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Testosterone decreases the potential for song plasticity in adult male zebra finches

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Abstract

Zebra finches are age-limited learners; males crystallize their songs at 90 days and do not subsequently alter those songs. However, a variety of interventions, including deafening and syringeal denervation, result in long-term changes to the crystallized song. These changes can be prevented by lesioning nucleus LMAN. As different social contexts for song production result in differential activation of LMAN, we asked whether the social context experienced by adult males would affect their ability to alter their songs in response to syringeal denervation. Males able to see and direct their songs to females made fewer changes to their songs than did males that could hear but not see females, but this trend was not significant. The volume of a male's HVC, a forebrain song control nucleus, also failed to predict the degree to which a male would change his song. However, testis mass was significantly correlated with the number of changes made to the song, indicating that variations in testosterone modulate adult song plasticity. We directly tested the effect of circulating testosterone on adult song plasticity by implanting adult males with either testosterone or flutamide, a testosterone receptor blocker, and tracking song changes triggered by ts nerve injury. As predicted, males implanted with testosterone changed their songs less than did males that received flutamide implants. These results suggest that the high testosterone concentrations associated with sexual maturity and song crystallization in zebra finches continue to act in adult males to reduce the potential for vocal plasticity.

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Introduction

Some species of oscine songbirds are open-ended learners, capable of relearning or adding to their vocal repertoires as adults, whereas other species are age-limited learners (or critical-period learners) that acquire a song model and learn to imitate that model early in life. Age-limited learners do not normally change their songs after the first year of life, and, in the case of zebra finches (*Taeniopygia guttata*), the end of the sensitive period for learning is reached at 80–90 days of age in normally reared birds (Immelmann, 1969; Price, 1979). Although adult male zebra finches normally have fixed songs, they do change their songs after experimental interventions that interrupt the vocal-auditory feed-

back loop; the interventions that have proved effective include deafening (Nordeen and Nordeen, 1992), denervation of the vocal organ (Williams and McKibben, 1992), muting (Pytte and Suthers, 2000), and delayed auditory feedback (Leonardo and Konishi, 1999). The basal ganglia circuit of the song control system (sometimes called the anterior forebrain pathway, or AFP), which is necessary for song learning but not for maintenance of adult song, is important in mediating the adult plasticity that results from syringeal denervation or deafening (Williams and Mehta, 1999; Braïnard and Doupe, 2000).

The plasticity that can be induced in the songs of adult male zebra finches (by deafening, syringeal denervation, muting, or delayed auditory feedback) arises over a relatively long period, sometimes becoming apparent only several months after the intervention. There is also individual variation in the degree of adult song plasticity: the effects of deafening upon song are more profound for some birds than

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for others (Nordeen and Nordeen, 1992), and the number of song changes in birds that receive syringeal denervation varies widely (Williams and McKibben, 1992). Because the induction of plasticity in a neural system responsible for age-limited learning is a phenomenon of potentially wide interest, it would be useful to understand the sources of this variability in behavioral and neural plasticity.

One correlate of variability in adult plasticity is age. The songs of older male zebra finches appear to degrade less quickly and/or less profoundly after deafening than do those of younger males (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001); this phenomenon may be related to the number of song repetitions that have been sung during the bird's lifetime. Three other potential correlates of variability in adult plasticity include (1) the "brain space" available for reconfiguring the neural program for song, (2) the hormonal status of the bird, and (3) opportunities for and aspects of song performance itself. The possibility that brain space is important for plasticity is suggested by results in the canary (Nottebohm et al., 1981) and marsh wren (Canady et al., 1984) showing that increased volume of an important song control nucleus, HVC, allows birds to acquire a larger repertoire. Similarly, the proportion of a young zebra finch's learned song that is an accurate imitation of an adult model increases with HVC volume (Ward et al., 1998). Hormonal status is closely correlated to the end of the sensitive period for song learning and the crystallization of a stereotyped song in both open-ended and age-limited song learners (Pröve, 1983; Marler et al., 1987). Experimental conditions that reduce testosterone concentrations prevent song crystallization in song and swamp sparrows (Marler et al., 1988), and although castration does not affect the stereotypy of adult zebra finch song (Arnold 1975), increasing hormone concentrations during development leads to early crystallization (Korsia and Bottjer 1991). Finally, although adult zebra finches have a fixed song, that song does have two "forms" (Sossinka and Böhner 1980), one of which is directed at females and does not induce expression of the immediate early gene ZENK in the anterior forebrain pathway, whereas the other, undirected song, is quieter and more variable in syllable order and induces strong ZENK expression in the anterior forebrain pathway (Jarvis et al., 1998).

This study examines the consequences of the behavioral context of singing and the hormonal status of adult zebra finch males on the ability of the birds to respond to the challenge of unilateral syringeal denervation by changing their stereotyped song patterns.

Materials and methods

Subjects

A total of 24 adult male and 12 female adult zebra finches (*Taeniopygia guttata*) ranging in age from 13 to 60

months were used. Twelve males and twelve females were used to examine the effects of song context and twelve males were used to examine the effects of hormonal status. The birds were raised and housed in the animal care facility at Williams College in Massachusetts, where they were maintained on a 14:10 light:dark schedule at 24°C. Food, water, grit, and cuttlebones were available continuously ad lib. All procedures were approved by the Williams College IACUC and were carried out in accordance with the standards defined by the National Institutes of Health.

Potential subjects were identified from among intact members of the colony by first identifying groups of male nestmates. The song of each of these birds was then recorded, and pairs of nestmates with similar songs were chosen to be included in the study. This allowed us to assign birds to treatment groups so that each subject had an age-, lineage-, and song-matched counterpart in the other group. For both the song context and the hormonal status studies, one male was lost prior to the beginning of the experiment and was replaced by another male matched for age and song complexity (but not for lineage). Female nestmates were used to provide age- and lineage-matched consorts for males (females do not sing).

Song context study

To study the influence of song experience on adult plasticity, 12 males were individually housed in one half of a Prevue Double Breeder cage, and a female consort was housed in the other half of each cage. Two age- and lineage-matched females were assigned to similarly matched males, so that each male's consort was the sister of the consort assigned to the matched male in the other experimental group. Six of the male/female pairs were separated only by a wire barrier and the birds could see each other readily (allowing the males to perform directed song). For the remaining six pairs, an opaque barrier was placed between the birds so that they could hear but not see each other. As a consequence, the males in this group could not perform the courtship dance oriented toward the female that is the defining characteristic of directed song (although songs can be triggered by female vocalizations, we have never observed directed song in the absence of a zebra finch that is the object of courtship; in contrast, undirected song may be given in the presence of other finches). All cages were placed in sound-attenuating chambers (Industrial Acoustics IAC-1). After being housed in these conditions for 1 to 6 weeks, the males received right tracheosyringeal nerve transections with removal of the distal nerve stump, a procedure that prevents regeneration of the peripheral nerve (Williams and McKibben, 1992).

The males' songs were recorded immediately before and after surgery and every 10 days thereafter. During song recording, and only during song recording, the opaque barrier separating birds in the undirected song group was removed. This ensured that all song recordings were of di-

rected song, so that any differences observed in the songs of the males in the two groups were not due to the type of song that was sung. The time of day of song recording was rotated among the birds to avoid any biases due to circadian rhythms in song production.

After 16 weeks, the subjects were euthanized and perfused. Their brains were sectioned and stained and HVC volumes measured (see “Histology”). The testes were also removed and their mass determined.

Hormonal manipulation study

As for the song context study, the subjects were 12 adult male zebra finches, forming two groups of age-, lineage- and song-matched males. One member of each pair of matched males received a subcutaneous silastic implant of testosterone (T; 6-mm implant), whereas the other received an implant containing flutamide (F; 10-mm implant; flutamide is a synthetic antagonist of testosterone receptors and was obtained from Schering-Plough). Birds were not castrated prior to receiving implants. Previous reports in the European robin (*Erithacus rubecula*), a bird that is similar in size to the zebra finch (16 g for the robin vs 12 g for the zebra finch) have indicated that this dose of flutamide does not cause overproduction of testosterone by blocking receptors in the hypothalamus in that species (Schwabl and Kriener, 1991).

Two weeks after receiving implants of testosterone or flutamide, each male's right tracheosyringeal nerve was transected and the distal stump removed to prevent regeneration. The males' songs were recorded immediately before surgery, immediately after surgery, and weekly thereafter. After 16 weeks, the birds were euthanized and perfused, the brains sectioned and stained, and HVC volumes measured.

To assess the effects of the implants on circulating concentrations of testosterone, we placed T and F implants in a separate set of adult male zebra finches ($n = 10$, five for each treatment) and measured circulating testosterone present 1 month later (ELISA analysis using the IBL kit from Research Diagnostics, Flanders NJ; we relied on the information to be found at <http://www.researchd.com/rdikits/re52151.htm> for the procedure, antibody, sensitivity, and statistical validation). Birds with flutamide implants had an average testosterone level of 296 pg/ml (SD = 64 mg/ml), which is at the low end of the normal range for adult male zebra finches as measured by Hutchison et al. (1984). Birds that received T implants had circulating testosterone concentrations more than ten times higher than those with flutamide implants, averaging 3.5 ng/ml (SD = 0.9 ng/ml). These T concentrations are within the physiologically appropriate range, similar to those in egg-laying females and lower than the T concentrations measured during the post-hatching testosterone peak by Hutchison et al. (1984).

To provide a comparison to untreated birds, we reanalyzed data from a reference group of eight birds that had

undergone the identical nerve transection and stump removal procedure plus a sham forebrain lesion as part of a previous study (Williams and Mehta, 1999).

Song recording and analysis

To record directed (courtship) song, a microphone was placed inside the sound-attenuation chamber (song context study) or the male's cage was placed inside a Lucite chamber (75 × 60 × 50 cm) containing a microphone and lined on four sides with acoustic foam. In both cases, the males could see females—in the other half of the cage for the song context study (the opaque barrier was replaced by a wire barrier for the males in the undirected during song recording) and through one Lucite wall for the hormonal status study. This ensured that all recordings were of courtship song directed at a female, giving consistency to all song recordings.

Songs were recorded with a dynamic microphone (Marantz EC-7) and a Marantz PMD-201 cassette recorder onto Maxell MS-60 studio tape and then digitized (16 bits at 22 kHz using SoundEdit on a Macintosh computer). Zebra finch song normally consists of a sequence of distinct and harmonically complex syllables, usually but not always separated by intervals of silence, that are delivered in a stereotyped order to form a 0.5- to 1.5-s motif. A song bout consists of a series of identical introductory notes followed by one or more motifs (Sossinka and Böhner 1980). Several song bouts, including at least 30 motifs, were recorded from each bird within the 3 weeks preceding surgery. The target for each post-op song recording session was also 30 song motifs; in some cases birds did not sing readily and recording sessions were repeated until an adequate representation of song was obtained.

We identified syllables according to the criteria described in Williams and Staples (1992), which are consonant with behavioral measures of how birds split song into discrete units (Cynx, 1990). Syllables were defined as those sounds separated from other sounds by short periods of silence or by abrupt transitions in amplitude and/or frequency. This delineation sometimes results in defining more syllables for a given song than do other methods, but it should also be noted that birds from our colony have songs with a length and complexity closely approximating those of wild zebra finches (see Zann, 1993).

Following right-side tracheosyringeal nerve injury (ts injury), the syllable structure of zebra finch songs is disrupted, sometimes severely (Williams et al., 1992). However, syllables can be readily identified by matching the relative position, length, and amplitude of each sound to those of the syllables in the intact bird's song. For each postoperative recording, the status of each song syllable was assessed. Syllables were scored as deleted if they were omitted from all song bouts in the final two-song recording sessions (for examples, see Fig. 1). Syllables were considered to be added to the song if they appeared with a con-

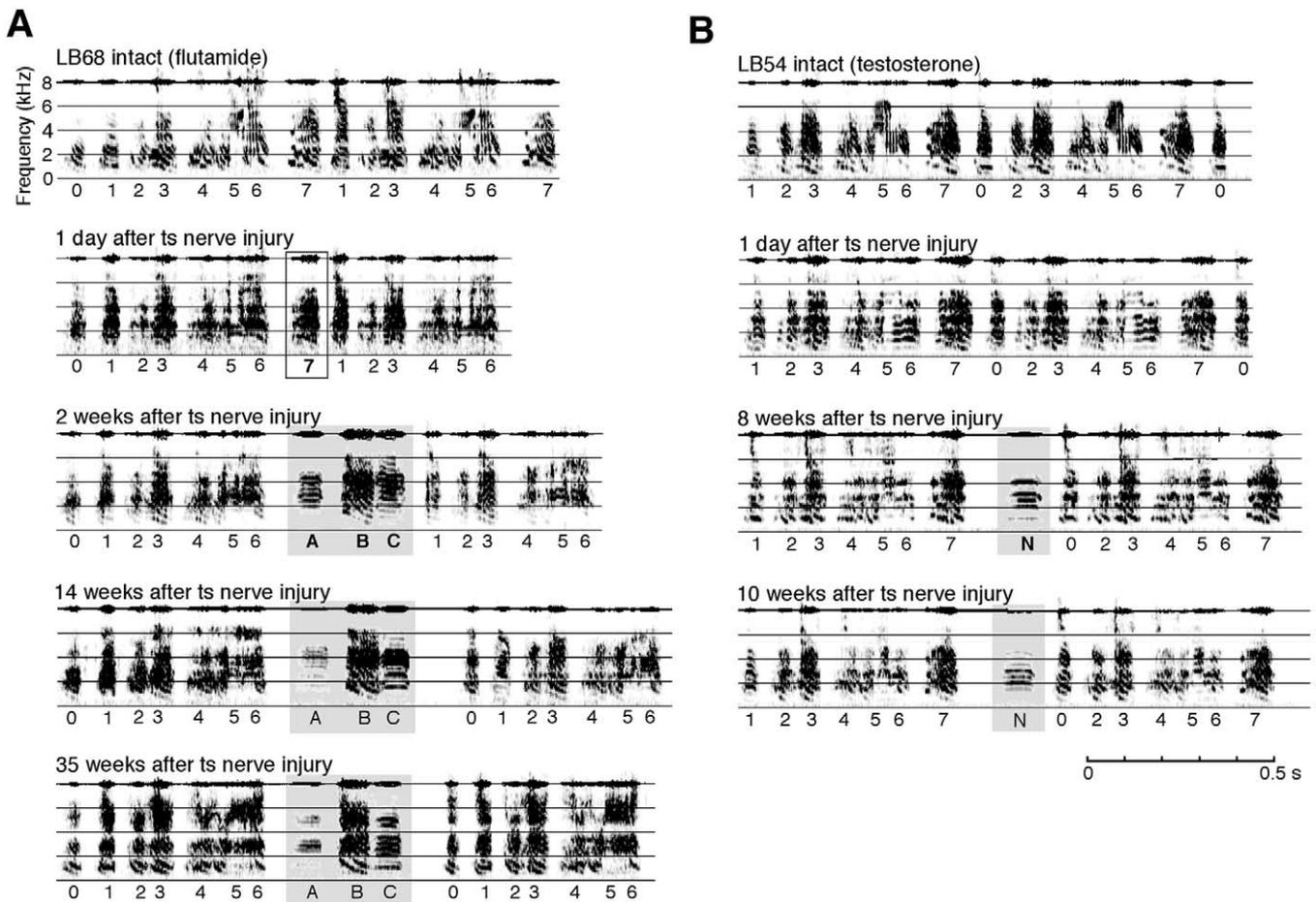


Fig. 1. Example of song changes after nerve injury. The songs of (A) LB68, a male that received a flutamide implant, and (B) LB54, a male that received a testosterone implant. These two birds were “matched”; their original songs are similar, and they were hatched in the same clutch. Syllables are numbered comparably for the two birds. The songs recorded immediately after nerve injury were degraded and relatively quiet, (with higher background noise levels). Nevertheless, syllables in each postoperative recording can easily be matched to those in the intact song. Deleted syllables are enclosed in a box in the recording preceding their disappearance, and added syllables are highlighted in all recordings in which they appear. Panel A shows that LB68 lost one syllable (7) after the first postoperative recording, whereas three syllables (A, B, and C) were scored as additions to the song, first appearing in the recording made 2 weeks after nerve injury and sung consistently thereafter. In later recordings, syllable 0 was sung at the beginning of each song rather than only at the beginning of the first song of a bout; this alteration was not scored as a change, as the syllable and its position relative to other song syllables were consistent with the recording from the intact bird. The one deleted syllable and three added syllables totaled four syllable changes. The length of the deleted syllable was 0.140 s and the length of the added syllables 0.382 s, for a total of 0.522 s of changed song time; this amounted to 80.8% of the length of the original song (which was 0.646 s long). Panel B shows changes in the song of LB54, the matched male; in this case one syllable (N) was added to the song, first appearing 8 weeks after nerve injury. The proportion of the original song’s length that was changed was 31.8% (208 ms added to a song that was originally 639 ms long).

sistently stereotyped structure and position within the motif in at least one-third of the complete song strophes recorded in both of the final two-song recording sessions. To provide a simple overall index of syllable changes, the number of syllables added to and deleted from the song were summed.

A change in a single long syllable within a short song has a proportionately greater effect on the overall song than would altering a single short syllable in a long song. We measured the length of entire songs and of all individual syllables to the nearest millisecond for at least five songs from each recording session. As silent periods preceding each syllable are copied along with the syllable during song learning (Williams and Staples 1992) and help to define the

rhythm of the song, intersyllable silences were included in all measurements (each syllable’s length included the immediately preceding silent period). The lengths of the syllables added to and deleted from a song were summed and then divided by the original song length; this measure, the proportion of the original song that was changed, provided a more refined measure of the degree of changes in a bird’s song.

Data analysis and statistics

The programs StatView 4.5 and SuperAnova (Abacus) were used to calculate means and SEMs and to perform tests

of statistical significance. Statistical significance was assessed using a criterion of $P < 0.05$. Tests for the song context study were two-tailed, based on the correlational results from the song regime study and the many published results showing that high testosterone is associated with a reduction in song plasticity (e.g., Marler et al., 1987, 1988; Nottebohm et al., 1987) we made a priori predictions that the birds with testosterone implants would be less likely to change their songs and thus used one-tailed tests for statistical analysis in the hormonal manipulation study.

Surgery (ts nerve injury)

Food was removed from the bird's cage for 1 h prior to surgery. Birds were then injected intramuscularly with 0.05 ml of a 50:50 mixture of ketamine (10 mg/ml) and xylazine (20 mg/ml), supplemented when appropriate by inhaled Metofane. All birds received injections of yohimbine to reverse the effects of the anesthetic after surgery was completed (Kilander and Williams, 1992).

The bird's head was placed in a Kopf stereotaxic apparatus with the beak held at 45° below horizontal using a specially designed bill clamp. The bird's body was rotated 180° counterclockwise and gently restrained on a water-filled pad maintained at 40°C. An incision was made over the trachea, and the right tracheosyringeal (ts) nerve was dissected free of the surrounding connective tissue and muscle. The nerve was then cut and the distal stump pulled out to prevent regeneration (see Williams and McKibben, 1992). The right ts nerve was injured because it is more easily accessible and because injuring the right side has a greater effect on song, as zebra finches are right-side dominant for song control (Williams et al., 1992). The incision was sutured with 5-0 silk and sealed with collodion or acrylic tissue adhesive.

Histology

After the song context study was completed, the 12 males were given an overdose of Metofane (inhaled) and perfused with 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4). At this time, tracheosyringeal nerve stumps were inspected for evidence of regeneration; in all cases, a short (<1 mm) fanlike group of fibers was seen emerging from the stump, but no fibers extended beyond the area of transection (approximately 1 cm from the syrinx).

Brains were sectioned on a Vibratome, and 50- μ transverse sections were mounted on slides and stained with cresyl violet. Alternate sections were digitized (Dage MTI camera, Data Translations board) and the area of HVC measured (NIH Image 1.53). The volume of HVC was reconstructed by multiplying by the section interval and the section thickness, and these absolute volumes were used for analysis. One brain did not produce usable sections, so for the comparison of HVC sizes of related males one pair of males was omitted.

Results

Song context

In this study, six adult male zebra finches could see and hear (and thus court through directed song) their female companions, whereas six matched males could hear but not see (and so could not direct their songs to) their female companions. We predicted that the opportunity to direct courtship song to females would influence the males' propensity to change their songs after receiving ts nerve injuries.

Five males added syllables to their songs (range: one to three syllables), and seven males deleted syllables from their songs (range = one to five syllables). A total of nine birds made changes to their songs, with the number of changed syllables ranging from one to five. Two of the birds with unchanged songs were in the directed song group and the remaining bird with no song changes was in the undirected song group. Although birds in the undirected song group did, on average, change more song syllables (SEM = 3.0 ± 0.86) than did the birds with the opportunity to sing directed song ($1.33 \pm .62$), this difference did not reach significance.

When the song changes were expressed as a percentage of the original song length, results were similar to the analysis of syllable changes. The songs of birds housed in the directed song conditions changed less ($16.2 \pm 8.5\%$ of the original song) than the songs of birds housed in the undirected song condition ($26.4 \pm 11.8\%$); however, once again, the difference was not significant.

Neither the bird's age nor its HVC volume was significantly related to the proportion of the bird's song that was changed. Another factor that might have affected the results is the amount of time a bird spent housed in the experimental conditions (and outside the group cage/aviary environment) prior to surgery, which varied somewhat between birds. However, the number of days spent in experimental housing prior to surgery had no effect upon the proportion of the song that was changed.

The mass of the bird's testes, as measured at the conclusion of the experiment, was significantly correlated with the number of syllables changed ($r = .67$, $n = 12$, $P < 0.05$; Fig. 2), and, even more strongly, with the proportion of the bird's song that changed after ts nerve injury ($r = .73$, $n = 12$, $P < 0.01$; Fig. 2). Birds with smaller testes were more likely to change their songs, whereas birds with large testes made few or no changes to their songs. Although the birds in the directed song condition had, on average, larger testes (41.8 ± 5.9 mg) than did the birds in the undirected song group (32.2 ± 6.3 mg), this difference did not reach significance. The tendency for males that could not see females to have smaller testes parallels these birds' propensity for making more changes to their songs, suggesting that differences in testis mass (and hence in hormonal status) account for the weak effect of housing condition on song changes.

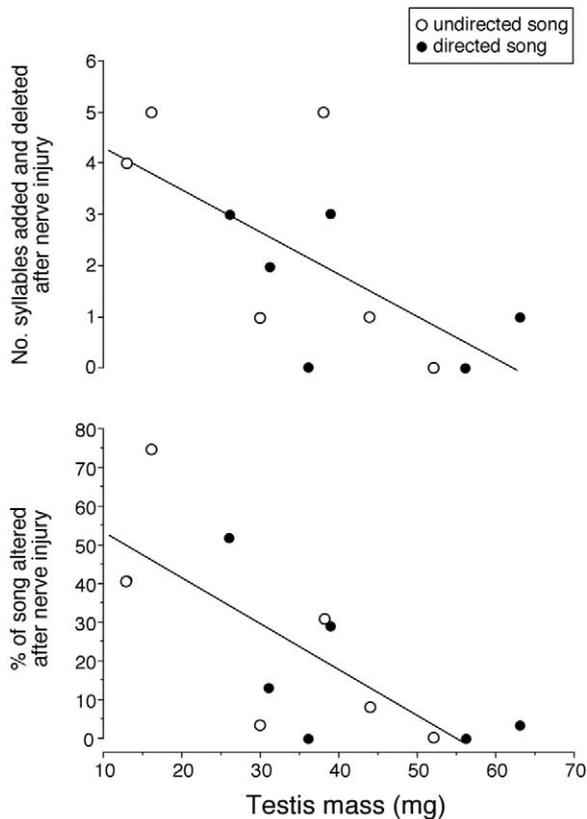


Fig. 2. Birds with smaller testes made more extensive song changes after nerve injury. The mass of a male's testes at the conclusion of the study was significantly correlated both to the number of syllables that were changed ($r = 0.67$, $n = 12$, $P < 0.05$) and, more strongly, to the percentage of the song that was altered after nerve injury ($r = 0.73$, $n = 12$, $P < 0.01$).

The importance of the relationship between testis mass and the proportion of the song that was changed was tested with a linear model that included housing condition, testis mass, HVC volume, and time in experimental housing prior to surgery. Only testis mass was significantly related to the proportion of the song that was changed ($F(1, 6) = 6.271$, $P < 0.05$; for all other factors, $P > 0.5$).

Comparisons of nestmates' HVC volume and testis mass

For each of five paired sets of males that were nestmates (one pair was omitted from this analysis because of poor sectioning of the HVC region of one brain), the average difference in the volume of HVC was calculated. Nestmates are presumed to be siblings (although extrapair copulations and egg-dumping are known in zebra finches). The differences in HVC volumes of nestmates were strikingly small (Fig. 3). The average difference in HVC volume for birds from different nests ($0.115 \pm .011 \text{ mm}^3$) was more than five times that of nestmates ($0.022 \pm .008 \text{ mm}^3$), a statistically significant difference ($t = 6.256$, $df = 9$, $P = .0001$). The minimum difference in HVC volumes for the birds in this

sample was also determined, by pairing each HVC volume measurement with its nearest neighbor; the actual differences in nestmates' volumes was nearly identical to and statistically indistinguishable from the minimum difference (Fig. 3).

In contrast to the tight match of nestmates' HVC volumes, the difference in nestmates' testis mass (Fig. 3) was indistinguishable from differences in unrelated males' testis mass ($t = .28$, $df = 17$, $P > .78$) and significantly greater than the minimum pairwise difference in testis mass ($t = 2.75$, $df = 17$, $P < .05$).

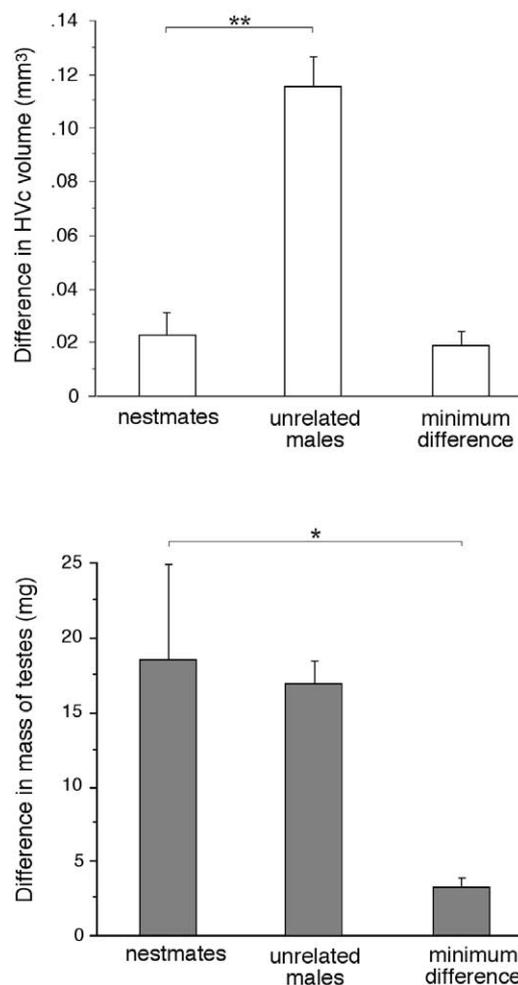


Fig. 3. The volume of the song control nucleus HVC, but not testis mass, was similar in male nestmates. The average differences between the HVC volumes ($n = 5$ pairs) and testis mass ($n = 6$ pairs) of nestmates were compared to the average of differences between each male and all unrelated males in the sample ($n = 11$ for HVC volume and $n = 12$ for testis mass) and also to the minimum values obtained when nearest neighbors for each measurement were paired ($n = 5$ pairs for HVC volumes and $n = 6$ pairs for testis mass). A single asterisk denotes a significant difference at the $P < 0.05$ level between designated groups, and a double asterisk denotes a significant difference at the $P < 0.001$ level. Error bars represent the standard error of the mean.

Hormonal manipulation

In this portion of the study, the hormonal status of 12 different adult male zebra finches was manipulated by administering either testosterone or flutamide (a testosterone receptor antagonist). The birds' right ts nerves were then injured, and we followed their songs for a period of 14 weeks. Seven of the 12 males added syllables to their songs (range = one to three syllables), and 2 males deleted syllables from their songs (range = one to four syllables). A total of 8 birds made changes to their songs, with the number of changed syllables (added + deleted) ranging from one to five. Three of the birds with unchanged songs had received testosterone implants, and one bird with an unchanged song had a flutamide implant. Birds with flutamide implants did, on average, change more song syllables (2.0 ± 0.58 ; Fig. 4) than did birds with testosterone implants (1.0 ± 0.63), but this difference did not reach significance. Neither the flutamide nor the testosterone group differed significantly from the group of untreated reference birds from a previous study (which changed 2.5 ± 2.27 syllables).

The more refined measure of the proportion of the song (in terms of time) represented by the added and deleted syllables was more informative (Fig. 4). The songs of birds with testosterone implants changed less (added and deleted syllables represented an average of $13.9 \pm 6.4\%$ of the original song) than did the songs of birds with flutamide implants (where changes averaged $46.1 \pm 15.2\%$ of the original song). This difference was statistically significant ($t = 1.95$, $df = 10$, $P < 0.05$). The proportion of the song changed by the untreated reference group from a previous study (which changed $29.7 \pm 8.4\%$ of the original song) was intermediate between the flutamide- and testosterone-treated groups and did not differ significantly from either.

Discussion

Although adult zebra finch males with the opportunity to direct their songs to females made fewer changes to their songs after ts nerve injury than did males that could not see females, the difference between the two groups did not reach significance. This trend was most probably secondary to the correlation between testis size and the preservation of song: birds with larger testes were significantly less likely to change their songs, and birds housed in visual contact with females were more likely to have larger testes (although again the relationship between song context and testis size was not significant). We also examined the effect of age and HVC volume on adult male song plasticity after nerve injury, but testis size, which is related to reproductive condition and to circulating testosterone, was the best and only significant predictor of whether and how much a bird changed its song. Testis size accounted for over half of the

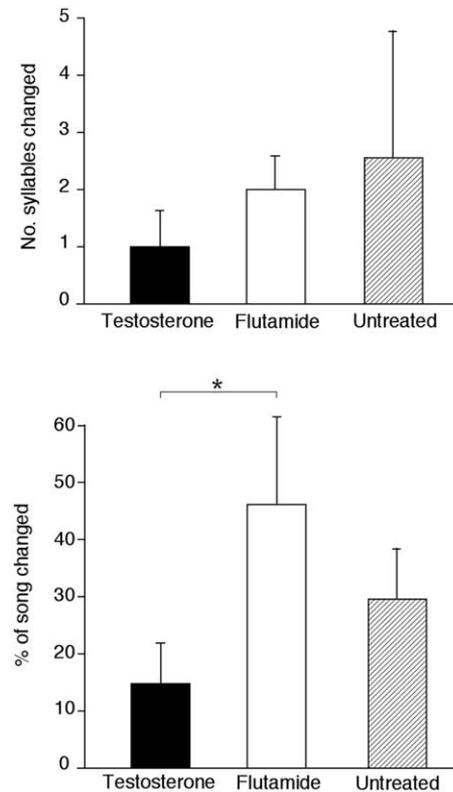


Fig. 4. Flutamide-treated birds changed a larger proportion of their songs than did testosterone-treated birds after ts nerve injury. A single asterisk indicates a significant difference ($P < 0.05$) between the testosterone ($n = 6$) and flutamide ($n = 6$) groups in the percentage of the song changed. Comparisons with untreated animals ($n = 8$) did not reach significance. Error bars represent the standard error of the mean.

variance in the amount of song changes observed ($r^2 = 0.53$).

The direct test of the effect of testosterone on experimentally induced song plasticity in adult male zebra finches confirmed the correlation between testis size and adult song plasticity. Birds treated with flutamide, a testosterone receptor antagonist, showed more song plasticity after ts nerve injury than did birds that received testosterone implants of a size that induced high, but still physiological, T levels. The proportion of song that was changed in a reference group of untreated birds was intermediate between the measures for the experimental groups; although the reference group did not differ significantly from either of the treatment groups, its intermediate value suggests that both testosterone and flutamide may have affected song plasticity, in opposite directions.

The timing and type of song changes seen in these two studies were similar to those seen previously in other studies using ts nerve injury (Williams and McKibben, 1992) and to those seen after deafening (Nordeen and Nordeen, 1992) and introduction of altered auditory feedback (Leonardo and Konishi, 1999). The song changes often were delayed, occurring several weeks after ts nerve injury. The song changes did not include the learning of new material, but

rather consisted of alterations to the existing stereotyped song—a form of plasticity that does not normally occur in adult zebra finches. Song plasticity in response to ts nerve injury or deafening does not occur in LMAN-lesioned birds (Williams and Mehta, 1999; Brainard and Doupe, 2000), and thus we can infer that adult plasticity requires the participation of the song system's basal ganglia circuit, which is necessary for song learning but not song production in zebra finches (Bottjer et al., 1984; Scharff and Nottebohm, 1991).

Although there are minor structural differences between directed and undirected song in zebra finches, the importance of distinguishing between these two types of singing behavior was not fully appreciated until the demonstration of dramatic differences in the pattern of ZENK induction in the song system. The expression of ZENK, an “early immediate gene,” increases dramatically in the song system within minutes after bouts of singing, but appears in LMAN and Area X (which are both part of the basal ganglia circuit of the song system) at high levels only after undirected song—and not after courtship song directed at a female (Jarvis et al., 1998). We had expected that differences in song context afforded by different housing conditions might result in differences in song plasticity, acting through long-term differences in the patterns of gene activation in the basal ganglia portion of the song circuit. However, our data indicate that differences in testis size and concomitant differences in circulating T concentrations are a more important factor in determining the potential for adult song plasticity in zebra finches.

Testosterone and song plasticity

Testosterone has long been known to affect song behavior; the first reports that increased T levels were associated with increased song production in female canaries were published over 60 years ago (Shoemaker, 1939; Baldwin et al., 1940). In seasonally breeding species such as canaries, the highest T levels in the yearly cycle correspond to the peak of the breeding season, when song is most stereotyped (Nottebohm et al., 1987). As open-ended learners, canaries remodel their songs each year; this song plasticity peaks in late summer and early fall, when testosterone levels are at their lowest and song nuclei are correspondingly small. Further, the seasonal changes in hormone concentrations are paralleled by hormonally mediated seasonal changes in singing behavior and in the volume of forebrain nuclei that mediate song production: song system nuclei are largest during the peak of the breeding season when males have maximal T concentrations and sing their loudest, most stereotyped songs (Nottebohm et al., 1986). In age-limited learners such as zebra finches, song crystallization marks the end of song learning and hence of plasticity, and this point in development is also marked by a surge in circulating T concentrations (Pröve, 1983). Experimentally raising circulating T concentrations in developing birds results in

premature song crystallization in zebra finches (Korsia and Bottjer, 1991) and in white-crowned sparrows (Whaling et al., 1995) and a reduced repertoire size in juncos (Titus et al., 1997). In the absence of T swamp sparrow song does not crystallize (Marler et al., 1988). Thus there is ample evidence relating high testosterone concentrations to reduced song plasticity in seasonal learners and to the end of plasticity in age-limited learners. Our results demonstrate that testosterone continues to affect adult male zebra finches' potential for song plasticity well beyond crystallization, when plasticity normally ceases in these age-limited learners.

HVc volume

The classic demonstration of steroid hormone effects on the avian brain is the sexual dimorphism in the volume of forebrain song nuclei. Females of many songbird species sing less than males and have correspondingly smaller song nuclei (Nottebohm and Arnold, 1976), and it has long been known that administering testosterone to adult female canaries masculinizes singing behavior (Shoemaker, 1939; Baldwin et al., 1940) as well as the volumes of HVc and RA (Nottebohm, 1980). Larger volumes of HVc are correlated with larger song repertoires in canaries (Nottebohm et al., 1981) and zebra finches (Airey and DeVogd, 2000). Thus one might predict that birds with larger HVc volumes would show greater song plasticity as adults. However, no such relation was found in our study; instead, a male's HVc volume was strongly predicted by his nestmate's HVc volume, even when the nestmate had very different T and adult song plasticity levels. This observation echoes previous results showing song nucleus volumes are heritable, particularly for HVc (Airey et al., 2000). Although higher concentrations of testosterone did affect song production and adult plasticity in our study, this plasticity was probably not mediated by large changes in HVc volume similar to the seasonal changes associated with song learning in the canary.

Potential mechanisms for testosterone effects on adult song plasticity

Neurons in several nuclei within the song system, including HVc, RA, LMAN, and nXIIIts, concentrate steroid hormones (Arnold et al., 1976) and express androgen receptor mRNA (Metzdorf et al., 1999) and androgen receptors (Gahr, 1990). Testosterone may also affect song system nuclei through its metabolites, in particular estradiol—the avian brain has high concentrations of aromatase (Schlinger and Arnold, 1991) and neurons in the song system are known to have estrogen receptors (Gahr et al., 1987). Experimental evidence suggests several mechanisms for a role of testosterone and its metabolites in regulating neural plasticity within song circuits, including (1) myelination, (2) modulation of norepinephric inputs, (3) changes in synaptic

properties, and (4) birth and incorporation of new neurons. Some of these T effects have been demonstrated only during development, but in the absence of any information about their potential for modulation of adult plasticity they must be considered.

Myelination of projections between song system nuclei and within these nuclei occurs at around 90 days (Herrmann and Bischof, 1986), the time of song crystallization in zebra finches, and appears to be regulated by the surge of testosterone that coincides with sexual maturity and the end of song learning (Kafitz et al., 1992; Stocker et al., 1994). All studies to date have investigated the regulation of myelination of the song system during development, but if the degree of myelination in adults is regulated by steroid hormone concentrations, the ability to remodel axonal trajectories might be a possible testosterone-modulated mechanism for regulating song plasticity.

Avian song nuclei have higher levels of catecholamine innervation than surrounding brain tissue (Lewis et al., 1981; Bottjer, 1993), and steroid hormones modulate catecholamine levels and turnover within the song system (Barclay and Harding, 1988), presumably by acting on steroid receptors in the catecholamine-producing neurons of brainstem nuclei (Maney et al., 2001). Increased norepinephrine (NE) within the song system is correlated with increased levels of courtship song (Barclay et al., 1992, 1996) and a reduction in the strength of auditory inputs to HVC and RA (Dave et al. 1998). As accurate auditory feedback is necessary for song learning and for song maintenance over the long term in adult zebra finches (Nordeen and Nordeen, 1992; Leonardo and Konishi, 1999), this testosterone-mediated gating of auditory activity within HVC might reduce the potential for plasticity in birds with high testosterone levels. When testosterone concentration is low, the NE level within HVC and RA would also be reduced, increasing the strength of auditory inputs to these areas—this augmented auditory input might then be permissive for the types of song changes we observed.

Testosterone alters the electrophysiological properties of synaptic potentials by regulating NMDA receptor activity within the song system of developing male zebra finches (White et al., 1999). Testosterone also increases the number of dendritic spines on which these synapses form (Canady et al., 1988). The underlying mechanism for these electrophysiological changes is most probably a testosterone-induced alteration in the expression of mRNA for specific NMDA receptor subunits (Singh et al., 2000, 2003; Heinrich et al., 2002); the distribution of NMDA receptor subunit types also changes during normal development in male zebra finches. These results are particularly intriguing because they occur in the LMAN-RA projection, involving the portion of the songbird brain circuitry that is associated with song development but not adult song production in zebra finches. Adult song plasticity of the type demonstrated here does not take place in birds with LMAN lesions (Williams and Mehta, 1999; Brainard and Doupe, 2000), which sug-

gests that this portion of the circuit is necessary for song changes in the adult brain. However, the developmental shift in NMDA receptor properties is unlikely to be the basis for change in song plasticity, as young male zebra finches deprived of social song tutors retain the ability to learn songs beyond the normal age for song crystallization, even though their NMDA receptor properties mature normally (Livingston et al., 2000).

Perhaps the most intriguing potential mechanism for testosterone-mediated regulation of adult plasticity is the possibility that song changes rely upon the birth and incorporation of new neurons. In all songbird species that have been examined, neurons continue to be born and incorporated into the HVC of adults (Goldman and Nottebohm, 1985; Nottebohm, 1985; Barnea and Nottebohm, 1994). In zebra finches adult neurogenesis does not normally have any function in song relearning or plasticity, as adult zebra finches do not change their songs after reaching sexual maturity. Interestingly, the Bengalese finch, a closely related species, has a greater degree of adult song plasticity and also incorporates new neurons into HVC at a much higher rate than the zebra finch (Scott et al., 2000). This finding suggests that the incorporation of new neurons into song circuitry might play a role in the remodeling of the crystallized song in the type of adult plasticity described here. If so, the relationship between testosterone and the birth, recruitment, and survival of new neurons would bear directly on the potential for song plasticity. Higher concentrations of circulating testosterone are associated with greater survival of new neurons within the canary brain (Rasika et al., 1994). Exogenous testosterone also leads to elevated BDNF levels and higher survival of new neurons in canaries (Rasika et al., 1999). The interactions among testosterone, BDNF, and new neuron survival may be mediated by local effects of T upon angiogenesis (Louissaint et al., 2002). Regardless of the exact mechanism, testosterone ultimately increases the survival of new neurons and thus has the consequence of reducing turnover of cells within HVC. This neuronal stability could then lead to increased song stereotypy, which would in turn reduce the potential for plasticity in birds with high circulating T concentrations.

It has been suggested that some of the effects of testosterone and its metabolites on the avian song system may be secondary to the physiological effects of singing (Ball et al., 2002). In this scenario, steroid hormones act on motivational centers in the brain, increasing the amount of song production, and this increase in neural activity within the song system nuclei induces increased expression of transcription and growth factors such as ZENK and BDNF, which in turn increases survival of new neurons in the song circuit. Higher song rates increase the survival of new neurons in the HVC of castrated canaries (Alvarez-Borda and Nottebohm, 2002), indicating that song rates affect survival of new neurons independently of testosterone. In the first portion of our study, male zebra finches' opportunities to sing directed song were controlled by regulating their visual

access to females. We noticed, but did not systematically quantify, one behavioral effect of this regime: males housed without visual access to females sang less often and with a longer latency to their first song when they were exposed to females during the weekly recording sessions. As Arnold first showed in 1975, T treatment increases song and courtship rates in adult male zebra finches; the T-treated males in our hormonal manipulation study were vigorous singers. Thus it is possible that reduced opportunities for courtship song result in reduced singing and also contribute to reduced testicular growth and thus to lower circulating T concentrations. It may be the reduced song rate rather than the lower T concentrations that engages the brain mechanisms that allow for increased song plasticity. Testosterone might then affect brain mechanisms for plasticity either directly, by increasing neuronal survival through its effect on BDNF levels, or indirectly, by modulating the rate of singing and thus engaging the activity-related mechanisms within the song circuit.

Although the mechanism responsible for testosterone's effect on adult plasticity of age-limited learners is not clear, establishing that testosterone (and/or its metabolites) is related to adult song plasticity is an important step toward understanding how processes that are normally not accessible by adults might be reengaged in response to injury or intervention within the song system.

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