Male song quality affects circulating but not yolk steroid concentrations in female canaries (*Serinus canaria*)

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Summary

Male song complexity is a sexually selected trait found in many songbirds, including strains of the domestic canary. Studies on several species have shown that male song can affect the hormonal state of females and may also influence concentrations of maternal hormones in the yolk of their eggs. In this study, we show that the level of circulating androgens and oestrogens of female canaries, as measured in faeces, varies with the quality of male song to which they are exposed. The female-perceived quality of male canary song depends on the production of special 'sexy syllables' to which females respond with more sexual displays. Using playback of synthetic song we show that females hearing songs with sexy syllables have higher levels of faecal testosterone than control females hearing songs without them. However, unlike previous studies on the canary, we found no evidence that such females laid eggs with more testosterone (or other steroids) in their egg yolks. We discuss these results in relation to the evolution of male signalling and maternal investment strategies.

Key words: song, faecal steroid, HVC, female choice, mate quality, yolk hormone.

Introduction

The song of male birds is a sexually selected character used by females to select a mate (Catchpole, 1987, 2000; Searcy and Yasukawa, 1996). The song attributes to which females pay attention when gauging quality vary between species and include the number of song types (Krebs, 1977), syllable repertoire size (Catchpole, 1980) and the complexity of the syllables themselves (Vallet et al., 1998). In all cases, the complexity of song is thought to be an honest signal of a male's quality (Grafen, 1990; Zahavi, 1975), which has in turn been linked with the physiological constraints on production and availability of brain space (Catchpole, 1996, 2000).

Comparisons of playback treatments containing either conspecific song, heterospecific song or no song have demonstrated that song has a stimulatory effect upon the female reproductive axis in canaries (*Serinus canaria* L.), song sparrows (*Melospiza melodia*; Bentley et al., 2000) and ring doves (*Streptopelia decaocto risoria*; Cheng, 1986; Cheng et al., 1998). Previous studies showed that song quality affects reproductive development in canaries (Kroodsma, 1976) and that song affects female faecal oestrogen levels in zebra finches (*Taeniopygia guttata;* Tschernichovski et al., 1998). But, to our knowledge, no studies have investigated the effect of song quality on the secretion of reproductive hormones. The brain regions involved in avian song perception are hormone sensitive (Brenowitz and Arnold, 1992) although the

mechanism by which the song stimulus results in an endocrine response in female birds remains unclear. Recently, two pathways have been suggested, the first involving an indirect neural connection from the higher vocal centre (HVC), the area of the brain involved in female perception of song (Brenowitz, 1991; Leitner and Catchpole, 2002), to the thalamus (Foster et al., 1997), and the second involving control of gonadotrophin releasing hormone in the HVC itself (Bentley et al., 2004).

Female canaries respond to specific attractive ('sexy'; A-phrase) syllables (Vallet et al., 1998) with copulation solicitation displays. Syllables that do not elicit such a response may be artificially transformed into the attractive form by shortening the period of silence between the two elements comprising a syllable or broadening the frequencies (Draganoiu et al., 2002). Although such sexy syllables induced female copulation solicitation displays, they had no stimulatory effects on egg laying (Leboucher et al., 1998). We investigated whether sexy syllables influence female hormone secretion.

Female hormone levels depend on a wide range of factors, from time of day to the stage of the reproductive cycle (Sockman and Schwabl, 1999). The eggs of female canaries contain varying amounts of testosterone (Schwabl, 1993), higher concentrations leading to an increase in nestling growth and begging as well as correlating with dominance and social rank in later life (Schwabl, 1996b). This has led to the

hypothesis that females may be able to influence the quality of their offspring beyond their initial choice of a mate by actively varying the amount of specific resources within each egg in accordance with their perception of a partner's quality (e.g. Gil et al., 1999; Cunningham and Russell, 2000; Ellegren et al., 1996). Canary eggs contained higher concentrations of testosterone when the female was exposed to attractive syllables during yolk formation than when she was exposed to songs without such syllables (Gil et al., 2004). Similar experiments in zebra finches (using manipulation of coloured leg-rings) have produced mixed results, one study showing that eggs of females mated to more attractive males contain higher levels of testosterone (Gil et al., 1999), while a recent study found no such effect (Rutstein et al., 2004a), although in the latter study the authors investigated only the second egg from each brood.

We tested the following two hypotheses: (1) male song quality has an acute affect upon circulating levels of testosterone and oestradiol in female canaries, as measured by their concentrations in faeces, and (2) the concentrations of maternal steroids in the egg yolks change in response to the presence or absence of attractive syllables.

Materials and methods

Canaries

All experiments were conducted under Government licence at the Max Planck Research Centre for Ornithology, Radolfzell, Germany. Unrelated domestic canaries were obtained from a commercial supplier and placed in aviaries with a light:dark cycle of 9 h:15 h light:dark. Food and water were provided *ad libitum*, supplemented with fresh vegetable and plant matter twice per week. Prior to the experiments, females were moved to individual cages and the light:dark cycle was switched to 15 h:9 h. We used one-year-old females, entering their first breeding phase, as previous research has shown that they are most responsive to differences in male song at this stage (Leboucher et al., 1998).

Song playback

Song was recorded from 12 males using a Marantz (Longford, Middlesex, UK) CP430 cassette recorder with a Sennheiser (Wedemark, Lower Saxony, Germany) MD211U microphone. Song was edited using Avisoft-SASLab Pro (Avisoft Bioacoustics, Berlin, Germany). A maximum of two attractive syllables from each of 12 males was selected to compile the playback treatment, selected to maximise variation between syllables. Syllables were computer-edited to increase their attractiveness to females by reducing the gap between the elements of individual syllables (Draganoiu et al., 2002). Playback procedure was similar to that described by Gil et al. (2004). Each song lasted 30 s, followed by 90 s of silence. Song order was randomised using the Random Order Continuous Repeat function of the Sony Minidisc system (Sony MZ R700) and included one 'silent' song lasting 15 min to avoid habituation to the stimulus. The control song contained five different syllables, which were repeated for 30 s,

none of which was attractive. 21 attractive syllables were used in constructing the attractive playback. Syllables were used in a randomised order, each 30-s segment of attractive song occurring only three times in each playback session. Playbacks lasted for 3 h in the morning (immediately after feeding/cleaning) and 3 h in the evening, finishing 1 h before the lights were turned off.

Experimental design

Yolk hormone experiment

22 individually housed females were randomly divided into two groups, one in each of two experimental rooms, where they were in auditory but not visual contact with members of the same group. Each group received playback treatment in a different order (attractive–control or control–attractive). Nesting material was provided from the commencement of playback. Eggs were removed and replaced with dummy eggs on the day that they were laid, and yolks were frozen for later analysis. Nests were removed 3 days after the last egg was laid, and the female was moved to the other room, where she was exposed to playback of the alternate treatment immediately. Nest cups were returned after 3 days in the new room. 13 of the 22 females laid one clutch in each treatment, and the analysis of yolk hormones presented here is limited to these clutches. Since no males were present, the eggs were unfertilized.

Acute faecal hormone experiment

After completing two broods for yolk analysis, females were moved to a room without playback for one week. The same 22 females that were used for analyses of yolk steroids were then assigned to one of two experimental rooms where they were in auditory but not visual contact with members of the same group. Each group received its playback treatment in a different order (attractive–control or control–attractive). During the 3 h of playback, faeces were collected on paper placed on the cage floor. At the end of each playback period, the collection sheet was removed and the faeces scraped from it. There was a 1-h period of silence between treatments during which faeces were not collected. Thereafter, the treatment was reversed and faeces collected again. We used non-invasive measurements of steroids in faeces to avoid handling effects. Faecal steroid concentrations correlate with circulating levels in canaries, injected levels of radiolabelled hormone correlating with the faecal profile (Sockman and Schwabl, 1999). Our method of faecal steroid measurement has been validated for the canary, the concentrations of immunoreactive steroid in faecal buffer solution correlating strongly with the figures obtained after steroids were separated from faeces by hydrolysis, organic extraction and chromatographic separation from other metabolites (Schwabl, 1996a; Sockman and Schwabl, 1999).

Hormone analysis

Collection and storage of faeces was standardised, and all faeces were collected at the same time and allowed to dry at

room temperature for 24 h before being frozen until analysis (Cockrem and Rounce, 1994; Goymann, 2005; Sockman and Schwabl, 1999). Analyses of faecal androgens and oestrogens were conducted as described elsewhere (Schwabl, 1993; Sockman and Schwabl, 1999). The limited quantity of faeces led us to restrict analyses to androgen [testosterone (T) and 5α-dihydrotestosterone (5α-DHT)] and oestrogen (oestradiol) only. Briefly, faeces were homogenized with phosphate-buffered saline with glucose (PBSg) and assays were performed without any further extraction or purification. These assays have been validated for the canary (Schwabl, 1996a; Sockman and Schwabl, 1999). All samples were assayed for androgens (A) and oestrogens (E) in two single assays. The antibody (Wien Laboratories, Succasunna, NJ, USA) used to measure faecal A cross-reacts with T and 5α-DHT. The E antibody (Arnel Products Co., New York, NY, USA) cross-reacts with 17β-oestradiol and to a low extent with oestrone and oestriol. Yolk steroids were measured according to Schwabl (1993). Briefly, whole yolks were homogenised with dH_2O, and steroids were extracted from the homogenate and partially purified and separated using mini-chromatography columns before radioimmunoassay for androstenedione (A_4), 5α-DHT, T and 17β-oestradiol were performed. All eggs were assayed for A_4, 5α-DHT, T and 17β-oestradiol in a single assay. The antibodies used to measure yolk concentration of T, 5α-DHT and 17β-oestradiol were the same as those used for faecal assays; the A_4 antibody was purchased from Wien Laboratories.

Analysis

Brood size ranged from three to six eggs, most females laying four or five. We used mean values of neighbouring eggs in the laying sequence in accordance with the matrix in Table 1 in order to produce a standardised brood size focussed upon the beginning, middle and end of the laying cycle (Rutstein et al., 2004b). Females differed significantly in the levels of all four hormones in their eggs, in some cases by factors of 10 or more (data not shown). All results were analysed on a within-female basis, with eggs as repeated measures, each female thereby acting as her own control.

Results

Acute faecal hormone experiment

Faecal concentrations of E and A were significantly higher during playback of song containing 'attractive' syllables than during the playback of song lacking such syllables (E, $P=0.04$, $t=2.08$, $N=25$; A, $P=0.04$, $t=2.22$, $N=25$). Analysis of variance (ANOVA) for A incorporating the effects of treatment (song quality), treatment order and the associated interaction showed significance for all three factors (treatment, $F=5.0$, $P=0.03$; order, $F=5.8$, $P=0.02$; interaction, $F=6.1$, $P=0.02$). A similar ANOVA for E reduced the effect of treatment to a non-significant trend as a result of a strong interaction effect (treatment, $F=2.87$, $P=0.098$; order, $F=0.87$, $P=0.36$; interaction, $F=15.62$, $P=0.0003$).

Table 1. *Matrix used to standardise brood size by amalgamating eggs in neighbouring positions in the lay order*

Clutch size	Egg number					
	1	2	3	4	5	6
3	1	2	3			
4	1	2	2	3		
5	1	1	2	3	3	
6	1	1	2	2	3	3

Although the main effect of song treatment on faecal E was not significant, Fig. 1 indicates that this was probably due to a carry-over effect from the previous treatment with attractive song in one of the two experimental groups (indicated by the highly significant interaction term). Comparing the two control playback regimens directly, levels of E were significantly lower when control playback was received before the attractive song treatment than when it was played after the attractive treatment ($t=3.8$, $P=0.001$, $N=22$). E levels were lower in faeces from the control treatment than from those during the subsequent attractive playback when control playback was the first treatment, although marginally non-significant ($t=2.01$, $P=0.071$, $N=10$).

The levels of faecal A and E were strongly correlated with each other within each playback treatment (attractive, $r=0.658$, $P=0.001$, $N=22$; control, $r=0.814$, $P<0.001$, $N=22$). We found a similar significant correlation between A and E levels when comparing the level of each steroid in the control treatment as a proportion of that in the attractive treatment, controlling for treatment order ($r=0.6557$, $P=0.001$, $N=22$).

Yolk hormone experiment

Mean yolk steroid levels calculated across treatments and all females were as follows: $A_4=9.63\pm7.79$ mg pg^{-1} (mean ± S.D.), $N=117$; 5α-DHT=13.53 ± 6.83 mg pg^{-1}, $N=119$; T=143.41 ± 79.47 mg pg^{-1}, $N=119$; $E_2=8.81\pm5.65$ mg pg^{-1}, $N=119$. All
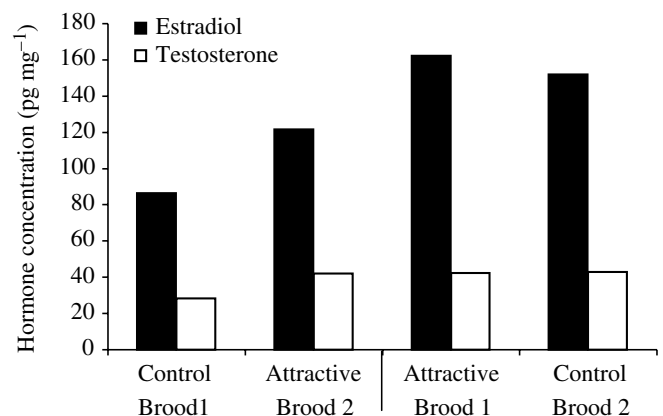

Fig. 1. Effect of song quality on female hormone levels: mean concentrations for androgen (testosterone) and oestrogen (oestradiol; pg mg^{-1} faeces) showing effect of song treatment and treatment order. See text for explanation of statistical significance.
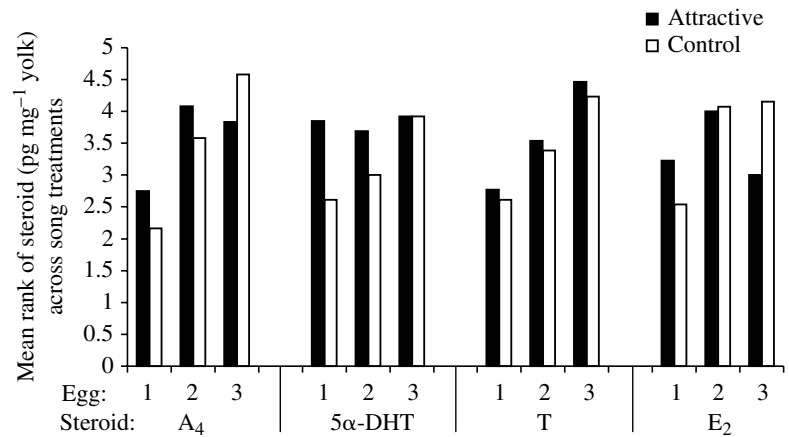
Fig. 2. Effect of song quality on yolk steroid levels across the lay order. Females varied by orders of magnitude in their means, thus the figure shows mean ranked concentrations of androstenedione (A_4), 5α-dihydrotestosterone (5α-DHT), testosterone (T) and 17β-oestradiol (E_2) for ease of interpretation (pg steroid mg^{-1} yolk): all analysis was conducted upon calculated levels.

yolk steroids, except for E_2 and A_4, were correlated with each other: T vs A_4, $F=116.442$, $P=0.0001$; 5α-DHT vs A_4, $F=16.755$, $P=0.0001$; E_2 vs A_4, $F=3.354$, $P=0.696$; 5α-DHT vs T, $F=31.113$, $P=0.0001$; E_2 vs T, $F=16.011$, $P=0.0001$; E_2 vs 5α-DHT, $F=22.987$, $P=0.0001$.

There was a significant effect of lay order on the concentrations (pg mg^{-1} yolk) of all four steroid hormones measured in the egg yolks, the hormone level rising in each subsequently laid egg (ANOVA; A_4, $F=8.47$, $P=0.0008$; T, $F=5.27$, $P=0.009$; 5α-DHT, $F=3.22$, $P=0.049$; E_2, $F=9.28$, $P=0.0002$; Fig. 2). However, there was no effect of treatment on these concentrations (A_4, $F=0.08$, $P=0.78$; T, $F=0.27$, $P=0.61$; 5α-DHT, $F=0.09$, $P=0.36$; E_2, $F=0.004$, $P=0.95$). Only for E_2 was the interaction of lay order and treatment significant ($F=9.93$, $P=0.0001$; all others $P>0.5$).

Since the amount of yolk as well as the concentrations of yolk steroids may vary, we also analysed the ratio of yolk mass to whole egg mass. As with the steroid concentrations, we found a significant effect of lay order upon this ratio, yolks becoming smaller in relation to the size of the egg towards the end of a brood ($F=28.42$, $P<0.0001$). However, there was no effect of song treatment upon this ratio ($F=0.3$, $P=0.59$).

Correlation of yolk and faecal steroid concentrations

There were no significant correlations between treatment effects on levels of steroids in yolks and faeces: yolk 5α-DHT vs faecal androgens, $r=0.243$, $P=0.423$; yolk T vs faecal androgens, $r=-0.039$, $P=0.9$; T+5α-DHT vs faecal androgens, $r=0.079$, $P=0.798$; yolk E_2 vs faecal oestrogens, $r=-0.13$, $P=0.672$.

Discussion

This study demonstrates an acute effect of male song upon female hormonal state. However, it does not show an influence on overall concentrations or within-clutch patterns of yolk steroids. The behavioural preference of females of many species of birds for males that sing songs of higher complexity is well established both in the field and under controlled laboratory conditions (Catchpole, 1987; Searcy and Yasukawa,

1996). In both wild female song sparrows (*Melospiza melodia*) and domesticated canaries, conspecific and heterospecific song enhanced follicular development in comparison with no-song treatments, the effect of conspecific song being strongest (Bentley et al., 2000). Ours is the first study, to our knowledge, to show different female hormonal responses to song quality. Whereas Gil et al. (2004) found a strong increase of yolk testosterone concentrations in this species in response to sexy song, we found little evidence for such an effect.

The link between song perception and an endocrine response remains unclear, although a neural pathway between the HVC and the thalamus (Foster et al., 1997) and the release of gonadotrophin releasing hormone direct from the HVC itself (Bentley et al., 2004) have been suggested. The HVC is the most important nucleus of the passerine neural song system for song production in males (Nottebohm et al., 1976) and is present as a smaller feature in female birds as well (Brenowitz, 1991; Leitner and Catchpole, 2002). Female canaries that both respond stronger to male songs and discriminate more accurately among songs of different quality have a larger HVC compared with less sensitive and discriminative females (Leitner and Catchpole, 2002). We propose that our results suggest an evolutionary feedback mechanism that maintains the honesty of song as a signal under sexual selection; female canaries solicit more copulations from males that sing more elaborate songs, containing more attractive syllables (Vallet et al., 1998). If male song is an honest signal of quality (Draganoiu et al., 2002) then it is necessary that females, as receivers of the signal, are able to discriminate amongst the variance in signal quality (Guilford and Dawkins, 1991), a task in which the HVC is heavily implicated (Leitner and Catchpole, 2002). Here, we have shown that high quality song promotes higher levels of circulating (faecal) androgens and oestrogens in females.

Since testosterone and oestradiol both increase the recruitment and survival of HVC neurons in female canaries (Rasika et al., 1994; Hidalgo et al., 1995; Harding, 2004), the increased levels of androgens and oestrogens in response to high-quality song may enhance female abilities of discrimination and accuracy, allowing them to select high-

quality males as mates based on their song. Thus, by singing attractive songs, males may be indirectly improving their selective advantage, and/or that of their progeny, over males who sing less attractive songs. Such a system would benefit both signaller and receiver, selecting for more attractive songs whilst preserving signal honesty and enhancing the discriminatory power of the receiver. The requirement to signal is maintained as males must sing in order to elicit a copulatory solicitation display and copulate.

Although our findings demonstrate an immediate effect of male song quality upon female steroid secretion, we did not find an effect of song quality upon the levels of these hormones in their eggs. While the first- and middle-laid eggs of a clutch showed a trend towards higher levels of the three measured androgens in the attractive song treatment, this was not the case for the late-laid eggs (Fig. 2). Overall androgen concentrations (A$_4$, 5α-DHT and T combined) were not different in our study (data not shown). We also did not find significant differences when using non-standardised brood size in the analyses (data not shown). These results conflict with those of Gil et al. (2004), who showed higher yolk T in all eggs when females were presented with attractive songs and implied significance for 5α-DHT and A$_4$ based on a correlation of these androgens with T in a small sub-sample of their yolks. Although sexy song affected female endocrine state, it affected neither overall nor intra-clutch patterns of yolk steroids in our study, which used a similar design. Such a lack of repeatability seems also to be the case in another song bird, the zebra finch. While Gil et al. (1999) found increased yolk androgens when females were mated to red-banded as opposed to green-banded males, a similar study by Rutstein et al. (2004a) was unable to find such an effect, although females did lay larger eggs for more attractive males in the first breeding round (Rutstein et al., 2004a). Thus, it is premature to accept the generality of the hypothesis that females adjust yolk steroid concentrations in response to male quality.

An emerging feature of experimental investigations of differential allocation are carry-over effects of previous experience into subsequent reproductive bouts. In our study, this was evident as higher yolk steroid concentrations in the second exposure to song regardless of its quality. Such carry-over was also present in the zebra finch studies of Gil et al. (1999) and Rutstein et al. (2004a). Future tests of the differential allocation hypothesis need to take such physiological inertness into account. Despite claims that female birds regulate yolk steroid concentrations, the extent to which females exert control over yolk steroid sequestration remains unclear (Birkhead et al., 2000; Gil, 2003). It has previously been shown that yolk androgen levels reflect the hormonal state of the female canaries (Schwabl, 1996a) during egg formation. Although we cannot directly address the association of female circulating and yolk steroid concentrations in our study, the comparison of faecal and yolk steroids in the two experiments revealed no correlation. Thus, the possibility for regulation of circulating and yolk androgens remains an unanswered question. We can conclude, however,

that among-female variation in circulating steroids does not necessarily predict yolk steroid concentrations and *vice versa*.

In summary, findings from an increasing number of studies present a mixed picture as to whether females can 'manipulate' the hormone levels in their eggs. We found no support for the hypothesis that female canaries allocate reproductive resources in relation to the perceived attractiveness of their mate. Facultative manipulation of yolk hormone levels by females, if it exists, may not be linked to male quality, as has previously been suggested. Nonetheless, we find that variations in song quality of canaries can have a significant and immediate effect upon the female endocrine state and suggest a feedback mechanism for maintaining signal honesty and improving discrimination amongst signals by the receiver.

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