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Associated and disassociated patterns in hormones, song, behavior and brain receptor expression between life-cycle stages in male black redstarts, *Phoenicurus ochruros*

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ABSTRACT

Testosterone has been suggested to be involved in the regulation of male territorial behavior. For example, seasonal peaks in testosterone typically coincide with periods of intense competition between males for territories and mating partners. However, some species also express territorial behavior outside a breeding context when testosterone levels are low and, thus, the degree to which testosterone facilitates territorial behavior in these species is not well understood. We studied territorial behavior and its neuroendocrine correlates in male black redstarts. Black redstarts defend territories in spring during the breeding period, but also in the fall outside a reproductive context when testosterone levels are low. In the present study we assessed if song output and structure remain stable across life-cycle stages. Furthermore, we assessed if brain anatomy may give insight into the role of testosterone in the regulation of territorial behavior in black redstarts. We found that males sang spontaneously at a high rate during the nonbreeding period when testosterone levels were low; however the trill-like components of spontaneously produced song contained less repetitive elements during nonbreeding than during breeding. This higher number of repetitive elements in trills did not, however, correlate with a larger song control nucleus HVC during breeding. However, males expressed more aromatase mRNA in the preoptic area a brain nucleus important for sexual and aggressive behavior - during breeding than during nonbreeding. In combination with our previous studies on black redstarts our results suggest that territorial behavior in this species only partly depends on sex steroids: spontaneous song output, seasonal variation in trills and non-vocal territorial behavior in response to a simulated territorial intruder seem to be independent of sex steroids. However, context-dependent song during breeding may be facilitated by testosterone – potentially by conversion of testosterone to estradiol in the preoptic area.

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1. Introduction

Most animals live in highly seasonal environments that vary, for example, in temperature and precipitation and consequently in the availability of resources such as food. To survive and maximize their reproductive success animals need to cope with these largely predictable changes and time their life-cycle accordingly. Hormones play a major role in the development and transition between life-cycle stages (e.g. Jacobs and Wingfield, 2000; Wingfield et al., 2001) and testosterone has been identified as an important player in regulating the breeding life-cycle stage of male vertebrates: it is required for spermatogenesis, the development of secondary sex characteristics and it facilitates sexual and territorial behaviors (Adkins-Regan, 2005; Nelson, 2005). Accordingly, testosterone levels are often highest at the beginning of the breeding season when interactions among males, song and sexual activities are most intense (Ball and Wingfield, 1987; Dawson, 1983; Morton et al., 1990; Silverin, 1993; Silverin et al., 1986; Van Duyse et al., 2003; Wingfield et al., 1990). However, the correlation between behaviors expressed in a territorial context and testosterone levels is not always that straight-forward: many temperate-zone songbird species, for example, defend territories and sing outside the breeding season when testosterone levels are low (Apfelbeck and Goymann, 2011; Canoine and Gwinner, 2002; Landys et al., 2010; Schwabl, 1992; Wingfield, 1994). Testosterone could still facilitate territoriality in these species: testosterone precursors may be derived from non-gonadal sources and metabolized to testosterone

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directly in the brain (e.g. Soma et al., 2000; Soma and Wingfield, 2001) or the brain may have a higher sensitivity for low levels of the hormone (Canoine et al., 2007). In some species, however, testosterone facilitates territorial behavior during the breeding period, but does not appear to facilitate territorial behavior during the nonbreeding season (Canoine and Gwinner, 2002; Hau and Beebe, 2011; Hau et al., 2000; Landys et al., 2010; Marasco et al., 2011; Schwabl and Kriner, 1991). In rufous-collared sparrows (Zonotrichia capensis) territorial behavior seems to be independent of testosterone even during breeding (Moore et al., 2004; Moore et al., 2004). In other species, that defend territories and sing both in- and outside the breeding season, these behaviors seem to differ in these two contexts (Moore, 1988). For example, during breeding song may contain more repetitive elements (DeWolfe et al., 1974; Leitner et al., 2001; Smith et al., 1997; Voigt and Leitner, 2008), longer songs (Riters et al., 2000) or song may be more stereotyped (Smith et al., 1997) than during nonbreeding. In the latter cases testosterone during breeding may activate these changes in territorial behaviors and song. In song sparrows (Melospiza melodia), for example, seasonal changes in song have been correlated with the size of the HVC, a brain nucleus of the song control system in songbirds (Nottebohm et al., 1976) that is considered to control motor output during singing (Brenowitz et al., 1997; Yu and Margoliash, 1996). The HVC is sensitive to androgens (Gahr and Metzdorf, 1997) and its size is thought to depend at least partly on circulating testosterone levels (Nottebohm, 1980; Sartor et al., 2005). Furthermore, the sensitivity to testosterone may change within the HVC depending on season (Gahr and Metzdorf, 1997; Soma et al., 1999). It has been argued that a larger HVC during breeding is related to a larger song repertoire, a higher song rate and facilitation of a more complex song in song sparrows (Brenowitz, 1997; Smith et al., 1997, but see (Gahr, 1997). In other species such as canaries (Serinus canaries) and black-capped chickadees (Poecile atricapillus), however, seasonal changes in song are not related to HVC size (Fusani et al., 2000; Smulders et al., 2006). Furthermore, testosterone may influence the motivation to sing and the song rate in a reproductive context by activating song areas outside the song control system, e.g. by aromatization of testosterone to estrogens in the preoptic area (Foidart et al., 1998; Riters et al., 2000; Soma et al., 2003). The preoptic area has been shown to play an important role in the regulation of estrogen-dependent aggressive behavior (Schlinger and Callard, 1990; Silverin et al., 2004).

Hence, although it is well accepted that testosterone plays a role in the organization and activation of song (Bolhuis and Gahr, 2006) and territorial behavior (Wingfield et al., 2006) in the breeding season, it is still unclear to what extent testosterone facilitates these behaviors in species that sing and defend territories outside the breeding season.

The black redstart (Phoenicurus ochruros) is a temperate-zone song bird species that defends a territory and sings during the breeding season in spring and also during nonbreeding in fall. Black redstarts are socially monogamous and both females and males provide parental care (Draganoiu et al., 2005; Landmann, 1996). Males defend a territory and sing during the breeding season in spring and early summer. During late summer (mid-August–mid-September) they molt and show a decrease in singing activity. Afterwards they express a pronounced period of fall territoriality and song activity until the end of October just before they start migration (Nicolai, 2005; Weggler, 2000).

To investigate whether testosterone may facilitate song and territorial behavior in different life-cycle stages, we compared testosterone (obtained via blood samples), territorial behavior, spontaneous song, and the distribution of hormone receptors in the brain of male black redstarts during breeding and nonbreeding territoriality. We focused on brain areas relevant for singing (forebrain song control nuclei) and aggressive behavior (diencephalon). We describe the expression pattern of androgen receptor-, estrogen receptorand aromatase mRNA of male black redstarts during breeding and nonbreeding territoriality and determine whether HVC size and aromatase expression in the preoptic area differ between life-cycle stages and correlate with testosterone levels, territorial behavior and spontaneous song. If song output and structure differ with life-cycle stage, we expected males to have a larger HVC during breeding than outside the breeding season (e.g. Smith et al., 1997). As aromatase expression in the preoptic area has been shown to play an important role in the expression of reproductive behaviors (Balthazart et al., 2010), we expected a higher expression of aromatase mRNA in that area during breeding compared to the nonbreeding fall territorial phase.

We compared song output and song structure between breeding and nonbreeding territoriality to test if they differ between life-cycle stages. If testosterone activates song during the breeding season we expected that males produce more spontaneous song during breeding in spring than during nonbreeding in fall. Similar to other species, parts of the song of black redstarts contain repetitive elements and males increase the number of these elements in an agonistic context (Apfelbeck et al., 2012). If testosterone changes the structure of song during breeding, we expected to find significant differences in the structure of spontaneous song between the breeding and the nonbreeding season.

2. Methods

2.1. Study period and study site

Free-living male black redstarts were challenged with simulated territorial intrusions and caught in 2008 (April 1-June 12; September 19-October 6), 2009 (July 3-August 13) and, 2010 (June 28–July 31) in Upper Bavaria, Germany (N 47°, E 11°, 500–600 m above sea level). These males contributed to different experiments (see below); however most of them were bled and contributed to the seasonal testosterone profile presented. Furthermore, some of the hormone data from the early breeding season and the fall nonbreeding period as well as the behavioral responses to simulated territorial intrusions were collected as part of a different study and have been previously published (Apfelbeck and Goymann, 2011). The comparison between the two territorial phases (during breeding in spring and nonbreeding in fall) was conducted in a cross-sectional manner (studying different birds in each phase). Breeding stages for the seasonal hormone profile were determined by behavioral observations of males and females during capture. Before capture, we mapped territories by determining boundaries through frequently used singing posts. After capture, territorial males were followed throughout the breeding season and the presence of focal males in the respective territories was confirmed through observation or the use of playback (i.e. we played back black redstart song until the territory owner responded by approaching the loud speaker or by singing or for at least 10 min). The main focus of this study was a comparison of hormone levels, behavior, song and brain between early breeding season territory establishment and fall nonbreeding territoriality. In addition, hormonal data were obtained in more detail throughout the whole breeding season and during molt. Therefore, for the hormone part of the manuscript, the breeding period was further divided into several substages (incubation, nestlings and fledglings of first brood, nestlings and fledglings of second brood).

2.2. Simulated territorial intrusion experiments

Territorial behavior was assessed by simulated territorial intrusion experiments (STI). For a full description of the territorial intrusion experiments see (Apfelbeck and Goymann, 2011). Briefly, a stuffed decoy (in full adult plumage, three specimens) along with black redstart song was placed into the territory and the behavioral responses of the territory owner were recorded from a distance. We used five different playback strings with species-typical song in random order (wav.files, each repeated at a rate of eight strophes per min) that were played back from a loudspeaker at a sound pressure levels of 65 dB SPL at 1 m (as measured with a CEL 573.B1 Sound Level Analyser) directly below the decoy. We recorded the following behaviors of the territory owner during 10 min of vocal playback: (1) latency to respond to the STI either by singing or approaching the decoy, (2) the first time the male was in a 5 m radius around the decoy, (3) the total time the male spent in this 5 m radius. (4) the total time the territory owner fluffed its feathers, and (5) the number of head nods. Furthermore, we noted whether the male attacked the decov or sang at any time during the STI.

2.3. Capture and blood sampling

Males were caught after the STI experiments or while searching for food to obtain blood for hormone analysis (see also Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011). They were caught after STI experiments to obtain brains for mRNA expression analysis. Birds were caught in mealworm-baited ground traps or tree traps (in the case of simulated territorial intrusion experiments, some traps were attached close to the decoy and were opened after ten minutes and the playback continued until capture of the territory holder). Immediately upon capture ($178 \pm 112 \text{ s}$), a blood sample ($\sim 120 \,\mu$ I) was taken from the wing vein and collected into heparinized capillaries. We checked if males were molting body, wing, and/or tail feathers and banded them with a numbered aluminum ring (Vogelwarte RadolfzeII) and a combination of two color rings. All experimental procedures were approved by the governmental authorities of Upper Bavaria.

2.4. Recording and analysis of spontaneous song

Adult (≥ 2 years) territorial male black redstarts were recorded with a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660 (sampling frequency: 44.1 kHz; resolution: 16 bit) during breeding territorial establishment in spring 2009 (April 9–27, n = 20) and a different set of individuals during the nonbreeding territorial phase in fall 2009 (September 22–October 7, n = 12). Songs were analyzed using Avisoft-SASLab Pro software, version 4.51. Recordings were visualized in spectrograms (settings: sample rate 22,050 Hz, FFT = 256 points, Hamming-Window, Overlap: 50%). We determined the number of songs by visual inspection and selected songs of sufficient quality (low background noise) for further sound analysis. Each song of black redstarts can be divided into three distinct parts (part A, B, and C, e.g. (Cucco and Malacarne, 1999) with a pause of varying length between part A and B. We measured the duration of part A, B, C, the total song and the duration of pauses between A and B. We counted the number of elements of part A and C (mean of max. 20 songs). We also determined the frequency bandwidth and the maximum frequency of part A, B, and C using the automatic parameter measurement function (threshold -20 dB) in Avisoft (mean of max. 10 renditions of high-quality songs).

2.5. Plasma separation and hormone analysis

Plasma was immediately separated by centrifugation with a Compur Minicentrifuge (Bayer Diagnostics). The amount of plasma was measured with a Hamilton syringe and stored in 500 μ l ethanol (Goymann et al., 2007). After returning from the field samples

were stored at -80 °C. Testosterone concentration was determined by direct radioimmunoassay (RIA, following Apfelbeck and Goymann, 2011; Goymann et al., 2006). Samples were measured in duplicates in four assays. Mean ± SD efficiency of the extraction with dichloromethane was 92.5 ± 0.1%. The lower limits of detection of the assays were determined as the first values outside the 95% confidence intervals for the zero standard (B_{max}) and were 6.7, 6.6, 6.0, and 8.0 pg/tube respectively. The intra-assay coefficients of variation were 4.7%, 4.2%, 3.1%, and 7.9%, respectively. The inter-assay variation was 6.4 ± 1.8%. As the testosterone antibody shows significant cross-reactions with 5 α -dihydrotestosterone (44%) our measurements may include a fraction of this potent androgen.

2.6. Tissue collection

Brains and testes were collected between April 14–28 in 2008 (n = 8) and between September 21–24 in 2009 (n = 8). Upon capture, birds were immediately killed by decapitation and brains removed from the skull, frozen on dry ice and stored at -80 °C until further analysis. We also recorded the length and width of the left and right testis using calipers and calculated testis volume as the volume of an oval body $(4/3*\pi*(width/2)^2*(length/2))$. Frozen brains were cut into 20 µm sagittal sections on a cryostat microtome (Leica Microsystems GmbH, Wetzlar, Germany) and collected on Superfrost object slides (Menzel GmbH, Braunschweig, Germany) in five parallel series. One series of brain sections was selected for Nissl staining and used to provide anatomical landmarks for later interpretation of in situ hybridization results. The remaining series were used for in situ hybridization of adjacent sections for androgen receptor, estrogen receptor α and aromatase.

2.7. In-situ hybridization

Riboprobes were synthesized from cDNA previously cloned from zebra finch androgen and estrogen receptor α mRNA (Gahr and Metzdorf, 1997) and canary aromatase mRNA (Metzdorf et al., 1999). Antisense and sense ³⁵S-CTP-labeled probes were transcribed from the T7 and SP6 promoter region of a pGEM7Zf + vector using the Riboprobe System (Promega, Madison, WI). Brain sections were fixed in a 4% formaldehyde solution in phosphate-buffered saline (PBS; 0.01 M; pH 7.4) for 5 min, washed in DEPC-treated PBS, and incubated in 0.25% acetic anhydride in ethanolamine (TEA; 0.1 M; pH 8.0) for 10 min to reduce nonspecific binding. After a washing step in $2 \times$ standard saline citrate (SSC), sections were dehydrated in serially increasing percentages of ethanol, and left to dry at room temperature. Sections were hybridized under a cover slide with ³⁵S-CTP-labeled sense or antisense riboprobes (0.4×10^6 cpm/slide) in hybridization buffer with 50% formamide and 10% dextran sulfate overnight at 55 °C. After hybridization, slides were immersed in $2 \times$ SSC at room temperature to remove the cover slides and incubated in RNase A (20 ug/ml) for 30 min at room temperature. Sections were then consecutively washed for 30 min in 2× SSC at 50 °C, 0.2× SSC at 55 °C, and 0.2× SSC at 60 °C, dehydrated in ethanol containing 0.3 M ammonium acetate, and dried for 1 h at room temperature. Finally, slides were exposed to Kodak BioMax MR film (Sigma-Aldrich Co., St. Louis, MO) in lightproof boxes for 3 weeks at room temperature, developed in Kodak D-19 developer, washed in tap water, and fixed with Kodak fixer.

2.8. Brain data analysis

Brightfield photomicrographs of the Nissl stained brain sections were made with a Leica DM6000B digital microscope (Leica Microsystems), equipped with a Leica DFC420 5 megapixel CCD digital



Fig. 1. Post-capture testosterone levels (ng/ml) of male black redstarts caught during different life-cycle stages. Testosterone levels are presented on a log-scale. Territory establishment: n = 60, male testosterone levels during female incubation: n = 17, nestlings first brood: n = 21, fledglings first brood: n = 12, from July on: nestlings second brood: n = 33, fledglings second brood: n = 38, molt: n = 3, nonbreeding territoriality: n = 15. Most birds caught after the 21 July had already initiated molt of the wing feathers even if they were still feeding young. Each individual is represented only once.

color camera. The Nissl sections were manually co-registered with the in situ hybridization autoradiograms of parallel brain sections to reliably determine the location of brain areas, such as the robust nucleus of arcopallium (RA) and the preoptic area (POA), which were difficult to distinguish from the autoradiograms directly. The data analysis of mRNA expression patterns was carried out similarly as described in Voigt et al. (2009). Autoradiograms were scanned with an Epson scanner using SilverFast Ai software as 16 bit grey values and with a resolution of 2400 dpi for later analysis in ImageJ. The system was calibrated by scanning a calibrated optical density step tablet (part #T2115, Stouffer Graphic Arts Equipment Co., Mishawaka, USA) and a calibration curve was calculated based on the Rodbard function in ImageJ. All autoradiogram images were saved in ImageJ with this calibration. HVC volumes were estimated based on androgen receptor expression (see also Fusani et al., 2000; Gahr and Metzdorf, 1997). For each androgen receptor-labeled brain section we delineated HVC, summed the area measurements and multiplied them with 100 µm (interval between sections). Optical densities of androgen receptor, estrogen receptor α and aromatase mRNA expression levels in the preoptic area were measured in two different ways using an ellipsoid with fixed dimensions for all sections and individuals, and again using an ellipsoid covering most of the stained area (therefore with changing size between individuals). To control for background staining the optical density in a control area just adjacent to the preoptic area was subtracted from the value for receptor expression. Optical density measures were averaged across all sections that were labeled for the respective mRNA. All measurements were carried out blind to the seasonal group of the bird.

2.9. Statistical analysis

Data were analyzed in *R* Development Core Team (2009). Hormonal data were analyzed using linear models. In a first step we used a priori set contrasts to compare the breeding substages (incubation, nestlings, and fledglings) and the nonbreeding period with the breeding substage territory establishment. In a second step we only compared testosterone levels of males caught in different phases within the breeding season (first and second brood vs. territory establishment). Spontaneous song and brain data were only collected during breeding territory establishment and nonbreeding territoriality. Spontaneous song data were tested for seasonal differences using *t*-tests and were transformed, if necessary, to meet assumptions of equality and normality of variances. Brain data did not meet these assumptions and were, therefore, tested for seasonal differences using non-parametric Mann–Whitney-*U*-tests.

3. Results

3.1. Seasonal testosterone profile

Testosterone levels of males caught at various times of the year (see next sentence) differed significantly ($F_{5,193} = 34$, p < 0.0001, Fig. 1). A priori set contrasts revealed that testosterone levels of males during all other phases of the life-cycle were significantly lower than during territory establishment (incubation: t = -2.3, p = 0.02, nestlings (first and second brood combined): t = -6.4, *p* < 0.0001; fledglings (first and second brood combined): *t* = -8.5, *p* < 0.0001; molt: *t* = -3.1, *p* = 0.002; nonbreeding territoriality: t = -11.7, p < 0.0001). Within the breeding life-cycle stage, testosterone levels also differed significantly ($F_{2.178} = 99$, p < 0.0001). Testosterone levels of males caught during their first brood did not significantly differ from those of males caught during territory establishment (t = -1.8, p = 0.08), but males caught during the second brood had significantly lower testosterone levels than males caught during territory establishment (t = -13.3, p < 0.0001). During early breeding in April testes were fully developed (volume of the left testis (mean $\pm 95\%$ CI): 76.1 ± 14.0 mm³, n = 8) and significantly larger than during nonbreeding territoriality in September, when testes were completely regressed $(0.5 \pm 0.5 \text{ mm}^3, n = 8, U = 0, p = 0.0002).$

3.2. Behavioral response to simulated territorial intrusions

Territorial male black redstarts did not significantly differ between life-cycle stages in the time they spent within 5 m of a simulated territorial intruder, the time they spent with feathers fluffed in response to a simulated territorial intruder or the percentage of individuals attacking a simulated territorial intruder (Fig. 2, Apfelbeck and Goymann, 2011). However, a significantly higher percentage of individuals responded to a territorial intruder with song during the breeding life-cycle stages than during nonbreeding territoriality (Fig. 2, Apfelbeck and Goymann, 2011).

3.3. Spontaneous song: differences between life-cycle stages

Territorial male black redstarts sang significantly more songs with shorter pauses between songs during nonbreeding than during breeding (Table 1). Songs during breeding had more elements in part A and C than those sang during nonbreeding (Table 1). All other structural song parameters did not differ significantly between life-cycle stages (Table 1).

3.4. Expression patterns of androgen receptor (AR), estrogen receptor (ER α) and aromatase

3.4.1. Telencephalon

Dense androgen receptor mRNA staining was found in the song control nuclei HVC and in the lateral and medial nucleus magnocellularis (IMAN and mMAN, Fig. 3, Table 2) independent of life-cycle stage. However, in contrast to previous studies in other songbirds, androgen receptor expression in the robust nucleus of arcopallium (RA) was weak (Fig. 4). Instead, RA was surrounded by a band of androgen receptors, as demonstrated by relatively dense androgen receptor expression that was contained to the caudal arcopallium and did not extend rostrally (Fig. 4). Weak estro-



Fig. 2. Time spent within 5 m (as % of time seen, A), time spent feather fluffing (as % of time seen, B), number of individuals attacking (%, C) and number of individuals singing (%, D) in response to a simulated territorial intruder. Horizontal axes depict percent. Breeding territoriality is split into territory establishment (bars to the left) and feeding of nestlings or fledglings of the first brood (bars in the middle). Bars represent means \pm 95% CI, numbers within bars represent sample sizes. Asterisks indicate significant differences between breeding stages (** $\underline{z} < 0.01$). For statistics and a description of the territorial intrusion experiments see (Apfelbeck and Goymann, 2011).

gen receptor α mRNA expression was only found in the so called paraHVC (Table 2, see Gahr and Metzdorf, 1997 for a description of this region). Aromatase expression was not found in any of the song control nuclei (Table 2). However, similar to other songbirds, dense staining for aromatase mRNA occurred in the caudo-medial nidopallium (NCM, Table 2, Shen et al., 1995). Similar to canaries, aromatase and estrogen receptor α expression in NCM did not overlap (Metzdorf et al., 1999), but aromatase and androgen receptor expression did. Estrogen receptor α in NCM was specifically expressed around the lateral ventricle.

3.4.2. Diencephalon: preoptic and hypothalamic areas

During both breeding and nonbreeding territoriality co-expression of androgen receptor, estrogen receptor α and aromatase was found in the preoptic area (Fig. 5, Table 2). Androgen receptor, estrogen receptor α and aromatase were also co-expressed in the nucleus lateralis hypothalami posterioris (PLH, Fig. 5, Table 2), but similar to other species no estrogen receptor α expression was found in the nucleus medialis hypothalami posterioris (PMH,

Table 1

Comparison of song parameters during bouts of spontaneous song between breeding and nonbreeding territoriality. Significant results are highlighted in bold.

Song parameter	t – Statistic p – Value (df = 30)		Mean ± 95% CI		
			Breeding $(n = 20)$	Non-breeding $(n = 12)$	
Song rate (songs/min)	3.9	0.0005	3.1 ± 1.1	6.1 ± 1.5	
Pauses betw. songs (s)	-3.2	0.004	11.3 ± 5.1	6.0 ± 1.8	
Duration A (s)	0.7	0.5	0.9 ± 0.1	0.9 ± 0.1	
Duration B (s)	0.6	0.6	0.9 ± 0.2	0.9 ± 0.2	
Duration C (s)	-1.8	0.09	0.8 ± 0.1	0.7 ± 0.1	
Duration pause A-B (s)	-0.4	0.7	1.3 ± 0.4	1.2 ± 0.5	
No. of elements in A	2.0	0.05	8.4 ± 1.5	6.9 ± 1.1	
No. of elements in C	2.8	0.009	6.8 ± 1.1	5.2 ± 1.0	
Max frequency A (kHz)	-0.6	0.6	6.2 ± 0.2	6.1 ± 0.2	
Freq bandwidth A (kHz)	0.5	0.6	3.2 ± 0.3	3.3 ± 3.9	
Max frequency B (kHz)	0.1	0.9	8.2 ± 3.7	8.2 ± 2.6	
Freq bandwidth B (kHz)	0.4	0.7	6.0 ± 3.9	6.1 ± 3.3	
Max frequency C (kHz)	1.7	0.09	5.9 ± 2.7	6.2 ± 2.2	
Freq bandwidth C (kHz)	1.9	0.07	3.2 ± 3.5	3.5 ± 3.5	

Fig. 5, Table 2, (Fusani et al., 2000; Metzdorf et al., 1999). The tuberal region was densely stained for estrogen receptor α and androgen receptor mRNA and weakly stained for aromatase mRNA (Fig. 5, Table 2). Further staining for androgen receptor, estrogen receptor α and aromatase mRNA was found in the bed nucleus of the stria terminalis (BNST, Table 2).

3.5. HVC volume

The HVC volume determined by androgen receptor expression did not significantly differ between breeding and nonbreeding territoriality (mean \pm 95% CI: breeding: 0.6 \pm 0.1 mm³, *n* = 8, non-breeding: 0.8 \pm 0.08 mm³, *n* = 8; Mann–Whitney *U* test: *U* = 50, *df* = 12, *p* = 0.06).

3.6. Optical density in the preoptic area

As both methods to measure optical density produced similar results, we only present results for one of the methods (i.e. ellipsoid fitted to area with staining). Aromatase mRNA expression in



Fig. 3. Example of androgen receptor mRNA expression in two song control nuclei during the early breeding season. HVC: proper name, IMAN: lateral nucleus magnocellularis.

Table 2

Brain areas in the telencephalon and diencephalon expressing androgen receptor (AR), estrogen receptor (ER α) or aromatase (ARO) depending on life-cycle stage. IMAN, lateral nucleus magnocellularis; mMAN, medial nucleus magnocellularis; NCM, caudo-medial nidopallium; POA, pre-optic area; PMH, nucleus posterioris hypothalami lateralis; BNST, bed nucleus of the stria terminalis; RA, robust nucleus of arcopallium. We visually graded the expression in the different areas into ++ strong expression, + weak expression or – no expression. Only in the preoptic area aromatase expression seemed to differ with life-cycle stage (bold areas).

Brain area	AR breeding	AR non-breeding	ARO breeding	ARO non-breeding	ER α breeding	ER α non-breeding
Telencephalon						
HVC	++	++	-	_	++	++
IMAN	++	++	-	_	-	-
mMAN	++	++	-	_	-	_
RA	+	+	-	_	_	_
Arcopallium	++*	++*	-	_	_	_
NCM	++	++	++	++	++	++
Diencephalon						
POA	++	++	++	+	++	++
PMH	++	++	++	++	-	-
PLH	++	++	++	++	++	++
Tuberal region	++	++	+	+	++	++
BNST	++	++	++	++	++	++

* Expression was restricted to the caudal arcopallium.

the preoptic area was significantly higher during breeding territoriality than during the nonbreeding territoriality (U = 45; p = 0.007, n = 8 per season). Expression of estrogen receptor mRNA (U = 12, p = 0.1) and androgen receptor mRNA (U = 26.5, p = 0.9) did not differ significantly between life-cycle stages.

4. Discussion

4.1. Non-vocal territorial behavior and testosterone

In black redstarts, the expression of non-vocal territorial behaviors did not correlate with differences in testosterone levels and aromatase expression in the preoptic area. Because males vigorously defended territories both during breeding and during fall nonbreeding while testosterone levels and aromatase expression in the preoptic area were higher during breeding than during fall nonbreeding territoriality. Thus, territorial behavior does not seem to be maintained by an increased sensitivity for sex steroids during nonbreeding; i.e. upregulation of aromatase (higher levels of aromatase during nonbreeding compared to breeding) metabolizing low levels of testosterone (Canoine et al., 2007). Instead the increased expression of aromatase during breeding in the preoptic area is similar to other temperate-zone bird species (Foidart et al., 1998; Silverin et al., 2000; Soma et al., 2003; Wacker et al., 2010) and suggests that also in black redstarts the preoptic area plays a role in estrogen-dependent regulation of male reproductive behavior (Ball and Balthazart, 2004; Balthazart et al., 2010).

Similar to black redstarts, song sparrows aggressively defend territories in a nonbreeding context and have higher testosterone levels and higher aromatase expression and activity in the preoptic area during breeding than during nonbreeding territoriality (Soma et al., 2003; Wacker et al., 2010). Although song sparrows respond to a simulated territorial intruder during all life-cycle stages, the response is much reduced during molt (Wingfield and Hahn, 1994). Wacker et al. Wacker et al. (2010) propose that in song sparrows aggressive behavior is regulated by aromatase in the ventromedial hypothalamus because a weak response to a territorial intruder during molt correlates with low expression of aromatase in that area. In addition, aromatase inhibitors reduced aggression during nonbreeding in song sparrows and the aggressive response was rescued by estrogen treatment (Soma et al., 2000; Soma et al., 2000). However, although we have not assessed aggressive behavior and receptor expression during molt in male black redstarts we suggest that in redstarts non-vocal territorial behaviors are independent of sex steroidal control as treatment with an androgen receptor blocker and an aromatase inhibitor during breeding did not reduce non-vocal territorial behaviors (Apfelbeck et al., submitted).

4.2. Vocal behavior, brain and testosterone

Similar to black redstarts male canaries spontaneously sing more syllable types with trills (Leitner et al., 2001; Voigt and Leitner, 2008) and male song sparrows sing trills with more elements (Smith et al., 1997) during breeding than during nonbreeding. These changes have been suggested to be mediated by testosterone as they follow seasonal changes in plasma testosterone levels (Leitner et al., 2001; Smith et al., 1997; Voigt and Leitner, 2008). More specifically, these seasonal changes in song structure seem to be regulated by estrogen-dependent mechanisms in HVC in canaries (Fusani and Gahr, 2006; Fusani et al., 2003; Gahr and Metzdorf, 1997; Rybak and Gahr, 2004) and by testosterone-dependent seasonal changes in HVC size in song sparrows and Gambel's whitecrowned sparrows (Zonotrichia leucophrys gambelii, (Fraley et al., 2010; Smith et al., 1997; Smith et al., 1995; Soma et al., 1999). The expression pattern of androgen receptor, estrogen receptor and aromatase mRNA of male black redstarts is similar to the expression pattern found in canaries and other songbirds (but see expression in the caudal arcopallium, Fig. 4, (Brenowitz, 2008; Fraley et al., 2010; Gahr and Metzdorf, 1997). Thus, similar mechanisms may regulate seasonal changes in song. However, in male black redstarts HVC size and sensitivity for androgens or estrogens do not seem to differ between breeding and nonbreeding territoriality. Furthermore, in another experiment treatment of males with an antiandrogen and an aromatase inhibitor during breeding and fall nonbreeding did not reduce the number of repetitive elements produced (Apfelbeck and Kiefer, 2012). This suggests that the seasonal changes in the production of repetitive elements in the song are not directly induced by sex steroiddependent changes in nucleus HVC in black redstarts. This demonstrates that although the song system seems to be fairly conserved across song birds, the mechanistic control of song production may still differ between species and more studies especially on wild birds under natural conditions are necessary to understand this diversity of mechanisms in the production of song.

In contrast to the seasonal decrease in repetitive elements in spontaneous song, spontaneous song output was even higher during nonbreeding than during breeding territoriality. During breeding males were sampled during a longer period of time than during nonbreeding and some females had already started to incubate. Males of these females probably decreased their song rate (Landmann, 1996). A high song rate during the fall nonbreeding territo-



Fig. 4. Androgen receptor expression in the arcopallium and in HVC. (A, B): Nissl stained sagittal brain sections. C, D: In-situ hybridization autoradiogram for androgen receptor expression in brain sections adjacent to sections shown in (A, B). (E, F): Overlay of the Nissl stained sections (A, B) with sections stained for androgen receptor (C, D). Yellow circles indicate the border of HVC and red circles delineate RA as determined in the Nissl stained sections. (B, D, F) show magnifications of the arcopallium of the sections presented in (A, C, E), and closed arrowheads indicate the dorsal border of the arcopallium.



Fig. 5. Example of androgen receptor (A), estrogen receptor (B) and aromatase (C) mRNA expression during breeding territoriality. mMAN: medial nucleus magnocellularis, NCM: caudo-medial nidopallium, POA: preoptic area, PMH: nucleus posterioris hypothalami medialis, PLH: nucleus posterioris hypothalami lateralis.

rial phase also suggests that spontaneous song rate in male black redstarts is independent of testosterone. This is in contrast to most other temperate-zone song birds in which song rate decreases steeply after breeding and in concert with plasma testosterone levels (reviewed in Ball et al. (2002)). However, in contrast to spontaneously produced song, the likelihood of song produced in

Table 3

Summary of physiological, morphological and behavioral results comparing breeding and nonbreeding territoriality of male black redstarts.

	Breeding	Nonbreeding
Endocrine parameters Testosterone Testes	High Large	Low Regressed
<i>Response to STI</i> Non-vocal Probability of song	Strong High	Strong Low
Spontaneous song Song rate Song structure	Medium More elements in A and C	High Fewer elements in A and C
Brain anatomy HVC Aromatase pre-optic area	Large Strong	Large Weak

response to a territorial intruder was significantly reduced during nonbreeding territoriality (Fig. 2). This suggests that in male black redstarts seasonal changes in testosterone do not regulate song output in general, but in a context-dependent way. Similarly, European starlings (Sturnus vulgaris) sing at a high rate also during nonbreeding (Riters et al., 2000), but respond to females with an increase in song rate only during breeding (Riters et al., 2000). This sexually-motivated change in seasonal song production has been suggested to be facilitated by testosterone through indirect effects outside the song control system: it is correlated with high aromatase activity (Pintér et al., 2011; Riters et al., 2000) and immediate early gene expression in the preoptic area during breeding (Heimovics and Riters, 2005). Thus, the preoptic area supposedly does not only play a major role in the regulation of sexual behavior per se, but also the expression of other sexually motivated behaviors (Riters, 2012).

4.3. Testosterone and the regulation of non-vocal and vocal territorial behaviors

Combined evidence from this and previous studies suggests that testosterone and its metabolites regulate territorial behavior in black redstarts only partly and in a complex context-dependent manner. The non-vocal territorial response and spontaneously produced song seem to be independent of the control by sex steroids (Apfelbeck and Goymann, 2011), Fig. 2, Table 3). However, song produced in an agonistic context is probably facilitated by sex steroids (Apfelbeck and Goymann, 2011, Fig. 2, Table 3). These findings are similar to results obtained from two tropical songbird species. In staged male-male encounters during nonbreeding, captive spotted antbirds (Hylophylax n. naevioides) only responded with song when they were implanted with testosterone and during breeding, males implanted with blockers reduced the number of songs during such encounters (Hau et al., 2000). Costa Rican rufous collared sparrows (Zonotrichia capensis costaricensis) reacted - similar to male black redstarts - with equal intensity to a territorial intruder across seasons, however, they responded with significantly fewer songs during nonbreeding (Addis et al., 2010). In another rufous-collared sparrow population, Zonotrichia capensis, territorial behavior is completely independent of testosterone (Moore et al., 2004). Furthermore, these data corroborate similar findings in other bird species that defend territories outside the breeding season: males can respond to intruders and defend a territory even with low circulating levels of testosterone (Burger and Millar, 1980; Canoine and Gwinner, 2002; Hau and Beebe, 2011; Landys et al., 2010; Logan and Wingfield, 1990; Schwabl and Kriner, 1991; Soma et al., 1999; Wingfield, 1994) In addition to its signal function to other males, song may provide relevant cues for females during breeding. In most songbirds, song is not only important for territory defense, but also to attract and stimulate mates. In many species, including black redstarts (Landmann, 1996), during the breeding season song rate is highest when females are fertile (Gil and Gahr, 2002; Mace, 1987). Furthermore, it has been shown that females pay attention to the vocal output in singing interactions of males during agonistic encounters (Ballentine et al., 2004; Otter et al., 1999). Thus, seasonal changes in social context-dependent song output may be sexually motivated and selected and may be facilitated by the conversion of testosterone to estradiol in the preoptic area.

5. Conclusions

Our studies show that the relationship between testosterone and territorial behavior in male black redstarts is complex: testosterone does not seem to modulate non-vocal territorial behaviors, but the hormone may be involved in the regulation of contextdependent song through aromatization in the preoptic area. Our studies on black redstarts also suggest that there is no one unique mechanism by which sex steroids regulate territorial and song behavior in songbirds. Rather there seems to be a variety of solutions to the control of year round territoriality that we are just beginning to understand.

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