression level and no significant interaction (Table 6.1.5). Posthoc comparison showed that in dominant males ER was expressed at higher intensity in the paraHVC than in medial HVC

(Tukey's HSD test). In medial HVC, the expression level of ER was numerically higher in subordinate compared to dominant males. Without the applied Bonferroni correction the effect of status in the ANOVA would have approached significance (p = 0.047).

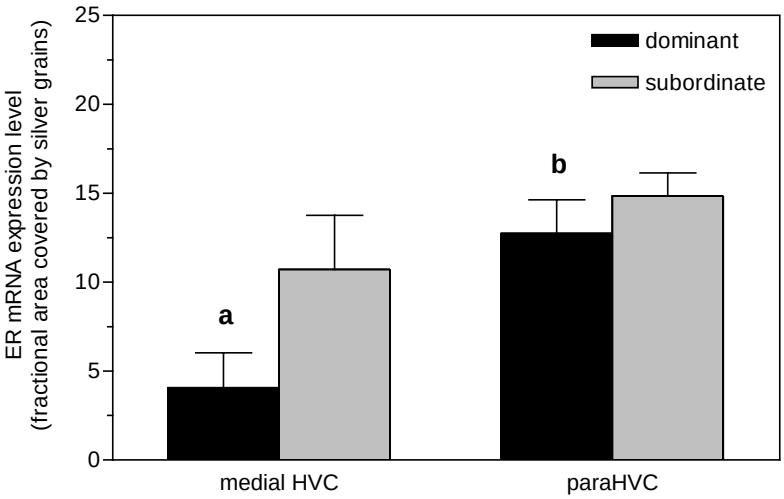


Fig. 6.1.10: Expression level of ER mRNA in medial HVC and in the paraHVC. In dominant males, the ER expression was significantly higher in the paraHVC than in the medial HVC (Tukey's HSD test, indicated by different letters).

Table 6.1.5: Two-Way Repeated Measures ANOVA of AR- and ER mRNA expression in dominant and subordinate males ($\alpha' = 0.025$).

Factor	df	F	P
AR-HVC volume			
status (A)	1,14	18.32	0.001
marker (B)	1,14	24.20	0.0001
AxB	1,14	1.26	NS
AR mRNA expression level in HVC			
status (A)	1,13	6.74	0.022
cell density (B)	1,13	7.38	0.018
region (C)	2,26	1.16	NS
AxC	2,26	1.81	NS
BxC	2,26	1.16	NS
AR mRNA expression level in neostriatum			
status (A)	1,13	12.09	0.004
cell density (B)	1,13	1.74	NS
region (C)	2,26	1.98	NS
AxB	2,26	1.87	NS
BxC	2,26	1.29	NS
ER mRNA expression level			
status (A)	1,12	4.88	0.047
region (B)	1,12	11.69	0.005
AxB	1,12	3.36	0.092

6.1.4. Discussion

In this study, I investigated inter- and intrasexual differences of the song control system by using cytochemical markers, i.e. the expression of AR and ER mRNA. 1) The general distribution of AR and ER in the song system of male and female white-browed sparrow weavers was monomorphic. AR was found in several forebrain vocal control areas such as HVC, RA, MAN and in the brain stem nuclei nXIIts and Ram. ER expression was restricted to HVC and paraHVC. 2) The volume of AR-HVC was significantly larger in dominant males than in dominant females. Compared to the volume of Nissl-HVC, AR-HVC was smaller in dominant males but not in dominant females. 3) There was no sex difference in the AR expression level throughout HVC and surrounding tissue. 4) The expression level of ER in medial HVC was significantly higher in dominant females than in dominant males. 5) The volume of AR-HVC was significantly larger in dominant males than in subordinate males. In both groups of males, AR-HVC was significantly smaller than Nissl-HVC. 6) Subordinate males had increased AR expression levels in medial HVC and in the neostriatum surrounding central and medial HVC. 7) The level of ER expression was higher in subordinates than in dominants, but the difference only approached significance.

6.1.4.1. Distribution of AR and ER mRNA in song nuclei of white-browed sparrow weavers

The present study shows that although plasma levels of steroid hormones of male and female white-browed sparrow weavers were very low (chapter 7), the forebrain song control nuclei exhibited pronounced steroid hormone sensitivity. This finding is particularly interesting when considering that circulating levels of gonadal steroid hormones are known to exert profound effects on song behaviour and the morphology of the song system both during development and in adulthood (for reviews, see Bottjer & Johnson 1997; Schlinger 1997b).

The occurrence of AR in the song control nuclei HVC, RA, lMAN, mMAN, nXIIts and Ram agrees with the pattern described for other songbird species such as the zebra finch, canary, starling, white-crowned sparrow and the slate-coloured boubou shrike (reviewed by Gahr 2001). In comparison to the intense AR expression in nucleus RA of canaries (Gahr & Metzdorf 1997), in white-browed sparrow weavers the expression in this area was very low and not present in every individual. This finding could relate to individual genetic differences in the distribution of AR or to inter-individual variability in physiological conditions, which induce changes in expression levels of steroid

hormone receptors in certain brain areas. Variable AR expression among individuals has also been described for song nuclei DLM, Nif and Area X (Gahr & Metzdorf 1997). A brain area of white-browed sparrow weavers, which frequently showed AR expression, was the paraHVC. This finding clearly contrasts with the definition of this area, originally proposed by Johnson & Bottjer (1995) for canaries. According to it, paraHVC contains Area X-projecting neurons, which are exclusively oestrogen-target cells and never concentrate androgen. Further, no RA-projecting neurons were found in this region (Johnson & Bottjer 1995). In the present study on white-browed sparrow weavers, it is possible that both types of projection neurons occur in paraHVC. Alternatively, Area X-projecting and/or non-projecting paraHVC-neurons express AR in addition to ER, although both receptor types never occur in the same cell (Gahr 1990b). These contrasting expression patterns indicate a species- or family-specificity in the cellular composition of paraHVC. AR expression in this region was also detected in another ploceid species, the yellow weaver (*Ploceus subaureus*, personal observation) and occurs occasionally in zebra finches (M. Gahr, personal communication).

The expression of ER in the song system of white-browed sparrow weavers was restricted to HVC and paraHVC and matched the general pattern found in songbirds (Gahr et al. 1993; Gahr 2001). However, the extent of ER expression in HVC varies between families. Whereas in the canary and other Fringillidae, ER is expressed throughout the nucleus, in Estrildidae such as the zebra finch, only the medial portion of HVC shows labelling (Gahr et al. 1993). Similar to the estrildid finches, in white-browed sparrow weavers the ER expression was found in the medial part of HVC, while the lateral and central part of the nucleus was devoid of labelling.

The observation that the general distribution of the AR and ER expression within the song system is monomorphic is not surprising, because this has already been reported for other songbird species with varying degrees of sexually dimorphic song behaviour (Arnold 1980; Brenowitz & Arnold 1992; Brenowitz et al. 1996; Gahr & Metzdorf 1997). The zebra finch, nevertheless, represents an exception to this pattern, because of its extremely sexually dimorphic song system and there, some nuclei lack AR expression in adult females (Kim et al. 2004).

6.1.4.2. Comparison of the volume of AR- and Nissl-HVC in dominant males and females

The HVC volume delineated by the distribution of AR mRNA was about 2.6 times larger in dominant males than in dominant females. Delineation of HVC

by cytoarchitecture (see chapter 5) revealed a similar sex difference. The finding that AR-HVC matches the size of Nissl-HVC in dominant females whereas in dominant males AR-HVC was smaller than Nissl-HVC, suggests a genetically determined sex difference in the spatial distribution of AR expressing cells rather than a measurement error. This is further supported by the fact that subordinate males showed the same pattern than dominant males. In zebra finches and canaries, the distribution of AR expressing cells in HVC is already sexually dimorphic at posthatching day 9, respectively 10, when AR is first expressed in that brain region (Gahr et al. 1996; Gahr & Metzdorf 1999; Kim et al. 2004). This expression pattern is independent of the action of gonadal hormones and most likely driven by brain-intrinsic factors (Gahr & Metzdorf 1999).

6.1.4.3. Sexually dimorphic expression of AR in the song nuclei of dominant birds?

The AR expression level, expressed as the fractional area covered by silver grains in HVC and surrounding tissue was similar in dominant males and females. In both sexes, the expression level within HVC was about six times higher than in the surrounding neostriatum, which made it possible to delineate the nucleus by this criterion. That the AR mRNA is indeed translated into protein can be inferred from localisation of the AR protein in HVC by immunocytochemical studies (Balthazart et al. 1992; Smith et al. 1996; Soma et al. 1999). The measurement of the area covered by silver grains constitutes an indicator of the amount of AR mRNA present in that brain area, yet it does not produce quantitative data about the amount of mRNA per cell. However, I have shown in the previous chapter that cell density is about 20 % higher in HVC of females than in males. Therefore, it can be concluded that females either express less AR mRNA per cell than males or when expressing similar amounts of AR mRNA per cell, females should have additional cells, which do not express AR. The lack of a significant influence of cell density on AR expression in the ANOVA together with the lack of a positive correlation between both factors support the second conclusion. This would mean the proportion of AR expressing cells is 20 % lower in females compared to males. It seems unlikely that an ‘activational’ effect of gonadal steroid hormones accounts for this sex difference because the female song system, in particular HVC, exhibits pronounced AR expression despite having undetectable plasma levels of testosterone (T, chapter 7). Furthermore, in dominant males, the AR expression level in HVC does not correlate with circulating plasma testosterone levels suggesting that the AR expression in HVC is independent of the acute hormone

levels that I have measured. However, it may be that the differences have been 'organised' at certain times of life.

Sex differences in androgen-accumulation of HVC cells have been investigated in a number of studies on songbirds by *in vivo* autoradiography (Arnold 1980; Brenowitz & Arnold 1985; Brenowitz & Arnold 1992; Brenowitz et al. 1996). It was found that in zebra finches, where only the male sings, the proportion of androgen-target cells was significantly higher in males than in females whereas in canaries and two duetting species, in which both sexes are able to produce vocalisations, no such sex differences were found. With the technique used in those studies, the amount of silver grains per cell corresponds to the amount of radioactive-labelled testosterone, which was previously injected in the live bird. However, the results obtained by *in vivo* autoradiography have to be considered with caution. The T injection immediately before sacrifice might have induced short-term effects at the level of the androgen receptor protein due to acute fluctuations of circulating T, which are not functionally relevant and which probably differ from long-term regulatory mechanisms. It has been shown in mice that in brain areas, which are sexually dimorphic in respect to AR density in untreated animals, T treatment induces a rapid increase in AR levels at similar intensity in both sexes (Lu et al. 1998). Further, for AR mRNA a biphasic regulatory pattern has been suggested which is time and tissue specific (Kerr et al. 1995; Handa et al. 1996). In all species studied so far, including the white-browed sparrow weaver, the absolute number of androgen-target cells in HVC is sexually dimorphic, resulting simply from volumetric sex differences (Gahr 2001).

From comparative analysis of the studies mentioned above it has been proposed that the proportion of androgen-target cells in HVC relates to the ability to learn and/or produce song, whereas the total number of these cells relates to song complexity (Brenowitz & Arnold 1992). It is conceivable that a minimum percentage of AR expressing cells must be present in HVC to facilitate song production. Concerning the results of the present study, it seems unlikely that the presumably 20 % lower proportion of AR expressing cells in HVC of females limited their song output. Furthermore, subordinate males have a similar proportion of AR expressing HVC cells as dominant males and even higher AR expression levels; still, they do not show the same song output as dominant males. However, based on the data available, it cannot be excluded that the total number of HVC neurons or the total number of AR expressing HVC neurons represents a limiting factor for the production of the solo song. Experiments involving the characterisation of HVC in testosterone-treated females that produce solo song, would further clarify this problem.

6.1.4.4. Sexually dimorphic expression of ER in the song nuclei of dominant birds?

The ER expression level in medial HVC was significantly higher in females than in males whereas no sex difference was found in paraHVC. It seems unlikely that the higher cell density in female HVC accounted for this sex difference because there was no significant correlation between both factors. Rather, the proportion of ER expressing cells is 20 % lower but female HVC cells express higher levels of ER mRNA per individual cell.

This sex difference could reflect a difference in the activity of the enzyme aromatase in the adjacent caudomedial neostriatum (NCM), which may result in different local levels of oestrogen. A negative feedback mechanism for the regulation of ER mRNA by oestrogen has been described in birds (Fusani 1999) and mammals (Lauber et al. 1991). According to it, female white-browed sparrow weavers might have reduced local levels of oestrogen in NCM compared to dominant males. NCM is an area of highest aromatase expression in the songbird telencephalon (Schlinger 1997a; Metzdorf et al. 1999). Aromatase activity (AA) has been found to be higher in the hypothalamus and preoptic area of male Japanese quails (*Coturnix japonica*) compared to females and similarly, its induction by T-treatment is higher in males than in females (Balthazart & Adkins-Regan 2002). In songbirds, females have about 70 % lower levels of AA in NCM than males (Fusani 1999).

The regulation of ER expression levels in the brain by circulating levels of oestrogens in the blood represents an alternative mechanism (Lauber et al. 1991; Fusani et al. 2000) but in the case of the white-browed sparrow weavers this possibility can be ruled out, because both sexes had basal plasma levels of oestradiol (chapter 7).

The functional significance of the observed sex difference in ER expression in medial HVC remains largely unclear. However, inhibition of brain oestrogen formation in T-treated female canaries leads to incomplete masculinisation of song behaviour, accompanied by increased levels of ER in HVC (Fusani et al. 2003), which suggest a role of local oestrogens in the song production pathway. It is also conceivable, that sex differences in the ER expression level relate to other sex-typical behaviours, which are controlled by the same brain area (Gahr 2001). Such an example is the copulation-solicitation-display (CSD) of female canaries, which is oestrogen-sensitive and HVC appears to be part of its control circuit (Del Negro et al. 1998).

6.1.4.5. The expression of AR and ER mRNA in relation to social status

The delineation of HVC by the distribution of AR mRNA revealed a similar degree of intra-sexual size difference as the measurements using a cytoarchitectural marker (see chapter 5). The finding that in both groups of males AR-HVC was smaller than Nissl-HVC and this pattern contrast with that in females, means that the distribution of AR-expressing cells within HVC is either based on a genetic or on an 'organised' sex difference.

Subordinate males had a higher AR expression level in medial HVC compared to dominant males. Cell density was similar in both groups of males and was not correlated with the AR expression level; this suggests that dominant and subordinate males have a similar proportion of AR expressing cells. Therefore, a higher amount of AR mRNA per cell must account for the higher AR expression level found in medial HVC of subordinates. Higher cellular mRNA levels could reflect a higher turnover of the AR protein in subordinate males relating to increased availability of local T in this brain region. Long-term castrated rats have reduced levels of AR mRNA in the medial preoptic area and this effect can be reversed by treatment with androgen (Handa et al. 1996). However, an 'activational' effect of gonadal androgen cannot account for the higher expression levels of AR in the brain of subordinates, because both groups of males had similar low levels of plasma testosterone and there was no correlation between the AR expression level and the circulating testosterone level (chapter 7). Furthermore, subordinates had several times smaller testes compared to dominants (see chapter 5). The similar proportion of AR expressing cells indicates that this is not a limiting factor for song production in subordinate males. Nevertheless, subordinates differ from dominants in having about 30 % fewer HVC cells and accordingly fewer AR expressing cells, which could represent a limitation.

Regarding the growth of HVC in subordinate males and assuming that those males have higher local levels of androgen in the brain the latter could be an indication for steroid hormone-induced neurogenesis (Rasika et al. 1994; Hidalgo et al. 1995). Testosterone increases the recruitment and/or survival of new HVC neurons (Rasika et al. 1994; Rasika et al. 1999). Also, testosterone stimulates the proliferation of glia and endothelial cells in HVC (Goldman & Nottebohm 1983; Louissaint et al. 2002). The fact that HVC of subordinate males is about 400 µm shorter in its latero-medial extension than HVC of dominant males (Fig. 6.1.8) supports the idea that HVC grows not only in its dorso-ventral and caudo-rostral extension but also latero-medial. The higher AR expression level in medial HVC and surrounding neostriatum in subordinates is probably an indicator that growth occurs mainly in the medial part of the

nucleus. Alternatively, an increase in HVC size might be attained by increasing the activity and soma size of already existing neurons in the adjacent neostriatum accompanied by increased levels of AR mRNA per cell. Such a scenario would be in line with the reduced cell density in the neostriatum of subordinates (chapter 5). T-treatment of adult male and female canaries was shown to increase selectively the soma size of RA-projecting neurons that accumulate androgen (Johnson & Bottjer 1993; Rasika et al. 1994). Furthermore, the cellular content of ARmRNA in HVC of canaries, measured as the number of silver grains per cell, can vary 3-5 fold between singing and non-singing periods (Gahr & Metzdorf 1997).

The ER expression level in medial HVC was numerically higher in subordinates than in dominants but the difference only approached significance. Because of equal cell density in both groups, the cellular amount of ER mRNA must be higher in subordinates. Assuming a negative feedback mechanism for the regulation of ER mRNA (Lauber et al. 1991; Handa et al. 1996; Fusani 1999) it could be, that aromatase activity in NCM of subordinates is selectively down regulated and thereby reducing the level of available oestrogen in this brain region. Given the complexity of the regulation and function of the enzyme aromatase and the increasing evidence for modulation by environmental cues (Balthazart & Ball 1998), such a mechanism seems possible.

The similar levels of ER mRNA in paraHVC across status indicate that this area is differently regulated than HVC and probably not involved in adult song production. Although paraHVC is interconnected with other song control nuclei (Foster & Bottjer 1998), its function within the song circuitry remains unknown.

6.2. Synaptic proteins as novel markers for the cytochemical characterisation of the song system – the zebra finch as model species

(Differential expression pattern and steroid hormone sensitivity of SNAP-25 and synaptoporin mRNA in the telencephalic song control nucleus HVC of the zebra finch)[2]

6.2.1. Introduction

Gonadal steroid hormones, androgens and oestrogens, play an important role for the plasticity of behaviours related to reproduction. Examples for gonadal hormone-controlled behavioural systems are the mate calling by South African clawed frogs, *Xenopus laevis* (Kelley 1980), the copulatory behaviour of male Japanese quail (Beach & Inman 1965) and the lordosis behaviour of female rodents (Pfaff 1980). In adult songbirds, the two main functions of song in male birds are mate attraction and territory defence against other males, for review see Catchpole & Slater 1995). Song patterns are sensitive to testosterone and its oestrogenic metabolites, the latter probably formed in the brain through aromatisation (Marler et al. 1988; Schlinger & Arnold 1991; Walters et al. 1991; Schlinger & Brenowitz 2002).

Because the brain circuit that controls the song pattern has been identified, the songbird is a distinct model to study the neural mechanisms of individual behavioural differences and behavioural plasticity of adult vertebrates. Most of the vocal areas contain sex steroid receptors, i.e. androgen (ARs) and/or oestrogen receptors (ERs) throughout life (Gahr & Metzdorf 1997). These receptors are thought to be key elements of neural circuits that are sensitive to sex steroids (Arnold & Breedlove 1985; Matsumoto 1991). Furthermore, many parts of the forebrain adjacent of vocal areas, in particular the caudo-medial neostriatum, have high capacities to produce oestrogen (Schlinger 1997a; Balthazart & Ball 1998; Metzdorf et al. 1999). The functional consequences of this oestrogen production are not understood but one target of these oestrogens might be nearby vocal areas such as HVC. In adult songbirds, sex steroids are known to affect the volume of song areas (Nottebohm 1980), the overall morphology (Gahr 1990a), neuronal recruitment and angiogenesis (Louissaint et al. 2002), neurotransmitter systems (Barclay & Harding 1990; Perlman et al. 1995; Singh et al. 2003), protein synthesis (Konishi & Akutagawa 1981), and changes in synapse anatomy (DeVoogd & Nottebohm 1981a; DeVoogd et al. 1985; Gahr & Garcia-Segura 1996).

[2] Original title published as: Voigt, C., Metzdorf, R. & Gahr, M. 2004 *J. Comp. Neurol.* **475**, 83-94. The layout of the paper has been adjusted. Methods, already described in chapter 3 were removed.

Despite intensive work on hormone-dependent neural features and song behaviour, the functional significance between both remains controversial. For example, the neuroanatomical features of two forebrain areas of the song circuit shown to be involved in song patterning were not correlated with differences in the overt song repertoire in wild canaries (*Serinus canaria*) whereas the latter correlated with the testosterone levels (Leitner et al. 2001b). This mismatch is further supported by a study of testosterone-induced singing of female canaries (Fusani et al. 2003). While untreated females did not sing, testosterone-treated females developed male-like song. In testosterone-treated females in which the oestrogen formation was inhibited through systemic treatment with an aromatase inhibitor, syllable repetition rates were reduced. These behavioural differences between the two groups of hormone-induced singing females were not reflected in overall HVC morphology but in differences of gene expression such as brain derived neurotrophic factor (Fusani et al. 2003). Syllable repetition rate and complexity are signals for female copulation solicitation display (Kreutzer & Vallet 1991; Vallet et al. 1998). These data suggest (1) that certain phenotypes of the vocal system are under oestrogenic control while others are under androgenic control, (2) that not all hormone-dependent changes in gene-expression are linked with hormone-dependent gross-morphology, and (3) that neural phenotypes determining the sexual quality of the overt song phenotype need to be studied close to processes of neurotransmission. Such hormone-dependent dynamics can be found in the number of synapses, the type of synapses or the efficiency of the synaptic machinery. Sex steroids are, indeed, an important factor in the regulation of synaptic plasticity (Lustig et al. 1993; Jacobsson et al. 1998; Brake et al. 2001). For example, oestrogen treatment of female ovariectomized rats increases the levels of synaptic proteins (Brake et al. 2001) and dendritic spine density in hippocampal neurons (Woolley & McEwen 1992). Furthermore, increased hippocampal synaptophysin levels are associated with oestrogen-dependent spatial memory in rats (Frick et al. 2002). Since the expression of proteins associated with synapses are useful markers to investigate synaptic plasticity, we used a neurochemical approach to study the mRNA distribution and expression pattern of two synaptic proteins, synaptoporin (SPO) and synaptosomal-associated protein 25 kDa (SNAP-25) in the zebra finch brain under different conditions of oestrogen availability. Given the high level of oestrogen producing enzyme in the forebrain and the trophic action of oestrogens, the effect of oestrogen deprivation on gene expression in vocal areas was compared with its global effects on gene expression and its global and local effects on brain area morphology.

Information between nerve cells is transmitted by the release of neurotransmitter from small synaptic vesicles of the presynaptic nerve terminal

and binding to receptors of the postsynaptic membrane. In the process of neurotransmitter release, many different proteins are involved. Intensively studied proteins are those constituting small synaptic vesicles and plasma membrane proteins which are essential for fusion and exocytosis of synaptic vesicles (for review see Südhof 1995) Since the synaptic machinery involves many proteins we focused on SNAP-25 because of its ubiquitous role and on synaptoporin because of its differential expression pattern. The avian SPO (Bixby 1992), is a synaptic vesicle protein which most closely resembles rat SPO, a member of the synaptophysin family, also called synaptophysin II (Knaus et al. 1990; Fykse et al. 1993). The synaptophysins are thought to be involved in the formation of the fusion pore at the onset of exocytosis (for review, see Jahn & Südhof 1994). In difference to the ubiquitous synaptophysin I (Wiedenmann & Franke 1985; Leube et al. 1987), SPO is expressed in restricted areas of the rat brain, suggesting its association with specific neuronal pathways (Marqueze-Pouey et al. 1991; Singec et al. 2002). SNAP-25 is a plasma membrane protein of synaptic terminals that plays as part of the core complex an important role in synaptic vesicle exocytosis (Oyler et al. 1989; Südhof 1995).

We cloned the zebra finch SNAP-25 and SPO cDNA and used in situ hybridisation to investigate its expression, in particular in the areas of the song control system. We found that transcripts of both proteins were differently expressed in several forebrain areas. In HVC and in RA, SNAP-25 expression was elevated and SPO expression was similar or reduced relative to the surrounding tissue. Further, blocking of the oestrogen producing enzyme aromatase, significantly increased SPO expression level in HVC, whereas SNAP-25 expression was not affected, despite an absolute reduction in HVC volume. This decreased HVC size is not area-specific but correlates with an overall reduction of size and an overall increase of cell density of the forebrain.

6.2.2. Material and Methods

6.2.2.1. Animals

Adult male zebra finches (*Taeniopygia guttata*) were taken from a mixed-sex-colony kept in an aviary on 12/12 L/D cycle. For the experiment, they were transferred to individual cages and kept under the same photoperiod. One group of males (n = 8) was implanted subcutaneously with a time-release pellet containing 1.0 mg Fadrozole (30 day release, Innovative Research of America, USA; Fadrozole was kindly provided by Dr. Bhatnagar, Ciba-Geigy). A second

group of males (n = 6) was implanted with a placebo pellet and served as control. All birds were sacrificed four days after implantation.

6.2.2.2. Tissue processing

Birds were killed with an overdose of chloroform and perfused transcardially, first with 0.9 % saline, followed by 4 % FPBS. Brains were removed, weighed and postfixed in 4% phosphate buffered formaldehyde (pH 7.3) at 4°C until analysis. After fixation, one half of the brain was immersed in RNase-free phosphate buffered sucrose (pH 7.3; first in 10 %, followed by 30 %; 24 hours each) and cut on a freezing microtome into parasagittal sections. Serial sections were collected for processing with in situ hybridisation and Nissl staining. In situ hybridisation of synaptosomal-associated protein 25 kDa (SNAP-25), synaptoporin (SPO) and androgen receptor (AR) mRNA and Nissl staining were carried out as described in General Methods, section 3.2.

6.2.2.3. Morphometric analysis

The volume of HVC was estimated on the basis of the distribution of AR and SNAP-25 expressing cells and conventional Nissl staining. The distribution of AR allows the delineation of the forebrain vocal area HVC in congruence with its projections in the descending motor pathway. (Gahr et al. 1996; Bottjer & Johnson 1997; Soma et al. 1999). Similar to AR, SNAP-25 showed elevated expression in HVC and therefore was a suitable marker to delimit HVC boundaries. SPO expression in HVC was lower or at the same level as in the surrounding tissue and caused ambiguous delineation of HVC boundaries. Therefore, it was not considered as a marker for volume estimation. Measurements were done using an image analysis system on a video screen (Metamorph 4.6, Visitron, Germany). For volume measurements, the perimeter of the region of interest in each section was drawn on digitised images (0.729 µm/pixel) and the area was calculated by a built-in function of the software. The volume of HVC was calculated as the sum of these measurements multiplied with the section thickness and the interval between sections (120 µm). RA volume was measured in Nissl-stained sections as described for HVC. Telencephalon volume was estimated by sampling every eighth section throughout the extent of the brain hemisphere.

The density of cells in HVC and the surrounding tissue was estimated in Nissl-stained sections under a 100x oil-immersion objective with help of the image analysis system Metamorph 4.6. Although we did not use specific markers to distinguish between neurons and glia cells, the histological staining

used is likely to stain predominantly neurons (Ball & MacDougall-Shackleton 2001). In each animal at the lateral, central and medial level of HVC (see below) a counting frame of 10000 µm^2 was analysed within the borders of HVC and in the neostriatum adjacent to HVC using the digitised images. We counted all profiles that contained one or two nucleoli throughout the entire depth (30 µm) of the section that fell within the boundaries of the counting frame. Density measurements are presented as 10^4 cells/mm^3.

6.2.2.4. Estimation of mRNA expression level in HVC

Labelled areas were identified due to the elevated number of silver grains per visually inspected brain region in relation to homologous areas of sections processed with the sense probes. The expression level of such selected areas was than estimated with a 0-3 scale (- = no staining, 2-6 silver grains/cell; + = low intensity, 7-13 silver grains/cell; ++ = moderate intensity, 14-19 silver grains/cell; +++ = high intensity, 20-29 silver grains/cell). Quantitatively, we measured AR, SNAP-25 and SPO mRNA expression in HVC at the lateral, central and medial level of the nucleus (Fig. 6.1.1.). These levels were estimated according to the AR mRNA-defined boundaries of HVC. For SNAP-25 and SPO, the adjacent sections were measured. We counted the number of sections where HVC was visible. *Central* was the section N/2. This section divided the HVC in a lateral and medial portion. The *lateral* level was section $N_l/2$ and the *medial* level was section $N_m/2$ ($N_{l,m}$ = number of sections in the lateral and medial part, respectively). At each level, the mean of four measured areas (5211 µm^2 each) across HVC was taken. To quantify the level of mRNA expression in a selected area, the image was converted to a greyscale image. A threshold level was then adjusted to separate the silver grains from the background. The above thresholded area was calculated by a built-in function of the software and was expressed as fractional area covered by silver grains. This measurement was named mRNA expression level and represents the mean from the three regions of the nucleus that were measured. The same measurements were done in the adjacent neostriatum, in an area immediately outside HVC at its ventral border. To correct for different amounts of background labelling due to different sets of in situ hybridisations we measured the area covered by silver grains in a region of the same section lacking specific labelling. We chose the Tractus septomesencephalicus (TSM) as region for background labelling. Correction was done by subtracting the value for background labelling from the values of HVC and neostriatum, respectively.

6.2.2.5. Statistical analysis

Statistical analyses were performed with Systat 10.2. We used the General Linear Model (GLM) whenever possible and some data sets were transformed to meet the assumptions for the use of GLM. Post hoc comparisons of repeated measures were done using the Tukey's HSD test. Body condition, brain weight and telencephalon size were compared between groups with t-tests. All tests were two-tailed and the significance level was fixed at $p < 0.05$. Data are reported as means ± standard error.

6.2.3. Results

6.2.3.1. Distribution of SNAP-25- and SPO mRNA in the songbird brain

We found SNAP-25 and SPO expression throughout the brain. No specific labelling was visible using the sense probes. In general, SNAP-25 was expressed at higher intensity than SPO in all analysed brain areas (Table 6.2.1). Within the song system, all nuclei exhibited moderate or high expression of SNAP-25, except for Area X and lobus paraolfactorius (LPO, Fig. 6.2.1; medial nucleus magnocellularis and nucleus uvaeformis were not analysed). SNAP-25 was elevated compared to surrounding tissue in HVC and RA (Fig. 6.2.1 A, E). High expression was also found in the hippocampal region, the thalamus and the cerebellum (Figs. 6.2.1 A, 6.2.2 A, D, G). SPO showed a more restricted expression pattern (Figs. 6.2.1, 6.2.2). Among the song control nuclei, SPO was only detectable in HVC, in the lateral nucleus magnocellularis (lMAN) and in the nucleus interfacialis (Nif) / field L (Table 6.2.1, Fig. 6.2.1 B, J). Moderate to high expression was also found in the hippocampal region (Figs. 6.2.1 B, 6.2.2 B). Detailed inspection of expression in HVC revealed that SPO was evenly distributed throughout the nucleus whereas SNAP-25 occurred in clusters (Fig. 6.2.5 A, B). It is known that the different HVC neuron subpopulations form clusters, consisting of Area X-projecting neurons, RA-projecting neurons and interneurons (Hunger 1994). This suggests that SNAP-25 is probably expressed by all HVC neuron types and SPO only by a specific subpopulation.

Table 6.2.1: Relative abundance of synaptosomal-associated protein 25 kDa (SNAP-25), synaptoporin (SPO), and androgen receptor (AR) mRNA in different brain regions of the zebra finch[1]

Brain areas	**SNAP-25**	**SPO**	**AR**
HVC / neostriatum	+++ / ++	++ / ++	+ / -
RA / archistriatum	+++ / ++	- / ++	- / -
lMAN / neostriatum	++ / ++	+ / ++	+ / -
AreaX / LPO	+ / +	- / +	- / -
DM / MLd	++ / ++	- / +	- / -
Nif / field L	++ / ++	+ / +	- / -
DLM / thalamus	++ / ++	- / +	-
n.XIIts	++	-	+
AHP dorsale / ventrale	+++ / ++	+++ / +	- / -
Purkinje cells	+	-	-

[1]Intensity of hybridisation signals were estimated by visual inspection and classified on a 0-3 scale: - = no staining, + = low intensity, ++ = moderate intensity, +++ = high intensity.
Abbreviations: AHP, area parahippocampalis; DLM, dorsolateral nucleus of the medial anterior thalamus; DM, dorsomedial nucleus of nucleus intercollicularis; HVC, nucleus hyperstriatalis ventrale; lMAN, lateral nucleus magnocellularis; LPO, lobus parolfactorius; MLd, nucleus mesencephalicus lateralis, pars dorsalis; Nif, nucleus interfacialis; n.XIIts, nucleus hypoglossus pars tracheosyringealis; RA, nucleus robustus archistriatalis.

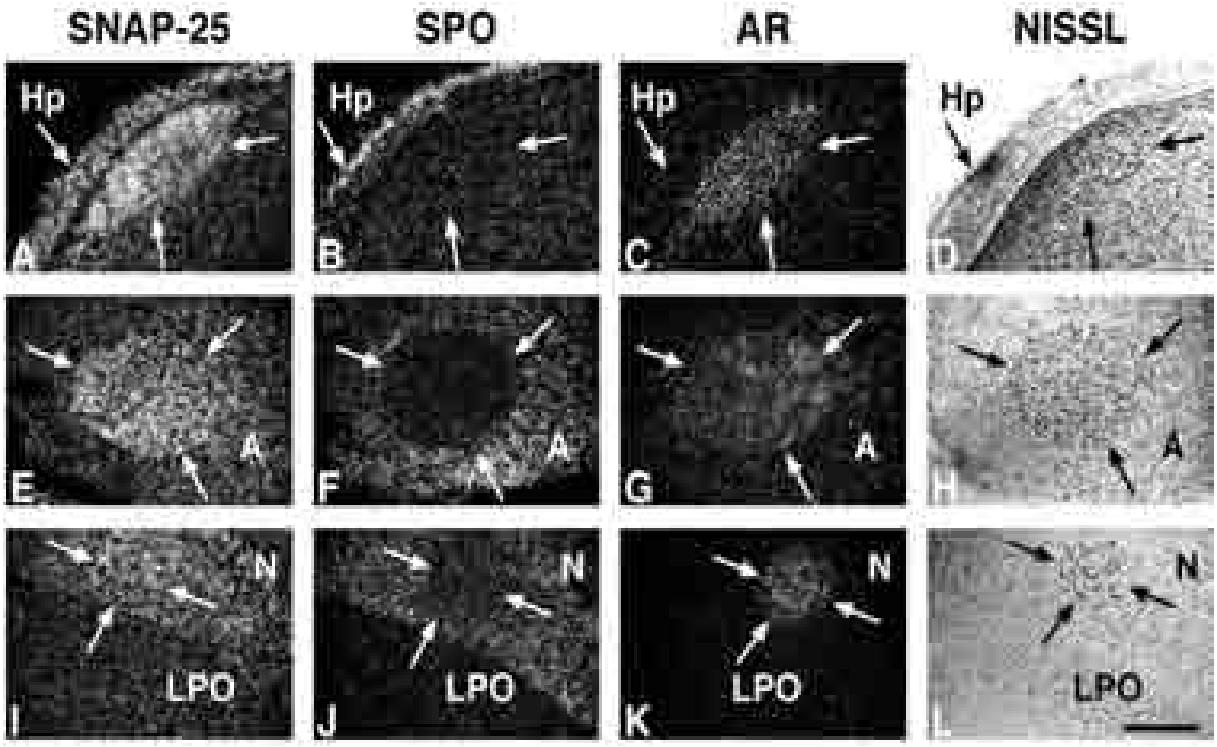


Fig. 6.2.1: Distribution of synaptosomal-associated protein 25 kDa (SNAP-25), synaptoporin (SPO) and androgen receptor (AR) mRNA in different regions of the song control system of the zebra finch. **A-C:** Darkfield photomicrographs of the vocal control nucleus high vocal center (HVC). Arrows indicate the ventral border of the nucleus. HVC showed elevated expression of SNAP-25 and AR mRNA compared to the surrounding neostriatum (N), whereas SPO mRNA expression in HVC was low. **E-G:** In the nucleus robustus archistriatalis (RA) only SNAP-25 mRNA showed elevated expression, SPO and AR mRNA were similar to background labeling. **I-K:** In the lateral nucleus magnocellularis (lMAN) SNAP-25 and SPO mRNA were expressed at moderate to low levels. Expression was not elevated compared to the surrounding neostriatum. AR mRNA showed elevated expression in lMAN. SNAP-25 and SPO mRNA expression were also low in the lobus paraolfactorius (LPO). AR mRNA was absent in LPO. The same brain regions are shown in adjacent Nissl-stained sections (**D**, **H**, **L**, brightfield photomicrographs). Dorsal is at the top and caudal is on the left in these sagittal sections. Scale bar = 300 µm. A, archistriatum; Hp, hippocampus.

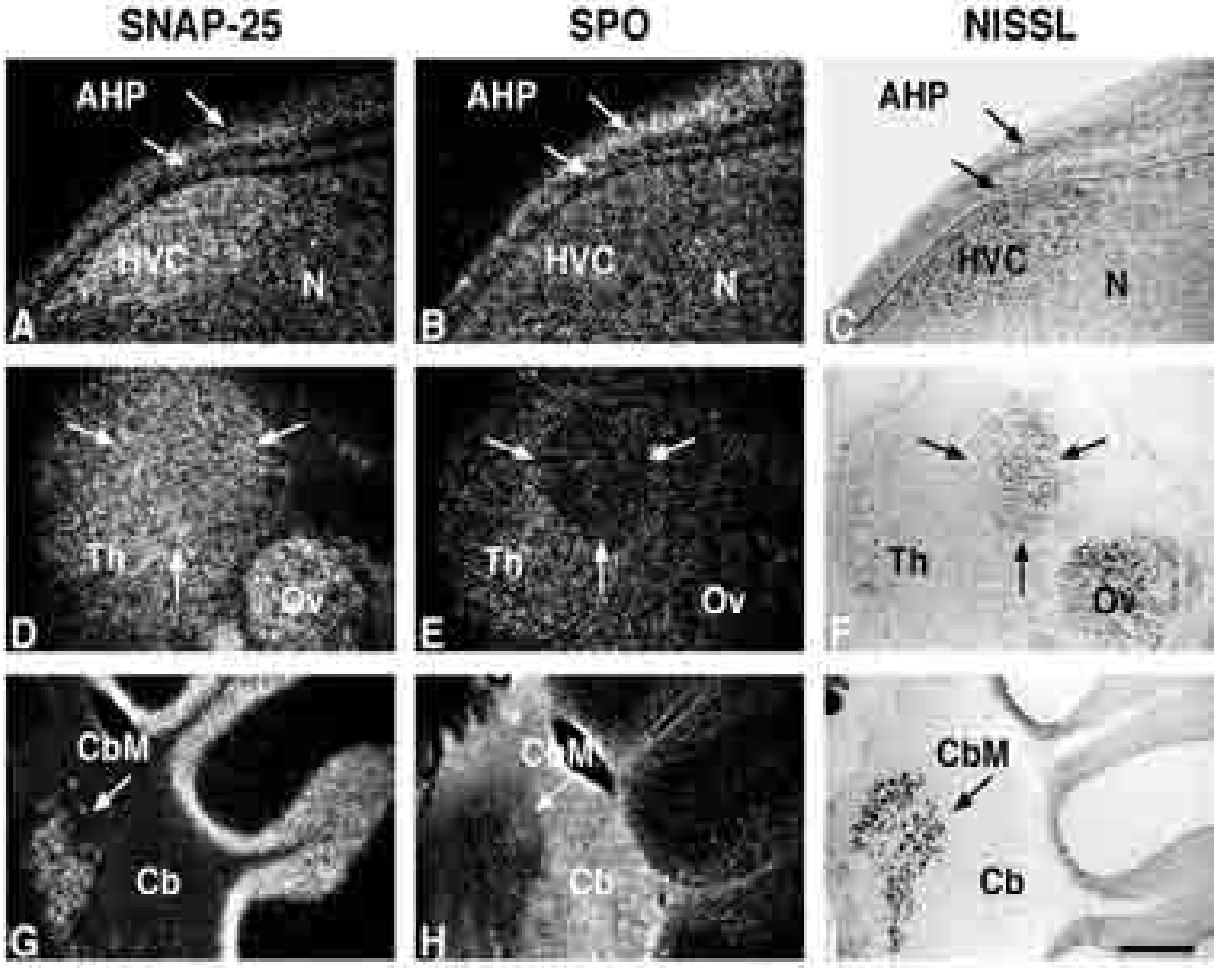


Fig. 6.2.2: Distribution of synaptosomal-associated protein 25 kDa (SNAP-25), synaptoporin (SPO) mRNA in different brain regions of the zebra finch. Darkfield photomicrographs of adjacent sections showing the hippocampal region (**A, B**), the thalamic region (**D, E**) and the cerebellum (Cb, **G, H**). The same brain regions were depicted in Nissl-stained sections (**C, F, I**, brightfield photomicrographs). **A, B**: Intense labeling with SNAP-25 and SPO mRNA were found in the area parahippocampalis (AHP, indicated by arrows). With both markers expression levels were higher in the dorsal compared to the ventral layer. **D, E**: Arrows indicate the outline of the dorsolateral nucleus of the medial anterior thalamus (DLM). **D**: High expression of SNAP-25 mRNA was present in DLM and in the surrounding thalamus (Th). **E**: SPO expression was absent in DLM and low in the thalamus. **G**: The cerebellar cortex and the nucleus cerebellaris medialis (CbM) showed intense labeling with SNAP-25 mRNA. Within the cerebellar cortex highest intensity of staining was found in the granular layer, whereas Purkinje cell layer and molecular layer were of lower staining intensity. **H**: SPO expression in the granular layer was low, in the Purkinje cell layer and molecular layer expression was absent and was also absent in CbM. Dorsal is at the top and caudal is on the left in these sagittal sections. Scale bar = 300 µm. HVC, high vocal center; N, neostriatum; Ov, nucleus ovoidalis.

6.2.3.2. Inhibition of oestrogen-producing enzyme aromatase

Telencephalon size and body measurements. Birds treated with Fadrozole (Fad) had significantly smaller telencephalon volumes ($t = 3.89$; $p < 0.01$) and lower brain weights ($t = 2.41$; $p < 0.05$) compared to controls. No significant differences between groups were found in body weight ($t = 0.30$; $p > 0.05$) and testes size ($t = 1.10$, $p > 0.05$; see Table 6.2.2).

Volume of song control nuclei HVC and RA. The volume of HVC was measured by the distribution of AR and SNAP-25 mRNA and conventional Nissl staining, while RA volume was estimated only by Nissl-staining. Fadrozole-treatment significantly reduced the absolute volume of HVC (Repeated-Measures ANOVA, effect of treatment: $F_{(1,12)} = 10.36$, $p < 0.01$; within subjects effect (Nissl, AR, SNAP-25): $F_{(2,24)} = 1.44$, $p > 0.05$; interaction: $p > 0.05$). Absolute volume of RA was not significantly different between groups, although there was a trend for smaller RA size in Fad-treated birds ($t = 1.93$; $p = 0.078$). HVC volume was positively correlated with the volume of the telencephalon, irrespectively of the marker used for delineation ($p < 0.01$ for all tests). There was also a positive correlation between RA and telencephalon volume ($r = 0.56$, $p < 0.05$).

To determine whether the treatment caused changes in the volume of these nuclei that were independent of changes in telencephalon volume, we included the latter as covariate in the ANOVA. The analysis revealed no significant effect of Fad-treatment on relative HVC volume (ANCOVA, effect of treatment: all tests $p > 0.20$, effect of telencephalon volume: all tests $p > 0.08$). Both groups did not differ in relative RA volume (ANCOVA, effects of treatment and telencephalon volume $p > 0.25$).

Cell density in HVC and surrounding tissue. Cell density was measured in HVC and in the surrounding neostriatum. Fad-treatment significantly increased cell density in both regions, HVC and neostriatum (Repeated-Measures ANOVA, effect of treatment: $F_{(1,12)} = 15.50$, $p < 0.01$; within subjects effect (HVC, neostriatum): $F_{(1,12)} = 4.89$, $p = 0.05$; interaction: $p > 0.40$). Cell density in HVC was negatively correlated with the volume of HVC ($p < 0.01$ for Nissl-, SNAP-25- and AR-HVC). Fadrozole did not affect the total number of HVC cells ($t = 1.40$; $p > 0.05$).

Table 6.2.2: Anatomical measurements of Fadrozole (Fad)-treated and control zebra finches (mean ± SE).

	Control (n = 6)	Fad-treated (n = 8)
Body weight (g)	15.5 ± 0.5	15.3 ± 0.6
Testes size (mg)	27.2 ± 3.9	33.3 ± 3.9
Brain weight (g)	0.656 ± 0.03	0.586 ± 0.02
Telencephalon volume (mm^3)	124.0 ± 3.0	102.2 ± 4.3
Nissl-HVC volume (mm^3)	0.263 ± 0.02	0.215 ± 0.01
Nissl-RA volume (mm^3)	0.211 ± 0.02	0.171 ± 0.01
HVC cell density (10^4 cells/mm^3)	24.30 ± 2.18	32.80 ± 1.29
Neostriatum cell density (10^4 cells/mm^3)	27.68 ± 1.59	34.30 ± 1.28

Expression level of AR-, SNAP-25- and SPO-mRNA. In both groups, expression of AR and SNAP-25 was significantly higher in HVC compared to the surrounding neostriatum (all tests $p < 0.001$). SPO expression in the Placebo group did not differ between regions ($t = 0.03$; $p > 0.05$), whereas in the Fad-treated group expression was significantly higher in HVC than in the neostriatum ($t = 4.99$; $p < 0.01$). To investigate whether the treatment caused area-specific changes in the expression of AR, SNAP-25 or SPO rather than simply an increase due to an overall increase in cell density, we included cell density as covariate in the ANOVA and analysed the expression level in HVC and neostriatum separately. In HVC, Fad-treatment significantly increased SPO expression (ANCOVA, effect of treatment: $F_{(1,11)} = 5.35$, $p < 0.05$; effect of cell density: $F_{(1,11)} = 6.99$, $p < 0.05$), while AR and SNAP-25 expression was not affected (all tests $p > 0.20$; Figs. 6.2.3.-6.2.5.). In the adjacent neostriatum, AR expression was significantly increased in Fad-treated birds (ANCOVA, effect of treatment: $F_{(1,11)} = 7.86$, $p < 0.02$, effect of cell density: $F_{(1,11)} = 0.30$, $p > 0.05$) but no effect was detected on the expression of SNAP-25 and SPO (all tests $p > 0.30$). Since AR mRNA expression in the adjacent neostriatum was very low, we do not discuss this finding any further. The results are also reflected when we calculated the ratio of the expression in HVC versus neostriatum (relative expression level) and controlled for cell density in the analysis. The relative expression level of SPO significantly increased due to Fad-treatment (effect of treatment: $F_{(1,11)} = 11.77$, $p < 0.01$, effect of cell density: $F_{(1,11)} = 0.02$, $p > 0.05$) whereas relative expression levels of AR and SNAP-25 did not change (all tests $p > 0.30$).

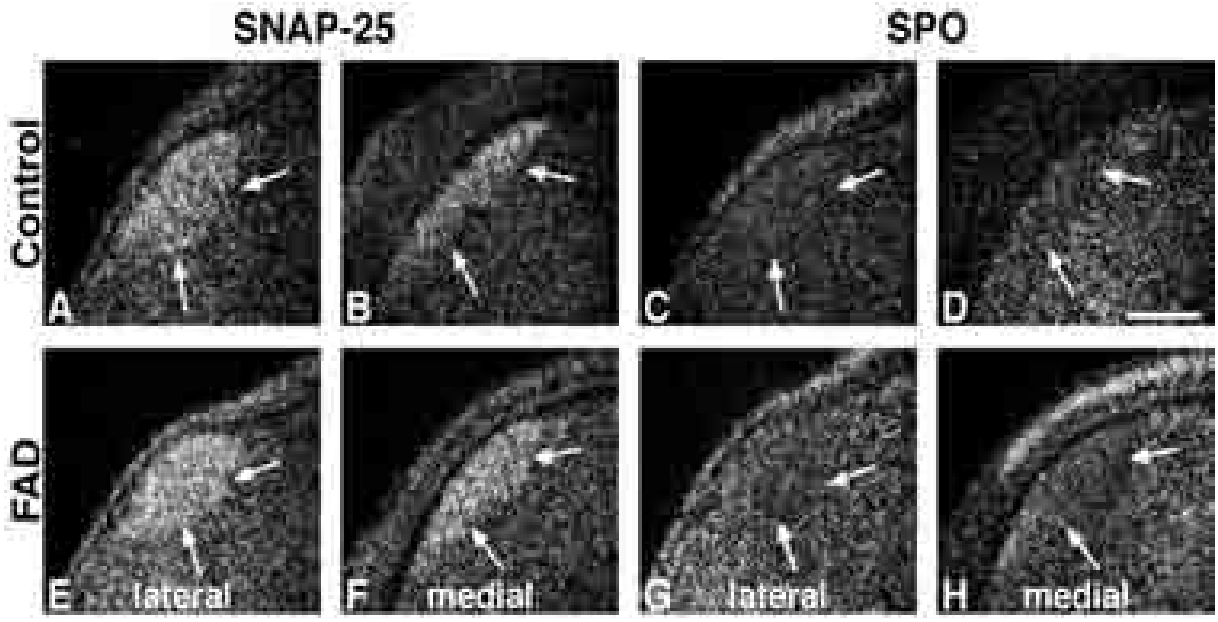


Fig. 6.2.3: Darkfield photomicrographs of the expression of synaptosomal-associated protein 25 kDa (SNAP-25) and synaptoporin (SPO) mRNA in the song control nucleus high vocal center (HVC) of control birds (**A-D**) and birds treated with the aromatase inhibitor Fadrozole (Fad, **E-H**). Arrows indicate the ventral border of HVC at the lateral and medial level. SNAP-25 was expressed at high intensity throughout HVC and irrespectively of the treatment (**A, B, E, F**). In contrast, mean SPO expression was significantly increased due to Fadrozole-treatment (**C, D, G, H**). Dorsal is at the top and caudal is on the left in these sagittal sections. Scale bar = 300 µm.

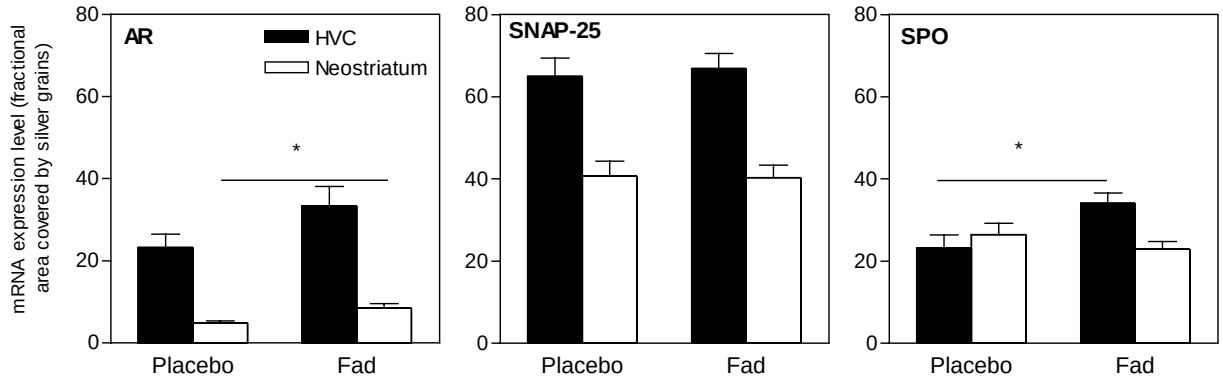


Fig 6.2.4: Expression level (fractional area covered by silver grains) of AR, SNAP-25 and SPO mRNA in HVC and neostriatum (Neo) of Fad-treated birds and controls. AR expression in the neostriatum and SPO expression in HVC were significantly increased due to Fadrozole-treatment, when controlled for changes in cell density, ANCOVA, * $p < 0.05$).

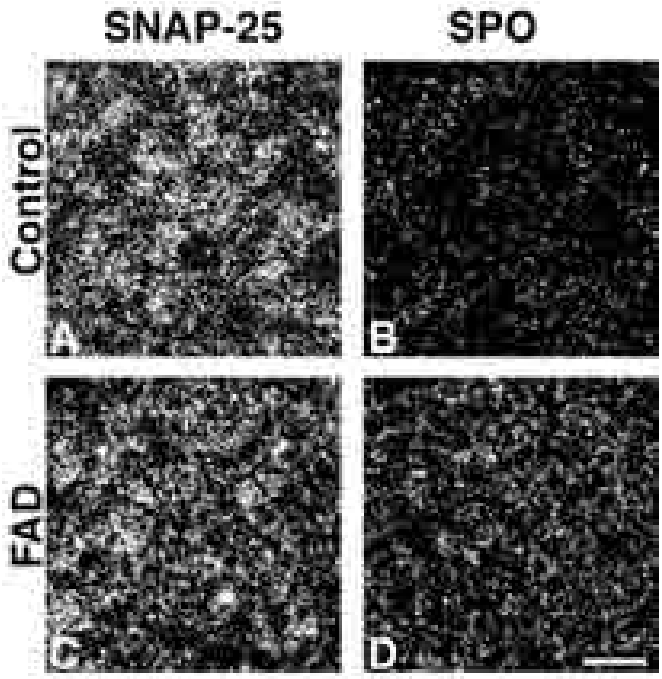


Fig. 6.2.5: Darkfield photomicrographs of the expression of synaptosomal-associated protein 25 kDa (SNAP-25) and synaptoporin (SPO) mRNA in the song control nucleus high vocal center (HVC). **A, B**: control birds. **C, D**: Fadrozole-treated birds. **D**: SPO expression was significantly increased in Fad-treated birds. Note that, SNAP-25 mRNA occurs in clusters whereas SPO mRNA is evenly distributed. Scale bar = 50 µm.

6.2.4. Discussion

We analysed the mRNA expression pattern of two synaptic proteins, SPO and SNAP-25 in neural song control areas and their sensitivity to oestrogen availability in adult male zebra finches. 1) SNAP-25 was expressed at high intensity throughout the telencephalon and was particularly enriched in the song control nuclei HVC and RA. 2) SPO expression in the song nuclei RA, lMAN and Area X was absent or low while it was moderate in HVC. 3) Inhibition of oestrogen producing enzyme aromatase led to increased SPO expression in HVC whereas SNAP-25 expression was not affected. 4) Although Fadrozole-treatment increased cell density in HVC and in the surrounding tissue and decreased both, HVC and telencephalon volume, this global effect of oestrogen deprivation does not explain the increase in SPO expression. These results suggest a differential role of SPO and SNAP-25 in the function of the song control system in relation to the status of the endocrine system.

6.2.4.1. SNAP-25 and SPO are differently expressed in the songbird brain

The brain distribution of both genes has been previously investigated in mammals (Knaus et al. 1990; Marqueze-Pouey et al. 1991; Boschert et al. 1996; Oyler et al. 1989) and in chicken (Catsicas et al. 1991; Bixby 1992). As expected from the mammalian studies, the expression of SNAP-25 mRNA is ubiquitous whereas that of SPO mRNA is limited to certain brain areas. In the

rat, SNAP-25 is highly expressed in most brain areas, including the olfactory bulb, cortex, hippocampus, thalamus, brainstem and cerebellum (Boschert et al. 1996). Similar, we found SNAP-25 throughout the hippocampus, thalamus, brainstem and cerebellum while both in rodents and birds SNAP-25 expression in the striatum appears to be low. The elevated expression of SNAP-25 in many brain areas correlates with the finding in rats that SNAP-25 does not coincide with any neurotransmitter or neuronal type (Oyler et al. 1989). The similar pattern of SNAP-25 expression in homologous areas of birds and mammals suggests an evolutionary conserved expression pattern and importance of this protein in the process of neurotransmitter release. Whether the moderate to high levels of SNAP-25 in most forebrain areas of the zebra finch are a conserved pattern, too, remains to be seen. These areas are homologous to parts of the dorso-ventricular ridge of reptiles with no direct mammalian homology. Data about the distribution of SNAP-25 in reptiles are currently not available. Interestingly, SNAP-25 was even higher expressed in the song control nuclei HVC, RA and the nucleus hypoglossus pars tracheosyringealis (nXIIts). This increased area-specific expression appears to be not due to differences in cell density. Since the expression of SNAP-25 might restrict the neurotransmitter release probability (Gibbins et al. 2003), the higher levels of SNAP-25 in the neurons of the descending vocal motor pathway compared to nuclei of the anterior forebrain pathway such as lMAN, Area X and DLM, might be related to its pre-motor and motor activity.

Studies of the rat involving in situ hybridisation and immunolabelling revealed that SPO is heterogeneously expressed in the brain, being enriched in restricted areas mainly belonging to forebrain regions, e.g. olfactory bulb, cortex, striatum, hippocampus In contrast, the related synaptophysin I is present in the majority of neurons throughout the brain (Marqueze-Pouey et al. 1991; Fykse et al. 1993). Similar to the mammalian data, we found high expression of SPO only in restricted areas of the brain. Among the vocal control areas, SPO expression was absent or relatively low despite moderate expression of SPO in the tissues surrounding the vocal areas, except for the HVC. This pattern implies that SPO is not a major synaptic vesicle protein of the song circuitry but instead is important in HVC and its efferences. However, it is possible that another protein of the synaptophysin family is abundant in certain song control nuclei, replacing the function of SPO (for review, see Jahn & Südhof 1993).

Although in situ hybridisiation and immunocytochemistry can result in different distribution patterns depending on the projections of neurons (Oyler et al. 1989; George et al. 1995), the mRNA level of presynaptic proteins in neuronal somata of the hippocampus correlates significantly positive with the protein abundance in the interconnected synaptic terminals (Melloni et al. 1993;

Eastwood et al. 1994). This supports the idea that the mRNA content of presynaptic proteins in neuron populations could, to a certain degree, predict the amount of presynaptic protein in their terminal fields. This suggests that SPO and SNAP-25 mRNA are synthesized in neuronal somata of the HVC with the protein translocated to the synaptic terminals, which could be onto Area X, RA or HVC neurons. However, the restricted expression of SPO mRNA in HVC suggests that it does not occur in all HVC neuron types. In the rat hippocampus, SPO occurs mainly in GABAergic neurons (Singec et al. 2002). It is tempting to speculate that, in HVC, too, SPO is expressed in GABAergic neurons.

6.2.4.2. Hormonal regulation of SPO in HVC

The increase of SPO mRNA expression level in the HVC of oestrogen-deprived males might be due to an ER-dependent mechanism regulating this gene or due to indirect action of oestrogen on the neural network properties of HVC. The latter could again involve ER- mediated changes of gene expression or various non-genomic mechanisms such as interference with second messenger pathways. Given the low abundance of ER, in particular in the lateral part of the zebra finch HVC, the oestrogen-ER mechanism underlying an altered expression of SPO would either involve neurons of the medial part of HVC or afferent areas such as catecholaminergic midbrain neurons, both of which frequently contain ER (Nordeen et al. 1987; Gahr & Konishi 1988; Gahr et al. 1993; Maney et al. 2001). The neurons of the lateral part of HVC that contain ER are a sub-population of Area-X projecting neurons (Gahr 1990a). In the canary, those neurons have dendritic arbors that extend through large parts of HVC and into the adjacent neostriatum (Benton et al. 1998). Note that one neural source of oestrogens is the aromatase activity of the caudal neostriatum ventromedial to HVC (Schlinger & Arnold 1992; Shen et al. 1995).

Oestrogen has stimulatory effects on several neurotransmitter systems in the rat brain (McEwen & Alves 1999) and up-regulates the neurotrophin BDNF in the HVC (Dittrich et al. 1999; Fusani et al. 2003). The ER-containing HVC neurons, which comprise at least partly Area X-projecting glutamatergic neurons (Dutar et al. 1998) are likely to stimulate inhibitory GABAergic HVC-interneurons (Burd et al. 1985; Grisham & Arnold 1994). In the rat hypothalamus and preoptic area, such stimulation involves oestrogen-dependent regulation of glutamic acid decarboxylase, $GABA_A$ receptor expression, GABA release and re-uptake (McCarthy 1995; Herbison 1997). Reduction of oestrogen levels due to aromatase inhibition could, therefore, lead to reduced activity of inhibitory HVC interneurons and disinhibition of their post-synaptic targets in HVC and simultaneously to increased levels of SPO expression in the latter

cells. Similar action of oestrogen is conceivable for other neurotransmitter systems in HVC, e.g. the dopaminergic input to HVC from the mesencephalon (Appeltants et al. 2000). This mechanism would be reminiscent of the work of Plunkett et al. (1998), who report an up-regulation of SPO in the chicken ciliary ganglion by blockade of synapses (no neurotransmitter synthesis and secretion) onto the ciliary ganglion neurons. They suggest that synaptic drive from other pre-ganglionic neurons inhibits mRNA expression and the loss of such drive leads to mRNA upregulation. The increased SPO expression in HVC might thus indicate a need for more synaptic vesicles due to higher neuronal activity following disinhibition. Such a scenario would, however, not be compatible with the idea that SPO is expressed in the GABAergic interneurons.

Alternatively to the genomic action via oestrogen receptors, oestrogen can act via non-genomic mechanisms. Aromatase occurs in neuronal somata as well as in dendrites, axons and terminal-like boutons (Saldanha et al. 2000), suggesting that oestrogen could directly influence synaptic transmission although the expression of aromatase in the HVC is low. Reduced synaptic transmission might then feedback to the SPO mRNA expression in case of a negative feedback between synthesis/release of neurotransmitter and SPO expression (Plunkett et al. 1998). Although it is likely that oestrogen-dependent direct or indirect control of neurotransmission might coincide with oestrogen-dependent homeostasis of synaptic vesicle release, the consequences of this regulatory mechanism on the song level need to be seen.

In the Fadrozole-treated birds, the absolute size of HVC was reduced. A first explanation would be that HVC cells died following oestrogen deprivation, but this was not reflected in the cell counts. Thus, oestrogens might be important for the overall protein synthesis related to neuronal size and neuronal activity, which could result in a smaller “active” HVC that expresses the phenotypes used to delineated HVC, as suggested previously for seasonal changes of the canary HVC (Gahr 1990a; Gahr 1997). A second explanation might be the low number ($n = 8$) of Fad-treated animals. Since the relative size of HVC, normalized against the forebrain, was similar in both groups of males, the smaller absolute HVC’s of Fad-treated males might be due to chance. However, random sampling of eight HVC volumes from our zebra finch database does not result in such a mean that is close to the minimum HVC volume of normal zebra finches (Gahr, unpublished data).

Alternatively, one needs to consider that oestrogen, surprisingly, affects the size of the entire telencephalon. One consequence of the reduced forebrain size is then the shrinking of HVC after aromatase inhibition. This effect is likely due to oestrogen deprivation because Fadrozole is a rather specific aromatase inhibitor with little known short-term side effects (Wade et al. 1994; Bonnefoi et

al. 1996; Bhatnagar et al. 2001). Aromatase activity is very abundant in many forebrain areas (Schlinger 1997a; Balthazart & Ball 1998; Metzdorf et al. 1999). Since the density of cell somata increased both in HVC and in the adjacent neostriatum following aromatase inhibition, oestrogens might sustain the dendritic arborisation, and/or the glia compartment, and/or the vascular system, and/or the extracellular volume throughout the forebain. The lack of classical oestrogen receptors in most forebrain areas (Gahr et al. 1993; Gahr & Metzdorf 1997; Bernard et al. 1999) is not contradictory with the proposed targets of oestrogens given the numerous non-receptor mediated actions of oestrogen (Küppers et al. 2001). Further, oestrogen receptor mediated mechanisms have been reported in glia (Jung-Testas & Baulieu 1998; Zsarnovszky et al. 2002; Lu et al. 2003; Platania et al. 2003) and in endothelial cells (Watanabe et al. 2003) of mammals. In the canary, previous work demonstrated that testosterone and/or oestrogens affect the glia compartment and the vascular system (Goldman & Nottebohm 1983; Louissaint et al. 2002). Thus, oestrogen deprivation might result in a less developed microvascular system, which in turn could affect the neuron and glia activity through reduced oxygen and glucose availability, or reduced production of growth factors originating from endothelial cells (Louissaint et al. 2002). Decreased activity of neurons and glia might lead to reduced osmotic and metabolic water and thus to reduced extracellular space. However, reduction of extracellular space alone might not be sufficient to explain the about 20 % decrease in forebrain volume if we consider that the extracellular volume accounts for 15-25 % of the brain volume (Nicholson & Sykova 1998).

Although the data suggest a role of forebrain aromatase for the homeostasis of overall forebrain morphology, we need to consider the pharmacological nature of the experiment. Thus, brains might adapt to oestrogen deprivation after some time. The lack of changes in HVC size in Fadrozole-treated adult canaries sacrificed after a long treatment period (four days vs. four weeks, Fusani et al. 2003) might point in this direction. Future efforts to establish a link of short-term, hormone-dependent changes in the synaptic gene expression of HVC to changes in singing require careful differentiation between global effects of oestrogens on overall brain morphology and local effects on neural networks.

6.3. The mRNA expression level of synaptic proteins in HVC of white-browed sparrow weavers

6.3.1. Introduction

In the previous chapter, I have described two synaptic proteins, SNAP-25 and SPO, as useful markers for the characterisation of the song control system. In the zebra finch brain, both proteins show distinct distributions and expression pattern at the mRNA level and exhibit differential steroid hormone sensitivity. Whereas the expression of SNAP-25 in HVC was independent of the availability of local oestrogens, the expression level of SPO in this area was increased due to a reduction in oestrogen formation.

In the present chapter, I apply this approach to the song control system of male and female white-browed sparrow weavers. I will focus on the detection of sex differences in the synaptic composition of the song control nucleus HVC. Moreover, it is of interest, whether the behavioural and anatomical differences of the song system as well as the differences in local steroid hormone sensitivity between dominant males and females and subordinate males are reflected at the level of neurotransmission. We have extended our approach to another synaptic protein, syntaxin 1. Similar to SNAP-25, syntaxin 1 is a plasma membrane protein of synaptic terminals and functions as part of the core complex in fusion and exocytosis of synaptic vesicles (Bennett et al. 1992, for review, see Südhof 1995). During the process of neurotransmitter release, SNAP-25 and syntaxin 1 bind to each other and form a binding site for synaptobrevin, a membrane protein of synaptic vesicles (Südhof 1995, Fig. 6.3.1). Despite these functional similarities, it has been shown that the mRNA expression of syntaxin 1 can be selectively increased by induction of long-term potentiation (LTP) in the hippocampus of the rat, leaving expression levels of SNAP-25 and of several other synaptic proteins unaffected (Hicks et al. 1997). Further, in the same brain area, increased levels of syntaxin 1 were associated with learning of a spatial memory task (Davis et al. 1996; Davis et al. 1998). These data suggest a distinct role of syntaxin 1 in the process of learning and formation of memory. Moreover, oestrogen treatment of female ovariectomised rats significantly increased syntaxin expression in the dorsal hippocampus and enhanced the performance of spatial memory tasks (Li et al. 2004). This shows that syntaxin exhibits steroid hormone sensitivity and that it is involved in oestrogen-regulated synaptic plasticity (Woolley & McEwen 1992; Brake et al. 2001; McEwen 2002). These properties make syntaxin 1 a suitable candidate to study the morphological dynamics of vocal control areas, such as HVC, which is

involved in learning and expression of song behaviour, in relation to the action of epigenetic factors, i.e. steroid hormones or social cues.

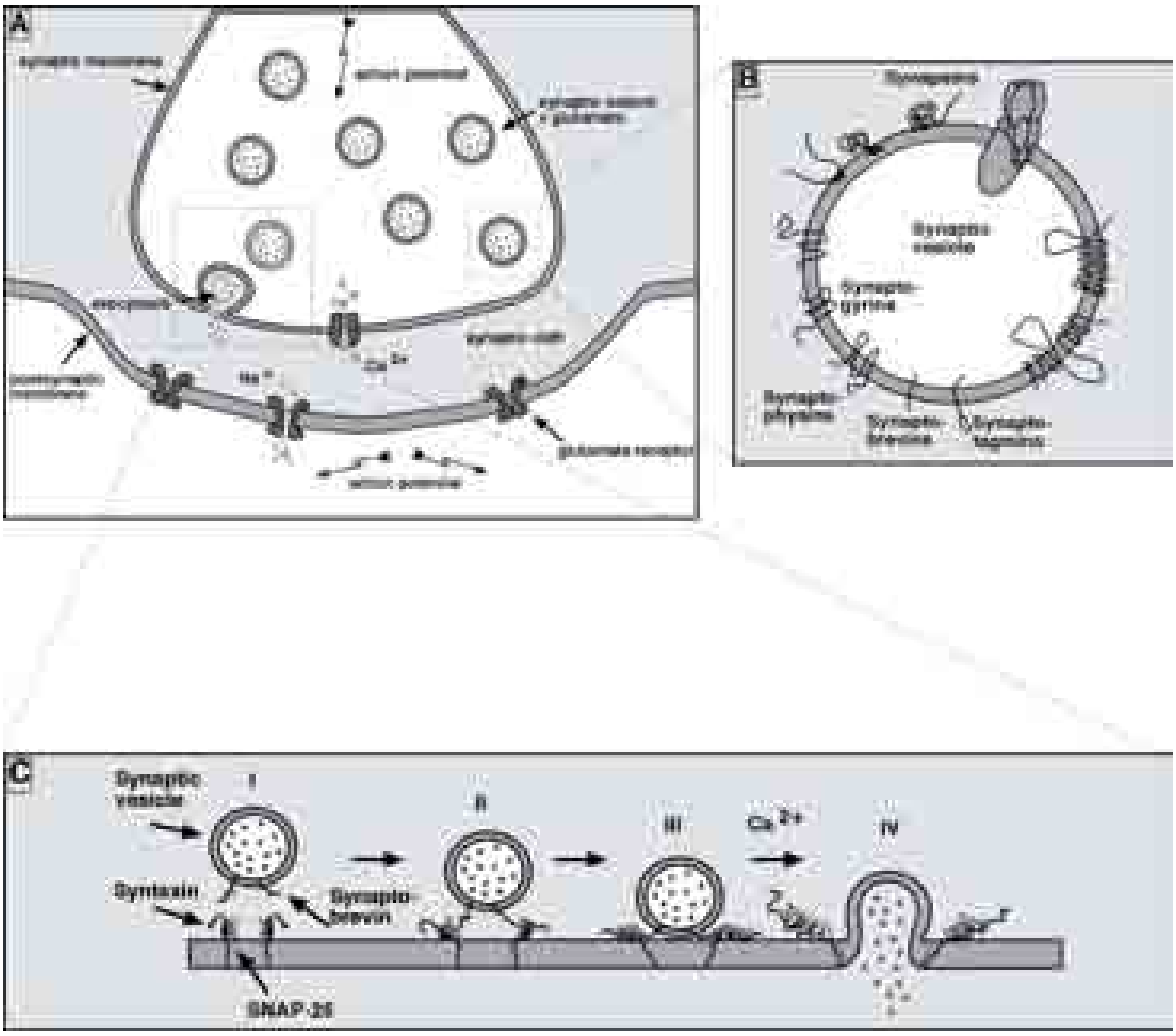


Fig. 6.3.1: Schematic drawing illustrates the information transfer at chemical synapses. **A**: Synapses are formed between the axon terminal of a sending nerve cell and the dendrite or cell body of a receiving nerve cell. Signals are transmitted by neurotransmitters (e.g. glutamate), which are stored in synaptic vesicles (B). Stimulation of the presynaptic cell leads to calcium (Ca^{2+}) influx through voltage-dependent Ca^{2+}-channels, which triggers the fusion of synaptic vesicles with the plasma membrane (C). During exocytosis neurotransmitter is released from the vesicle and binds to receptors at the postsynaptic cell, thereby propagating the signal. **B**: Enlargement of a synaptic vesicle showing some of the major membrane protein families involved in exocytosis. Synaptoporin (SPO) represents an isoform of synaptophysin. **C**: Enlargement of the process of vesicle fusion and exocytosis. During docking of the vesicle, SNAP-25 forms a complex with syntaxin at the plasma membrane thereby creating a binding site for the synaptic vesicle protein synaptobrevin, which results in the synaptic core complex. After fusion, Ca^{2+}-dependent neurotransmitter release occurs. Modified from material kindly provided by R. Jahn.

6.3.2. Material and Methods

6.3.2.1. Animals

The brains of eight dominant females, eight dominant males and eight subordinate males were analysed. For details, see section 5.2.1.

6.3.2.2. Tissue processing

In situ hybridisation of synaptosomal-associated protein 25 kDa (SNAP-25), synaptoporin (SPO) and syntaxin 1B (STX) mRNA was carried out as described in General Methods, section 3.2.3. For processing with SNAP-25 and SPO mRNA the third, respectively fourth, series from the right hemisphere was used and for STX mRNA, the third series from the left hemisphere was used.

6.3.2.3. Estimation of mRNA expression level in HVC

Sections were examined using a microscope (Leitz Aristoplan) under darkfield illumination (63x magnification), connected to an image analysis system (Metamorph 4.6, Visitron, Germany). Labelled areas were identified due to the elevated number of silver grains per visually inspected brain region and were compared with adjacent sections processed with Nissl stain. The expression level of such selected areas was than estimated with a 0-3 scale (- = no staining, 2-6 silver grains/cell; + = low intensity, 7-13 silver grains/cell; ++ = moderate intensity, 14-19 silver grains/cell; +++ = high intensity, 20-29 silver grains/cell). The same scale was used as in chapter 6.1 for the estimation of AR and ER expression.

Quantitatively, mRNA expression in HVC was measured at the lateral, central and medial level of the nucleus. These levels were estimated according to the Nissl-defined boundaries of HVC. I counted the number of sections where HVC was visible. *Central* was the section N/2. This section divided the HVC in a lateral and medial portion. The *lateral* level was section $N_l/2$ and the *medial* level was section $N_m/2$ ($N_{l,m}$ = number of sections in the lateral and medial part, respectively). At each level, the mean of four measured areas (13100 μm^2 each) across HVC was taken (Fig. 6.1.1). To quantify the level of mRNA expression in a selected area, the image was converted to a greyscale image. A threshold level was then adjusted to separate the silver grains from the background. The above thresholded area was calculated by a built-in function of the software and was expressed as fractional area covered by silver grains. This measurement was

named mRNA expression level. It represents the mean from the measurements at the lateral, central and medial level of HVC.

The same measurements were done in the adjacent neostriatum, in an area immediately outside HVC at its ventral border. To correct for different amounts of background labelling due to different sets of in situ hybridisations I measured the area covered by silver grains in a region of the same section lacking specific labelling. I chose the Tractus septomesencephalicus (TSM) as region for background labelling. Correction was done by subtracting the value for background labelling from the values of HVC and neostriatum, respectively.

6.3.2.4. Statistical analysis

Statistical analyses were done with Systat 10.2. General Linear Models (GLM) were used whenever possible. If necessary, data were transformed to meet the assumptions for the use of GLM. Data are presented as means ± standard error. All tests were two-tailed and the significance level was fixed at $\alpha = 0.05$. For analysis of mRNA expression level of SNAP-25, SPO and STX, ANCOVA (Analysis of Covariance) was used with sex or status as between-subjects factor and cell density as covariate. Relationships between mRNA expression levels, cell density and Nissl-HVC volume, respectively, were analysed with a Pearson correlation. Correlations between mRNA expression levels and plasma levels of steroid hormones were analysed with non-parametric Spearman rank correlation. Because data of dominant males were used in two comparisons, first with dominant females, second with subordinate males, the standard Bonferroni technique was applied ($\alpha' = 0.05/2$). On these data sets the significance level α' was fixed at 0.025 and is indicated in the respective sections.

6.3.3. Results

6.3.3.1. Brain distribution of SNAP-25, SPO and STX mRNA

Expression of SNAP-25, SPO and STX mRNA was found throughout the brain of the white-browed sparrow weaver. No specific labelling was found using the sense probes. The overall distribution did not differ between the sexes and between males of different status. Therefore, the expression is exemplified for dominant males (Table 6.3.1). SNAP-25 and STX showed moderate to high expression in several areas of the song control system, i.e. HVC, RA, lMAN, mMAN and nXIIts. In contrast, SPO expression was absent in the nuclei RA, lMAN, mMAN and nXIIts and was moderate in HVC. The expression of all three mRNA's was low in Area X and Nif / field L. The general distribution of

SNAP-25 and SPO found in white-browed sparrow weavers fitted the pattern described for zebra finches (chapter 6.2.). However, in contrast to the zebra finch, lMAN appeared to be devoid of labelling and DLM showed low expression of SPO in white-browed sparrow weavers. In general, SNAP-25 and STX were expressed at moderate to high levels in the nuclei of the song production pathway, whereas SPO expression in these areas was absent, except for HVC. Apart from the song system, SNAP-25, SPO and STX were expressed in moderate to high levels in the diencephalon, e.g. the thalamic and preoptic-hypothalamic region.

Table 6.3.1: Distribution of SNAP-25, SPO and STX mRNA in different brain areas of the white-browed sparrow weaver [1]

Brain areas	**SNAP-25**	**SPO**	**STX**
Telencephalon			
HVC / neostriatum	+++/++	++/++	+++/++
paraHVC [a]	++	++	++
RA / archistriatum	+++/++	-/++	++/++
lMAN / neostriatum	++/++	-/++	++/++
mMAN / neostriatum	+++/++	-/++	+++/++
Area X / LPO	+/+	+/+	+/+
Nif / field L	+/+	+/+	+/+
AHP dorsale / ventrale	+++/++	++/++	++/++
Diencephalon			
DLM / thalamus	+/++	+/++	++/++
preoptic area	++	+	++
hypothalamus	+++	++	++
Mesencephalon			
DM / MLd	++/++	-/++	+/+
Rhombencephalon			
n. hypoglossus pars tracheosyringealis	++	-	++
n. retroambigualis	++	-	n.a.
Cerebellum			
Purkinje cells	+	-	-

[a] Region according to definition by Johnson & Bottjer (1995)

[1] Distribution is shown for dominant males. Intensity of hybridisation signals were estimated by visual inspection and classified on a 0-3 scale: - = no staining, + = low intensity, ++ = moderate intensity, +++ = high intensity, n.a. = data not available. *Abbreviations*: AHP, area parahippocampalis; DLM, dorsolateral nucleus (n.) of medial anterior thalamus; DM, dorsomedial n. of n. intercollicularis; HVC, n. hyperstriatalis ventrale pars caudale; lMAN, lateral n. magnocellularis; LPO, lobus parolfactorius; mMAN, medial n. magnocellularis; MLd, nucleus mesencephalicus lateralis, pars dorsalis; Nif, n. interfacialis; RA, n. robustus archistriatalis.

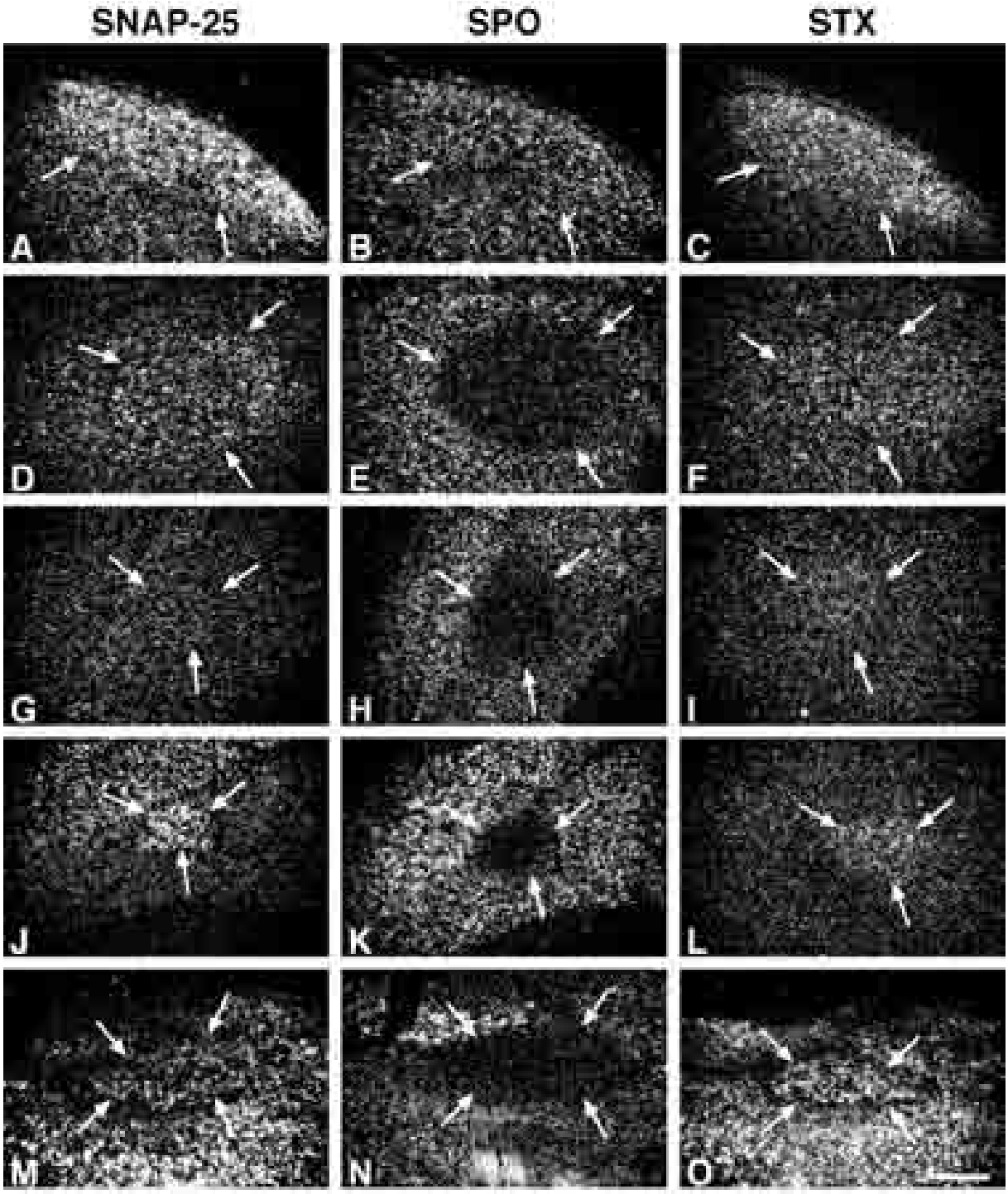


Fig. 6.3.2: Distribution of SNAP-25, SPO and STX mRNA in different regions of the song control system of the white-browed sparrow weaver. Expression is shown for dominant males. **A-C**: Darkfield photomicrographs of HVC. Arrows indicate the ventral border of the nucleus. HVC showed elevated expression of SNAP-25 and STX compared to the surrounding neostriatum. In RA (**D-F**) and lMAN (**G-I**), SNAP-25 and STX were expressed at moderate to high intensity, whereas SPO expression was absent. Moderate to high expression of SNAP-25 and STX were also found in mMAN (**J-L**) and in the brain stem nucleus nXIIts (**M-O**). Both regions showed no expression of SPO mRNA. Dorsal is at the top and caudal is on the right in these sagittal sections. Scale bar = 300 µm.

6.3.3.2. Expression level of SNAP-25, SPO and STX in dominant males and females

The expression levels of SNAP-25, SPO and STX in HVC and adjacent neostriatum were not significantly different between the lateral, central and medial region. Therefore, the means were used in all further analyses. Because cell density could potentially influence the expression, I included this variable as covariate. For SNAP-25, the ANOVA revealed a significant effect of sex and of cell density on the expression level in HVC (Table 6.3.2). Dominant females had significantly higher expression of SNAP-25 mRNA in HVC than dominant males, when adjusted for variation of cell density (Fig. 6.3.2a). Both variables cell density and SNAP-25 expression level in HVC were negatively correlated and reached significance in males ($r = -0.720$, $p = 0.044$), but not in females ($r = -0.663$, $p = 0.073$). Mean SNAP expression did not correlate significantly with Nissl-HVC volume in either sex (all tests, $p > 0.13$). For SPO, the ANOVA revealed neither a significant effect of sex nor of cell density on the expression level in HVC (Table 6.3.2, Fig. 6.3.2a). Also, SPO expression did not correlate with cell density or with HVC volume in both sexes (all tests, $p > 0.20$). For STX, the ANOVA revealed a significant effect of sex but not of cell density on the expression level in HVC (Table 6.3.3.). Mean STX expression was higher in HVC of dominant females than of dominant males and this effect was independent of cell density (Fig. 6.3.2a). STX expression in HVC did not correlate with cell density or HVC volume ($p > 0.14$ for both sexes).

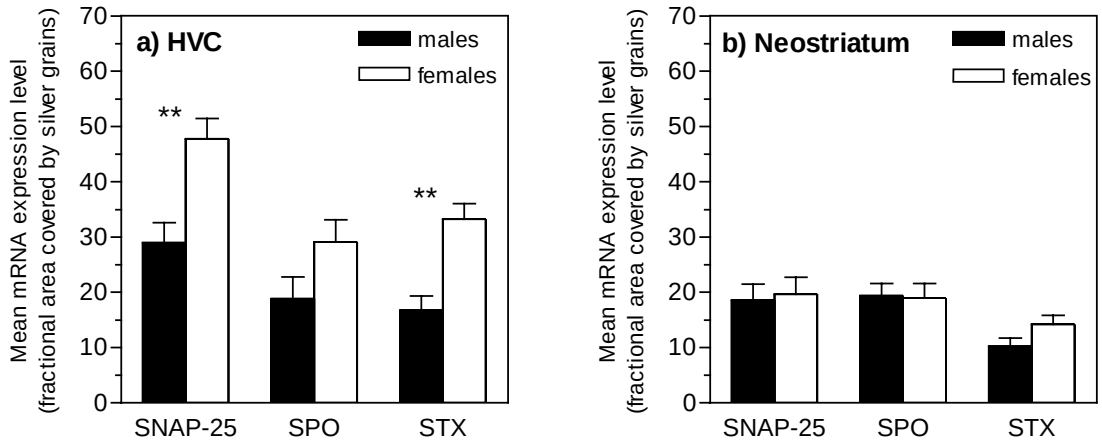


Fig. 6.3.2: Mean expression levels of SNAP-25, SPO and STX mRNA in HVC (a) and in the adjacent neostriatum (b). In HVC, expression of SNAP-25 was significantly higher in dominant females than in dominant males due to the effect of cell density. STX expression was significantly higher in females than in males, independently of cell density. In the neostriatum, there were no sex differences in the expression levels of SNAP-25, SPO and STX and no influence of cell density (** = $p < 0.01$, $\alpha' = 0.025$).

For SNAP-25, SPO and STX in the neostriatum, the ANOVA revealed neither a significant effect of sex, nor of cell density on the expression level (Table 6.3.2). The mean expression level did not differ between dominant males and females (Fig. 6.3.2b). The expression level of SNAP-25 was significantly negative correlated with cell density in the neostriatum in dominant males ($r = -0.831$, $p = 0.011$) but not in females ($r = -0.057$, $p = 0.893$). There were no correlations between cell density and the expression level of either SPO or STX (all tests, $p > 0.17$).

Table 6.3.2: Analysis of Covariance of SNAP-25-, SPO and STX mRNA expression in dominant males and females ($\alpha' = 0.025$).

Factor	df	F	P
SNAP-25 mRNA expression level in HVC			
sex	1,13	10.66	0.006
cell density	1,13	9.52	0.009
SPO mRNA expression level in HVC			
sex	1,13	2.74	NS
cell density	1,13	0.83	NS
STX mRNA expression level in HVC			
sex	1,12	14.82	0.002
cell density	1,12	2.14	NS
SNAP-25 mRNA expression level in neostriatum			
sex	1,13	0.07	NS
cell density	1,13	1.24	NS
SPO mRNA expression level in neostriatum			
sex	1,13	0.01	NS
cell density	1,13	0.06	NS
STX mRNA expression level in neostriatum			
sex	1,12	3.27	NS
cell density	1,12	3.63	NS

6.3.3.3. Expression level of SNAP-25, SPO and STX in dominant and subordinate males

For SNAP-25, the ANOVA revealed a significant effect of status and of cell density on the expression level in HVC (Table 6.3.3). SNAP-25 expression was significantly higher in HVC of subordinate males than in dominants (Fig. 6.3.3a). This effect was only partly influenced by cell density. There was a

negative correlation between cell density and the mean SNAP-25 expression level in HVC, which approached significance in dominant males ($r = -0.720$, $p = 0.044$) but not in subordinates ($r = -0.456$, $p = 0.258$). Mean SNAP expression did not correlate significantly with HVC volume in either group of males (all tests $p > 0.13$). For SPO and STX, the ANOVA revealed a significant effect of status but not of cell density on the expression level (Table 6.3.3). SPO and STX expression in HVC was significantly higher in subordinates compared to dominants (Fig. 6.3.3a) and this effect was independent of cell density. The expression level of both SPO and STX did not correlate with cell density (all tests $p > 0.14$).

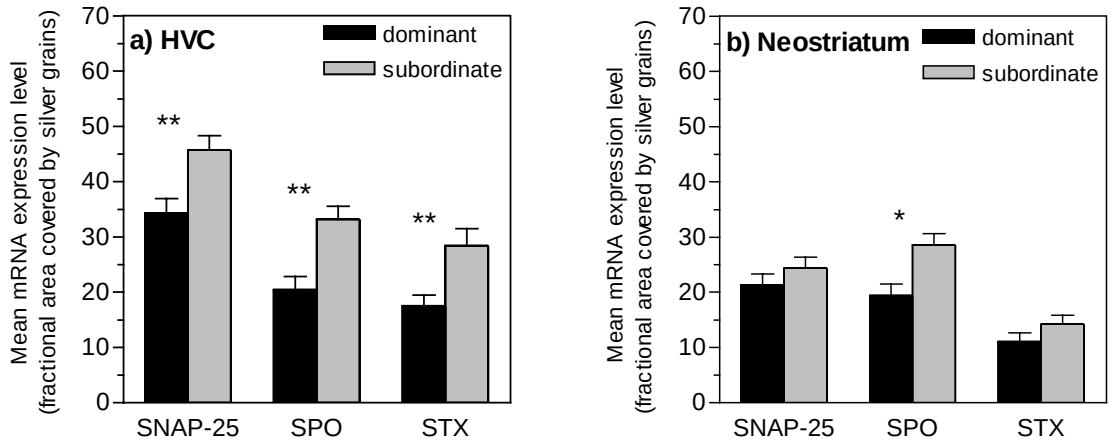


Fig. 6.3.3: Mean expression levels of SNAP-25, SPO and STX mRNA in HVC (a) and in adjacent neostriatum (b). In HVC, subordinate males had higher expression levels of SNAP-25, SPO and STX than dominant males. HVC cell density influenced the expression level of SNAP-25, but not of SPO and STX. In the neostriatum, subordinates had significantly higher expression of SPO independently of cell density. SNAP-25 and STX expression was not different between groups. However, the expression level of SNAP-25 was affected by cell density (** = $p < 0.01$, * = $p < 0.025$, $\alpha' = 0.025$).

In the adjacent neostriatum, the ANOVA revealed no significant effect of status, but a significant effect of cell density on the expression level of SNAP-25 (Table 6.3.3). Therefore, SNAP-25 expression did not differ between the two groups of males, when corrected for differences in cell density (Fig. 6.3.3b). SNAP-25 expression was significantly negative correlated with cell density in dominant males ($r = -0.831$, $p = 0.011$) but not in subordinates ($r = -0.206$, $p = 0.625$). For SPO, the ANOVA revealed a significant effect of status but not of cell density on the expression level (Table 6.3.5). Subordinate males had higher SPO expression in the neostriatum than dominant males, independently of cell

density (Fig. 6.3.3.b). For STX, the ANOVA revealed neither a significant effect of status nor of cell density on the expression level (Table 6.3.3). The expression level did not differ between dominant and subordinate males. Further, the expression levels of both SPO and STX did not correlate significantly with cell density (all tests, $p > 0.17$).

Table 6.3.3: Analysis of Covariance of SNAP-25, SPO and STX mRNA expression in dominant and subordinate males ($\alpha' = 0.025$).

Factor	df	F	P
SNAP-25 mRNA expression level in HVC			
status	1,13	9.57	0.009
cell density	1,13	7.01	0.020
SPO mRNA expression level in HVC			
status	1,13	14.36	0.02
cell density	1,13	4.69	NS
STX mRNA expression level in HVC			
status	1,13	10.23	0.007
cell density	1,13	0.29	NS
SNAP-25 mRNA expression level in neostriatum			
status	1,13	1.00	NS
cell density	1,13	6.81	0.022
SPO mRNA expression level in neostriatum			
status	1,13	8.54	0.012
cell density	1,13	0.00	NS
STX mRNA expression level in neostriatum			
status	1,13	1.68	NS
cell density	1,13	2.12	NS

6.3.3.4. Comparison of the expression level of SNAP-25, SPO and STX between dominant females and subordinate males

The comparison of SNAP-25, SPO and STX expression levels between dominant females and subordinate males revealed no significant sex differences in HVC (Table 6.3.4, Fig. 6.3.4a). Further, cell density did not affect expression levels. However, in the adjacent neostriatum, a sex difference in the expression level of SPO was found (Table 6.3.4). Subordinate males had significantly higher expression of SPO in this region than females (Fig. 6.3.4). Cell density had no effect on the expression levels.

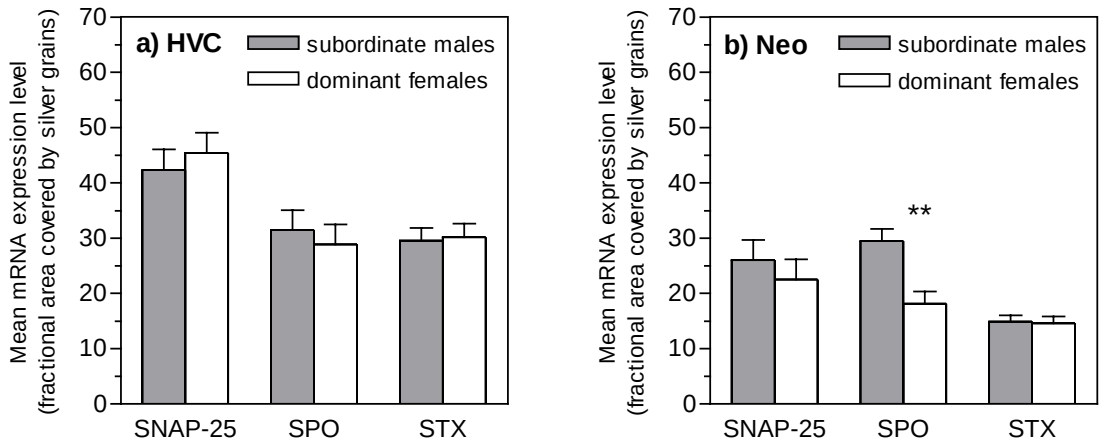


Fig. 6.3.4: Comparison of the mean expression levels of SNAP-25, SPO and STX mRNA in HVC (a) and in adjacent neostriatum (b, Neo) of subordinate males and dominant females. In HVC, no sex differences were found in the expression levels of SNAP-25, SPO and STX. In the neostriatum, subordinate males had a significantly higher expression level of SPO but not of SNAP-25 and STX compared to females. Cell density had no significant effect on the expression levels in HVC and neostriatum (** = $p < 0.01$, $\alpha' = 0.025$).

Table 6.3.4: Analysis of Covariance of SNAP-25, SPO and STX mRNA expression in dominant females and subordinate males ($\alpha' = 0.025$).

Factor	df	F	P
SNAP-25 mRNA expression level in HVC			
sex	1,13	0.28	NS
cell density	1,13	3.72	NS
SPO mRNA expression level in HVC			
sex	1,13	0.22	NS
cell density	1,13	0.90	NS
STX mRNA expression level in HVC			
sex	1,13	0.03	NS
cell density	1,13	0.65	NS
SNAP-25 mRNA expression level in neostriatum			
sex	1,13	0.41	NS
cell density	1,13	0.12	NS
SPO mRNA expression level in neostriatum			
sex	1,13	11.54	0.005
cell density	1,13	1.07	NS
STX mRNA expression level in neostriatum			
sex	1,13	0.03	NS
cell density	1,13	0.27	NS

6.3.4. Discussion

In the present study, I have described the distribution and mRNA expression pattern of the synaptic proteins SNAP-25, SPO and STX in the song control system of white-browed sparrow weavers. 1) SNAP-25 and STX were expressed at moderate to high intensity throughout the brain and were particularly enriched in nuclei of the song system, e.g. HVC, RA, mMAN. Expression of SPO within the song system was low or absent, except for HVC, which showed moderate expression. 2) The SNAP-25 expression level was significantly increased in HVC of dominant females compared to dominant males and this effect was influenced by cell density. No sex difference was found in the SPO expression level in HVC. The STX expression level was significantly increased in HVC of females but not in males and independently of cell density. 3) No sex differences occurred in the mRNA expression level of the three proteins in the surrounding neostriatum. 4) Male status significantly influenced the expression level of SNAP-25, SPO and STX in HVC. Subordinates had increased mRNA expression levels of all three proteins compared to dominants. In the adjacent neostriatum, SPO expression was increased in subordinates, whereas SNAP-25 and STX were not affected, when correcting for differences in cell density. 5) Comparison of dominant females with subordinate males revealed no sex differences in the expression levels of synaptic proteins in HVC. 6) In the neostriatum, SPO expression of subordinates was increased compared to females.

6.3.4.1. Distribution of SNAP-25, SPO and STX mRNA within the song system of white-browed sparrow weavers

The three synaptic proteins showed differential mRNA expression patterns within the song control system. Generally, the expression of SNAP-25 and STX were ubiquitous and more similar to each other than to SPO, which showed restricted expression. This observation can be explained by the different functional properties of the proteins in the process of neurotransmitter release. SNAP-25 and STX belong together with synaptobrevin to neuronal SNARE proteins (Jahn & Südhof 1999), which are involved in exocytosis. These three proteins form the SNARE or core complex that brings the synaptic vesicle and the plasma membrane together (Fig. 6.3.1). In contrast, SPO belongs to the family of synaptophysins, which constitute integral membrane proteins of synaptic vesicles (Greengard et al. 1993). The distribution of different isoforms of synaptic vesicle protein families can vary between neuron populations (Jahn & Südhof 1993). The restricted expression of SPO in the song nuclei could

suggest that another protein of the synaptophysin family is more abundant in synaptic vesicles of these brain areas. Besides the abundance of SNAP-25 and STX in most brain areas, mRNA expression of these proteins was particularly enriched in areas of the song production pathway relating to its pre-motor and motor activity. It has recently been shown in rats that the syntaxin isoform 1B, which I used in the present study, is specifically present in motor neurons (Aguado et al. 1999).

Comparison of the SNAP-25 and SPO expression in brain areas of white-browed sparrow weavers with the expression pattern found in zebra finches reveals an overall similarity and might be an indication for an evolutionary conserved expression pattern among songbirds.

6.3.4.2. Expression level of SNAP-25, SPO and STX in dominant males and females

The results of the present study revealed sex differences in the expression level of synaptic proteins in HVC of white-browed sparrow weavers. For SNAP-25 and STX, the mean mRNA expression level was significantly higher in dominant females compared to dominant males. However, the difference in SNAP-25 expression disappeared when HVC cell density was not included in the analysis. The SNAP-25 expression level was negatively correlated with cell density in dominant males but was not correlated in females, which indicates that there is a sex difference in the regulation of the density of SNAP-25 proteins in HVC.

Although functionally related to SNAP-25, the STX expression appeared to be differently regulated than SNAP-25 because its expression was independent of overall cell density. This suggests that STX is only expressed in HVC subpopulations and higher amounts of the STX protein in these cells are present in dominant females compared to dominant males. Previous studies have shown that the mRNA level of presynaptic proteins in neuronal somata is positively correlated with the protein abundance in the interconnected synaptic terminals (Melloni et al. 1993; Eastwood et al. 1994). Higher mRNA expression levels found in females could therefore relate to higher turnover or higher abundance of the protein and could be an indication for higher rates of neurotransmitter release from certain neuron populations. The increased labelling intensity of the HVC efferent target RA compared to the other target Area X suggests a predominant role of STX in RA-projecting HVC neurons related to song production. Alternatively, the sex differences in the expression levels of STX and in the regulation of SNAP-25 could indicate an ‘organised’ sexual dimorphism in synapse density and morphology in HVC.

No significant sex difference was found in the mRNA expression level of the synaptic vesicle protein SPO and there was no correlation with cell density. In the previous chapter, I have shown that SPO in the zebra finch song system exhibits oestrogen sensitivity. However, the basal levels of circulating plasma oestradiol (chapter 7) of dominant male and female white-browed sparrow weaver cannot explain the SPO expression levels and provide no information about the local levels of oestrogen in the brain.

Based on the present data, I cannot exclude, that the in situ hybridisation technique used in this study also labelled non-neuronal cells. Synaptic proteins were originally thought to be specific for the control of neurotransmitter release from neurons. However, the presence of many presynaptic proteins, including SNAP-25, STX and SPO in cultured oligodendroglia and astroglia has recently been demonstrated (Madison et al. 1999; Maienschein et al. 1999).

6.3.4.3. Expression level of SNAP-25, SPO and STX in relation to social status

There was a pronounced intra-sexual difference in the mRNA expression level of synaptic proteins in HVC. Expression of SNAP-25, SPO and STX were significantly increased in subordinate males compared to dominant males and these effects existed regardless of overall cell density. It can be assumed that subordinates expressed higher amounts of mRNA of all three proteins per individual cell than dominant males because cell density was not correlated with mRNA expression levels in subordinates. This finding relates to increased turnover rates of the proteins in synaptic terminals, suggesting increased neuronal activity throughout HVC in subordinates. The ability of these birds to further increase the size of HVC combined with the ability to enhance song production would fit such a hypothesis (Bottjer & Johnson 1997). Furthermore, the lack of a negative correlation between the SNAP-25 expression level and overall cell density in HVC of subordinates but not of dominants shows that males not only differ in the expression level of synaptic proteins but that these proteins could also be subject to different regulatory mechanisms. This is further supported by the presence of a strong negative correlation between SNAP-25 expression and cell density in the adjacent neostriatum of dominant males, which was absent in subordinates.

In the neostriatum, subordinates had probably a higher proportion of cells expressing synaptic proteins than dominants because cell density was reduced and not correlated with expression levels. The increased expression of SPO in this region could relate to low local levels of oestrogen.

In the previous chapter, I have shown in zebra finches that the expression of SPO in HVC is hormone sensitive. However, the intrasexual differences in

mRNA expression levels of synaptic proteins in HVC and neostriatum cannot be attributed to a simple difference in circulating gonadal hormones because these did not differ between both groups of males (chapter 7). Further, this finding does not support an ‘activational’ action of gonadal hormones on brain structure except when assuming that either the brain is sensitive to subtle changes in hormone levels or ‘activation’ by gonadal hormones occurs in peaks which I missed in measurements.

Comparison of dominant females with subordinate males revealed no sex differences in the expression levels of the synaptic proteins in HVC despite a sex difference in circulating levels of testosterone (chapter 7), which further supports the finding that the expression and regulation of these proteins in the brain is not simply sexually dimorphic and gonadal hormone-dependent but is influenced by other factors such as social status.

7. STEROID HORMONE LEVELS OF WHITE-BROWED SPARROW WEAVERS IN RELATION TO MALE SONG BEHAVIOUR AND STEROID HORMONE SENSITIVITY OF SONG CONTROL NUCLEUS HVC

7.1. Introduction

There is substantial evidence that steroid hormones activate song production in adult birds (for review, see Schlinger 1997b). In temperate zone birds, plasma testosterone (T) levels generally rise at the onset of the breeding season and decline when reproduction terminates in response to environmental cues (Hahn et al. 1997). These seasonal variations in circulating T levels appear to correlate with the seasonal production and pattern of vocal behaviour in songbirds (Nottebohm et al. 1987; Rost 1992; Smith et al. 1997; Leitner et al. 2001b). For example, male wild canaries (*Serinus canaria*) sing an increased number of sexually attractive syllables during the breeding season, which are thought to be driven by elevated T levels (Leitner et al. 2001b). However, song can also be produced during the non-breeding season when circulating steroid hormone levels are basal (Schwabl & Kriner 1991; Leitner et al. 2001b). Experimentally it has been shown that castration of male songbirds, which reduces the circulating T level, decreases or eliminates song production, whereas T treatment restores singing (Pröve 1974; Nottebohm 1980). Further, T administration induces song behaviour in adult female songbirds (Nottebohm 1980; Fusani et al. 2001). Song patterns are sensitive to both androgens and oestrogens (Marler et al. 1988; Walters et al. 1991; Fusani et al. 2003). Androgen receptors and oestrogen receptors are found in the song control system (Gahr 2001), and the caudomedial neostriatum (NCM) adjacent to HVC (high vocal center) is an area of high aromatase activity (Schlinger 1997a).

So far, little is known about the regulation of song behaviour by steroid hormones in tropical songbirds. However, the presence of steroid hormone receptors within the song control nuclei in several tropical species suggests that the song system is responsive to androgens and oestrogens (Brenowitz & Arnold 1989; Brenowitz et al. 1996; Gahr et al. 1998, this study). Because in the tropics seasonality is not as pronounced as in temperate zones, breeding in many species can occur almost year-round, associated with year-round territoriality and song production. A recent comparative analysis shows that circulating T levels are significantly lower in tropical birds than in species of northern temperate zones. Within tropical species, the length of the breeding season is the major determinant of plasma T levels (Goymann et al. 2004). Because prolonged elevated T levels are accompanied by costs that potentially reduce

lifetime fitness, it is conceivable that tropical birds have evolved mechanisms to avoid those costs (Wingfield et al. 2001; Gil & Gahr 2002).

Studies investigating the mechanisms of hormone-behaviour interactions have mainly focused on the regulation of territorial aggression, a behaviour known to be T-dependent in temperate zone birds in the context of reproduction (for review, see Wingfield 1999). There is evidence that this behaviour is indeed independent of circulating T levels in several tropical species (Levin & Wingfield 1992; Wiley & Goldizen 2003; Moore et al. 2004).

Concerning song behaviour, there exists only one study on duetting bush shrikes (*Laniarius funebris*) stating that song pattern and song activity of males and females were not correlated with circulating steroid hormone levels (Schwabl & Sonnenschein 1992). The same result can be inferred from earlier studies on white-browed sparrow weavers and on another duetting species, the bay wren that reported baseline T levels in males and females during most times of the year (Wingfield et al. 1991; Levin & Wingfield 1992). However, in white-browed sparrow weavers, Wingfield et al. (1991) found a slight, but significant increase in circulating T levels of dominant males in the middle of the breeding season. This peak could coincide with the production of the solo song by dominant males, which is thought to be restricted to the breeding season. Unfortunately, the authors provide no information on song behaviour in this study.

The focus of the present study was to investigate the relationship between song expression and circulating sex steroid hormone levels in both male and female white-browed sparrow weavers in more detail and to relate these data to the steroid hormone sensitivity of the song control system. Another interesting aspect is the influence of social status on these factors. Subordinate males have significantly reduced testes compared to dominant males (Wingfield et al. 1991, this study) and the increased androgen sensitivity found in medial HVC (see chapter 6.1) might therefore be reflected in the circulating T levels.

7.2. Material and Methods

7.2.1. Animals

Blood samples from white-browed sparrow weavers comprised 22 dominant females, 27 dominant males and 14 subordinate males. These birds included the individuals, which were used for neuroanatomical analysis (chapters 5, 6.1, 6.3) and analysis of song behaviour (chapter 4). For details about determination of social status, see section 3.1.3. Blood samples were collected during the study

periods in 2000 and 2001 (see section 2.3.). Data from both years were lumped. Additionally, I collected blood samples from eight male grey-headed sparrows (*Passer diffusus*, Family Passeridae), and from single males of four ploceid species, the village weaver (*Ploceus cucullatus*), the masked weaver (*Ploceus velatus*), the lesser-masked weaver (*Ploceus intermedius*) and the red-billed buffalo weaver (*Bubalornis niger*).

7.2.2. Blood sampling

Blood sampling was done within 5 minutes after capture by puncture of the wing vein. For details, see General Methods, section 3.1.1.

7.2.3. Radioimmunoassay of plasma levels of steroid hormones

The androgens 5α-dihydrotestosterone (DHT) and testosterone (T) and the oestrogen 17β-oestradiol (E2) were measured by radioimmunoassay (RIA) as described in General Methods, section 3.2.3.

7.2.4. Statistical analysis

Statistical analysis was done with Systat 10.2. Data were analysed with non-parametric statistics and the results are presented as box plots showing median, 1^{st} and 3^{rd} quartiles and range. All tests were two-tailed and the significance level was fixed at $\alpha = 0.05$. Hormone levels were compared between groups and between years by Mann-Whitney U-test. Spearman rank correlation was used to analyse relationships between hormone levels and testis size and AR expression levels in HVC, respectively. The Fisher's exact test was used to compare the presence or absence of detectable T levels between dominant males that produced solo song and those that did not. Because some data sets were used in two comparisons, the standard Bonferroni technique was applied ($\alpha' = 0.05/2$). On these data the significance level α' was fixed at 0.025 and is indicated in the respective sections of the results.

7.3. Results

7.3.1. Plasma levels of T, DHT and E2 in dominant males and females

The detection limits (pg/ml plasma) of the radioimmunoassays were 12.5 for T, 19.0 for DHT and 4.0 for E2. In males, T was detectable in 18 out of 27 individuals, DHT in four individuals and oestradiol in none. Plasma levels of T showed large variation among dominant males. The highest concentration of T measured in males was 1125 pg/ml. In females, T was undetectable in all individuals; DHT was detectable in one individual and E2 in two individuals.

The plasma levels of T were significantly higher in dominant males than in dominant females (Table 7.1, Fig. 7.1a). Plasma levels of DHT and E2 were low in both sexes and did not differ significantly between dominant males and females (Table 7.1, Fig. 7.1b, c).

Table 7.1: Median (quartiles) hormone concentrations (pg/ml) of dominant individuals ($\alpha' = 0.025$)

	Dominant males (N = 27)	Dominant females (N = 22)	U	P
T	69.57 (12.50, 506.80)	12.50 (12.50, 12.50)	99.0	0.0001
DHT	19.00 (19.00, 19.00)	19.00 (19.00, 19.00)	267.5	0.259
E2	4.00 (4.00, 4.00)	4.00 (4.00, 4.00)	324.0	0.113

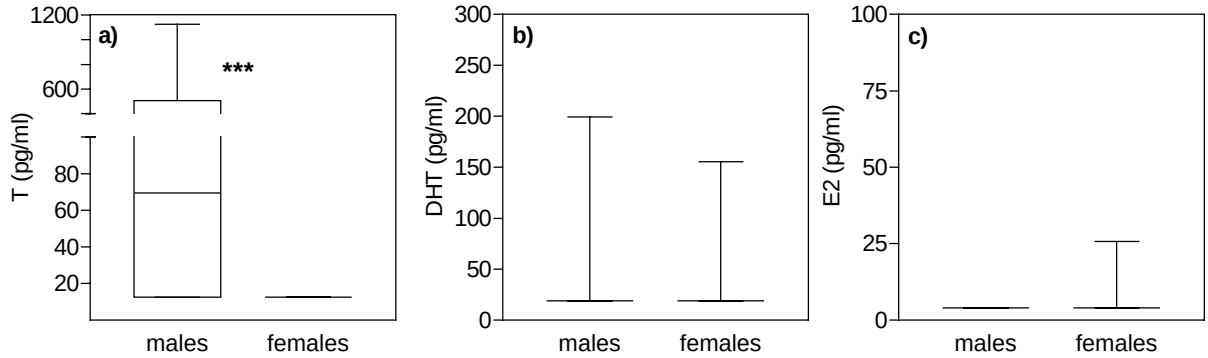


Fig. 7.1: Median plasma levels (quartiles, range) of T (a); DHT (b) and E2 (c) in dominant males (N = 27) and dominant females (N = 22) from the study population in Zimbabwe during the breeding season. Plasma levels of T were significantly higher in dominant males than in dominant females. Plasma levels of DHT and E2 were similar in both sexes (Table 7.2). There was large variation in plasma levels of T among dominant males. The highest concentration of T measured was 1125 pg/ml (Fig. 7.1.a). *** = $p < 0.001$, Mann-Whitney *U* test. Note that the y-axes representing hormone concentration have different scales.

In dominant males, the plasma level of T did not significantly correlate with the mean AR expression level in HVC (Fig. 7.2.). In females, T was undetectable despite similar AR expression levels in both sexes.

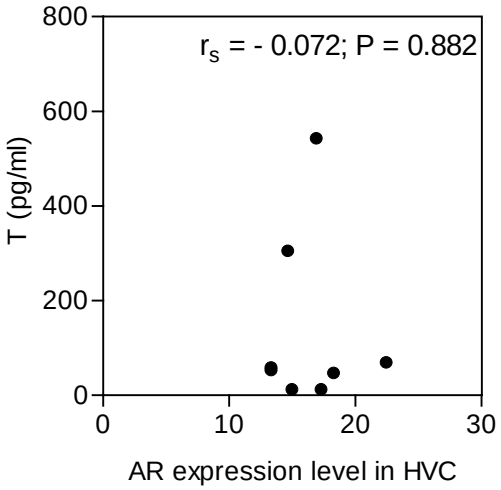


Fig. 7.2: There was no significant correlation between plasma T levels and the mean AR expression level (fractional area covered by silver grains) in HVC of dominant males (N = 8).

Among dominant males, not every individual produced solo song during both study periods. For 22 males, the occurrence of solo singing at the time of blood sampling was known. Therefore, I compared males that sang solo song and males that did not for the presence of detectable T levels with the Fisher's exact test. The analysis showed that the performance of solo song was not associated with elevated T levels (Fisher's exact test, p = 1.00, N=22, Table 7.3).

Table 7.3: Presence (yes) or absence (no) of detectable T levels in dominant males that differed in respect to the production of solo song.

Song behaviour of dominant males	T level detectable	
	yes	no
Males that produced solo song	7	4
Males that did not produce solo song	8	3

7.3.2. Plasma levels of T, DHT and E2 in relation to social status

In subordinates, T was detectable in six out of fourteen individuals, DHT in one individual and E2 in none. Similar to dominant males, there was large variation in plasma T levels among subordinates. However, the range of plasma T levels was larger in dominant males than in subordinates and the highest plasma level measured was found in dominants.

The plasma levels of T, DHT and E2 did not differ significantly between males of different status (Table 7.4, Fig. 7.3).

Table 7.4: Median (quartiles) hormone levels (pg/ml) of dominant and subordinate males ($\alpha' = 0.025$).

	Dominant males (N = 27)	Subordinate males (N = 14)	U	P
T	69.57 (12.50, 506.80)	12.50 (12.50, 251.80)	233.0	0.209
DHT	19.00 (19.00, 19.00)	19.00 (19.00, 19.00)	202.5	0.514
E2	4.00 (4.00, 4.00)	4.00 (4.00, 4.00)	189.0	1.000

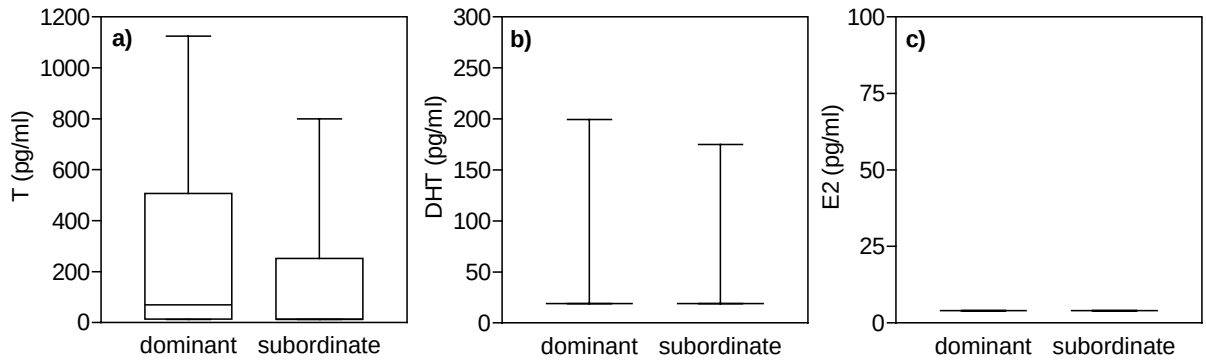


Fig. 7.3: Median plasma levels (quartiles, range) of T (a), DHT (b) and E2 (c) in dominant males (N = 27) and subordinate males (N = 14) from the study population in Zimbabwe during the breeding season. Concentrations of all three steroid hormones were very low and did not differ between males of different status (Table 7.2). Plasma levels of T showed large variation within both groups. The highest concentration of T measured, was 1125 pg/ml and occurred in dominant males. Note the different scales of the y-axes.

When analysing T levels separately for the two study years, I found among dominant males that T levels were significantly higher in 2001 than in 2000 (U = 33.0, p = 0.012, N = 27). In subordinate males, no such difference was found (U = 15.0, p = 0.268, N = 14). However, in both years, T levels were not

significantly different according to male status (2000: U = 58.0, p = 0.194, N = 22; 2001: U = 60.0, p = 0.082, N = 18).

In dominant males, the plasma levels of T did not correlate with the mean AR expression level in HVC (Fig. 7.2). Similar, there was no correlation between plasma T levels and AR expression in HVC of subordinate males (Fig. 7.4). Plasma levels of E2 were undetectable in both groups.

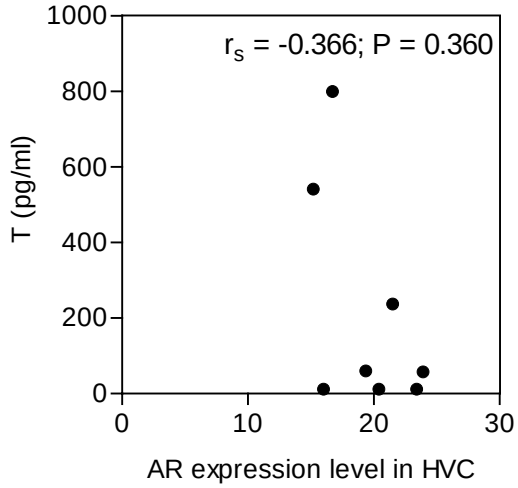


Fig. 7.4: There was no significant correlation between plasma T levels and the mean AR expression level (fractional area covered by silver grains) in HVC of subordinate males (N = 8).

Both groups of males differed significantly in testis weight. Dominant males had about three times larger testes than subordinates (see section 5.3.5). However, testis weight did not correlate with the plasma level of T in either group of males (Fig. 7.5).

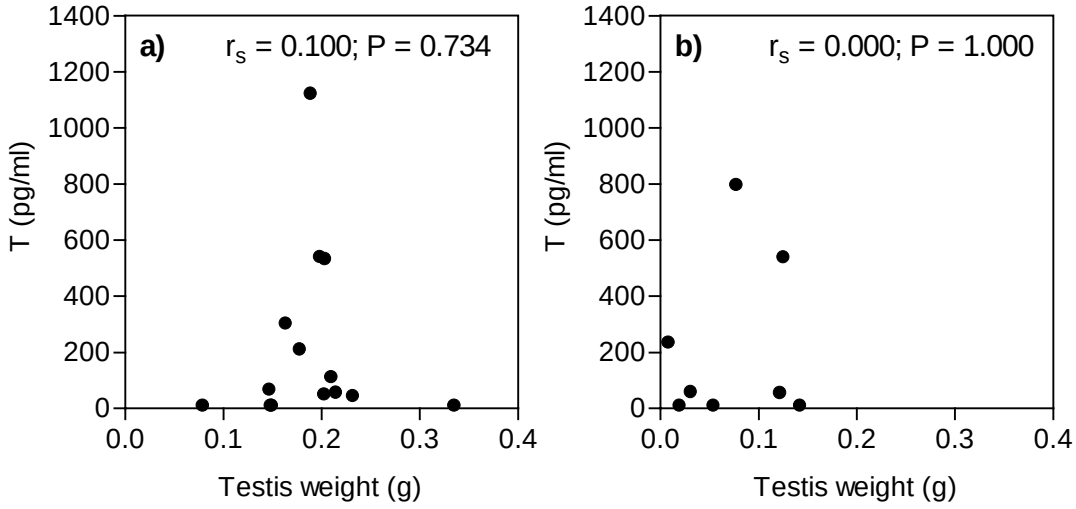


Fig. 7.5: Correlation between plasma T levels and testis weight in dominant males (**a**; N = 14) and subordinate males (**b**; N = 8). Both correlations were not significant.

7.3.3. Comparison of plasma levels of T, DHT and E2 between dominant females and subordinate males

Subordinate males had significantly higher plasma T levels than dominant females (Table 7.4, Fig. 7.6.a). No significant differences between groups were found in plasma levels of DHT and E2 (Table 7.5, Fig. 7.6.b, c).

Table 7.5: Median (quartiles) hormone levels (pg/ml) of subordinate males and dominant females ($\alpha' = 0.025$).

	Subordinate males (N = 14)	Dominant females (N = 22)	U	P
T	12.50 (12.50, 251.80)	12.50 (12.50, 12.50)	88.0	0.001
DHT	19.00 (19.00, 19.00)	19.00 (19.00, 19.00)	149.5	0.713
E2	4.00 (4.00, 4.00)	4.00 (4.00, 4.00)	168.0	0.253

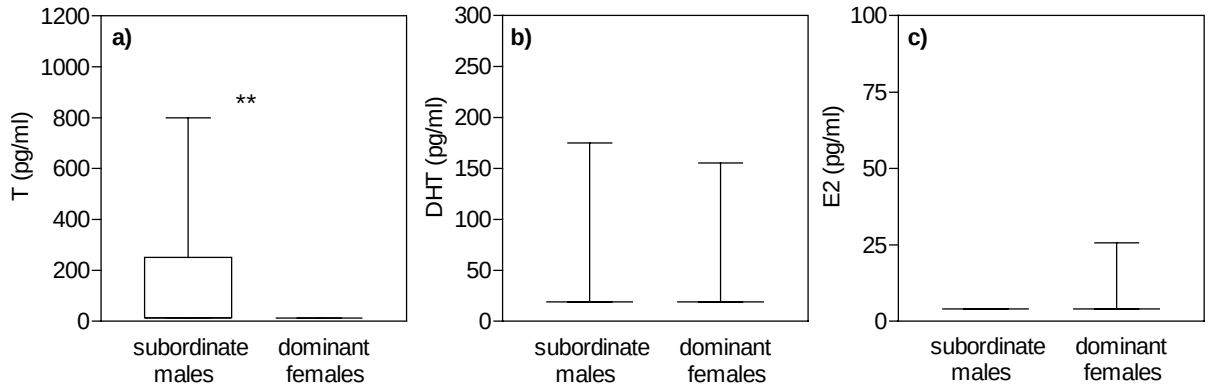


Fig. 7.6: Comparison of plasma levels of T (a), DHT (b) and E2 (c) between subordinate males (N = 14) and dominant females (N=22). Subordinate males had significantly higher T levels than dominant females. No differences were found in plasma levels of DHT and E2. ** = $p < 0.01$, Mann-Whitney *U* test. Note the different scales on the y-axes.

7.3.4. Plasma levels of T, DHT and E2 from males of closely related species

Additionally to the white-browed sparrow weavers, I analysed blood samples of single males from four other ploceid species and from one closely related species, the grey-headed sparrow (Family Passeridae), which were taken at the same time of the year as the samples of the white-browed sparrow weavers. These species were in breeding condition, which was confirmed by plumage

colour (*Ploceus* species) and nest building behaviour. Plasma levels of T, DHT and E2 from those males were within the range of the concentrations measured in male white-browed sparrow weavers (Fig. 7.7). T was detectable in the village weaver (*Ploceus cucullatus*), the red-billed buffalo weaver (*Bubalornis niger*) and most individuals of the grey-headed sparrow (*Passer diffusus*). DHT was detectable in males from two species only and oestradiol in none (Fig. 7.7).

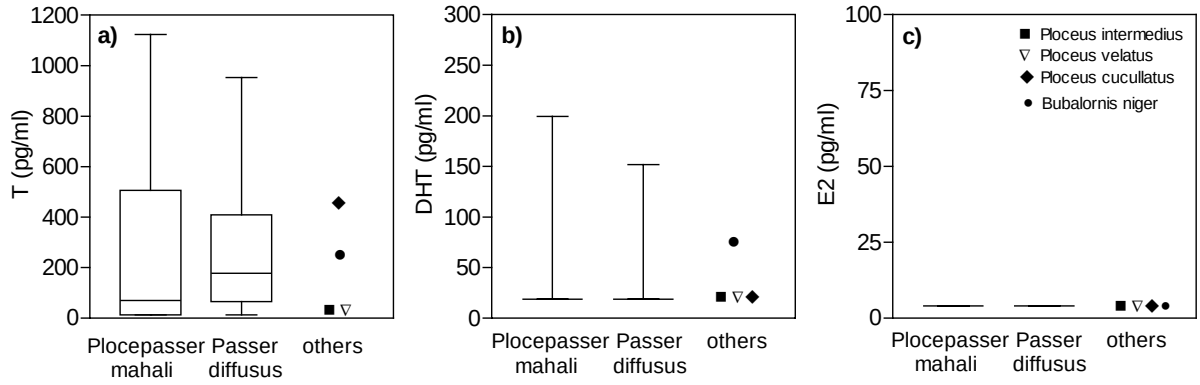


Fig. 7.7: Comparison of plasma levels of T, DHT and E2 (pg/ml) between males of white-browed sparrow weavers (*Plocepasser mahali*), males (each N=1) of four other species of the family Ploceidae and males (N = 8) of the grey-headed sparrow (*Passer diffusus*) from the study area in Zimbabwe during the breeding season. Levels of all three hormones were within the range of concentrations measured in white-browed sparrow weavers. Note the different scales of the y-axes.

7.4. Discussion

In the present chapter, I investigated plasma levels of the steroid hormones testosterone (T), 5α-dihydrotestosterone (DHT) and 17β-oestradiol (E2) in white-browed sparrow weavers in relation to sex and social status. I correlated these data to male song behaviour, testis size and to the androgen receptor (AR) level in song control nucleus HVC. 1) Among dominant individuals, T levels were significantly higher in males than in females, there was no sex difference in DHT and E2 levels. 2) Levels of all three hormones did not differ in respect to male status. 3) Subordinate males had significantly higher T levels than females; DHT and E2 level did not differ. 4) The performance of male solo song was not associated with elevated T levels. 5) T levels were not significantly correlated with testis size in either group of males. 6) T levels were not significantly correlated with the expression level of AR in HVC in either group. 7) Steroid hormone levels of closely related species were of similar magnitude than those measured in white-browed sparrow weavers.

7.4.1. Steroid hormone levels in relation to sex

In white-browed sparrow weavers, I found a significant sex difference in plasma T levels but not in DHT and E2 levels, which were baseline. However, T levels in almost all males were below 1.0 ng/ml and the sex difference was due to undetectable T levels in all females. Of similar magnitude were the plasma levels reported by (Wingfield et al. 1991) who studied the same species in Zambia. There, T levels peaked (<1.0 ng/ml) in breeding males at mid-breeding season but were baseline for the rest of the year. In females, T levels were basal year-round and E2 levels were detectable only in few individuals around egg-laying. Furthermore in the present study, I measured T levels from males of five closely related species, which were in breeding condition, but T levels in all males were below 1.0 ng/ml. Very low levels of testosterone were also found in males and females of several neotropical passerine species throughout the year (Levin & Wingfield 1992; Wikelski et al. 2000; Wikelski et al. 2003). In contrast, from temperate-zone species it is known that reproductive activity in spring, often associated with increased song production and territorial aggression depends on elevated T levels and prior gonadal recrudescence (Wingfield & Farner 1978; Silverin & Wingfield 1982; Balthazart 1983; Wingfield 1985; Rost 1992; Logan & Wingfield 1995).

White-browed sparrow weavers maintain territories year-round (Lewis 1982) accompanied by high levels of duet and chorus song production by all colony members (Ferguson 1988a) despite low T levels. Even experimentally induced territorial challenges, which significantly increased aggressive behaviours and chorus vocalisations, did not elicit a significant rise in plasma T levels in either sex (Wingfield et al. 1992; Wingfield & Lewis 1993). In another tropical duetting species, the slate-coloured bou-bou shrike (*Laniarius funebris*) changes in the hormonal state of males and females were not correlated with duet singing (Schwabl & Sonnenschein 1992). Furthermore, I could show that the performance of male solo song was not associated with increased T levels. I have argued (chapter 4) that the solo song of dominant males mainly functions in inter-sexual communication related to breeding activities. The peak testosterone levels of breeding males measured by (Wingfield et al. 1991) in the mid-breeding season, therefore, could have coincided with male solo singing. However, this is not supported from the plasma T levels measured in the present study. Together, these data suggest, that song behaviour and territorial aggression in both sexes is independent of the acute plasma T levels. It cannot be excluded that steroid hormone levels have influenced these behaviours at earlier times or that tropical birds in general are sensitive to very low levels of circulating steroid hormones or that androgen precursors such as

dehydroepiandrosterone (DHEA) play a more important role (Hau et al. 2004). I was able to show experimentally, that the administration of testosterone to adult females induces the performance of solo singing, which resembles the behaviour seen in males (chapter 8). This suggests indeed a role of testosterone for song production in this species. It remains to be seen however, which threshold levels are actually necessary for the induction and maintenance of song behaviour.

7.4.2. Steroid hormone levels of males in relation to status

In the present study, dominant and subordinate males did not differ significantly in respect to the plasma levels of all three hormones measured. Levels of DHT and E2 were undetectable in most individuals. Regarding T levels, the results contrast with previous findings in a population in Zambia, where dominant males had significantly higher T levels than subordinates during the mid-breeding season (Wingfield et al. 1991). However, at this point the authors sampled only five breeding males, whereas the data of the present study derived from 27 males. Variation in dominant males was large and T was not even detectable in all individuals. Furthermore, I found a significant effect of study years on T levels among dominant males. Males sampled in 2001 had higher T levels than those sampled in 2000. Nonetheless, no status difference was found in either year. Also, in 2001, none of the dominant males sampled did sing solo song. This was maybe due to a difference in peak rainfall between study years, which could have influenced breeding activities. White-browed sparrow weavers have a bimodal breeding rhythm (Lewis 1982, Earle 1983a) with a first peak around the start of the rainy season in October/November and a second peak when rainfall peaks, usually in January/February. In both study years, I started blood sampling at the beginning of February. However, in January 2001 there was virtually no rainfall compared to 2000 (Fig. 2.4) and most rainfall in 2001 occurred from the middle to the end of February. Therefore, it could have been that in that year the second peak of breeding activities was delayed. I know of two males, which started singing solo song by the end of February. In males of a variety of tropical bird species (Schwabl & Sonnenschein 1992; Seiler et al. 1992; Lormee et al. 2000; Wiley & Goldizen 2003) and in dominant males of cooperatively breeding species (Schmidt et al. 1991; Schoech et al. 1996; Khan et al. 2001) plasma T levels peak during the pre-laying period. For white-browed sparrow weavers, there is so far no evidence that elevated T levels relate to a particular phase of the breeding cycle (J. Wingfield, personal communication), nevertheless it is likely that in 2001 more dominant males were in the pre-laying period.

Alternatively, higher T levels in some dominant males in 2001 relate to a period of group instability or to a change in male status, i.e. males being for the first time in a dominant position. This would support the 'challenge hypothesis', which states that T levels in males may rise above baseline only during periods of social instability, i.e. male-male interactions over territories or mates (Wingfield et al. 1990). At least for half of the colonies sampled in 2001 it is known that compared to 2000 either the colony was newly established or the dominant male had changed. Interestingly, three of four subordinates with elevated T levels (T > 0.2 ng/ml) came from colonies where the dominant male had elevated T levels as well. The 'challenge hypothesis' was confirmed in a study on neotropical spotted antbirds (*Hylophylax n. naevioides*), where social instability caused T levels in males to rise from baseline to maximally 1.5 ng/ml (Wikelski et al. 1999). This increase was only slightly larger than the range observed in white-browed sparrow weavers. However, results from previous experimental studies in the latter species do not support this hypothesis, because territorial challenges did not elevate plasma T levels in males (Wingfield et al. 1992; Wingfield & Lewis 1993).

Although no status difference was found in plasma T levels, subordinates had significantly smaller testes than dominants, which indicates that they were reproductively suppressed. It was proposed that the absence of breeding in subordinate males could result from delayed maturity or from behavioural inhibition (Reyer et al. 1986). According to the latter scenario, subordinates are 'psychologically castrated' through dominance interactions. Such inhibition could be at the level of the gonad or the brain, affecting the activity of pituitary and/or hypothalamus. In cooperatively breeding pied kingfishers (*Ceryle rudis*), male related helpers are behaviourally dominated by breeders, which results at the physiological level in smaller gonads, lower T levels and no sperm production compared to unrelated helpers and breeders (Reyer et al. 1986). For white-browed sparrow weavers Wingfield et al. (1991) derived similar conclusions, but suggested that the inhibitory effect was at the level of pituitary/hypothalamus because subordinates had lower levels of luteinising hormone. The data of the present study support this view though smaller gonads in subordinates were not associated with lower T levels. There was great variation in testes size among subordinates and seven out of eight males had testes that were several times larger than fully regressed testes. This variation probably reflects a difference between related and unrelated subordinates, which could not be distinguished in the present study. It remains to be investigated whether related and unrelated subordinates differ in their ability to produce fertile sperm. No difference in T levels between breeding and non-breeding helper males have been reported for cooperatively breeding red-cockaded

woodpecker (*Picoides borealis)* (Khan et al. 2001) and Australian magpies (*Gymnorhina tibicen Latham)* (Schmidt et al. 1991). In both species, subordinate adult males are physiologically capable of reproducing. Yet, in another cooperative breeder, the Florida scrub-jay (*Aphelocoma coerulescens*) testis size increases with age and 1-year-old breeding and nonbreeding males had testes of similar size (Schoech et al. 1996). Observation of solo song and courtship behaviour in young white-browed sparrow weaver males in captivity indicate that these males are fully capable to reproduce though this remains to be experimentally confirmed.

7.4.3. Steroid hormone levels in relation to the steroid hormone sensitivity of the song system

Inter- and intrasexual comparisons of plasma levels of T, DHT and E2 with the expression levels of androgen receptors (AR) and oestrogen receptors (ER) in song nucleus HVC did not suggest a correlation between both factors. The sex difference found in T levels was not reflected in the AR expression level, which was similar in males and females. Furthermore, there was no status difference in T levels among males, whereas subordinates had significantly higher AR expression in medial HVC. Despite a sex difference in ER expression in medial HVC, all birds had baseline plasma levels of oestradiol. It has been suggested that the brain could respond to low circulating steroid hormone levels by increasing its sensitivity due to an up-regulation at the level of the steroid hormone receptor (Wingfield et al. 2001). The present data do not support this hypothesis. Also, expression levels of AR and ER measured in white-browed sparrow weavers were of similar magnitude as those obtained in the domesticated canary (Fusani et al. 2000; Fusani et al. 2003) and the zebra finch (chapter 6.2) providing no evidence that such a mechanism has evolved in tropical birds to avoid the costs of high steroid hormone levels, especially testosterone. Clearly, more tropical species have to be investigated to verify this. Alternatively, the regulation of behaviours such as song or territorial aggression could be mediated by the presence of short peaks in circulating steroid hormones at particular times in life, by the increased local activity of steroid conversion enzymes, e.g. aromatase (Fusani et al. 2001) or by steroid hormones synthesised de novo in the brain (Schlinger & Brenowitz 2002).

8. TESTOSTERONE-INDUCED MALE-LIKE SOLO SONG IN FEMALE WHITE-BROWED SPARROW WEAVERS?

8.1. Introduction

Singing in many songbirds is a sexually dimorphic behaviour and the sex differences in vocal behaviour are thought to correlate with sex differences in the neural structures controlling the behaviour (Ball & MacDougall-Shackleton 2001). For example, in species such as the zebra finch (*Taeniopygia guttata*) only the male sings but never the female. In other species such as canaries (*Serinus canaria*) and starlings (*Sturnus vulgaris*), females occasionally sing but not as complex and frequently as males do (Pesch & Guettinger 1985; Henry 1998). Yet, in others, males and females regularly engage in duet singing (Helversen & Wickler 1971; Wickler 1972; Wickler & Seibt 1980; Slater 1997; Mann et al. 2003).

Steroid hormones have permanent effects on the sexual differentiation of brain and behaviour during ontogeny and play an important role in the induction of transient sex-typical behaviour patterns in adulthood (Arnold & Breedlove 1985). In zebra finches, early organisational effects of oestrogen establish sex differences in brain and behaviour. The song nuclei of adult females, which never produce any song, are about five times smaller than those of males (Nottebohm & Arnold 1976). The responsiveness of these brain areas to steroid hormones is determined early in life. In adult females, which were not treated as juveniles with oestrogen, exogenous testosterone has no effect on the activation of song behaviour (Gurney & Konishi 1980). In contrast, in canaries and starlings the sex differences in song behaviour and in the morphology of the song control system are not as pronounced (Nottebohm & Arnold 1976; Bernard et al. 1993) and are thought to be attributed to different activational effects of testosterone. Plasma testosterone levels are indeed much higher in males than in females during the breeding season (Weichel et al. 1986). Treatment of such females with testosterone induces male-typical song behaviour, both in terms of structure and activity (Shoemaker 1939; Hausberger et al. 1995; Vallet et al. 1996). This indicates that in these species sex differences in vocal behaviour are not based on organisational effects of steroid hormones but are the result of an adult sex difference in circulating testosterone levels.

The activation of male-like song behaviour by sex steroids in females is accompanied by structural changes in brain areas controlling the behaviour, e.g. increase in the size of song nuclei (Nottebohm 1980; Brenowitz & Arnold 1990), increase in synapses (DeVoogd et al. 1985; Gahr & Garcia-Segura 1996) or neurotransmitter systems (Appeltants et al. 2003). The presence of androgen

receptors in song nuclei responsible for song production, i.e. HVC, RA, (Gahr 2001) suggests that testosterone acts directly on these nuclei. Despite the demonstrated effect of testosterone on behaviour and on brain morphology, the mechanisms of such steroid hormone induced changes are not fully understood.

In the white-browed sparrow weaver (*Plocepasser mahali*) males and females engage regularly in duet singing throughout the year and both sexes have duet repertoires of similar size (chapter 4). Interestingly, males also produce typically at dawn a complex solo song, which is restricted to the breeding season and comprises a different syllable repertoire than that of the duet songs. Regarding the structure of the song control system, song nucleus HVC, responsible for song production is significantly larger in males than in females (chapter 5). I have suggested that the production of solo song is testosterone-dependent (chapter 4). In line with this is the finding that the plasma level of testosterone during the breeding season is significantly higher in males compared to females (chapter 7). However, circulating testosterone levels in males are an order of magnitude lower than those of male songbirds from temperate zones.

During the development of duet singing, young females in captivity were observed to produce a number of syllable types belonging to the male-typical solo song (personal observation). Later in life and under natural conditions this type of song was never heard from females. Therefore, because females are capable to produce this song the intended experiment with females is suitable to answer the question whether this particular type of song is sensitive to circulating testosterone levels. If so, then females treated with testosterone should start to produce solo song. Furthermore, this will show that the sex difference in the production of solo song in adult birds is not the result of ‘organised’ neural differences in the sensitivity to testosterone but results from a sex difference in circulating levels of testosterone. At the same time the experiment should reveal which levels of plasma testosterone are actually necessary to maintain the production of solo song. This is an important issue in the light of comparing the regulation of song behaviour between tropical and temperate zone species. In the latter, song and other behaviours are known to be associated with elevated testosterone levels. It remains unclear how these behaviours are controlled in tropical birds despite of having lower circulating steroid hormone levels (Goymann et al. 2004). The experiment further explores the question whether the type of behaviour induced in females resembles in its complexity the behaviour normally seen in males. Testosterone-treated female starlings show only partly male-typical song behaviour suggesting that some aspects of this behaviour have been permanently determined in a sex-specific way during early development (De Ridder et al. 2002). I conducted a

preliminary experiment with three female white-browed sparrow weavers and I addressed the following questions:
1) Can treatment with exogenous testosterone induce the production of male-typical solo song in female white-browed sparrow weavers?
2) How does song behaviour change in relation to changes in steroid hormone levels?
3) Is testosterone-induced female solo song similar in structure to the solo song of males?

8.2. Methods

8.2.1. Animals

Three adult female white-browed sparrow weavers were used in this experiment. *Females No. 1* and *No. 2* were 12, respectively 14 months old, *female No. 3* was wild-caught and thus the exact age was unknown but it was at least four years old. During the experiment, they were housed individually in an aviary with acoustic contact to other birds but not to other conspecifics under a 12L:12D photoperiod. For each bird, the experiment lasted 4 weeks.

8.2.2. Testosterone implantation

Each female received a single subcutaneous silastic implant (length: 8 mm, inner diameter: 0.76 mm, outer diameter: 1.65 mm, Silastic Tubing; Dow Corning, Midland, MI, USA) filled with testosterone propionate (Sigma). The implantation procedure was as follows: A small incision (about 4 mm) was made with scissors above the left flank and the silastic capsule was inserted under the skin with forceps. The incision was closed with surgical glue (Brown).

8.2.3. Blood sampling

From *female No. 1* blood (about 100 µl) was collected at 1-week intervals starting two days after implantation until the end of the experiment. Blood was taken and processed as described in section 3.1.1. In total, five blood samples were collected. No blood sample was taken before implantation. Because blood samples from 22 females in Zimbabwe revealed baseline plasma levels of T, DHT and E2, I used these values as the data point before implantation.

8.2.4. Radioimmunoassay of plasma levels of steroid hormones

The androgens 5α-dihydrotestosterone (DHT) and testosterone (T) and the oestrogen oestradiol (E2) were measured by radioimmunoassay (RIA) as described in section 3.2.4.

8.2.5. Song recording and analysis

Vocalisations were recorded three to six times a week starting at the day of implantation until the end of the experiment using a Sony DC 6 tape recorder and a Sennheiser ME 67 directional microphone. Recordings were made at dawn for 45 minutes. Vocalisations were analysed with the sound analysis software Canary 1.2.1. (Cornell University) on a Macintosh computer. Song activity was calculated as the proportion of time spent singing during a 45 minute-recording session. Printouts of song sequences were generated and syllable types were sorted according to its structure as either belonging to duet or solo song. From this, the repertoire size of solo song syllables was estimated.

8.3. Results

8.3.1. Song activity

Before treatment with testosterone, no female was heard to produce solo song. All females started to produce solo song between day 3 and day 4 post-implantation. Song activity rapidly increased during the following days and remained high until week 4 post-implantation. At day 27, all females had stopped singing. Interestingly, song activity of *female No. 3* increased much higher compared to the other two females (Fig. 8.1). For each female, I calculated the mean song activity between day 3 and day 26 post-implantation, because this was the period where females were singing. When comparing these data with the mean song activity of dominant males in Zimbabwe no significant difference was found (Table 8.1). Therefore, T-implantation induced females to sing with a similar activity pattern as normal males.

Table 8.1: Comparison of song activity of T-implanted females and dominant males in Zimbabwe

	T-implanted females (N = 3)	Dominant males (N = 7)	U	P
Percent time spent singing	20.5 ± 3.7	27.1 ± 4.1	15.0	0.305

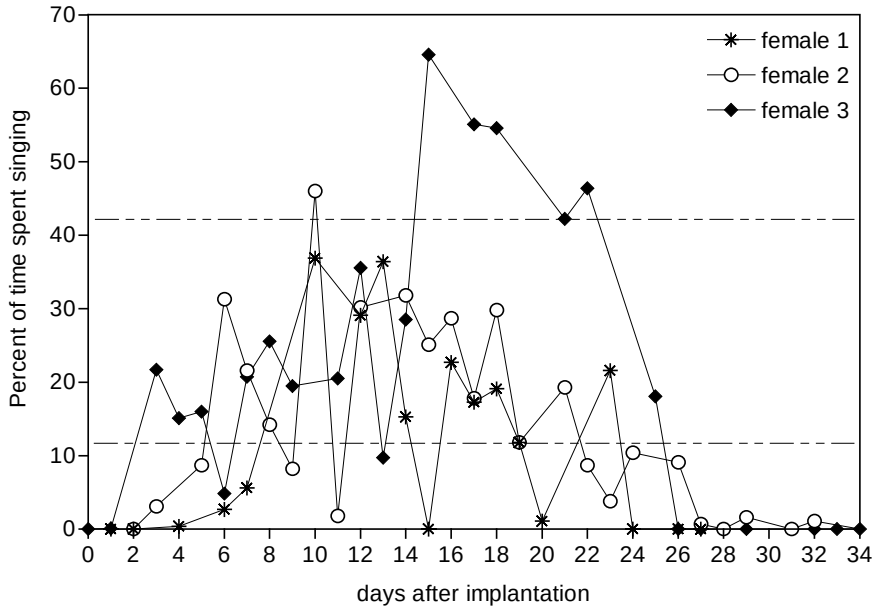


Fig. 8.1: Proportion of time spent singing solo song of three T-implanted females. Before implantation, no female produced solo song. Song activity started around day 3 after implantation and persisted until around day 26. Dashed lines indicate minimal and maximal song activity of males in Zimbabwe (N =7). Female song activity was not significantly different from that of normal males (Table 8.1). The reduced song activity at day 11 and 15 can be attributed to bad weather conditions during the recording session.

Besides an increase in song activity, I noticed increased nest building activity of the T-implanted females. Usually, without contact to conspecifics this type of behaviour is hardly performed. Additionally, the beak colour of the females became more blackish in the course of the experiment. In this species, normally females have horn-coloured and males have black beaks (chapter 2). Adult beak colour is attained at an age of about five months.

8.3.2. Repertoire size and syllable structure

Recordings were analysed throughout the 4-week experimental period. For each female, two to three recording sessions per week were analysed. However, at the start and at the end of the experiment song activity was rather low. In these cases, only a single recording session per week was used. *Females No. 1* and *No. 3* produced exclusively syllables belonging to solo song. *Female No. 2* combined in her songs duet syllables with syllables of the solo song. In this case, the duet syllables were discarded from further analysis. The three T-implanted females produced solo song, consisting of 48 to 57 different syllable types. The largest increase in repertoire size occurred during the first two weeks post-implantation. The asymptotic shape of the curves indicates that the majority of the females' repertoire was obtained (Fig. 8.2).

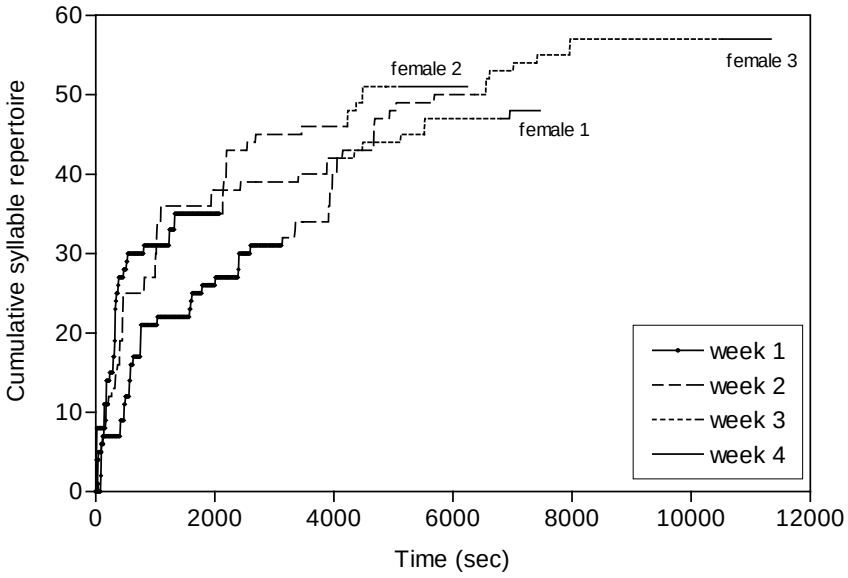


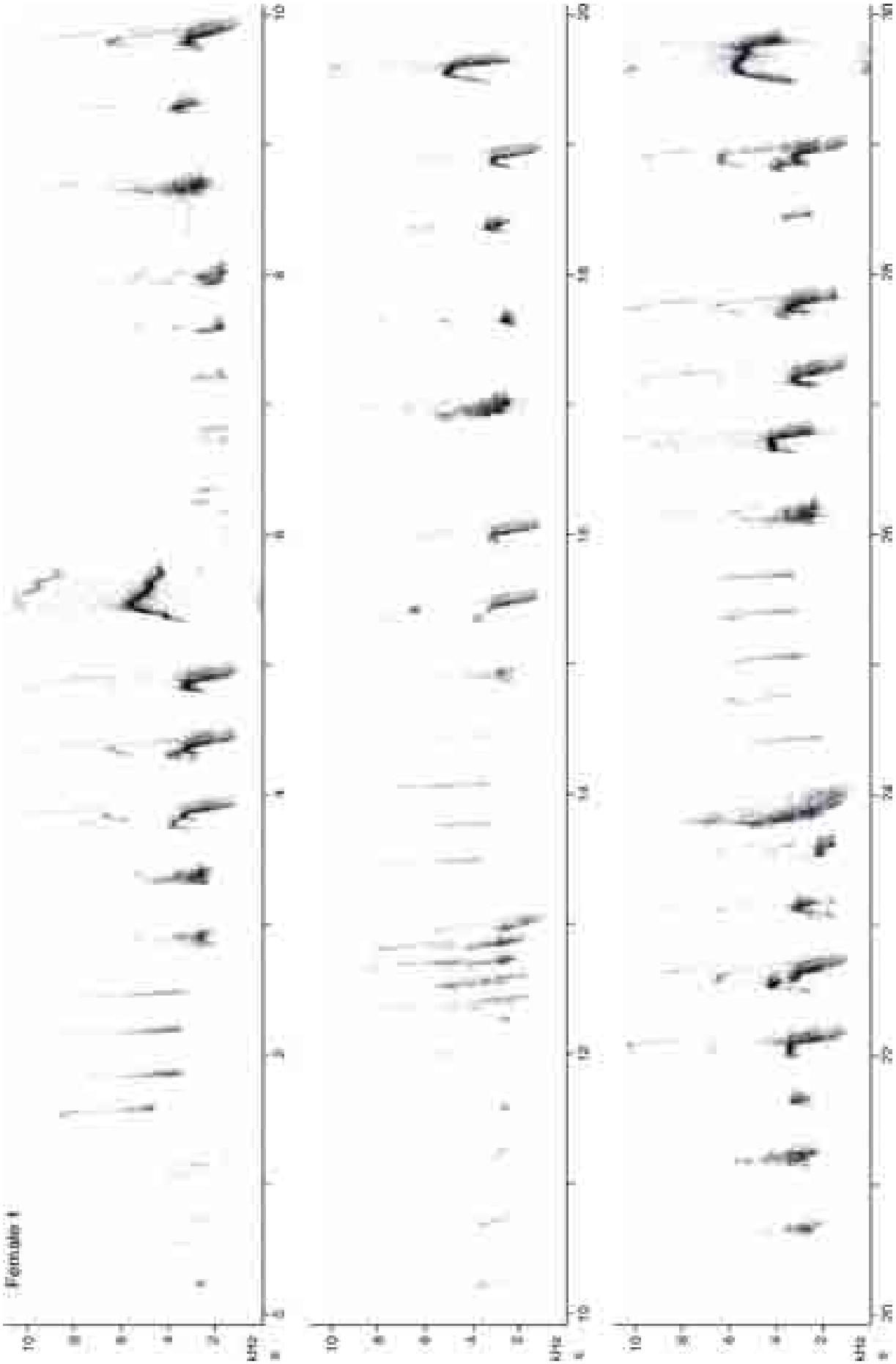
Fig. 8.2: Cumulative syllable repertoires of the solo song of three T-implanted females recorded throughout the experimental period. Repertoire size increased largely during the first two weeks post-implantation, whereas during week 3 and 4 there was only a slight increase. All curves show asymptotic shape suggesting that total repertoire size is obtained. Repertoire sizes were not significantly different from those of males (Table 8.3).

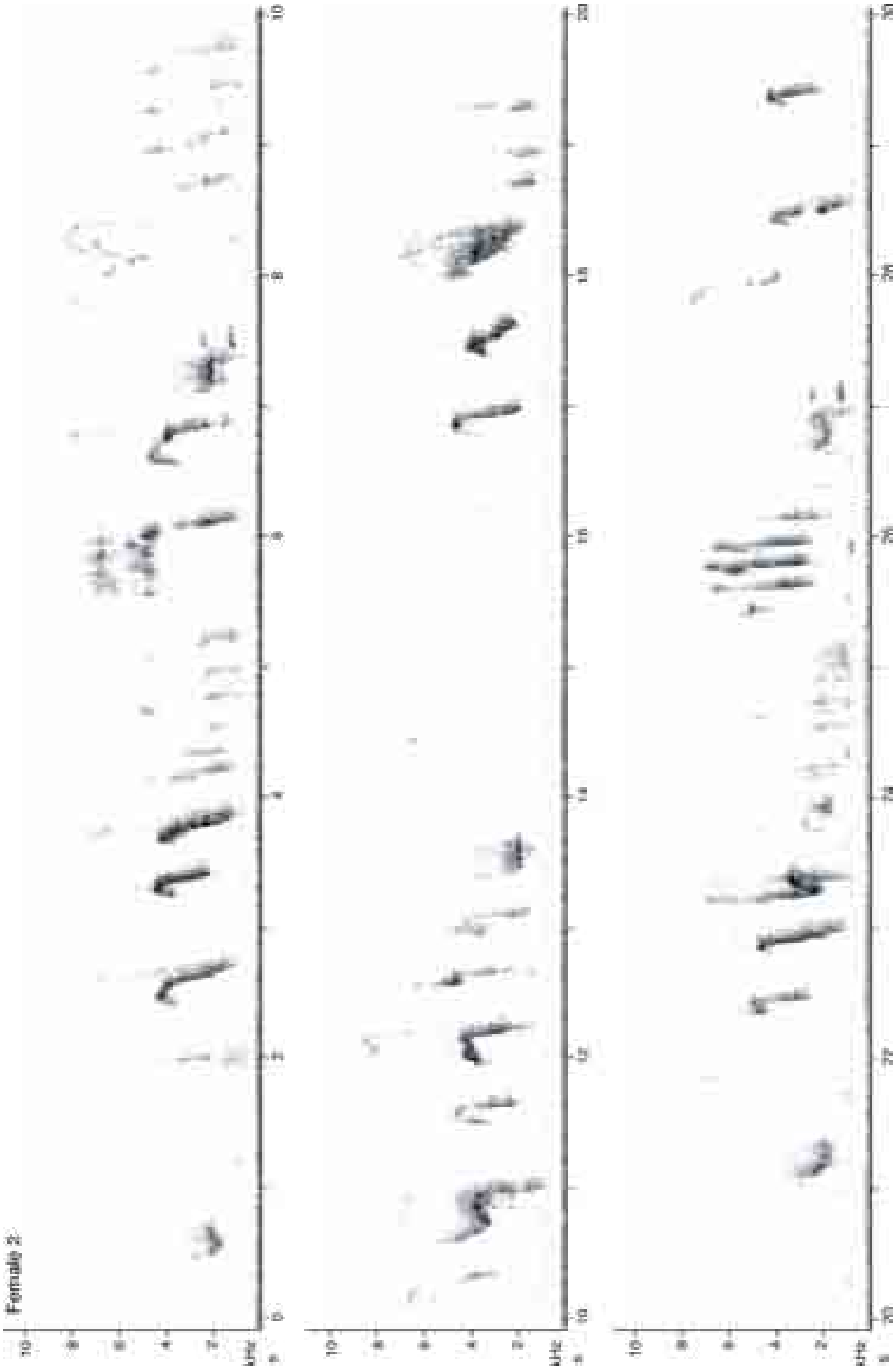
Compared with the repertoire size of dominant males recorded in Zimbabwe (section 4.3.2.2) the number of syllable types produced by T-implanted females was not significantly different (Table 8.2). Concerning syllable structure, both sexes differed in the types of syllables produced. Whereas repertoires of males contained 30 to 50 % of syllables, which were produced with repetitions of three or more, in females, these syllable types accounted for less than 20 % of the repertoire (Fig. 8.3, compare with Fig. 4.5, a sequence of male solo song). However, repetition rate of such syllables was not significantly different between the sexes.

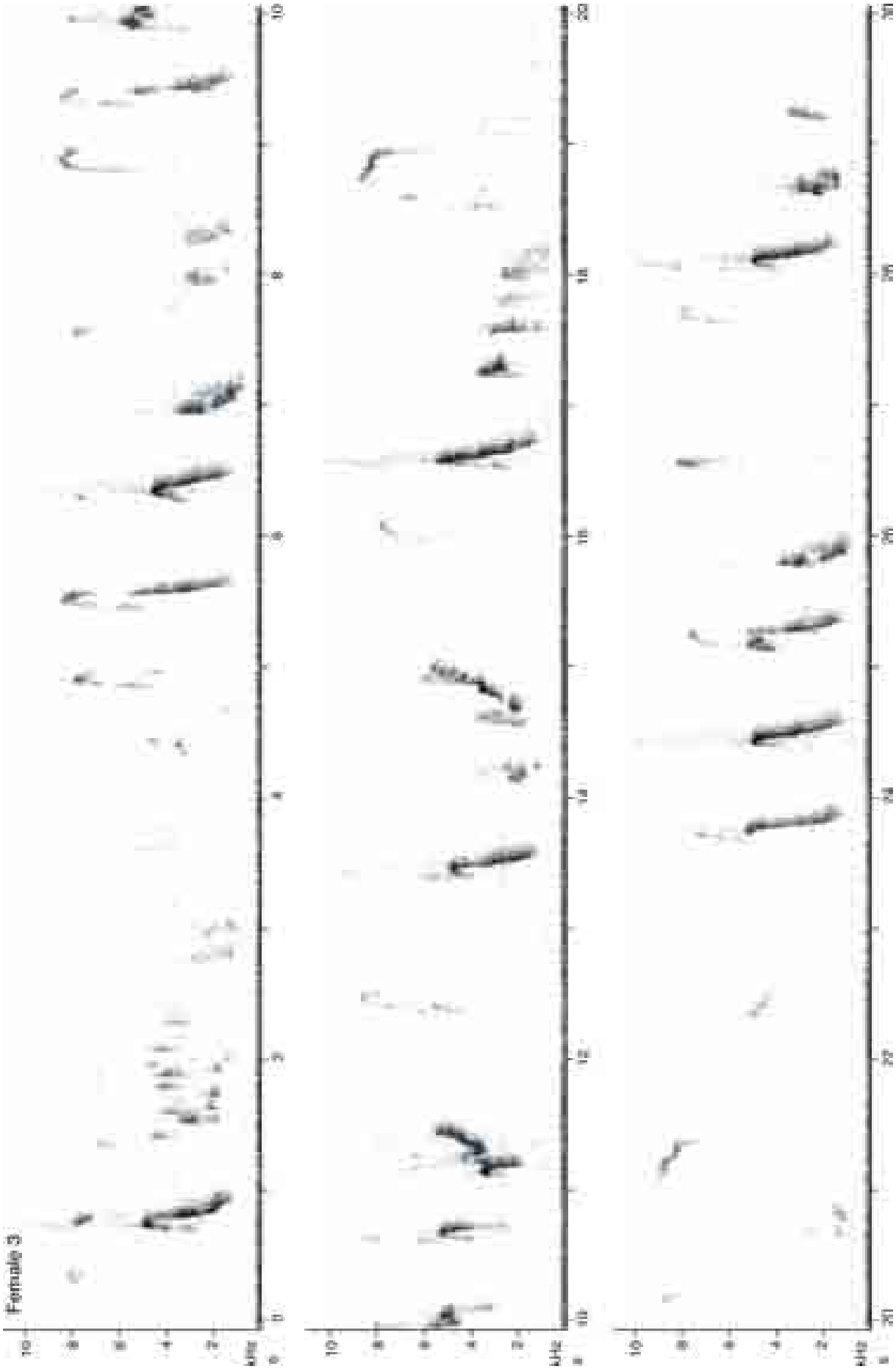
Table 8.2: Comparison of the syllable repertoire of T-implanted females and dominant males in Zimbabwe.

Song parameter	T-implanted females (N = 3)	Dominant males (N = 7)	U	P
No. of different syllables	52.0 ± 2.6	70.1 ± 6.8	3.5	0.117
Proportion of syllables with repetitions ≥ 3	16.6 ± 0.5	40.5 ± 3.4	0.0	0.016
Repetition rate	8.6 ± 1.0	10.1 ± 0.4	4	0.183

Fig. 8.3: (next pages): A sequence of 30 seconds out of the solo song of each of the three T-treated female white-browed sparrow weavers is shown. Repertoire size of female solo song was similar to that of males. However, females produced fewer syllable types with repetitions of three or more but repetition rate was identical in both sexes. Because recordings were made indoors, syllables show slight reverberations.


Female 1
kHz
s


Female 2:
kHz
kHz
kHz


Female 3
kHz
kHz
kHz

8.3.3. Song activity in relation to circulating steroid hormone levels

From *female No. 1* plasma levels of T, DHT and E2 were measured in weekly intervals throughout the experiment starting at day 2 after T-implantation. Already after two days, plasma levels of all three hormones reached peak values (Fig. 8.4). Levels of T and DHT were about 8 to 10 times higher than the highest levels measured in males in Zimbabwe. Therefore, the T implants caused steroid hormone levels of females to raise much above the physiological range of this species. However, plasma levels rapidly declined during the first week post-implantation and were about 80 % reduced at day 9 when the second measurement was taken. Thereafter, plasma levels declined further and by the third week post-implantation they had returned to baseline levels.

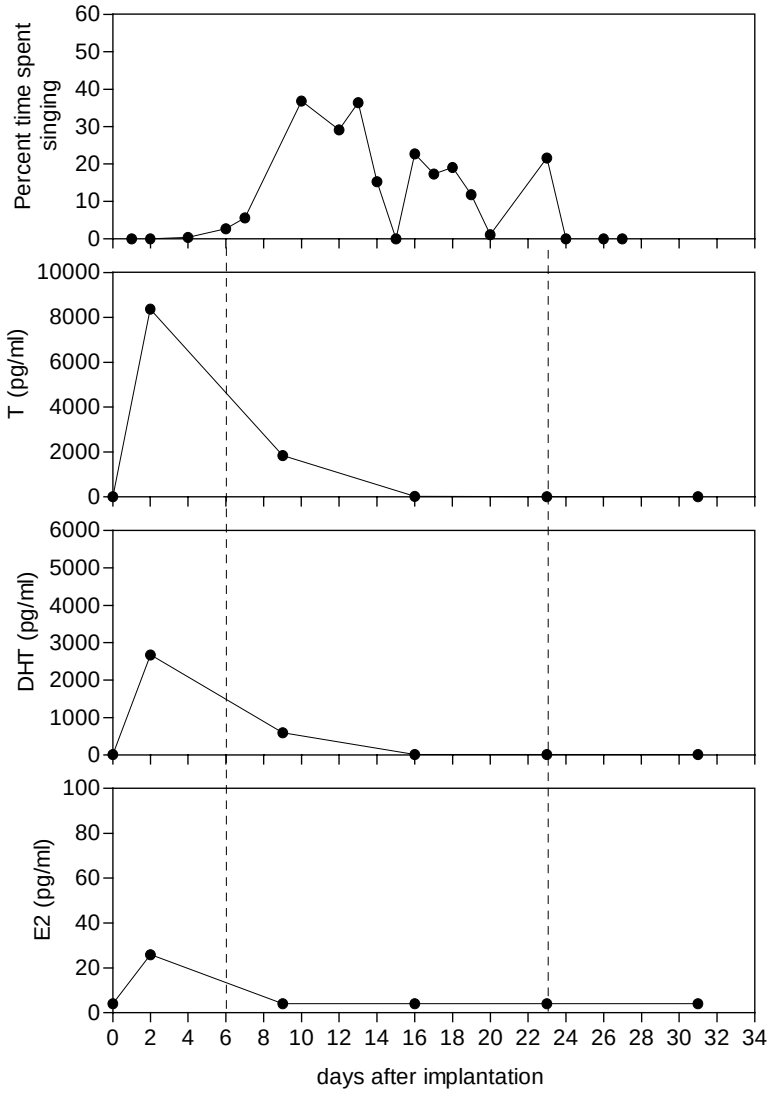


Fig. 8.4: Plasma levels of T, DHT and E2 in one female white-browed sparrow weaver before (day 0) and after (days 2, 9, 16 and 23) testosterone implantation. Levels of all three hormones peaked two days after implantation and reduced by about 80 % during the first week post-implantation. In the third week, levels had returned to baseline. Dashed lines mark the period of song activity.

Interestingly, the female started to produce solo song when steroid hormone levels were already declining. Song activity then persisted for 17 days although plasma levels returned to baseline. This pattern suggests that the performance of solo song can be induced once circulating hormone levels reach a threshold level. The behaviour is then performed for a certain period independently of the actual hormone levels.

8.4. Discussion

In the present chapter I have described a preliminary experiment with three female white-browed sparrow weavers that asked the question whether exogenous testosterone could induce females to produce male-typical solo song. The following results were obtained. 1) All females started to produce solo song in response to the T-implant. 2) Song activity lasted for about three weeks and during this time was of similar intensity as in dominant males recorded in Zimbabwe. 3) Females produced repertoires of 48 to 57 different syllable types, which resembled repertoire sizes of males. 4) Females sang fewer syllable types with a high number of repetitions but repetition rate of such syllables was identical. 5) The T-implant caused steroid hormone levels to peak two days after implantation and to return to baseline by week 3 post-implantation. 6) Song activity started when hormone levels were declining and lasted for 17 days independently of hormone levels.

8.4.1. Induction of male-like song behaviour in females

The present study shows that the performance of solo song is not a sex-specific behaviour but is sex-typical, which means it can be induced under certain conditions in the other sex (Gahr 1994). Female white-browed sparrow weavers retain throughout life the ability to respond to circulating testosterone in a similar way as males do. This finding adds to a number of studies in which female songbirds are found to sing under the influence of testosterone, e.g. domesticated canaries (Leonard 1939; Weichel et al. 1989), starlings (Hausberger et al. 1995), white-crowned sparrows (Kern & King 1972) and one non-songbird species, the budgerigar (*Melopsittacus undulatus*), which exhibits vocal learning (Nespor et al. 1996). In contrast to these species stands the zebra finch, where adult females are not able to sing under testosterone-treatment and therefore a sex difference in the responsiveness to testosterone must have been determined permanently early in life (Gurney 1982). However, early oestrogen treatment masculinises the song system and enables such females to respond as adults to exogenous T with song output similar to males (Gurney 1982).

The T-induced singing in adult females of the species mentioned above is accompanied by profound changes on the neural structure of the song system (Nottebohm 1980; DeVoogd et al. 1985; Gahr & Garcia-Segura 1996). It has been shown recently in female domesticated canaries, that testosterone acts directly on HVC via androgen receptors (AR) and indirectly by conversion to oestrogen in the caudomedial neostriatum (NCM), which in turn activates oestrogen receptors (ER) in HVC. The activation of both, AR and ER in HVC is necessary to develop all aspects of typical male-like song (Fusani 1999; Fusani et al. 2003). Therefore, the presence of AR and ER in HVC in adult females (Gahr & Metzdorf 1997; Fusani et al. 2003; this study) represents an important requirement for the ability to respond to exogenous testosterone. In adult female zebra finches the expression of AR and ER in HVC is greatly reduced compared to males or even absent (Gahr 1996; Kim et al. 2004) but the early oestrogen treatment enhances this expression (Gahr 1996; Gahr & Metzdorf 1999) and thus provides the basis for activation by testosterone in adulthood.

8.4.2. Repertoire and structure of female song

Female white-browed sparrow weavers started to produce solo song within 3 or 4 days following T-implantation. Because they were kept without conspecifics, these females must have acquired their auditory template at earlier times and testosterone now induced motor learning. Alternatively, both sensory and sensorimotor learning have taken place long before the experiment. The age composition of the group of females (*females No. 1 + 2* one year old, *female No. 3* at least four years old) revealed two findings. Females acquire an auditory template of solo song syllables already during their first year of life and they retain this repertoire throughout adulthood. Repertoire sizes of females were not significantly different from those of males. It is therefore most likely that young females learn similar amounts of song and during the same time window as young males and probably possess similar sized total repertoires when about one year old. This is especially interesting when having in mind that sexes differ significantly in the size of HVC (see chapter 5). However, it is likely that in females the neural substrate for auditory memory is not HVC but other structures such as the caudomedial part of the neostriatum (NCM) or of the hyperstriatum ventrale (CMHV, Bolhuis & Eda-Fujiwara 2003). From the present experiment, it can also be concluded that the overt duet repertoire recorded in adult females in Zimbabwe represents only a part of what the birds actually have in memory. It would be interesting to study T-induced singing in young, subordinate males to confirm that large parts of the solo song are learned early during development and to reveal the repertoire beyond the overt duet

repertoire produced at this time. Female starlings under the influence of testosterone produce similar repertoires of warbles and whistles as males and even under such conditions females produce often not all syllable types they actually memorise (Hausberger et al. 1995).

The recordings of female white-browed sparrow weavers nevertheless revealed a sex difference in the composition of solo song. Females sang fewer syllable types, which had repetitions of three or more times. Also, female T-treated canaries, although singing fully functional male-like songs, lack the bipartite syllable types typically found in males (Vallet et al. 1996). Similar results were reported from female starlings where the song lacks clicks and high-pitched trills typical of male song (Hausberger et al. 1995). An explanation at least for white-browed sparrow weavers could be that females, and probably males too, learn during their first year of life a “simple” version of solo song, which males improve later on when reaching a dominant position and females do not. Alternatively, females simply lack the capacity to learn more complex syllable types. However, females do not have a reduced capacity of motor control because syllable repetition rate was not different between sexes.

8.4.3. Song activity in relation to circulating hormone levels

The present study reveals the important result that one type of song produced by white-browed sparrow weavers, namely the solo song, which is restricted to the breeding season, depends on circulating steroid hormone levels. In contrast, the other type of song in this species, the duet song, which occurs throughout the year, is produced independently of actual hormone levels (chapter 7). When comparing with the song behaviour of other species this result comes not as a surprise. Seasonal breeding birds often retain high song activity also during the non-breeding season, when plasma T levels are basal (Schwabl & Kriner 1991; Smith et al. 1997; Leitner et al. 2001b). Furthermore, male starlings produce different types of song during the breeding season, which are context-dependent. Only the courtship song performed in the presence of females is activated by testosterone (Pinxten et al. 2002). However, tropical birds, including the present species, have circulating steroid hormone levels during the breeding season, which are an order of magnitude lower compared with temperate zone-birds. The plasma levels of T, DHT and E2 measured in dominant male white-browed sparrow weavers provided no evidence for steroid hormone-dependent song production. Furthermore, dominant males with detectable plasma T levels were not more likely to produce solo song than those males with undetectable T levels (section 7.3.1). The song-hormone profile found in the present study on captive T-treated females could possibly explain the lack of a direct relationship

between solo song production and circulating steroid hormone levels in males. The female pattern suggests that the induction of solo singing requires a single surge in circulating testosterone and afterwards the behaviour is performed for a certain time despite baseline plasma levels. If this is also true for males, then blood sampling during the breeding season will mostly reveal low steroid levels, because the peak might occur some time before breeding starts. Also, a single peak is likely to be missed if no sequential blood sampling is conducted. However, in males sampled in 2001 I might have hit such a period of elevated plasma T, because males had significantly higher T levels compared to 2000 and such males produced no solo song.

The experiment does not reveal the actual threshold levels of circulating hormones because of the sudden increase and decline of plasma levels, which was due to the type of implant used. However, it showed that the maintenance of the behaviour does not necessarily depend on high circulating steroid hormones. This is an important result for studies on hormone-behaviour relationships in tropical birds, which often do not fit the pattern seen in temperate zone species.

9. GENERAL DISCUSSION

The present study was undertaken to investigate the neural basis of male and female song behaviour in white-browed sparrow weavers. The existence of a behavioural polymorphism makes this species an especially interesting candidate for comparative studies. Whereas all group members engage in duet and chorus songs, only the dominant male produces the solo song. For the comparison of the song control system I aimed to go beyond the most often used cytoarchitectural approach by studying functionally defined neuron subpopulations such as cells expressing steroid hormone receptors. I further extended this cytochemical approach by introducing a new class of markers, synaptic proteins, which allow investigating synaptic plasticity within the song system in response to hormone-behaviour interactions.

Adult song production in this species is clearly sexually dimorphic when making comparisons among dominant individuals (chapter 4). This result, however, is not obtained when comparing dominant females with subordinate males and probably not when comparing male and female subordinates. Subordinate females were not subject of this study but personal observations revealed that they participate in chorus songs similar to all other colony members. Such a pattern emerges because there exist two adult male phenotypes, dominants and subordinates, which differ in the production of solo song. This behavioural polymorphism was found to be reflected in the structure of the song control system at all levels of analysis.

Concerning the gross morphology of song nuclei, in particular of HVC and RA, the degree of sexually dimorphic song behaviour (male to female ratio of total syllable repertoire) among dominant individuals matches well the degree of sex differences in the size of HVC and to a lesser extent that of RA (chapter 5). These data fit the hypothesis of (Brenowitz 1997) and (Schlinger & Brenowitz 2002) stating that “the degree to which the sexes of any species differ in the size of the song nuclei corresponds closely with the extent to which they differ in the complexity of song behavior” and “in those species in which females are able to sing, however, the brains of males and females have the same network of song nuclei”. Their hypothesis is supported by data from zebra finches, canaries and four duetting species. In this respect, white-browed sparrow weavers resemble approximately two other duetting species, the white-browed robin chat (*Cossypha heuglini*) and the rufous-and-white wren (*Thryothorus rufalbus*) with higher song repertoires in males, where the sizes of RA and HVC vary between sexes approximately 1.7 to 2.2 fold (Table 9.1).

However, the present study further shows first, that subordinate males do not fit this pattern (Table 9.1) and second, that females can produce under the

influence of steroid hormones a similar solo song as dominant males (chapter 8). Both findings question the functional significance of the proposed brain-behaviour relationship. Moreover, females have not just a 'smaller version' of the same song system network as males as the more detailed analysis at the cytoarchitectural and the cytochemical level revealed (chapters 5, 6.1, 6.3). Female HVC differs from HVC of dominant males despite having a smaller volume in a number of features such as having a higher overall cell density, higher ER expression level, different spatial distribution of AR expressing cells and different regulation and expression level of synaptic proteins. Interestingly, the cytochemical sex differences disappear when comparing female HVC with HVC of subordinate males.

Table 9.1: Male-to-female ratios for relative song repertoire sizes and volumes of song nuclei HVC and RA[1]

	Song repertoire	HVC volume	RA volume
Zebra finch	Male only	5.01	5.53
Canary	Male >>> Female	4.28	2.88
White-browed sparrow weaver[2]	**Male >> Female**	**2.96**	**1.97**
White-browed robin chat	Male >> Female	2.18	1.67
Rufous-and-white wren	Male > Female	2.16	1.70
White-browed sparrow weaver[3]	**Male = Female**	**2.00**	**1.44**
Bay wren	Male = Female	1.50	1.10
Buff-breasted wren	Male = Female	1.28	1.49

[1] Table according to Brenowitz (1997) with the data of the present study included.

[2] Comparisons among dominant individuals

[3] Comparisons among dominant females and subordinate males

When relating the similar production of duet / chorus song among all three groups of birds to their neural characteristics of HVC no common feature emerges and it is striking that HVC volume and total cell number are different in all groups, which only means that above a minimum size a further increase in size and neuron number is not functionally related to the production of this type of song. Such an increase could nevertheless be functional for the production of solo song.

The existence of two different male phenotypes in white-browed sparrow weavers allowed to identify features of the song control system, such as overall cell density in HVC and the spatial distribution of androgen receptor (AR) expressing cells in HVC, that were either 'organised' in a sex-specific way

during early life or genetically determined, because these properties were similar in all males but different from females. On the other hand, differences between males of different phenotypes revealed features that are most likely subject to 'activation' later in life, such as volume and cell number of song nuclei and expression level and regulatory mechanisms of AR and synaptic proteins in HVC and surrounding tissue (chapters 5, 6.1, 6.3).

It is conceivable that such activation could be triggered by a change in social status from the subordinate to the dominant position. Thereby the reproductive system is stimulated, which results in an increase in gonad size and in the production of short peaks of testosterone. Such peaks in circulating hormone levels would be sufficient to *activate* appropriate brain structures, which are restructured in the way as mentioned above and facilitate the production of solo song.

The original organisational-activational hypothesis, predicting the sexually dimorphic differentiation of brain structures and behaviour, however, states that in adulthood gonadal hormones activate differentiated brain structures to mediate behaviour and that these effects are reversible (Phoenix et al. 1959). And the 'relative plasticity hypothesis', which was proposed to fit the original concept to polymorphic species, proposes that an activational effect is reflected in different hormone profiles between adult individuals of different phenotypes (Moore 1991). The data of the present study are not perfectly conform to both statements. First, although there is evidence from other studies (Nottebohm 1980; Fusani et al. 2003) and my own (chapter 6.2) that at least some of the neural features, which differ between both groups of males, are sensitive to steroid hormones and although the production of solo song was shown to be testosterone-sensitive in females (chapter 8), the hormone profiles of dominant and subordinate males were not different (chapter 7). However, because tropical birds have generally lower circulating testosterone levels than temperate species (for review, see Goymann et al. 2004), the brain may be sensitive to more subtle differences or alternatively, activation by testosterone occurs in short peaks, which are likely to be missed in measurements. Second, there are at present no data, which would support a reversion of males from the dominant to the subordinate position and similarly, males retain the ability to produce solo song throughout life. Together with the pronounced restructuring of brain areas within the song system this would be indicative of an organisational instead of activational action of gonadal hormones in adulthood. On the other hand, assuming that short testosterone peaks persist throughout life in adult dominant males, thereby keeping the responsible brain structures in an activational state would still be consistent with the original concept of brain-behaviour differentiation.

Perspectives for future studies

The present study has further contributed to understand the neural mechanisms of sex differences in song production. Studying the control of female song behaviour has been largely ignored during the last years although this approach has the potential to uncover how male and female brains differ in the mechanisms mediating specific behaviours, which is of interest to several fields of research. The existence of behavioural and morphological polymorphism in the study species has challenged the traditional view about the sexual differentiation of brain and behaviour in vertebrates and requires further investigation. It seems likely from the present data that an 'activational' action of gonadal hormones accounts for the induction of the dominant male phenotype in adulthood but experiments involving castration of dominant males and hormone treatment of subordinates would be necessary to proof this. Furthermore, extending the current approach to hormonal manipulations of males and females during development allows unravelling the action of genetic and epigenetic factors during sexual differentiation.

Specifically for the study of birdsong, the results obtained from white-browed sparrow weavers raise interesting questions regarding the hormonal control of song production in tropical birds. Which circulating steroid hormone levels are actually necessary to maintain song behaviour? Do other hormones such as dehydroepiandrosterone (DHEA) or do steroid hormones synthesised by the brain itself play a more important role? Another intriguing aspect represents the mechanism by which the song nuclei of subordinates possibly grow. Are neurogenesis and neuronal recruitment involved? Similarly, is the testosterone-induced song production in females accompanied by profound restructuring of the song system as it is the case in males? Finally, the introduction of synaptic proteins as new cytochemical markers broadens further the possibilities to study the plasticity of the song system.

10. SUMMARY

Birdsong represents an attractive model for studying the neural mechanisms that cause sexually dimorphic behaviour patterns in vertebrates. In the current view of brain-behaviour differentiation of songbirds, it is thought that the degree of sex differences in the size of the song control nuclei in the brain correlates with the degree of sex differences in song complexity. The aim of the present study was to investigate the neural basis of male and female song behaviour in duetting white-browed sparrow weavers (*Plocepasser mahali*). This species, a cooperative breeder of eastern and southern Africa, which lives in groups of up to 10 individuals, exhibits polymorphism in terms of song behaviour. Two main types of song can be distinguished, duet (when produced by more than two birds called chorus) and solo song. Whereas all male and female group members produce duet and chorus songs, the solo song is restricted to the dominant male of the group. Such system allows making inter- and intrasexual comparisons of brain structure and behaviour.

Animals and study area
The study was conducted in Zimbabwe in two consecutive years during the breeding season of the birds. Data were collected from fourteen dominant females, fourteen dominant males and eight subordinate males. To complement the data on song behaviour obtained in the field some individuals were transferred to Germany and studied in detail in captivity. Further, captive pairs reproduced successfully and their offspring was used to investigate the development of duetting.

Song behaviour
Per single performance of solo song, dominant males in Zimbabwe have repertoires similar in size to males recorded in captivity, ranging from 48 to 92 different syllable types. A single performance represents on average 88 % of a male's total solo song repertoire. Captive males sing, however, longer solo songs than males in Zimbabwe. Duet repertoires of pairs recorded in Zimbabwe range from 45 to 61 different syllable types. Analysis of captive pairs reveals that both sexes have duet repertoires of similar size and share about 95 % of syllable types. Per individual duet about 60 % of the syllables are produced in unison. Duet syllables are arranged in phrases and certain whistle-like syllable types always precede certain phrase types. From data on captive birds, I estimated total repertoire sizes of birds recorded in Zimbabwe. The total repertoire of dominant males comprises on average 128 and that of dominant females 50 different syllable types. For subordinate males, although no

quantitative data are available, I conclude from observations in the field and in captivity that duet repertoires are of similar size as those of dominant individuals.

Song system cytoarchitecture

Intersexual comparison among dominant individuals reveals three, respectively two times larger volumes of song control nuclei HVC and RA in males than in females. Cell density in HVC is higher in females compared to males, whereas no sex difference is found in cell density of the surrounding tissue. Further, no sex differences exist in telencephalon size and syrinx weight. Total number of HVC cells is about 2.6 times higher in males than in females. Comparison among subordinate males and dominant females reveals two, respectively 1.4 times larger volumes of song nuclei HVC and RA in males than in females. Intrasexual comparison among males reveals smaller testes, smaller volumes of HVC and RA, lower total number of HVC cells and lower cell density in the surrounding neostriatum in subordinate males compared to dominant males. No differences between males are found regarding body size, telencephalon size, syrinx weight and HVC cell density.

Song system cytochemistry

Expression of AR and ER mRNA

The distribution of androgen receptor (AR) and oestrogen receptor (ER) mRNA in the song system is monomorphic. AR is found in forebrain song nuclei HVC, RA, MAN and in brainstem nuclei nXIIts and Ram. ER expression is restricted to medial HVC. ParaHVC contains expression of both, AR and ER mRNA. Intersexual comparison reveals larger volume of AR-HVC in dominant males than in dominant females. Also, both sexes differ in the spatial distribution of AR expressing HVC cells. No sex differences exist in AR expression levels in HVC and surrounding, whereas ER expression in medial HVC is higher in females than in males. Intrasexual comparison among males reveals larger volume of AR-HVC in dominant than in subordinate males. The distribution of AR expressing HVC cells is similar among males. Subordinates have higher AR expression level in HVC and surrounding.

Synaptic proteins as novel markers

The expression pattern of synaptic proteins SNAP-25 (synaptosomal-associated protein 25 kDa) and SPO (synaptoporin) and their steroid hormone sensitivity are studied in the song control system of the male zebra finch. SNAP-25 mRNA is expressed at high intensity throughout the telencephalon and is enriched in

song nuclei HVC and RA. SPO mRNA is expressed at moderate intensity in HVC and is low or absent in song nuclei RA, lMAN and Area X. Following treatment with the aromatase inhibitor Fadrozole, which blocks the conversion of androgen to oestrogen, SPO expression in HVC is increased, whereas SNAP-25 expression is not affected. Fadrozole-treatment also increases cell density in HVC and surrounding and decreases both, HVC and telencephalon volume.

Expression of SNAP-25, SPO and STX mRNA

The distribution and expression pattern of three synaptic proteins, SNAP-25, SPO and STX (syntaxin) are studied in male and female white-browed sparrow weavers. SNAP-25 and STX are expressed at moderate to high intensity throughout the brain and are enriched in song nuclei HVC, RA and mMAN. SPO expression in HVC is moderate and low or absent in other areas of the song system. Intersexual comparison among dominant individuals reveals higher expression level of SNAP-25 and STX in HVC of females compared to males, whereas no sex difference is found in SPO expression. The sex difference in SNAP-25 expression can be solely attributed to an influence of overall HVC cell density. In the surrounding neostriatum the expression level of all three proteins are not sexually dimorphic. Intrasexual comparison among males reveals higher expression level of SNAP-25, SPO and STX in HVC of subordinates compared to dominants. In the neostriatum, SPO expression is increased in subordinates. Intersexual comparison of subordinate males and dominant females reveals no sex differences in the expression level of synaptic proteins in HVC, but increased SPO expression in the neostriatum of males.

Steroid hormone levels

Plasma levels of the steroid hormones testosterone (T), 5α-dihydrotestosterone (DHT) and 17ß-oestradiol (E2) are investigated in male and female white-browed sparrow weavers. Intersexual comparison among dominant individuals reveals higher T levels in males compared to females but no sex difference in DHT and E2 levels. Intrasexual comparison among males reveals no sex differences in plasma levels of steroid hormones. T levels are neither correlated with testis size nor with the AR expression level in song nucleus HVC and are not associated with the performance of solo song among dominant males. Intersexual comparison of subordinate males and dominant females reveals higher levels of T, but not of DHT and E2, in males compared to females. Steroid hormone levels of males from closely related species are of similar magnitude than those of male white-browed sparrow weavers.

Testosterone-induced solo song in females

A preliminary experiment with three female white-browed sparrow weavers was conducted to study the effect of exogenous T on the production of male-like solo song. All females produced solo song in response to the implant. Song activity was similar to that in dominant males recorded in Zimbabwe and lasted for about three weeks. Females had solo song repertoires similar to males, ranging from 48 to 57 syllable types. Females sang, however, fewer syllable types with high repetitions than males. Steroid hormone levels peaked two days after implantation and returned to baseline levels by the third week post-implantation. Song activity started when hormone levels were declining and lasted up to seventeen days despite baseline levels of steroid hormones.

Conclusion

Intersexual comparisons of the song and song system cytoarchitecture in white-browed sparrow weavers reveal different relationships depending on which male phenotype, dominant or subordinate, is used and thus question the initially proposed hypothesis relating sex differences in song behaviour to sex differences in the size of the song system. The cytochemical approach seems to reflect the differences in song behaviour more closely. Comparing dominant individuals reveals profound sex differences in HVC structure, which go far beyond differences in song nucleus volume. Such sex differences are absent when comparing females and subordinate males that are similar in song production.

Intrasexual comparisons among males allow to distinguish between neural features that are likely to be permanently ‘organised’ during development and those that are reversible and ‘activated’ in adulthood by the action of gonadal hormones. The data obtained in the present study are, however, not fully conform to the original organisational-activational hypothesis that predicts the sexually dimorphic differentiation of brain and behaviour in vertebrates. The similar plasma levels of gonadal hormones in dominant and subordinate males do not support an ‘activational’ action except if the brain is sensitive to subtle changes in hormone levels or if activation occurs in short peaks of hormone secretion. Alternatively, the changes in the song control system of dominant males and the production of solo song are permanent features, resulting from an ‘organisational’ action of gonadal hormones in adulthood.

11. ZUSAMMENFASSUNG

Der Gesang der Singvögel stellt ein attraktives Modell dar, um neurale Mechanismen zu untersuchen, die für das Auftreten von Geschlechtsunterschieden in bestimmten Verhaltensweisen verantwortlich sind. Die gegenwärtige Vorstellung vom Zusammenhang zwischen Gehirn- und Verhaltensdifferenzierung im Singvogel geht davon aus, dass das Ausmaß der Geschlechtsunterschiede in der Größe der gesangskontrollierenden Gehirnzentren mit dem Ausmaß der Geschlechtsunterschiede im Gesangsverhalten korreliert. Die vorliegende Arbeit wurde durchgeführt, um die neuralen Grundlagen des Gesangsverhaltens von Männchen und Weibchen des duettierenden Mahaliwebers (*Plocepasser mahali*) zu untersuchen. Dieser Vogel, eine kooperativ brütende Art, kommt im Osten und Süden Afrikas vor, lebt in Gruppen mit bis zu 10 Individuen und besitzt einen ausgeprägten Polymorphismus in Bezug auf das Gesangsverhalten. Es können zwei Gesangstypen unterschieden werden, Duettgesang (genannt Chorusgesang, falls von mehr als zwei Individuen produziert) und Sologesang. Während alle Gruppenmitglieder Duett- und Chorusgesang singen können, wird der Sologesang nur vom dominanten Männchen einer Gruppe vorgetragen. Dieses System ermöglicht, inter- und intrasexuelle Vergleiche von Gehirnstruktur und Verhalten durchzuführen.

Tiere und Untersuchungsgebiet
Die Arbeit wurde in Simbabwe in zwei aufeinanderfolgenden Jahren während der Brutzeit der Vögel durchgeführt. Daten wurden von 14 dominanten Weibchen, 14 dominanten Männchen und 8 subdominanten Männchen erhoben. Um die Gesangsdaten aus dem Freiland zu vervollständigen, wurden Vögel nach Deutschland gebracht und dort detailliert in Gefangenschaft untersucht. So gehaltene Paare pflanzten sich erfolgreich fort und ihre Nachkommen konnten für Untersuchungen zur Entwicklung des Duettgesangs herangezogen werden.

Gesangsverhalten
Dominante Männchen in Simbabwe, genauso wie Männchen in Gefangenschaft, singen in einem einzigen Vortrag des Sologesangs zwischen 48 und 92 verschiedene Silbentypen. Ein Vortrag eines Männchens stellt im Durchschnitt 88 % seines gesamten Repertoires an Sologesangs-Silben dar. Männchen in Gefangenschaft unterscheiden sich jedoch von denen im Freiland durch das Singen längerer Sologesänge. Repertoires des Duettgesangs, aufgenommen von Paaren in Simbabwe, bestehen aus 45 bis 61 verschiedenen Silbentypen. Die detaillierte Analyse des Duettgesangs an Paaren in Gefangenschaft ergab, dass

beide Geschlechter gleichgroße Repertoires an Duett-Silben besitzen und dass 95 % der Silben bei Männchen und Weibchen eines Paares identisch sind in. In einem Duett werden ca. 60 % der Silben unisono, d.h. gleichzeitig von beiden Paarpartnern gesungen. Silben des Duettgesangs sind in Phrasen angeordnet und bestimmte Pfiff-ähnliche Silbentypen treten immer vor bestimmten Phrasentypen auf. Generell sind die Silben des Duettgesangs von denen des Sologesangs strukturell verschieden. Die Daten aus den Gefangenschaftsuntersuchungen ermöglichen die Abschätzung des tatsächlichen Gesamtrepertoires an Silbentypen der in Simbabwe aufgenommenen Individuen. Demnach besitzen dominante Männchen ein Repertoire von durchschnittlich 128 verschiedenen Silbentypen und dominante Weibchen eines von etwa 50 verschiedenen Silbentypen. Von subdominanten Männchen sind keine quantitativen Gesangsdaten vorhanden, aber aus Beobachtungen im Freiland und in Gefangenschaft schließe ich, dass ihr Duettrepertoire genauso groß ist wie das dominanter Tiere.

Cytoarchitektur der gesangskontrollierenden Gehirnzentren im Vorderhirn

Ein Vergleich der Größe der Gesangszentren zwischen dominanten Männchen und Weibchen zeigt, dass die Gesangsregionen HVC und RA in Männchen dreimal, beziehungsweise zweimal so groß sind wie im Weibchen. Dagegen haben die HVC's der Weibchen eine höhere Zelldichte als die der Männchen. In dem den HVC umgebenden Gewebe unterscheiden sich beide Geschlechter nicht in Bezug auf die Zelldichte. Weiterhin gibt es keinen Geschlechtsunterschied in der Größe des Vorderhirns und in der Größe des gesangsproduzierenden Organs, der Syrinx. Die Gesamtzahl an HVC-Zellen ist ungefähr 2,6 mal größer im Männchen als im Weibchen. Vergleicht man subdominante Männchen mit dominanten Weibchen zeigt sich, dass die Gesangszentren HVC und RA zweimal, beziehungsweise 1,4 mal größer sind im Männchen als im Weibchen. Ein intrasexueller Vergleich innerhalb der Männchen zeigt, dass subdominante Männchen durch kleinere Gonaden, geringere Größe von HVC und RA, niedrigere Gesamtzellzahl im HVC und geringere Zelldichte im umgebenden Gewebe gekennzeichnet sind im Vergleich zu dominanten Männchen. Dagegen unterscheiden sich beide Gruppen von Männchen nicht in der Körpergröße, der Größe des Vorderhirns, der Syrinxgröße und der Zelldichte im HVC.

Cytochemie der gesangskontrollierenden Gehirnzentren

Expression der mRNA des Androgenrezeptors (AR) und Östrogenrezeptors (ER)
Es existieren keine Geschlechtsunterschiede in der Verteilung der AR und ER mRNA in den gesangskontrollierenden Gehirnzentren. Der Androgenrezeptor wird in den Vorderhirn-Gebieten HVC, RA, MAN sowie in den Hirnstamm-Regionen nXIIts und Ram exprimiert. Die Expression des Östrogenrezeptors ist begrenzt auf den medialen Teil des HVC. Im sogenannten paraHVC, der sich caudomedial an den HVC anschliesst, findet man sowohl den Androgen- als auch den Östrogenrezeptor. Ein Vergleich zwischen dominanten Männchen und Weibchen zeigt, dass das Volumen des HVC, ermittelt anhand der Verteilung der Androgenrezeptoren, im Männchen größer ist als im Weibchen. Ebenso unterscheiden sich beide Geschlechter in der räumlichen Verteilung der Androgenrezeptor-exprimierenden Zellen. Jedoch besteht kein Geschlechtsunterschied in der Dichte der Androgenrezeptoren im HVC und dem umgebenden Gewebe. Dagegen ist im Weibchen die Dichte der Östrogenrezeptoren im HVC höher als im Männchen. Ein intrasexueller Vergleich innerhalb der Männchen zeigt, dass das Volumen des HVC in subdominanten Männchen kleiner ist als in dominanten. Die räumliche Verteilung der Androgenrezeptor-exprimierenden Zellen ist gleich in beiden Gruppen. Die Dichte der Androgenrezetoren im HVC und in der Umgebung ist jedoch höher im subdominanten Männchen.

Synaptische Proteine als neue cytochemische Marker des Gesangssystems
Die Expressionsmuster der mRNA der Synapsenproteine SNAP-25 (Synaptosomal-associated protein 25 kDa) und SPO (Synaptoporin) sowie deren Sensitivität gegenüber Steroidhormonen wurde in einem Experiment im Gesangssystem des männlichen Zebrafinken untersucht. SNAP-25 wird mit hoher Intensität im gesamten Vorderhirn exprimiert und ist besonders in den Gesangszentren HVC und RA angereichert. SPO ist dagegen hauptsächlich im HVC zu finden. Die Behandlung der Tiere mit dem Inhibitor Fadrozole, der das östrogen-produzierende Enzym Aromatase blockiert, führte zu einem Anstieg der Expression von SPO aber nicht von SNAP-25 im HVC verglichen mit der Kontrollgruppe. Die Behandlung führte ebenfalls zu einer Erhöhung der Zelldichte im HVC und dem umgebenden Gewebe und zu einer Verkleinerung des HVC's und des gesamten Vorderhirns.

Expression der mRNA der synaptischen Proteine SNAP-25, SPO and STX
Die zuvor im Zebrafinken charakterisierten Synapsenproteine SNAP-25 und SPO wurden nun im Mahaliweber untersucht und durch ein weiteres

synaptisches Protein STX (Syntaxin) ergänzt. SNAP-25 und STX werden im gesamten Gehirn mit starker Intensität exprimiert und sind besonders angereichert in den Gesangszentren HVC, RA und MAN. SPO wird hauptsächlich im HVC exprimiert und fehlt in den meisten anderen Gesangsregionen. Ein Vergleich zwischen dominanten Männchen und Weibchen ergibt eine höhere Expressionsdichte von SNAP-25 und STX im HVC von Weibchen, wohingegen es keinen Geschlechtsunterschied in der Expression von SPO gibt. Die höhere SNAP-25-Expression im HVC der Weibchen resultiert allerdings aus einem Einfluss der Zelldichte. Im umgebenden Gewebe existiert kein Geschlechtsunterschied im Expressionsmuster der drei Proteine. Ein intrasexueller Vergleich innerhalb der Männchen zeigt, dass im HVC von subdominanten Männchen die Expressionsdichte aller drei Proteine höher ist als im HVC von dominanten. Im umgebenden Gewebe dieser Männchen ist lediglich die Expression von SPO erhöht. Vergleicht man subdominante Männchen mit dominanten Weibchen, findet man keine Geschlechtsunterschiede im HVC bezüglich der Expression der drei Synapsenproteine.

Konzentration von Steroidhormonen im Blutplasma

In Männchen und Weibchen des Mahaliwebers wurden die Blutplasmawerte der Steroidhormone Testosteron, 5α-Dihydrotestosteron und 17ß-Östradiol untersucht. Dominante Männchen unterscheiden sich von dominanten Weibchen durch eine höhere Konzentration an Testosteron im Blutplasma. Die Konzentrationen von Dihydrotestosteron und Östradiol sind basal und weisen keine Geschlechtsunterschiede auf. Ein intrasexueller Vergleich der Männchen zeigt keine Unterschiede in der Steroidhormonkonzentration zwischen dominanten und subdominanten Männchen. Die Blutplasmawerte von Testosteron korrelieren weder mit der Gonadengröße noch mit der Expressionsdichte der Androgenrezeptoren im HVC und sind nicht mit dem Singen des Sologesangs in dominanten Männchen verknüpft. Vergleicht man subdominante Männchen mit dominanten Weibchen ergibt sich ebenfalls eine höhere Konzentration von Testosteron im Blutplasma der Männchen. Die Blutplasmawerte der Steroidhormone von Männchen nahe verwandter Arten sind in derselben Größenordnung wie die der männlichen Mahaliweber.

Testosteron-induzierter Sologesang in Weibchen

In einem vorläufigen Experiment mit drei Mahaliweber-Weibchen wurde untersucht, ob die Behandlung mit Testosteron die Produktion des Männchen-typischen Sologesangs induzieren kann. Alle Weibchen beginnen durch die Testosteron-Behandlung mit dem Singen des Sologesangs. Die Gesangsaktivität

hält etwa drei Wochen an und ist in der Zeit identisch mit der dominanter Männchen im Freiland. Das Silbenrepertoire des Sologesangs umfasst 48 bis 57 verschiedene Silbentypen und hat damit dieselbe Größe wie das von Männchen. Weibchen singen jedoch weniger Silbentypen, die häufig wiederholt werden. Durch die Testosteron-Behandlung erreichen die Blutplasmawerte der Steroidhormone ihre Höchstwerte zwei Tage nach dem Beginn der Behandlung und fallen bis zur dritten Woche wieder auf die basalen Ausgangswerte zurück. Die Gesangsaktivität steigt an, wenn die Blutplasmawerte bereits wieder abnehmen und sie dauert trotz basaler Hormonkonzentration etwa 17 Tage an.

Schlußfolgerung

Intersexuelle Vergleiche des Gesangs und der Cytoarchitektur des Gesangssystems im Mahaliweber zeigen verschiedenartige Beziehungen auf, je nachdem welcher männliche Phänotyp, dominant oder subdominant, herangezogen wird. Dieses Ergebnis stellt daher die ursprünglich vorgeschlagene Hypothese, die eine lineare Beziehung zwischen den Geschlechtsunterschieden im Gesang und in der Größe der Gesangszentren beschreibt, in Frage. Der cytochemische Versuchsansatz dagegen spiegelt die tatsächlichen Unterschiede im Gesangsverhalten exakter wider. Vergleicht man dominante Männchen und Weibchen miteinander, zeigen sich tiefgreifende Geschlechtsunterschiede in der Struktur der Gesangsregion HVC, die weit über Unterschiede in der Arealgröße hinausgehen. Solche Geschlechtsunterschiede findet man dagegen nicht, wenn man subdominante Männchen mit dominanten Weibchen vergleicht, die auch dieselbe Art von Gesang produzieren.

Intrasexuelle Vergleiche innerhalb der Männchen ermöglichen die Unterscheidung zwischen neuralen Charakteristika, die einerseits durch die Wirkung der Gonadenhormone in der frühen Entwicklung dauerhaft ‚organisiert' wurden und andererseits solchen, die reversibel sind und im adulten Tier ‚aktiviert' werden können. Die Daten der vorliegenden Studie stimmen jedoch nicht vollständig mit der originalen ‚Organisierungs-Aktivierungs'-Hypothese überein, die die sexualdimorphe Differenzierung von Gehirnstruktur und Verhalten in Vertebraten beschreibt. Die Vorstellung, dass eine aktivierende Wirkung der Gonadenhormone im Adultvogel verantwortlich ist für die Unterschiede in der Gehirnstruktur zwischen dominanten und subdominanten Männchen wird durch die identischen Blutplasmawerte der Steroidhormone in beiden Gruppen nicht unterstützt. Es sei denn, man nimmt an, dass das Gehirn bereits auf kleine Schwankungen im Hormonspiegel empfindlich reagiert oder dass eine Aktivierung durch eine kurzzeitige starke Hormonausschüttung erfolgt. Alternativ dazu ist es denkbar, dass die Änderung der Gehirnstruktur und die Produktion des Sologesangs in dominanten Männchen dauerhafte Eigenschaften sind, die aus einer ‚organisierenden' Hormonwirkung im Adulttier resultieren.

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13. APPENDIX

The standard nomenclature of the avian telencephalon and brainstem structures is based on flawed assumptions of homology to mammals (for review, see Reiner et al. 2004). Concerning the telencephalon of birds, it has been thought that it is a hypertrophied basal ganglia. It is now clear that most of the avian telencephalon is comparable to the mammalian neocortex, claustrum and pallial amygdala. Because the misnaming caused confusion among researchers working on other vertebrates, a recent effort was made to revise the avian brain nomenclature (Reiner et al. 2004).

Of the songbird brain regions named in the present study telencephalic regions are affected by the changes in terminology. I have listed below those regions by their old term and their new latin name.

Table A1. Old and new terminology of avian telencephalic brain regions

Old term	**New latin name**
Neostriatum	Nidopallium
NCM (Neostriatum caudomediale)	Nidopallium caudomediale
HVC (Nucleus (n.) hyperstriatalis ventrale, pars caudale)	HVC (formal name)
lMAN (lateral n. magnocellularis of the anterior neostriatum)	N. lateralis magnocellularis nidopallii anterioris
mMAN (medial n. magnocellularis of the anterior neostriatum)	N. medialis magnocellularis nidopallii anterioris
Nif (N. interfacialis)	N. interfacialis nidopallii
RA (N. robustus archistriatalis)	N. robustus arcopallii
LPO (Lobus parolfactorius)	Striatum mediale
TSM (Tractus septomesencephalicus)	Tractus septopallio-mesencephalicus

14. ACKNOWLEDGEMENTS

During my study, I received support from many people whom I would now like to thank. Prof. Benedikt Grothe and PD Dr. York Winter are kindly acknowledged for supervising this thesis. I thank Prof. Wolfgang Wickler and Prof. Manfred Gahr for providing the topic and for giving me the great opportunity to conduct this study in Africa, which I found to be a fascinating place. I am very grateful to Prof. Wolfgang Wickler for financial support during the most part of the study. He introduced me to this interesting bird species and to the phenomenon of duetting. I also appreciate very much his scientific support and his immense knowledge in the field of birdsong. Moreover, he supported other projects of mine besides this thesis and allowed me to continue them. He also encouraged me to broaden my scientific background at international meetings. I am particularly grateful to Prof. Manfred Gahr for his invaluable scientific support and for his confidence in me. Manfred transmitted to me the fascination of being a scientist and provided the necessary freedom for me to develop my own ideas. He introduced me to the study of neural mechanisms of animal behaviour and to the anatomy of the avian song system. The many hours of critical discussions with him were always a source of inspiration and motivation and compensated for periods of reduced input due to the distance between Seewiesen and Amsterdam.

In Zimbabwe many people helped to make this project feasible. I thank the Research Council of Zimbabwe for permission to conduct this study. I am grateful to Dr. Peter Mundy from the Department of National Parks and Wildlife Management whose help was essential for obtaining permits, finding suitable study sites and solving various logistical problems. At his office in Magnet House I enjoyed many conversations with him, which were often amusing (excluding the first one at Sinamatella Camp). I thank Ron Hartley from Falcon College for arranging study sites at nearby farms. Ron was of great help in every respect and it was always a pleasure to meet him and his family with all the birds and dogs and other animals around. He also kindly provided the rainfall data. The Mylnes and the Robinsons kindly allowed me to conduct the study on their properties. I very much appreciated their hospitality. The Mylnes also provided housing, transportation and email access and helped keeping away snakes and bees. Bruce Davison, Officer-in-charge of the Sengwa Wildlife Research Institute and his family welcomed us at Sengwa, gave access to the research station, and took us along in dense miombo and mopane woodland in search of sparrow weavers. Ken Seiler kindly provided mealworms. I thank Mrs. Audrey Msimanga from the Natural History Museum in Bulawayo for access to their specimen collection. I kindly acknowledge Air Zimbabwe for smooth transport of birds to Germany.

For the part of the study, which was conducted in Seewiesen, I received help from the following people. Reinhold Metzdorf kindly provided probes for

in situ hybridisation, introduced me to this method and to its problem-solving. Judith Kammerlander ran our histology lab and assisted in tissue preparation and image analysis. I thank Prof. Eberhard Gwinner for permission to work in the endocrinology lab in Andechs. Dr. Wolfgang Goymann and Ingrid Schwabl introduced me to the procedure of radioimmunoassay and were most helpful in data analysis. Dr. Wolfgang Goymann patiently helped in solving statistical problems. Roswitha Brighton, Rositta Hirner, Jenny Minshull, Gabriele Rusch und Gudrun Schindlbeck are acknowledged for always keeping the sparrow weavers in good shape. Heidrun Bamberg and Dr. Theo Weber assisted in figure preparation. I thank Werner Mohren and Karsten Müller for support regarding problems with computers and software. Our librarians Rose Klein and Christine Fathelbab fulfilled all my literature requests. Hannes Sagunsky solved every problem I encountered with my recording equipment. I appreciate very much the help and advice of Karl-Heinz Siebenrock from Vogelwarte Radolfzell on keeping sparrow weavers in captivity. Dr. Gerhard Fries is acknowledged for lending a field centrifuge. I thank Prof. Wolfgang Wickler, Dr. Leonida Fusani and Reinhold Metzdorf for comments on various chapters of this thesis. Prof. John Wingfield and Prof. Willem Ferguson gave valuable hints on the biology of white-browed sparrow weavers. Prof. Clive Catchpole commented on sound analysis and on the title of the thesis. During my stay in the Wickler department I enjoyed many discussions with the people meeting at the “Wickler Cafe”: Cornelia Bock, Gerdi Dietz, Dr. Rudi Diesel, Prof. Hans Fricke, Rositta Hirner, Dr. Hans-Ulrich Kleindienst, Barbara Knauer, Ute Lauterfeld, Dr. Stefan Leitner, Dr. Lucie Salwiczek, Dr. Uta Seibt and Prof. Wolfgang Wickler. During the years of my study, many people made working in Seewiesen enjoyable and I appreciated various scientific and non-scientific discussions with them. Those people are Dr. Ute Abraham, Dr. Ninon Ballerstädt, Dr. Redouan Bshary, Dr. Virginie Canoine, Angelika Denk, Dr. Heather Fugger, Dr. Leonida Fusani, Dr. Wolfgang Goymann, Silvia Kuhn, Susann Rössel, Dr. Alexander Scheuerlein, Dr. Sabine Tebbich and Dr. Lindy Weilgart. A special thanks to Karen Hissmann and Jürgen Schauer who always had an open ear for any kind of problem in good and in less favourable times. They made working at the weekends in Seewiesen endurable and gave the feeling of not being alone. I really enjoyed watching soccer games and catching Fritzi together in the old Wohnhaus.

I am very grateful to my family, who always promoted my interest in biology, encouraged me to pursue my plans and supported my studies in every respect. Finally, I wish to express my deepest thanks to Stefan. His support was essential for many aspects of my study and he helped to overcome all difficult stages of this thesis. Last of all, I want to mention Grisalho, Trixi and Jumbo for having been great companions.

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LIST OF PUBLICATIONS

Voigt, C., Metzdorf, R., & Gahr, M. 2004 Differential expression pattern and steroid hormone sensitivity of SNAP-25 and synaptoporin in the telencephalic song control nucleus HVC of the zebra finch. *J Comp Neurol* **475**, 83-94.

Leitner S., Voigt C., Metzdorf R., & Catchpole C. (in press) Immediate early gene (*Zenk, Arc*) expression in the auditory forebrain of female canaries varies in response to male song quality. *J Neurobiol*

Leitner, S., Voigt, C., Gahr, M., Dietzen, C., & Wink, M. (submitted) Phylogeography of Island Canary (*Serinus canaria*) populations. *J Ornithol*

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