Chronic Testosterone Treatment Impairs Vocal Learning in Male Zebra Finches during a Restricted Period of Development

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To study the effects of chronic exposure to testosterone on song behavior development, we administered various amounts of testosterone to juvenile male zebra finches during different periods of song acquisition. We report that testosterone exposure during development profoundly impairs song learning in juvenile males. The effects of the hormone do not seem to be dose related but vary according to the period of life during which testosterone is administered. Exposure to testosterone starting before day 40 and lasting until adulthood decreases the number of syllables in the birds' repertoire and increases the number of phrases per bout of singing. In addition to these changes in the "syntactical" features of song, acoustic abnormalities are also present in the song syllables of males that begin to receive testosterone during the first month of life. Administration of testosterone during only the first 3 weeks of life produces acoustic abnormalities but has no effect on repertoire size or bout structure. Birds receiving the hormone between 20 and 40 d of age develop both acoustic abnormalities and a reduced repertoire size, but their song bouts contain a normal number of song phrases. Exposure to testosterone starting on day 40 produces no behavioral abnormalities. These results indicate that normal song development requires a period of low levels of circulating testosterone and also provide support for the idea that vocal learning entails a progressive sequence of events.

Song production is a learned, sexually dimorphic behavior in many species of songbirds. In zebra finches (*Poephila guttata*), males learn to produce a species-typical courtship song during a restricted period of development (see Bottjer and Arnold, 1986, for review). Song learning begins with a sensory phase, during which juvenile males must hear the song they will subsequently produce. Auditory experience during the first 1–2 months of life is necessary for acquisition of a so-called auditory "template" (Immelmann, 1969; Bohner, 1983; Eales, 1985; Clayton 1987a,b). The second phase of song development is sensorimotor and overlaps partially with the phase of auditory learning. Around 25 d of age, juvenile male zebra finches begin to produce initial songlike vocalizations that are extremely variable and bear little resemblance to the bird's final song. During this period of subsong, vocal patterns are progressively adjusted to match the previously acquired song model (template). Temporal order and modulation of song syllables begin to be produced in a stereotyped fashion starting around day 50, and by 90 d of age, male zebra finches produce a highly stereotyped courtship song that remains unchanged throughout adulthood (Immelmann, 1969; Arnold, 1975a).

Both the development of song in juvenile males and the production of stereotyped song by adults are known to be dependent on plasma levels of androgens. Castration of adult males decreases the incidence of song production without changing song structure, and systemic administration of testosterone (T) to castrated adult males restores song production (Prove, 1974; Arnold, 1975b; Harding et al., 1983). In juvenile male zebra finches, plasma levels of T are elevated shortly after hatching, decrease at the end of the first week of life, and then remain low throughout the first 2 months of life (see Prove, 1983; Hutchison et al., 1984; Adkins-Regan et al., 1990). Transient increases in T levels have been observed around day 18 and day 35 (Prove, 1983; but see Adkins-Regan et al., 1990), although plasma levels of T do not seem to increase permanently until day 75 (Prove, 1983). Castration combined with antisteroid treatment of juvenile males disrupts normal development of vocal behavior by preventing acquisition of stereotyped song (Bottjer and Hewer, 1988). This effect can be reversed by delayed exposure to T even after the normal learning period (i.e., after day 95; Bottjer and Hewer, 1989). In addition, Nottebohm et al. (1987) reported that in canaries, a species in which adult males learn new vocal patterns each breeding season, a decrease in plasma levels of T tends to occur approximately 1 month prior to peak periods of new syllable acquisition. Conversely, increasing T levels seem to accompany the onset of song stereotypy in this species. Young male swamp sparrows that are able to learn their song from live tutors tend to have higher blood levels of estradiol (a metabolite of T) than males that cannot learn in similar conditions (Marler et al., 1987). However, the ability to acquire new vocal patterns is reduced when peak levels of estradiol are present (Marler et al., 1987; see also Marler, 1987). Furthermore, T levels rise in synchrony with the onset of song production in young swamp sparrows (Marler et al., 1987), and castrated juvenile male song and swamp sparrows fail to crystallize song (Marler et al., 1988). Taken together, the overall pattern of results concerning the relationship between hormone levels and vocal behavior suggests that low levels of T may be necessary for plasticity in song behavior, whereas high titers of T may contribute to the loss of behavioral plasticity (i.e., to the acquisition of stereotyped song behavior).

If testosterone acts to curtail the capacity for new learning, then chronic T exposure before the time of endogenous increase in hormone levels might prevent normal song development by

prematurely closing the period of plasticity in vocal behavior. In accord with this hypothesis, we report here that maintaining high levels of circulating testosterone in juvenile male zebra finches produces a dramatic impoverishment of adult vocal behavior. Furthermore, we show that exposure to testosterone must occur during an early, restricted period of development in order to impair vocal learning. This is the first demonstration of which we are aware in which chronic administration of an endogenous hormone has been used to assess its influence during behavioral development. Traditionally, studies of this type have tried to remove or block the effect of gonadal steroids in order to elucidate their roles. The alternative strategy of keeping circulating levels of hormone high (a "testosterone clamp") represents a novel and complementary approach that will increase our knowledge of the range of potential effects exerted by steroids and that may be better suited to studying the importance of timing of exposure to gonadal hormones.

Materials and Methods

Testosterone administration protocols

One hundred and nine male zebra finches, hatched in our aviary, were used in this study. Birds were divided into eight experimental groups and received systemic administration of testosterone (4-androsten-17B-ol-3-one, Steraloids) according to different protocols. Two types of T implant were used. For younger birds (less than 15 d old), T was mixed with Silastic (738RTV, Dow Corning), injected into polyethylene tubing (i.d. 0.58 mm, o.d. 0.97 mm; Clay-Adams) at a concentration of 50 μg of T/mm, and implanted subcutaneously in the rump region. Release of T occurs only from the cut end of these polyethylene implants (Gurney, 1981). For older birds (15 d and older), Silastic tubing (i.d. 0.76 mm, o.d. 1.65 mm; Dow Corning) was packed with 5, 10, or 20 mm of crystalline T, sealed with Silastic glue, incubated overnight in 0.75% saline, and implanted subcutaneously over the pectoral muscles. Release of T occurs along the entire length of the Silastic tubing (Smith et al., 1977).

Experiment 1: effects of continuous T administration and delays in onset of T treatment

In this experiment, birds received systemic administration of T beginning at different ages and lasting until the birds were fully adult (i.e., until at least 95 d of age). Once initiated, T exposure was continued throughout the experiment, including at the time song behavior was recorded in adults. To investigate whether the effects of chronic exposure to T are dose related, birds received Silastic implants that were either 5, 10, or 20 mm long.

T_{1-95} group (n = 19). These birds received exogenous T during their entire life in order to investigate the effects of continuous T administration on song behavior. Various lengths of polyethylene implants were given every 3 d beginning on the day of hatching (50 μg on day 1 and day 4, 75 μg on day 7, 100 μg on day 10, 125 μg on day 13, and 150 μg on day 17). These lengths of polyethylene implants were calculated so as to maintain a rate of release of roughly 0.01 mg T/gm/d (as measured by Gurney, 1981). On day 20, these birds received subcutaneous administration of a Silastic implant packed with either 5 (n = 3), 10 (n = 6), or 20 mm (n = 10) of crystalline T that was left in place throughout the experiment.

Other experimental groups. In order to determine the age at which high circulating levels of T would cease to exert a disruptive effect on vocal development, the onset of T administration was delayed until progressively later ages. Birds in these groups received a 5-, 10-, or 20-mm Silastic T implant at either

day 15 (T_{15-95} group, n = 14) (5 mm, n = 3; 10 mm, n = 6; 20 mm, n = 5),

day 20 (T_{20-95} group, n = 15) (5 mm, n = 6; 10 mm, n = 5; 20 mm, n = 4),

day 25 (T_{25-95} group, n = 11) (5 mm, n = 2; 10 mm, n = 5; 20 mm, n = 4),

day 30 (T_{30-95} group, n = 11) (5 mm, n = 3; 10 mm, n = 5; 20 mm, n = 3),

day 40 (T_{40-95} group, n = 8) (5 mm, n = 3; 10 mm, n = 3; 20 mm, n = 2).

These Silastic implants were left in place throughout the experiment.

Control group. Nineteen birds received empty implants of the same type and on the same schedules as the various treatments described above. Since no significant differences were observed between control subgroups, they will be considered as a single group hereafter. In addition, to assess the effects of high plasma levels of T on adult song behavior at the time of song recording, 5 of these 19 control males received a Silastic implant (10 mm, n = 2; 20 mm, n = 3) after 95 d of age, and their song behavior was recorded both before and after implantation.

Data collection

Birds were segregated from their parents at 60 d of age and housed in individual cages in a single room. Beginning on day 90, song recordings were made with a Tandberg 3300X or a TEAC X-300 tape recorder (3¾ inches/sec). Only song behavior produced in early morning was recorded (>10 min/bird). None of the songs recorded were directed toward females (see Sossinka and Bohner, 1980).

Between 95 and 110 d of age, after song behavior had been recorded, blood was collected from each bird (wing vein, >250 μl) using heparinized microhematocrit tubes and centrifuged for 10 min at 3200 rpm. Plasma was collected and immediately frozen at −20°C. Following blood sampling, birds were overdosed and perfused with 0.75% saline and 10% buffered formalin. Testes and syrinx were removed and stored in 10% buffered formalin. In some groups, syrinx wet weight was recorded after trimming the trachea and bronchi. Implants were removed before perfusion and checked for residual quantities of T. In all groups, implants were found to contain some residual amount of crystalline T at the end of the experiment.

Song analysis

An observer listened to the recorded behavior of each bird and assigned it a score between 1 and 5 indicating song quality in terms of stereotypy, syllable variety, and syllable modulation (1 = very poor, 5 = very good). The observer did not know the experimental treatment of any bird. Although subjective, this type of evaluation takes advantage of the capacity of the human ear for discriminating sound quality and reflects both tonal quality and overall structure of song behavior.

In addition, song recordings were analyzed on a Kay Elemetrics DS 5500 Sona-Graph. Male zebra finches have a relatively simple song composed of bouts of singing, which are separated by periods of silence lasting from a few seconds to several minutes. Each bout starts with a few typical introductory syllables, followed by repetitions of a single song phrase (see Fig. 1 for an example of normal song). This phrase is composed of different syllables (any continuous sound separated by a silent interval) that are usually not repeated within the song phrase (Price, 1979; for review and different terminologies, see Sossinka and Bohner, 1980; Slater et al., 1988; Cynx, 1990). For each bird, we recorded the average number of song phrases per bout as well as the maximal number of song phrases per bout of singing. In addition, 10–12 song phrases were randomly selected and printed (as plots of frequency over time). From these printed sonograms, we measured the number of different syllables in the repertoire of each bird, the average song phrase duration, the average duration of individual syllables, and the tempo of the song phrase (i.e., the ratio of number of syllables in the song phrase to its average duration). We also evaluated song stereotypy by visually comparing different renditions of each syllable and each song phrase.

Hormonal level measurements

Plasma levels of T were assayed using a solid-phase ^{125}I radioimmunoassay (Coat-A-Count Total Testosterone, Diagnostic Products Corp.) designed to measure total amount of T in unextracted plasma. This assay has a detection limit of approximately 0.04 ng/ml (0.21 nmol/liter) and an interassay variation of roughly 5% at most of the concentrations reported here. The antibody is highly specific for testosterone, showing approximately 3% cross-reactivity with 5-α dihydrotestosterone and 16–20% cross-reactivity with 4-estren-17-ol-3-one, 19-nortestosterone, and 11-ketotestosterone.
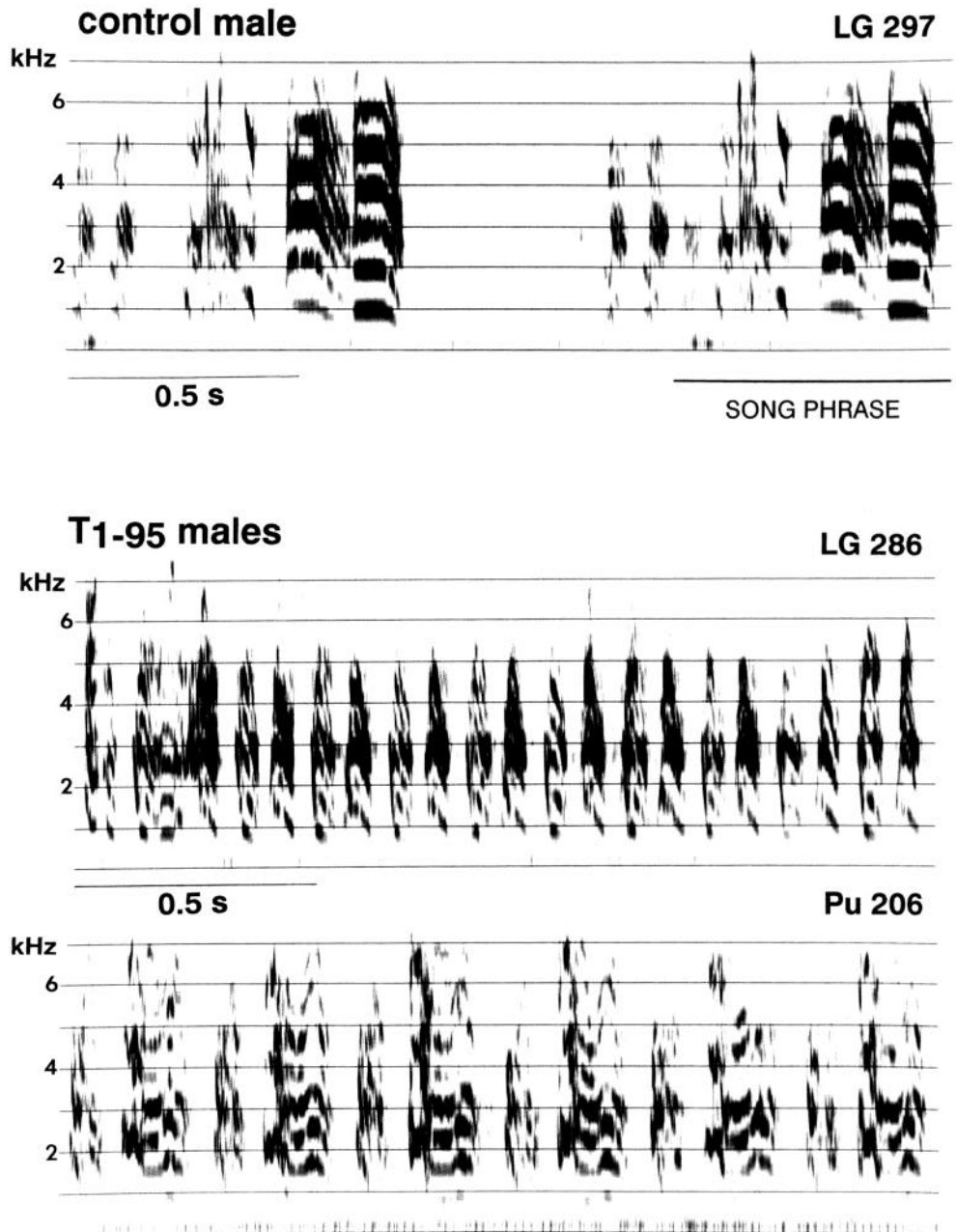
control male LG 297

kHz
6
4
2

0.5 s

SONG PHRASE

T1-95 males LG 286

kHz
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4
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0.5 s

Pu 206

kHz
6
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2

Figure 1. Examples of song behavior in control and T_{1-95} birds. Graphs are sonograms (*x-axis,* time; *y-axis,* frequency). A syllable is any continuous sound separated by a silent interval; a song phrase is composed of syllables arranged in a characteristic sequence. The *upper panel* shows two repetitions of the song phrase of a control bird; each iteration of the song phrase is preceded by two introductory syllables. The *lower panels* demonstrate that songs of two different T_{1-95} males show a dramatic reduction in the number of different syllables and an increase in the number of songs per bout.

Experiment 2: effects of transient administration of T on song development

In this experiment, we investigated whether effects of T on adult song behavior vary with the developmental timing of hormone administration. Birds received systemic administration of T during a restricted period of early development, either between hatching and day 20 or between day 20 and day 40. At the end of T exposure, implants were removed, and no implants were present at the time song behavior was recorded in adults (>95 d of age). Birds were killed immediately after song recording.

T_{1-20} *group (n = 5).* Birds in this group were initially treated identically to those in the T_{1-95} group, receiving a polyethylene implant every third day up until day 17. Implants were removed on day 20, and no further hormonal treatment was given.

T_{20-40} *group (n = 7).* These birds received a 5-mm Silastic implant on day 20 that was removed on day 40. No further hormonal treatment was given.

Data collection, song analysis and hormonal level measurements were performed as in Experiment 1. For two groups of birds (control and T_{1-20} groups), we printed the power spectrum (amplitude as a function of frequency) of five renditions of each syllable in each bird's song. In order to measure the use of well-defined harmonics in each syllable, we traced the outline of each power spectrum on a digitizing tablet interfaced with a microcomputer, which calculated the total length of the outline. This parameter is maximal when a syllable is composed of related harmonics and decreases when the syllable contains a broad range of frequencies (Fig. 2).

Statistical comparisons between groups in both Experiment 1 and Experiment 2 used a two-tailed one-way analysis of variance, except for the pre- versus postimplantation comparisons in five adult control males which used an analysis of variance for repeated measures.

Results

Plasma testosterone levels

As expected, we observed a direct relationship between the amount of T given to birds and their plasma levels (see Table
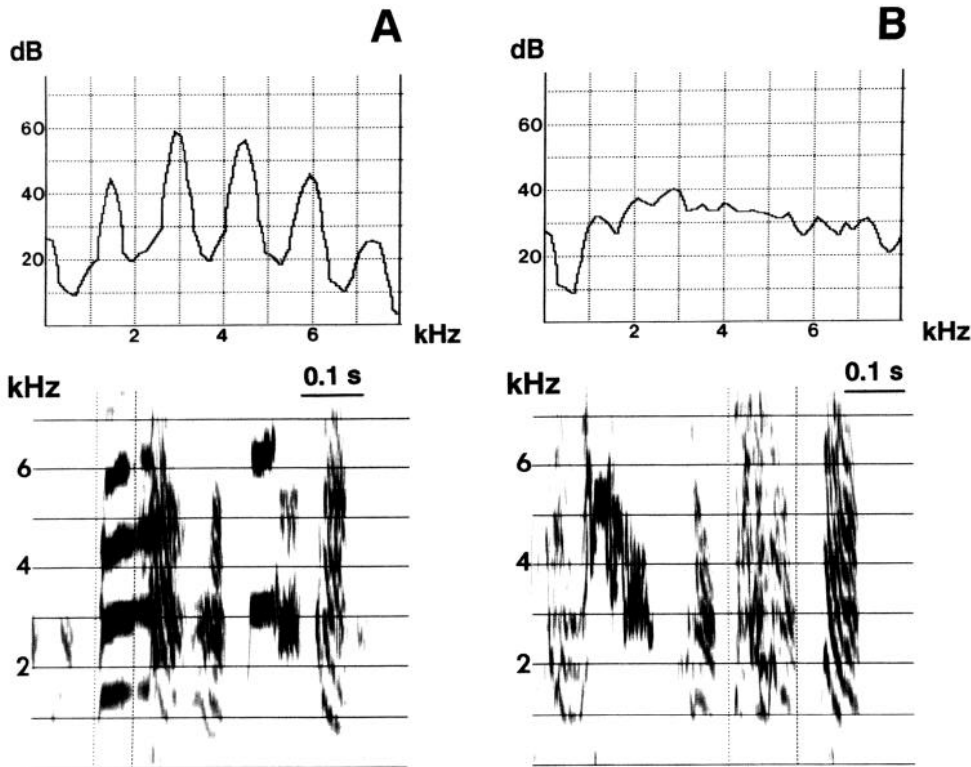
Figure 2. Two examples of syllables with their respective power spectra. The *lower graphs* are sonograms of two song phrases (*left*, control; *right*, T_{1-20} male; *x-axis*, time; *y-axis*, frequency). The *upper graphs* plot the power spectrum (*x-axis*, frequency; *y-axis*, amplitude) of one particular syllable in each song, isolated between the *dashed lines* on the sonogram. The syllable on the *left* is composed of frequencies that are harmonically related, whereas the syllable on the *right* contains many unrelated frequencies. Consequently, the *outline* of the *left power spectrum* is longer than the *outline* of the *right power spectrum*.

1, Experiment 1). It is interesting to notice that our control birds had very low levels of endogenous T compared to those reported elsewhere for normal adult male zebra finches. Reported T levels in adult males range from 0.3 ng/ml (Hutchison et al., 1984) to 1.0 ng/ml (Luine et al., 1980). Our males were housed singly in individual cages without contact with any female, and this non-breeding environment may have been responsible for the low T levels we observed. We failed to observe any significant difference in circulating T levels of birds that received the same size of Silastic implant regardless of whether the implant was given at 15, 20, 25, 30, or 40 d of age (data not shown). Thus, for the purpose of analyzing plasma hormonal levels, we considered all birds that received the same length of Silastic implant as a single group, regardless of age at implantation. In addition, for statistical comparisons, birds were divided into two populations depending on whether they had a Silastic implant at the time of blood collection (i.e., Experiment 1 vs. Experiment 2). By reducing the total variance in each population, this strategy enables the detection of small differences between groups that have low plasma levels.

Plasma levels of T in birds that had previously received poly-ethylene implants during the first 20 d of life (T_{1-20} group) were slightly higher than adult control birds despite the absence of implants in the T-treated birds at the time of blood collection [$F(1,22) = 8.4$, $p < 0.01$]. However, the ranges of plasma levels of T in these two groups were widely overlapping. As part of another study, we collected blood samples in six 15-d-old males that received the same treatment as the T_{1-20} birds. Plasma T levels were higher in these birds than in five 15-d-old control males [T-treated males: 1.07 ± 0.58 ng/ml; control males: all <0.1 ng/ml, $F(1,9) = 13.7$, $p < 0.01$]. Thus, polyethylene implants were effective in raising plasma T levels to physiological adult values during the first 20 d of life.

Plasma levels of T in birds that had received a 5-mm implant between day 20 and day 40 (T_{20-40} group) were not significantly different from adult control birds [$F(1,22) = 3.0$, $p > 0.09$]. Thus, we feel confident that T_{1-20} and T_{20-40} birds had physiological levels of circulating T at the time their song behavior was recorded.

For the birds that received a Silastic implant up to the end of the experiment, only birds that received 5-mm implants had plasma T levels that overlapped with the range of T levels observed in normal adult breeding males (Luine et al., 1980; Hutchison et al., 1984). However, their plasma levels of T were significantly larger than those observed in control, T_{1-20}, and T_{20-40} groups [$F(1,41)$ always > 43.1, p always < 0.0001]. Males that received 10- or 20-mm Silastic implants had T levels that were significantly higher than levels observed in males that received 5-mm implants [$F(1,52) = 11.3$, $p < 0.003$ and $F(1,52) = 52.5$, $p < 0.0001$, respectively]. Males that received a 20-mm implant had larger circulating levels of T than birds that received a 10-mm implant [$F(1,52) = 31.9$, $p < 0.0001$].

Table 1. Plasma levels of testosterone in adult male zebra finches

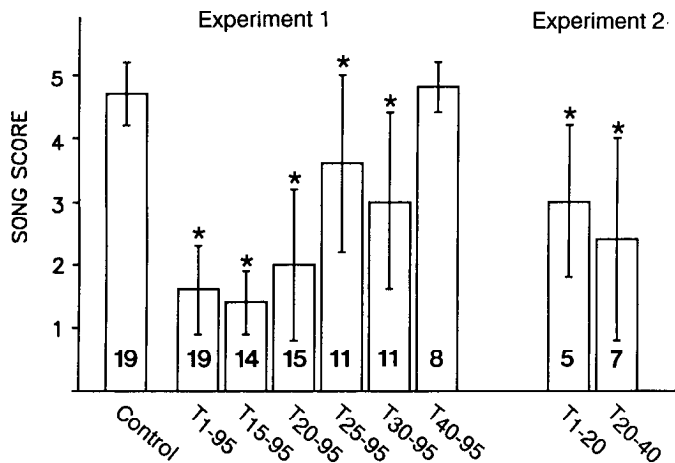| Type of implant	T levels[a] (ng/ml)	n
Control	0.17 ± 0.13 (<0.1–0.6)	13
Experiment 1		
5-mm Silastic	$2.68 \pm$ 96 (0.8–4.4)	20
10-mm Silastic	4.37 ± 2.01 (1.7–6.2)	18
20-mm Silastic	11.10 ± 5.90 (5.0–18.0)	17
Experiment 2		
Polyethylene (T_{1-20})	0.48 ± 0.22 (0.2–0.7)	5
5-mm Silastic (T_{20-40})	0.33 ± 0.30 (0.1–0.9)	7

[a] Mean $\pm$ SD (range).

Figure 3. Song scores for control and T-treated males (means ± SD). *Asterisks* indicate groups that were significantly worse than controls (*p* < 0.05). See Results for additional details.

To investigate peripheral effects of such elevated plasma T levels, we weighed the syringes of 8 control adult males and 14 T_{1-95} males (6 that received a 10-mm Silastic implant and 8 that received a 20-mm Silastic implant). Syringeal muscles are androgen-sensitive and sexually dimorphic in zebra finches (Luine et al., 1980). The average syringeal weights of these latter two groups (10 mm: 21.7 ± 3.7 mg; 20 mm: 22.9 ± 3.9 mg) were not significantly different from the average syringeal weight of control birds (23.8 ± 4.2 mg; *F* always < 1). All birds in Experiment 1 had drastically regressed testes, presumably as a result of high circulating levels of T. In Experiment 2, birds had fully developed testes that were similar in size and appearance to those found in control males.

We did not observe any symptoms of general toxicity linked to chronic exposure to testosterone. Rates of mortality did not differ between control and experimental groups; in both groups, almost all deaths occurred either shortly after hatching or during the fledgling period, and appeared to be associated with inadequate care by parents. All of the birds that survived appeared to be extremely healthy, and we could not detect any differences between control and experimental birds either during early development or during the period following 60 d of age when birds were isolated from breeding aviaries and housed in individual cages for subsequent recording. In addition, it should be emphasized that juvenile male zebra finches normally have quite high levels of circulating T during the first week of life, followed by low testosterone titers until the time of puberty. For example, both Hutchison et al. (1984) and Adkins-Regan et al. (1990) have reported T levels ranging from approximately 1.0 to 3.5 ng/ml in juvenile males between 1 and 4 d of age.

Experiment 1: effects of continuous T administration and delays in onset of T treatment

We did not observe any difference between birds that received 5-, 10-, or 20-mm implants for any behavioral parameter that we measured (*F* always < 2.9, *p* always > 0.07). Therefore, males within a single experimental group that received either 5, 10, or 20 mm of T according to the same schedule are collapsed across dosage in the following presentation of the results.

In the presentation of our results, we will distinguish two types of song abnormalities. "Acoustic" abnormalities will refer to

changes in acoustic properties of individual syllables as observed on a sonogram (e.g., the incidence of harmonics in each song syllable). Abnormalities in "syntax" will refer to structural changes in the overall song pattern, such as changes in the variety, arrangement, and interrelationship of syllables in song phrases and of phrases in song bouts. Decreases in the number of different syllables in song phrases and increases in the number of phrases per song bout are examples of syntactical abnormalities.

Song score. Chronic T administration had a striking effect on the overall quality of song, as indicated by song scores (see left part of Fig. 3). Birds that received continuous T treatment starting prior to day 40 had significantly poorer songs than both control birds [$F(1,100)$ always > 7.3, *p* always < 0.01] and males that received chronic T starting on day 40 [$F(1,100)$ always > 6.1, *p* always < 0.02]. Furthermore, delaying the onset of T administration seemed to decrease the tendency of the hormone to alter song quality: there was a gradual improvement in song quality when birds were exposed to T at progressively later ages. Birds that started to receive T on day 25 had a better song quality than birds that received T from day 20 onward [$F(1,100) = 18.2, p = 0.0003$] but a poorer song than birds that were implanted at day 40 [$F(1,100) = 6.1, p < 0.02$]. T_{30-95} birds were the only exception with a slightly poorer song than T_{25-95} birds [$F(1,100) = 4.1, p < 0.05$]. No significant difference was observed between birds that received T earlier than day 25 (*p* always > 0.1). Thus, chronic T treatment decreases song quality when exposure begins before 40 d of age, and there seems to be a progressive reduction in the efficacy of such treatment when its onset is delayed during the first month of life.

Song syntax. Since chronic T administration affected overall song quality as indicated by the difference in song scores, we tried to determine whether any change in the syntax of the overall song pattern was associated with the decrease in song quality. As shown in Figure 4*A,* birds that received continuous T administration starting prior to day 40 had shorter song phrases than control birds [$F(1,100)$ always > 6.4, *p* always < 0.02]. In T_{1-95} and T_{15-95} birds, song phrases were half the duration of those of control birds. Interestingly, only T_{1-95}, T_{15-95}, and T_{25-95} birds had shorter song phrases than T_{40-95} birds [$F(1,100)$ always > 4.0, *p* always < 0.05]. In contrast to the pattern seen for overall quality of song (Fig. 3), delaying the onset of exposure to testosterone did not seem to have a major effect on the shortening of song phrases as long as T administration began before day 40. Despite a significant increase in phrase duration in T_{20-95} birds compared to T_{15-95} birds [$F(1,100) = 8.5, p < 0.005$] and in T_{30-95} birds compared to T_{15-95} birds [$F(1,100) = 4.4, p < 0.04$], there was no significant difference between song phrase duration in T_{15-95} versus T_{25-95} birds or between T_{30-95} and T_{25-95} males [$F(1,100)$ always < 1.3, *p* always > 0.25].

A reduction in song phrase duration could be the result of either a faster song tempo (number of syllables sung per second) or a reduction in the number of different syllables in the bird's repertoire. However, as shown in Figure 4*B,* we did not observe any major effect of continuous T administration on song tempo. Although T_{15-95} and T_{25-95} birds had a significantly faster tempo than did controls [$F(1,100)$ always > 8.5, *p* always < 0.005], all other groups had song tempi similar to those of control birds [$F(1,100)$ always < 1.7, *p* always > 0.1]. We observed a similar absence of effect on the duration of individual syllables and silent intervals between them (data not shown).

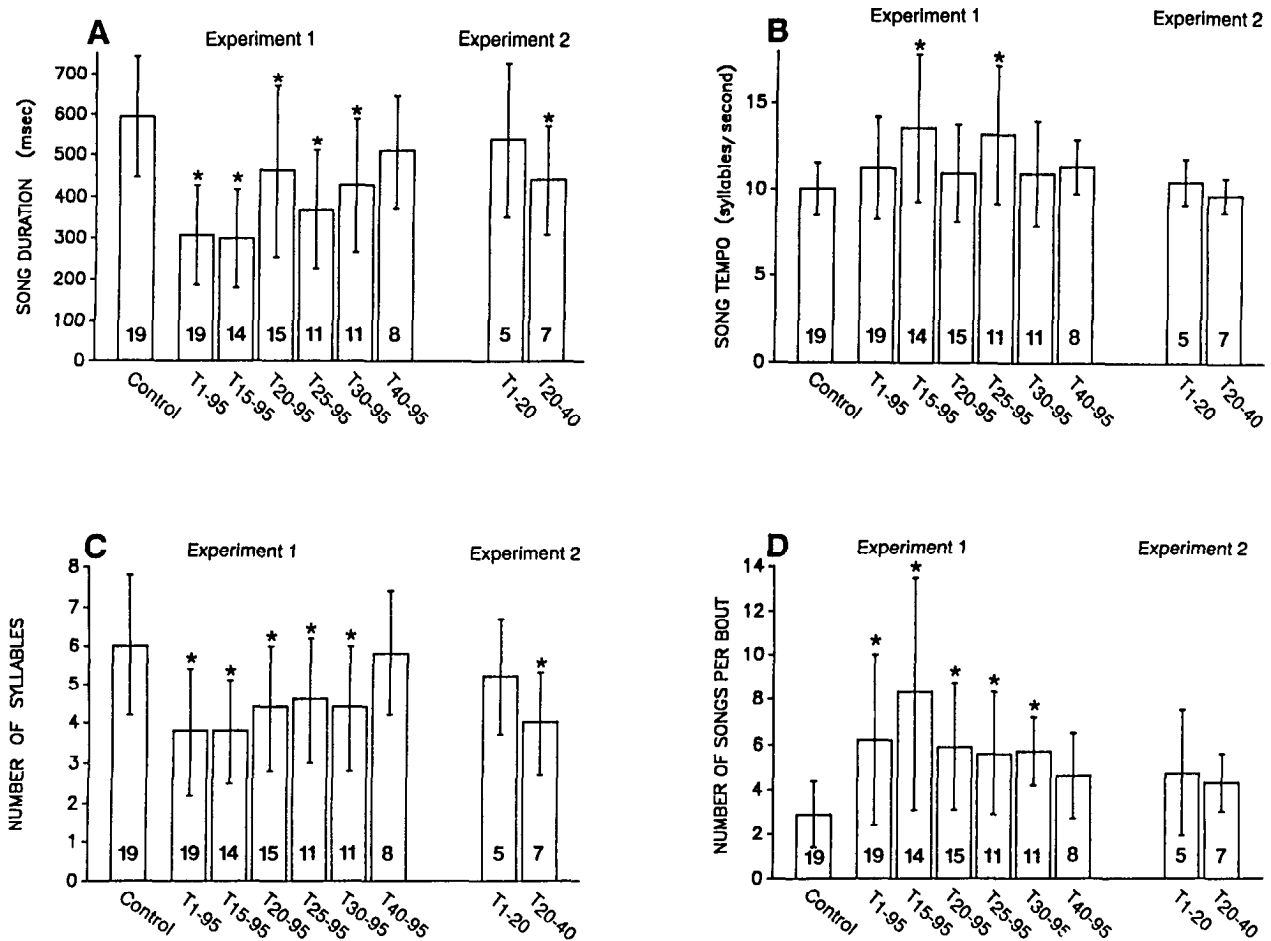As indicated in Figure 4*C,* the shorter duration of song phrases

Figure 4. Graphs showing differences in the syntactical characteristics of the overall song pattern for control and T-treated males. *Asterisks* indicate groups that were significantly different from control males ($p < 0.05$). Bars represent means ± SD. *A*, Average duration of individual song phrases; *B*, number of syllables produced per second; *C*, total number of syllables in individual song phrases; *D*, mean number of song phrases produced per bout. See text for additional details.

in T-treated birds is due to a decrease in the number of different syllables sung by these birds. Continuous T administration starting before day 40 tended to reduce the size of birds' repertoires: birds that received T starting prior to day 40 produced significantly fewer song syllables than did control birds [$F(1,100)$ always > 4.9, p always < 0.03]. Figure 1 shows sonograms of T_{1-95} birds having dramatically impoverished repertoires. Interestingly, only birds that received T prior to day 25 had significantly smaller repertoires than T_{40-95} males [$F(1,100)$ always > 3.8, p always < 0.05]. However, all birds that received chronic T starting prior to day 40 had similar repertoire sizes [$F(1,100)$ always < 1.8, p always > 0.18]. Thus, as described above for song phrase duration, delaying the onset of T exposure during the first month of life did not seem to reduce the effects of such treatment on song repertoire.

Bout syntax. Song phrases are produced in bouts of singing separated by periods of silence. Figure 4D shows the number of song phrases produced per bout for each bird. Birds that received continuous T treatment starting before 40 d of age tended to sing approximately twice as many song phrases per bout as control birds [$F(1,100)$ always > 5.5, p always < 0.02]. Onset of T administration at 15 d of age had a particularly striking effect, since T_{15-95} birds sang more phrases per bout than both T_{20-95} and T_{1-95} birds [$F(1,100)$ = 3.9 and 4.4, respectively;

p always < 0.05]. In some cases, T_{1-95} and T_{15-95} birds were able to sing more than 20 song phrases without interruption, which represents a considerable feat for a bird that usually sings 3–4 phrases per bout, even considering the shorter phrase duration in these birds. Figure 1 shows excerpts of long bouts produced by T_{1-95} birds.

Effects of T administration on songs of normal adult male zebra finches. In order to investigate whether the effects described above might be due to effects of high plasma levels of T at the time of recording, we implanted five fully adult (>95 days) control males with Silastic implants of T. Their song behavior was analyzed both before T treatment and 2–3 weeks after implantation. We found no significant changes between pre- and postimplantation recordings in any of the measures of song behavior we used in this study [$F(1,4)$ always < 1.4, p always > 0.2]. Thus, high plasma T levels at the time of recording are not responsible for the behavioral changes we described. These abnormalities are more likely due to chronic exposure to T during song development.

Experiment 2: effects of T on song behavior during restricted periods of development

Experiment 1 showed that continuous exposure to testosterone beginning before 40 d of age impairs normal song development.

The major syntactical changes responsible for the poor overall song quality of T-treated birds are a reduction in the number of different syllables in the song repertoire and an increase in the number of song phrases sung per bout. Although delaying the onset of exposure to testosterone during the first month of life resulted in progressively less disruption of the overall song score (Fig. 3), delaying exposure to T did not produce correspondingly less disruption of the syntax of song (Fig. 4). It therefore seems possible that exposure to adult levels of T during early development may produce some disruption in the acoustic properties of song, disruption that is not expressed in the song syntax but that is detected by the human ear. These acoustic characteristics may be modified only by early exposure to T, and their progressive improvement may be responsible for the improvement in song scores when the onset of T treatment is delayed. To investigate possible differences in the effects of chronic T treatment depending on the precise time of exposure, we administered T during two distinct, nonoverlapping periods of development, i.e., either from the day of hatching up until day 20 or from day 20 to day 40.

Effects of T administration during the first 20 d of life. Figure 3 shows that birds that received T during the first 3 weeks of life had a significantly poorer song score than control birds [$F(1,100) = 11.9$, $p < 0.002$] but were nonetheless better off than birds that received continuous exposure to T treatment starting before day 25 [$F(1,100)$ always > 4.6, p always < 0.04]. However, T_{1-20} birds did not show any significant changes in song duration and tempo, syllable duration, or song bout syntax when compared to control birds [$F(1,100)$ always < 1.4, p always > 0.24] (see Fig. 4). Thus, administration of T between the day of hatching and day 20 does not seem to alter song syntax. The poorer song scores of T_{1-20} birds therefore seem to suggest that the abnormalities perceived by the human ear are more acoustical than syntactical, i.e., that T_{1-20} birds tend to sing poorly modulated syllables.

As a preliminary investigation of this hypothesis, we analyzed the power spectrum of each syllable in the songs of both T_{1-20} and control birds. We evaluated the incidence of harmonics in each syllable by measuring the total length of the outline of the power spectrum (Fig. 2). T_{1-20} birds had a significantly shorter power spectrum outline than did control birds [186.9 ± 11.9 and 204.7 ± 16.0 mm, respectively; $F(1,22) = 5.3$, $p < 0.03$], suggesting that birds that receive T during the first 3 weeks of life use fewer harmonics in their song than control birds. This reduction in the incidence of harmonics may explain why the songs of T_{1-20} birds scored poorly despite their normal syntax.

Effects of T administration between 20 and 40 d of life. As shown in Figure 3, T_{20-40} birds had significantly poorer song scores than both control birds [$F(1,100) = 27.9$, $p < 0.0001$] and T_{40-95} birds [$F(1,100) = 22.2$, $p < 0.0002$]. Song scores in T_{20-40} males were similar to those observed in T_{20-95} birds [$F(1,100) = 1.3$, $p = 0.26$] and T_{1-20} birds ($F < 1$). Figure 4A shows that T_{20-40} birds produced song phrases that were shorter than those of control males [$F(1,100) = 5.4$, $p < 0.03$] but longer than those of T_{1-95} or T_{15-95} males [$F(1,100)$ always > 3.8, p always < 0.05]. T_{20-40} and T_{20-95} birds were not significantly different ($F < 1$). Figures 4B,C show that this shorter song phrase duration was not due to an increase in song tempo but to a decrease in repertoire size: T_{20-40} birds had fewer different syllables in their song than either control birds [$F(1,100) = 7.9$, $p < 0.007$] or T_{40-95} birds [$F(1,100) = 4.7$, $p < 0.04$]. T_{20-40} and T_{20-95} birds had a similar repertoire ($F < 1$). Interestingly, as

shown in Figure 4D, T_{20-40} birds did not have a larger number of songs per bout than control birds [$F(1,100) = 1$, $p = 0.31$].

Thus, chronic administration of T between 20 and 40 d of age seems to produce effects on song repertoire that are similar to those observed with longer treatments: vocal behavior is impoverished, as a result of a decrease in the number of different song syllables in the repertoire of each T-treated bird. However, unlike the pattern we observed in Experiment 1, transient T exposure during this period seems to have little effect on the number of phrases produced per bout of singing.

Discussion

Timing of exposure to testosterone reveals a chronology in song development

Keeping circulating levels of testosterone high for a period of ontogeny during which endogenous levels of hormone are usually low is a strategy that may produce many substantive changes in a learned behavior. This approach can be applied to the study of diverse hormone-dependent developmental events and provides an array of effects that may be complementary to more traditional approaches involving gonadectomy. Gonadectomy experiments may also suffer some disadvantages. For example, residual circulating levels of gonadal steroids persist even after castration (Luine et al., 1980; Marler et al., 1988; see Adkins-Regan et al., 1990), thus requiring the use of antisteroid substances in order to study the effects of steroid removal on development (Bottjer and Hewer, 1988, 1989). In addition, the effects of castration can be reversed only through the use of hormone implants, thus maintaining constant, unregulated circulating levels of steroids. Conversely, chronic administration of hormone can be stopped any time and seems to be followed by physiological plasma levels of T and regular testis size (as observed in T_{1-20} and T_{20-40} groups), such that endogenous levels of steroids may be regulated through the usual feedback mechanisms. Furthermore, as shown in this study, varying the schedule of application of this "hormonal clamp" may give valuable information concerning the developmental chronology of different components of a specific behavior. Exposure to hormone for distinct periods of time during development of a specific behavior may have different effects according to the timing of exposure and thus may increase our knowledge of both behavioral and neural processes occurring during these discrete periods of ontogeny.

We have shown that chronic exposure to testosterone during development profoundly impairs song learning in juvenile male zebra finches, and the effects of the hormone vary according to the period of life during which T is administered. Exposure to T starting before day 40 and lasting until adulthood decreases the number of different syllables in the vocal repertoire and increases the number of phrases in individual song bouts. In addition to these "syntactical" abnormalities, exposure to the hormone before the fourth week of life seems to produce acoustic abnormalities that are perceptible to the human ear (as indicated by the progressive improvement in song scores when the onset of T treatment is delayed). Administration of T during the first 3 weeks of life produced acoustic abnormalities (as evidenced by the decrease in song scores and the decreased incidence of harmonics) but failed to influence the number of different syllables in the repertoire and the number of song phrases per bout of singing. When birds received T between 20 and 40 d of age, the number of different syllables in their repertoire was reduced, but their song bouts contained a normal number of song phrases.

Although we did not attempt to quantify it, birds that received T between 20 and 40 d of age tended to show acoustical abnormalities similar to those observed in males that received hormone during the first 3 weeks of life. Birds that received T starting after day 40 did not show any behavioral abnormalities when compared to controls.

Overall, these findings show that juvenile male zebra finches require some time free from high levels of testosterone following the immediate posthatch interval when circulating levels of both testosterone and estradiol are quite high (Hutchison et al., 1984; Adkins-Regan et al., 1990). Thus, there appears to be a period of approximately 1 month following the first week of life when low titers of sex steroids are necessary for normal song development. The ability of testosterone to disrupt vocal learning during a restricted period of development may reflect either or both of two broad alternatives, i.e., that juvenile males learn separate components of song behavior at different ages and that testosterone actively interferes with those components of the learning process, or that testosterone exposure during a restricted period of time affects the development of behavioral and neural processes that are necessary for future learning to proceed. In both cases, our results suggest a chronology of sensitive periods of development during which exposure to T impairs acquisition of distinct characteristics of song behavior. Our study does not enable us to determine whether song features that are impaired by T administration during a specific period of development are acquired at that time or whether a sensitive period is a time during which T exposure has lasting consequences that impair song acquisition processes occurring beyond that period. Interestingly, other behavioral studies have yielded additional information that may help to distinguish between these two possibilities.

If testosterone acts on learning processes that are occurring at the time of exposure, our results would seem to indicate that acoustic characteristics of song syllables are acquired earlier than syntactical characteristics of song phrases and bouts. In addition, learning of the different elements within the bird's repertoire would seem to occur earlier than acquisition of the temporal sequence in which they are sung. These findings are reminiscent of the observations reported by Immelmann (1969). When varying the age at which young male zebra finches were isolated from their foster parents, Immelmann noticed that males that were isolated before day 40 tended to develop impoverished songs that were "uniform and thus resembled the song of males raised without a tutor." Males that were isolated between day 38 and day 66 developed a song containing syllables identical to those of the foster father, but the pattern of their song was not identical in the sequence of syllables nor in the total length of song bouts. From these observations, he concluded that juvenile male zebra finches learn acoustic characteristics of individual syllables first (before the onset of juvenile song) and that the sequence of syllables and the length and rhythm of bouts are fixed later in life. Similarly, Eales (1985) reported that male zebra finches isolated from other birds at day 35 have abnormal songs that, as in our study, included many repeated elements. Finally, in swamp sparrows, Marler and Peters (1977) reported that young males that were tutored with artificial songs during the sensitive period for song learning tended to learn only conspecific syllables, even when these syllables were arranged in the temporal pattern of another species. Furthermore, these young males were able to transpose these syllables into a phrase with a temporal pattern similar to that one observed in normal swamp sparrows,

even when reared in total isolation from adult conspecific songs. Thus, in swamp sparrows, temporal structure of the song can develop without access to an external model, but individual syllables are identified and learned during an early period of development.

Early acquisition of individual song syllables would explain why exposure to T during the first 3 weeks of life affects only the acoustic characteristics of song syllables but not the syntax of song phrases. In addition, since our results indicate that the ability of zebra finches to learn a normal, varied repertoire is most sensitive to T exposure between day 20 and day 40, it is likely that acquisition or retention of the proper number of song syllables extends into this period. Alternatively, it is possible that general, species-specific syllable characteristics are acquired during the first 3 weeks of life and that individual syllable characteristics are learned during the following weeks. It would be of particular interest to investigate whether, during this period, juvenile males begin to produce many different syllables that are subsequently pruned to select the bird's final repertoire (Marler and Peters, 1982). Testosterone might either decrease the development of varied syllables or increase syllable attrition, thus producing a final, impoverished repertoire. Finally, since only birds that had high circulating levels of T before *and* after day 40 showed abnormal bout structure, temporal sequence and pattern of song behavior seem to develop later during an extended period of time.

Possible mechanisms for the action of testosterone on song development

The effects of exposure to T during the first 3 weeks of life on the acoustic characteristics of song syllables might indicate that T is able to change the birds' ability to learn or produce specific acoustic patterns. It is difficult to separate a possible effect of T on central song-control nuclei from a more peripheral effect on the organization of the androgen-sensitive vocal organ, the syrinx. Testosterone might change the physical properties of the syrinx, thus explaining the loss of harmonics. In addition, Jordan et al. (1989a,b) recently showed that synapse elimination in an androgen-sensitive penile muscle of the rat may be prevented by high postnatal circulating levels of androgens. Similarly, it is possible that early administration of T in newly hatched male zebra finches might induce abnormal syringeal innervation and that this purely peripheral effect might be responsible for the acoustic abnormalities of song syllables in T_{1-20} birds. However, such peripheral effects cannot explain the syntactical changes in song patterns observed in this study. Little is known about the development of steroid receptors in the CNS of male zebra finches during the first 3 weeks of life. Although we have shown that 20-d-old males have a significant number of androgen-concentrating cells in brain nuclei that control vocal behavior (Bottjer, 1987; Korsia and Bottjer, 1989), it is still unclear whether brain nuclei of younger birds are sensitive to androgens. In addition, juvenile male zebra finches that receive an injection of T at day 16 begin to produce juvenile song at day 19 (instead of day 30 in controls), whereas the same injection at an earlier age fails to induce this shift in the onset of song production (Sossinka et al., 1975). Thus the behavioral effects of chronic testosterone administration during the first 20 d of life may be due to changes in the organization of the neuro-muscular vocal system, either centrally or peripherally.

Alternatively, it is possible that early administration of T during the first 20 d of life alters acquisition or maintenance of

the so-called "auditory template." During the first weeks of life, birds have to selectively memorize sounds produced by their tutor. In zebra finches, this process overlaps with the period when birds begin to produce songlike vocalizations. Testosterone might reduce the ability of the nervous system to extract, process, and store acoustic characteristics of model song syllables. The absence of an appropriate template would then be responsible for the development of a song composed of only a few syllables. In this regard, it is interesting that birds in our experiments produced songs that had characteristics similar to those of males raised in isolation starting before day 40 (i.e., uniform and with an abnormal number and sequence of syllables) (Immelmann, 1969; Price, 1979).

In juvenile zebra finches, the sensory phase of song acquisition overlaps with a sensorimotor phase during which birds begin to produce plastic song. The effects observed in birds that received T between 20 and 40 d of age might be due to the effects of T on both sensory and sensorimotor phases of song learning. An interesting possibility arises from the study by Sossinka et al. (1975) mentioned above. The observation that a single injection of T on day 16 shifts the onset of song production to day 19 (instead of day 30) suggests that chronic T exposure between day 20 and 40 might act indirectly by its ability to induce an abnormally early onset of song behavior. Impoverished vocal behavior in T-treated birds might then indicate that sensorimotor learning contributes to the closure of sensory acquisition. Through an early induction of motor production of juvenile song, T-treated males would experience a shorter phase of sensory acquisition that would result in an impoverished repertoire.

The sensorimotor phase of song learning seems to end with the development of song stereotypy. An additional possible mechanism for the effects of chronic T exposure on song learning comes from experiments studying the role of testosterone on the onset of stereotyped song in juvenile male zebra finches. Castration combined with antisteroid treatment of juvenile male zebra finches prevents acquisition of stereotyped song, and administration of T to these birds even after the normal learning period induces gradual development of song stereotypy (Bottjer and Hewer, 1988, 1989). It is thus possible that rising levels of T normally contribute to the loss of vocal plasticity in young adult males by promoting the production of stereotyped song (cf. Nottebohm, 1971). In this regard, it would be interesting to know whether T-treated birds develop stereotyped song behavior earlier than control birds. Early chronic exposure to T may influence the pace at which neural substrates of song learning develop (i.e., may accelerate their maturation), thus reducing the time available to acquire proper song characteristics. In rats, studies have suggested that increases in plasma T levels during puberty stabilize dendritic length of T-sensitive motoneurons in the spinal nucleus of the bulbocavernosus (Goldstein et al., 1990) and that the rate of synaptic replacement in denervated hippocampus declines rapidly after puberty (McWilliams and Lynch, 1983). Thus, exposure to high levels of T may promote the performance of behavioral consistency by curtailing the capacity for neural plasticity.

On a more general level, our results raise questions regarding the relationship between steroid hormones and learning processes. Interestingly, plasma levels of sexual steroids in higher vertebrates are low during infancy at the time most behavioral skills are acquired. In children, learning disabilities such as stuttering or dyslexia are more preponderant in males and tend to worsen at puberty (see Geschwind and Galaburda, 1985). In addition, male children with idiopathic precocious puberty (hence elevated levels of testosterone at a time when endogenous levels are normally low) show poorer verbal and spatial abilities than matched controls (Rovet, 1983). A possible hypothesis stemming from these results is that minimal circulating levels of gonadal hormones are permissive for behavioral learning during childhood, but higher plasma levels decrease learning abilities.

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