Enhanced testosterone levels affect singing motivation but not song structure and amplitude in Bengalese finches

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ABSTRACT

Song is a fundamental component of territory defense and mate attraction in birds, and androgens (like testosterone) are known to play a key role in controlling it. However, little is known about how differences in testosterone levels between males translate into inter-individual song variation. Indeed, testosterone could affect both the motivation to sing and the structure of song itself. Here, we tested whether experimentally elevated testosterone levels in adult Bengalese finches (Lonchura striata var. domestica), an oscine bird species, have an activational effect on 1) song performance, and 2) song structure. Our results show that testosterone-treated males, in contrast to sham–control males, sang more when confronted with a female. Other performance-related traits, however, such as latency to sing and song amplitude, were not affected. Testosterone-treated males also showed no differences in our two measures of song structure: fundamental element frequency and mean song frequency. Because song structure is known to be organizationally affected by testosterone, our results, synthesized together with findings from the current literature, suggest that in oscine birds, song contains multiple messages about the signaler's hormonal status. First, song performance may reflect current hormonal condition, and second, song structure may reflect the past hormonal state.

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1. Introduction

The sexual behaviour of many vertebrates is strongly influenced by testosterone, a steroid hormone produced in the gonads [1]. In oscine birds, song is one of the fundamental components of mate attraction and territory defense and there are many lines of evidence that suggest that singing is controlled by testosterone. For example, in the great tit (Parus major) and willow tit (Parus montanus) plasma testosterone levels co-vary with singing activity over the breeding season [2,3]. In zebra finches (Taeniopygia guttata) and canaries (Serinus canaria), castration of males decreases song rates, but normal singing activity is re-established after the castrated birds have been injected with testosterone [4–6]. In a number of species, such as white-crowned sparrows (Zonotrichia leucophrys) and canaries, females supplied with exogenous testosterone develop a male-like song and singing behaviour [7–9], even though in those species females normally do not sing. Finally, neurobiological studies demonstrated that songbirds have androgen receptors in their specialized song control nuclei in the forebrain [10,11] as well as in the ventralis muscle of the adult avian syrinx [12].

Even though the overall relationship between androgens and singing activity is well-documented, there is actually only a limited number of studies that have looked at how differences in testosterone levels between males translate into inter-individual song variation [13]. Two fundamentally different properties of song can be used to describe inter-individual song variation: song performance and song structure. First, song performance is the quantity and amplitude of song as well as the overall temporal pattern of song utterance. Second, song structure includes spectral and temporal properties of the sound waves that are emitted during singing. Song performance and song structure can be measured independently of each other [13].

Most studies that have looked at how differences in testosterone levels between males translate into inter-individual song variation have considered song performance only. The majority of these reported increased call or song rates in testosterone-treated oscines [14–21], as well as in a suboscine [22] and a non-oscine species [23]. Another study employing experimental manipulation of testosterone level did not find an effect on singing rates [24] and several investigations relating natural testosterone levels to inter-individual differences in song rate also failed to find such a relationship [25–27]. In some cases, this may be explained by seasonal variation in the level of the estrogen-synthesizing enzyme aromatase [28], which plays a crucial role in linking testosterone and behaviour [10,13]. Song bout length, another performance-related trait was not found to be positively related with experimentally increased androgen levels ([24], see also [29]). At the level of song element types, Galeotti et al.

[25] found that the length of the distinctive 'rattle' syllable in the song of the barn swallow (*Hirundo rustica*) as well as the number of pulses correlates with natural plasma testosterone levels.

One song performance parameter, amplitude, has been ignored among the several studies that have investigated the effect of testosterone on song performance. One reason for this may be that song amplitude can only be measured reliably in a highly controlled set-up. However, amplitude is important because 1) it determines the active space of the signal, 2) female birds prefer loud songs [30,31] and 3) song amplitude is controlled by air sac pressure [32], and it has been shown that certain muscles and other tissues that may affect the gas pressure in the syrinx are sensitive to androgens [12].

In contrast to song performance traits, there are very few studies that have addressed possible effects of sex steroids on song structure. From a proximate perspective such an effect is expected simply because of the high androgen sensitivity of the song control system (see [10] for review). From an ultimate perspective such an effect is expected because bird song is known to play a key role in male–male competition and mate choice [33]. As sex steroids strongly affect aggression and courtship behaviour [1,34], high testosterone levels may optimize song parameters for efficient sound transmission and mate attraction. Nevertheless, the current evidence for a link between testosterone and song structure is inconsistent. Repertoire size [35], rate of song type switching [35] and song versatility (repertoire size multiplied with the number of song type switches; [24]) were not affected by administration of exogenous testosterone. However, Cynx et al. [15] demonstrated that experimentally elevated testosterone levels in adult zebra finches decreased their fundamental song frequency. Remarkably this effect was only observed in the long term as treatment birds decreased their song pitch after five weeks post testosterone implantation. Moreover, the effect was still present one year later, indicating long term effects of sex steroids during adulthood (see [36] for a discussion of organizational vs. activational androgen effects). To date, no studies have demonstrated an activational effect of androgens on song structure.

In the present study we test for activational effects of testosterone implants (i.e. an increase of testosterone above baseline levels) on a thorough suite of both performance-related and structure-related song parameters, some of which have never (or rarely) been studied in the context of androgen sensitivity.

Our study species, the Bengalese finch (*Lonchura striata* var. *domestica*), belongs, as the zebra finch, to the Estrildidae family. Like many other members of that family, Bengalese finches display two types of song: directed song and undirected song [37,38]. Both types of song are structurally similar, but directed song is addressed at a female during courtship while undirected song is sung in contexts not clearly associated with a targeted receiver. Although it has been shown that testosterone levels in the Bengalese finch are significantly increased during the nest construction phase [39,40], overall levels and fluctuations of plasma testosterone are low compared to songbirds of temperate latitudes, which is a pattern commonly observed in tropical species [41,42]. As in the zebra finch [4], the occurrence of directed song in the Bengalese finch is controlled by testosterone, while undirected song is largely independent of androgen levels [37]. To test for activational effects of androgens, we measured performance-related song parameters and song structure of directed song in birds implanted with testosterone pellets and the control birds with sham pellets and compared their singing behaviour before and after implantation.

2. Methods

2.1. Subjects and housing

The Bengalese finch is a domestic strain of the white-rumped munia (*L. striata*), an estrildide finch which is distributed throughout tropical Asia [43]. White-rumped munias are gregarious birds often found in groups of several dozen individuals [43]. The domesticated form is highly gregarious, reaches sexual maturity at about 3 months of age, and may breed all year round [44]. Bengalese finches have relatively simple, stereotyped songs, similar to those of zebra finches. Songs consist of individual-specific phrases or element sequences repeated several times in a song bout [45,46]. A song element is a single note, visible as a continuous tracing in a spectrogram. A phrase is a stereotyped sequence of elements, whereas a song bout is a sequence of uninterrupted song (usually containing several phrases) which is separated by other song bouts by a clear break of at least a few seconds. While bird song often has a dual function of male–male competition and mate attraction, Bengalese finch song is never used in aggressive contexts but primarily functions in female attraction and during courtship [47].

The tested birds were obtained from local pet suppliers and kept for several months in a large aviary at the Max Planck Institute for Ornithology in Seewiesen to make sure that they had all reached sexual maturity. The experiments were conducted between June 2nd and June 21st. Two weeks before the experiment started, the males were moved to single cages (61×40 cm and 50 cm high) set up in a large outdoor aviary with three wooden walls, wooden ceiling and one wall made of wire mesh. Since Bengalese finches are highly social, all birds were kept in constant visual and acoustic contact with other males to allow some degree of social interactions. They were exposed to the outside light/dark cycle (ca. 16 h of full daylight during the time of the experiments) and temperature fluctuations. Cages were equipped with three perches and a cuttlefish bone, and birds had ad libitum access to a commercial tropical seed mixture and water. Their diet was supplemented with fresh lettuce once a week. The test birds could hear the songs of the other males kept in the same room and they could also see some of the other birds. No females were present in the room.

2.2. Testosterone implantation and hormone assay

A total of 34 birds were randomly assigned to the experimental groups, but we made sure that the same number of different colour morphs was included in the treatment and control group. Testosterone birds were implanted with testosterone pellets (1.5 mg testosterone, 60 day release, Innovative Research of America, Sarasota, Florida) subcutaneously through a small dorsal incision of the skin that was then closed with VetGlu tissue adhesive (Heiland Vet GmBH, Germany). For an assessment of release rates of pellets and a comparison with silastic tubes see [48]. The control birds were implanted with sham pellets consisting of pure binding material (Innovative Research of America, Sarasota, Florida). Blood samples were taken twice: 7 days before implantation and again 7 days after implantation. 70–150 µl of blood was collected with heparinized micropipettes following venipuncture of the alar vein. The samples were centrifuged and the plasma separated and stored frozen until further analysis. For each individual, the pre-implantation and the post-implantation blood samples were taken at the same time of day to control for circadian fluctuations of plasma testosterone levels [49–52]. Blood sampling took place between 1400 and 1630 h.

Plasma testosterone concentration was determined by direct radioimmunoassay (RIA) employing testosterone antiserum T3-125 (Esoterix Endocrinology, Calabasas, CA, USA), following the protocols described in Goymann et al. [53,54]. Cross reactivities of this antiserum are testosterone (100%), 5a-dihydrotestosterone (44%), d-1-testosterone (41%), d-1-dihydrotestosterone (18%), 5a-androstan-3b, 17b-diol (3%), 4-androsten-3b,17b-diol (2.5%), d-4-androstenedione (2%), 5b-androstan-3b, 17b-diol (1.5%), estradiol (0.5%), and less than 0.2% with 23 other steroids tested. Plasma samples were equilibrated with 1500 dpm of tritiated testosterone (Perkin Elmer, Wellesley, MA, USA) to calculate recoveries. Mean ± sd extraction efficiency for plasma

testosterone was $92 \pm 3\%$. Standard curves and sample concentrations were measured in duplicates and calculated with Immunofit 3.0 (Beckman Inc. Fullerton, CA), using a four parameter logistic curve fit. The lower detection limits of the standard curves was determined as the first value outside the 95% confidence intervals for the zero standard (B_{max}) and was 0.36 pg/tube. Samples were analyzed in two assays with an intra-assay coefficient of variation of 1.7 and 6.2% (determined from standard testosterone). The inter-assay coefficient of variation was 11.5%, the intra-extraction coefficient of variation of extracted plasma pools was 4.4 and 7.6%, and the inter-extraction coefficient of variation was 21.2%. Since the testosterone antibody used shows significant cross-reactions with 5a-dihydrotestosterone (44%), our measurement may include a fraction of 5a-DHT.

The experimental procedures were reviewed and approved by the administration of Upper Bavaria.

2.3. Song recording and song analysis

For each male, song was recorded 1–3 days after the first blood sampling (i.e. 4–6 days before implantation of the testosterone/control pellet) and again 1–3 days after the second blood sampling (i.e. 8–10 days after implantation).

For the song recordings, each bird was moved singly to the experimental cage, a small wire mesh cage (54×28 cm and 41 cm high) equipped with a single perch. The cage was placed in the center of an empty room (4.60×3.40 m and 2.45 m high) that was partly lined with anechoic foam to reduce reverberations. The back and side walls of the cage were foam-padded while the top and the front were open. A Sennheiser ME 62 omnidirectional microphone connected to a Marantz PMD 660 solid state recorder was installed 80 cm vertically above the center of the perch. This set-up allowed us to reliably measure sound amplitude independent of the singing direction of the subject [55,56]. A second cage with two females was placed in one meter distance from the uncovered front of the male cage to stimulate the test bird to sing. The females had access to ad libitum food and water. The sound recording was started as soon as the male was released into the experimental cage and lasted for 1 h. Simultaneously, the males' behaviours were recorded with a video camera (JVC Everio GZ-MG77E). This allowed us to later distinguish songs uttered from the perch from songs uttered from the floor of the cage. After 1 h, the male was moved back to its home cage.

Of the 34 males tested, one died between the first and the second session due to unknown causes. 12 (seven treatment birds and five control birds) sang during both recording sessions. Five males sang in only one of the two sessions, 16 birds did not sing at all. In our experience, the high rate of non-singers is typical for Bengal finches and related species. Zebra finches, for instance, show very similar singing rates with comparable testing set-ups when a female is kept in 1 m distance [57].

Sound analysis was performed both on the level of song bouts and on the level of single song elements. For the latter, we analyzed 3–8 different song element types per bird. We chose song elements depending on whether several good quality renditions of the same element were available. Only those songs which were uttered while the male was sitting on the perch were used in the analyses, as some song parameters, in particular song amplitude, can vary with the distance of the singing bird to the microphone. All recordings were high-pass filtered at 350 kHz to remove low-frequency background noise. The following song parameters were measured using Avisoft-SASLab Pro (R. Specht, Berlin, Germany): latency to sing (the time it took from the start of the experiment until the bird uttered the first song), time spent singing, element rate (number of elements per second), peak amplitude (the amplitude of the loudest element in the song, averaged over 1–10 renditions; root mean square (rms) values, averaging time 125 ms) and mean amplitude across the whole song bout (rms method, averaged over 1–10 song

bouts). Song element parameters included fundamental frequency (only measured in harmonic elements), mean frequency and amplitude (rms method with averaging time 125 ms), all averaged over 3–10 renditions. Recording settings were kept constant between birds, which allowed us to compare the sound levels of songs from different recordings. To calibrate the sound amplitude measurements, we played a calibration tone ($f_0 = 2$ kHz) with constant amplitude from inside the experimental cage (with a FOXPRO Scorpion X1A digital player, FOXPRO Inc, Lewistown, USA), which was recorded with the same recorder and settings as the song recordings. We then replaced the microphone with a CEL 314 precision sound level meter (integration time 125 ms) to measure the sound level at the location of the microphone. Mean of peak amplitude of the Bengalese finch songs during the pre-implantation session was 52.9 dB SPL re 20 μPa at 1 m (range: 44.1–61.7 dB), mean of mean amplitude was 49.3 dB (range: 43.1–55.9 dB).

2.4. Data analysis

All statistical tests were performed with R 2.8.1 [58] (R Development Core Team, 2008). The function *lmer* (R package lme4) was used to fit generalized linear mixed-effects models (GLMM). We used a Wald χ^2 test (see [59]) to investigate whether there was a significant interaction between the fixed factor "timing" (before/after the treatment) and the "group" (control/treatment group), i.e. whether the reaction of the individuals depended on the treatment. The variable "latency to sing" had a complex distribution because birds that did not sing during one session were assigned a maximum value. We were not able to fit a GLMM to the original data and therefore used a rank transformation following the procedures outlined by [60].

Birds that did not sing during at least one of the two recording sessions (i.e. 16 out of 33) were excluded from the analyses. One bird from the treatment group did not show an elevated testosterone level but a decrease by 30% (from 994 to 695 pg/ml), while all other treatment birds had a more than two-fold increase in testosterone levels. Therefore, we assumed that the implant probably fell off and the individual was removed from the analysis.

We accounted for the repeated sampling of the same individuals (one sampling before the treatment and one sampling after the treatment) by using individual subject as a random factor. For the analysis of the song element parameters (fundamental frequency, amplitude, mean frequency), we used a random factor "element type" that was nested in the individual subjects. Body weight was always included in our models as a fixed factor because the control birds tended to be heavier than treatment birds before implantation, but this difference was not significant (Welch Two Sample *t*-test: $t = -2.09$, $df = 7.85$, $P = 0.07$).

3. Results

Before implantation, the two experimental groups neither differed statistically in their testosterone levels ($t = -0.60$, $df = 13.90$, $P = 0.57$) nor in any of the measured song parameters: total time singing ($t = -1.20$, $df = 8.67$, $P = 0.26$), latency to sing ($t = -0.19$, $df = 14,00$, $P = 0.85$), peak amplitude ($t = -1.60$, $df = 8.41$, $P = 0.15$) and mean amplitude ($t = -0.87$, $df = 7.83$, $P = 0.41$). Similarly, baseline testosterone level was not correlated with body weight (Spearman's rank correlation, $r^2 = 0.01$, $t = 0.44$, $df = 14$, $P = 0.66$), nor were any of the song parameters ($0.22 \geq r^2 \geq 0.003$, $1.66 \geq t \geq -0.20$, $14 \geq df \geq 10$, $0.84 \geq P \geq 0.13$). Likewise, none of the song parameters varied with individual testosterone levels before implantation ($0.16 \geq r^2 \geq 0.01$, $1.38 \geq t \geq -0.37$, $14 \geq df \geq 10$, $0.72 \geq P \geq 0.20$).

After implantation, the treatment birds had considerably increased plasma testosterone levels compared to the control birds ($\chi^2 = 9812.1$, $df = 1$, $P < 0.001$, Fig. 1), which indicates that our hormone treatment was successful. Body weight was not affected by the treatment
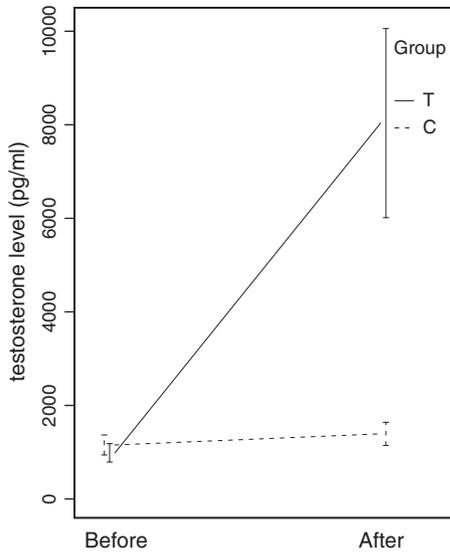
Fig. 1. Interaction plot of plasma testosterone levels before and after pellet implantation in male Bengalese finches. Means ± SE of testosterone levels are given for treatment birds (solid line; N = 8) and the control birds (dashed line; N = 8). In contrast to the control birds, treatment birds show significantly elevated testosterone levels after implantation (P < 0.001).

($\chi^2 = 0.35$, $df = 1$, $P = 0.55$), with both treatment group and control group showing a slight, but statistically insignificant, increase in weight.

3.1. Song performance

Our testosterone treatment did not affect the overall likelihood to sing ($\chi^2 = 0.09$, $df = 1$, $P < 0.76$); in the treatment group, a total of seven birds sang during the first session and eight birds during the second session, while in the control group, a total of six birds sang in the first session and seven birds (whereof two did not sing before) in the second session. In contrast, the treatment had a strong effect on the total singing duration ($\chi^2 = 206.63$, $df = 1$, $P < 0.001$) with testosterone birds spending more time singing and the control birds decreasing their total singing duration (Fig. 2). The decrease within the
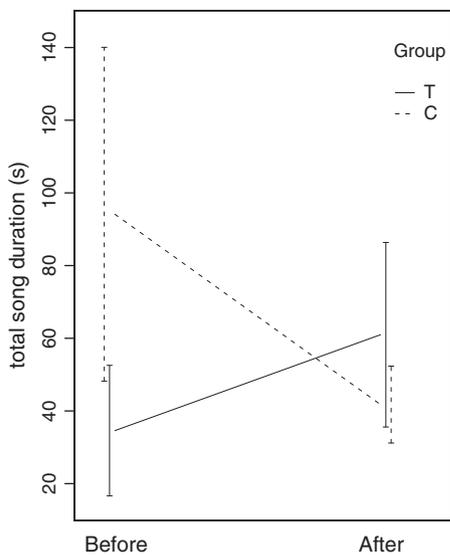

Fig. 2. Effect of testosterone on the total duration of courtship song in Bengalese finches. Interaction plot of the song duration before and after pellet implantation. Means ± SE of total song duration are given for treatment birds (solid line; N = 8) and the control birds (dashed line; N = 8). In comparison with the control birds, testosterone-implanted birds show a significant increase in total song duration (P < 0.001).

control birds suggests that the baseline singing motivation was lower in the second recording session compared to the first session. However, while the two slopes differed significantly, both the increase within the testosterone birds ($t = -0.76$, $df = 12.32$, $P = 0.46$) and the decrease within the control birds ($t = 1.10$, $df = 9.14$, $P = 0.30$) were statistically not significant. The testosterone-related difference in performance time was mainly due to the treatment birds producing more song bouts, but they also tended to increase their average song bout length compared to the control birds ($\chi^2 = 2.77$, $df = 1$, $P = 0.10$). Latency to sing ($\chi^2 = 0.44$, $df = 1$, $P = 0.51$) and element rate were not significantly affected by the testosterone treatment ($\chi^2 = 2.21$, $df = 1$, $P = 0.14$), nor were mean song amplitude ($\chi^2 = 0.30$, $df = 1$, $P = 0.58$), peak song amplitude ($\chi^2 = 0.15$, $df = 1$, $P = 0.70$) and element amplitude ($\chi^2 = 1.26$, $df = 1$, $P = 0.26$).

3.2. Song structure

We found no effect of the testosterone treatment on our two structural song measures, fundamental element frequency ($\chi^2 = 0.30$, $df = 1$, $P = 0.58$) and mean element frequency ($\chi^2 = 0.97$, $df = 1$, $P = 0.32$).

4. Discussion

We found that within 10 days after administration, testosterone implants increased the total time that male Bengalese finches spent singing. In contrast, no evidence for activational effects of testosterone on latency to sing and on song amplitude could be observed. Thus, we conclude that in Bengalese finches, high plasma testosterone levels augment the motivation to sing. A similar effect has been described in a number of avian species, including the pied flycatcher (*Ficedula hypoleuca*; [20]), song sparrow (*Melospiza melodia)*; [19]), dark-eyed junco (*Junco hyemalis*; [16]), rufous whistler (*Pachycephala rufiventris*; [18]), red grouse (*Lagopus logopus scoticus*; [23]), zebra finch [15], canary [14], golden-collared manakin (*Manacus vitellinus*; [22]), blue tit (*Cyanistes caeruleus*; [17]) and the house finch (*Carpodacus mexicanus*; [21]).

As singing functions in territory defense and mate attraction [33], a high motivation to sing is likely to be required during the periods when competition for resources and access to mates is high. Such periods may vary between species depending on their ecological requirements and life-history traits (see [42] for review). Peak androgen levels are correlated, among other factors, with, seasonality [2,3,41,61], mating system [41,62] and sociality [63,64]. In the Bengalese finch, testosterone levels are highest during the nest building stage, when males compete for access to females [40]. In combination, all these pieces of evidence strongly suggest that a direct physiological link exists between circulating testosterone levels and singing motivation.

Contrary to song output, song amplitude did not differ between testosterone birds and the control birds in our study. This indicates that song amplitude is not affected by variation in circulating testosterone levels, at least in the Bengalese Finch. It is possible that an effect of testosterone on song amplitude will be found in more territorial species that use their songs for long-range communication, because loud songs will be advantageous both in territorial defense and in passive female attraction, however we presume that modulation of song amplitude will generally tend to be under more direct (i.e. neuronal) control mechanisms (see later discussions).

The challenge hypothesis predicts short term increases of testosterone levels during aggressive encounters [65], which is particularly interesting with respect to the potential relationship between song amplitude, testosterone and territorial defense. In nightingales, territorial males have been found to increase their song amplitude during simulated challenges by rival males [66]. However, all nightingales increased their vocal effort within one minute after hearing a rival male (some individuals even within a few seconds) indicating

that the vocal regulation of song amplitude can be very fast. These brief reaction times suggest that testosterone may not be the immediate mediator of the increase in song amplitude because the secretion of testosterone is expected to take at least several minutes after stimulation [67]. Therefore short term changes in song amplitude are more likely to be mediated by other, i.e. neuronal, mechanisms.

In contrast to the effects on song performance, our testosterone treatment did not affect our two measures of song structure, fundamental frequency and mean frequency of song elements. This is in line with another study [15] that found organizational, but no activational effects of androgens on fundamental frequency of zebra finch song elements: the fundamental frequency of testosterone-treated males did not change within three weeks after implantation, but it was significantly lower five weeks after implantation and remained low for at least one year. However, Cynx et al. [15] did not assess plasma testosterone levels, so it is unclear how effective the treatment was and for how long testosterone levels remained elevated.

Although in our study administration of additional testosterone did not alter song structure on the short term, this does not exclude that testosterone below the observed baseline levels may have an impact on song structure. It might be that a comparably low testosterone level has activational effects on song structure and/or amplitude, but these song features may remain stable beyond a certain threshold of androgen concentration. This hypothesis can be tested with experiments using androgen receptor blockers or employing methods to reduce the amount of circulating testosterone [1].

Taken together, our study and the study of Cynx et al. [15] suggest that immediate, activational effects of enhanced plasma testosterone affect singing motivation, while long term effects may alter song structure. It is possible that some changes in song structure involve complex physiological processes altering neural and muscular actions and/or the structure of the syrinx. As a consequence, singing rate and perhaps song bout length, may reflect current hormonal state, while song structure may reflect past hormonal state, both during early development [68,69] and during adulthood [15]. However, this hypothesis regarding the hormonal basis of singing motivation and song structure needs to be substantiated by further experimental data. While there is a rather large body of evidence for a strong link between current androgen levels and overall courtship motivation in birds, activational and organizational effects of testosterone on song structure are still neglected. Therefore, future studies of testosterone-sensitivity of bird song should assess both short term and long term effects. Moreover, such studies may focus on song parameters which have been found to be under positive female preference, as testosterone during early development as well as during adulthood may have long-time effects and perhaps also short-time effects on song quality. High testosterone levels have been shown to be costly to maintain [70] and good singers may thus signal their ability to bear these costs. Song parameters which may be tested for testosterone-sensitivity include repertoire size [71,72] and specific song elements ('sexy syllables') like the broadband trills in canaries [73], which both have been shown to be important for female choice.

Acknowledgments

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