The Effects of Testicular Tissue and Prehatching Inhibition of Estrogen Synthesis on the Development of Courtship and Copulatory Behavior in Zebra Finches

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Received May 2, 1997; revised July 8, 1997; accepted July 8, 1997

As in many mammalian and avian species, testicular androgens or their metabolites activate courtship and copulatory behaviors in adult male zebra finches. However, studies of sexual differentiation of these behaviors and related anatomical structures provide conflicting results. For example, posthatching estradiol can both masculinize courtship and the neural structures involved in song in females and inhibit the development of masculine copulation in males. These and other results have led to the hypotheses that (1) testicular androgens are converted to estradiol in the brain of developing males, and estradiol serves to masculinize the song system, whereas (2) estradiol secretion by the female ovary allows feminine rather than masculine copulatory behavior to develop. Treating embryonic zebra finches with the estrogen synthesis inhibitor fadrozole causes functional testicular tissue to develop in genetic females. The present study investigated the effects of such treatment on the development of singing and copulatory behavior as well as song system anatomy in males and females. While exogenous testosterone facilitated the display of sexual behaviors in adult males, the testicular tissue in females had no masculinizing effect on the production of audible courtship song or copulation. Their song control nuclei were also not masculinized, even in individuals lacking ovarian tissue. In contrast, embryonic inhibition of estrogen synthesis in males significantly stimulated song production. These results suggest that while manipulations of steroid hormone exposure can influence the display of sexual behaviors, gonadal secretions may not be required for normal sexual differentiation of the song system in zebra finches.
synthesis before hatching is responsible for the development of song control nuclei, a series of studies place before embryonic day 8. The results of some experiments, however, are incompatible with the idea that estrogen is normally responsible for the masculinization of the song system in males. For example, if estradiol masculinizes males, then one might expect males to be exposed to more estradiol than females during the period of sexual differentiation. However, studies have been unable to detect consistent sex differences in either plasma estrogen levels (Hutchison, Wingfield, and Hutchison, 1984; Adkins-Regan, Abdelnabi, Mobarek, and Ottinger, 1990; Schlinger and Arnold, 1992) or telencephalic aromatase activity (Schlinger and Arnold, 1992; Wade, Schlinger, and Arnold, 1995) in developing zebra finches.

A variety of more direct tests have been used to determine whether estrogen masculinizes the song system in males. These studies attempted to inhibit estrogen secretion or action in males during development. Castration of hatchling zebra finches produced little or no effect on adult singing behavior (Arnold, 1975; Adkins-Regan and Ascenzi, 1990). Posthatching treatment of birds with anti-estrogens also failed to substantially inhibit masculine development of the neural song system in males (Mathews and Arnold, 1990, 1991). Treatment with the potent aromatase inhibitors fadrozole or vorozole during the first month of life had no effect on the development of song system anatomy in either males or females (Wade and Arnold, 1994; Balthazart, Absil, Fiasse, and Ball, 1995; but see Merten and Stocker-Buschina, 1995). Together these studies suggest that high levels of posthatching steroid hormone, estradiol in particular, are not critical to the normal development of the song system in males.

Since these posthatching treatments may have inhibited estrogen too late to yield dramatic effects on the development of song control nuclei, a series of studies was carried out to test the hypothesis that estrogen synthesis before hatching is responsible for the development of the song system in males. In addition, because estrogen is critical to ovarian development in birds, an aromatase inhibitor administered in ovo during gonadal differentiation causes testicular development in genetic females (Elbrecht and Smith, 1992). Treating zebra finches on embryonic day 5 with fadrozole produced genetic females with both ovarian and testicular tissue and males with apparently normal testicular tissue (Wade and Arnold, 1996). In most of the females this testicular tissue appeared to be endocrinologically active, functional enough to produce sperm and cause the growth of the syrinx (vocal organ; normally larger in males than in females, and grows in adulthood in response to androgen). This prehatching treatment had no significant effect on morphology of the song control regions of genetic females measured as either juveniles or adults or of juvenile males. In adult males, however, the treatment decreased the volume of one of the song control nuclei (RA) slightly but significantly. The data obtained from females suggest that functional testicular tissue is not sufficient to masculinize the song system. However, the data from males are consistent with the idea that aromatase activity in ovo plays some role in the masculinization of the song system.

A second study was designed to investigate the effects of inhibiting aromatase activity at a later embryonic stage on the structure of both the gonads and the song control nuclei. Eggs injected with fadrozole on embryonic day 8 (Wade, Springer, Wingfield, and Arnold, 1996) produced results similar to those found in the previous study with the same treatment on embryonic day 5 (Wade and Arnold, 1996). In the later study, functional testicular tissue was found in fadrozole-treated females, confirming the importance of the presence of estrogen on ovarian differentiation in birds. Although perhaps slightly less developed histologically than in the previous study, this testicular tissue did secrete androgens. Most females had developed spermatids, detectable levels of plasma testosterone were found in 3/5 of the fadrozole-treated females, but in no control females, and the androgen-sensitive syrinx was larger in fadrozole-treated females than in control females. Despite the fact that this testicular tissue appeared functional, the neural song system in these females again was not affected. Unlike the earlier study, no significant effect of fadrozole treatment in males was found. This result indicates that either embryonic aromatase activity is not critical to masculine development of the neural song system or that the critical period for song system differentiation takes place before embryonic day 8.

While these recent studies suggest that early estrogen (produced from testicular testosterone) does not induce
masculine development of the neural song system in males, other data indicate that estrogen does influence the development of other sexual behaviors in zebra finches. For example, estrogen treatment of males during the first few weeks after hatching leads to a decrease in adult copulatory behaviors (Adkins-Regan and Ascenzi, 1987). This finding suggests that in zebra finches, as in other birds, early estrogen, presumably secreted by the female ovary, prevents the development of normal masculine copulatory behaviors (Adkins-Regan, 1981; Adkins-Regan and Ascenzi, 1987).

The purpose of the present experiment was to repeat and extend the previous study in which fadrozole was given to male and female zebra finches on embryonic day 5 (Wade and Arnold, 1996). In particular we were interested in: (1) whether the decrease in RA volume in fadrozole-treated males would replicate, and (2) most importantly, investigating the effects on the development of singing and copulatory behavior of testicular tissue in females and inhibiting embryonic aromatase activity in males.

**METHODS**

**Animal Treatment**

Fertile zebra finch eggs were injected either with 20 μg fadrozole in 10 μl saline or saline on day 5 of incubation and returned to the nest. Hatchlings were raised by their parents in aviaries containing five pairs of normal adults and their treated young. Treated birds were removed from the breeding cages at 60 days of age and put in a flight cage containing birds of both sexes and treatments. The birds were subsequently removed from this holding cage after reaching sexual maturity (at least 105 days of age) and moved to individual cages (21cm d × 26cm w × 30cm h or 21cm d × 28cm w × 37cm h; the two sizes were randomly assigned across sexes and treatments) containing a single perch 7 cm from the bottom of every cage. The birds were isolated from the view of each other by black plastic dividers placed between the individual cages, but for most of each day could see and hear normal adult males and females.

**Behavior**

Beginning 10 days after their placement in individual cages, the birds were each given a 15-min test on alternate days with male and female stimulus zebra finches (6 consecutive days, three tests of each kind; sex of first stimulus animal randomly assigned). Subjects were paired with a different stimulus bird each time, and behaviors were recorded from behind a blind by an observer who had no knowledge of the treatment of the test birds. The number of song bouts and all copulatory behaviors (mount attempts and feminine receptivity, defined as allowing a cloacal contact movement to occur) were counted. The display of other courtship behaviors, such as dancing and beak wiping in the context of song, was also noted (detailed descriptions of behaviors in Adkins-Regan and Ascenzi, 1987; Adkins-Regan, Mansukhani, Seiwert, and Thompson, 1994). Following testing on the sixth day, subjects were implanted with a Silastic capsule (inner diameter 1.5 mm, outer diameter 2 mm, length 7 mm) containing testosterone propionate (TP; Steraloids). The implant was inserted subcutaneously over the breast muscle, and the incision was sealed with collodion. These hormone implants produce similar levels of testosterone in males and females that are equal to or slightly higher than the levels in untreated males (Adkins-Regan et al., 1990) and were designed to ensure that the animals had adequate levels of circulating testosterone to activate sexual behaviors (Adkins-Regan, Mansukhani, Seiwart, and Thompson, 1994). Subjects were not gonadectomized prior to implantation because the surgery does not reduce circulating estradiol (Adkins-Regan et al., 1990), which is important in the activation of sexual behaviors (Harding et al., 1983). Animals were tested again beginning 1 week after implantation. This time they were paired only with stimulus females for 3 consecutive days. Stimulus males were treated with the same TP capsules as test birds, and stimulus females were treated with capsules of the same size of which 2 mm was filled with cholesterol:estradiol benzoate (9:1; Steraloids).

**Tissue Preparation and Morphological Measurements**

A blood sample was taken from all birds on the day of perfusion for use in genetic sexing (see below). All birds were then given an overdose of Equithesin and perfused with saline and phosphate-buffered formalin. The presence of a capsule that still contained some crystalline hormone was confirmed in all animals at time of perfusion. The brains were removed and postfixed for at least 1 week. The syrinx and oviduct (if present) were removed and weighed, and the gonads were stored in Bouin’s fixative.

After postfixing, brains were embedded in gelatin, sectioned frozen at 30 μm, and stained with thionin. The volumes of two song control nuclei (RA and HVC) were estimated by tracing their outline in every third section using the NIH image analysis program, IMAGE, on a Macintosh Power PC. To obtain an estimate of
volume, the areas were summed and multiplied by the sampling interval (90 \mu m). An average neuron soma area was determined for each individual in RA and HVC using 50 cells (25 on each side of the brain). Brain sections were coded so that measurements were taken without knowledge of the sex or treatment of any individual. Volumes and soma sizes reported are averages from the two sides of the brain.

The gonads were dehydrated, embedded in paraffin, sectioned at 10 \mu m, and stained with hematoxylin and eosin. The relative development of ovarian and testicular tissues was both qualitatively and quantitatively assessed. That is, in the groups with testicular tissue, the extent of sperm development was noted, and the volumes of testicular and ovarian tissue were estimated by tracing their outline in every 10th section using IMAGE. To obtain an estimate of volume, the areas were summed and multiplied by their sampling interval (100 \mu m). Further, in fadrozole-treated females, a ratio of testicular to ovarian tissue volume was calculated.

**Analysis**

Statistical measures for variables other than behavioral measures were performed by ANOVA or t test using the Statview program (Abacus Concepts, Berkeley, CA) on a Power Macintosh. Pairwise post hoc comparisons following ANOVA were made using Fisher’s PLSD. The proportion of animals in all groups displaying song was analyzed by Fisher’s Exact Test, also using Statview. The frequency of behavioral displays was analyzed by ANOVA using SAS (SAS Institute Inc., Cary, NC). Effects of treatment were compared between subjects, while effects of testing condition (with stimulus male, with stimulus female, or with stimulus female following testosterone treatment) were assessed within subjects. Planned pairwise comparisons were then conducted when significant effects of testing conditions were found.

Syrinx, oviduct, and brain measures were obtained from 38 animals (6 fadrozole-treated males, 9 fadrozole-treated females, 14 saline-treated males, and 9 saline-treated females). Gonads were analyzed from 37 animals because the right gonad of 1 fadrozole-treated female was lost during histological preparation. Behavior testing was done using the 38 birds in that primary experiment, plus a second cohort of 12 fadrozole-treated females and 11 fadrozole-treated males. The two cohorts of fadrozole-treated birds were found not to differ on any behavioral measure (all \( P > 0.118 \)), so the data were combined.

Zebra finches generally have highly sexually dimorphic plumage. Typically, male zebra finches in our colony are gray or tan with rust-colored cheek patches and black stripes on their necks, whereas females are either gray or tan with none of those masculine markings. Occasionally we have birds with white patches mixed with either masculine or feminine plumage and even less frequently completely white birds. Although embryonic fadrozole treatment does not alter plumage (Wade and Arnold, 1996; Wade et al., 1996), the genetic sex of all fadrozole-treated animals and 13 saline-treated animals in the primary experiment, as well as 13 fadrozole-treated animals in the second cohort, was determined by Zoogen Inc. (Davis, CA). These determinations included samples from all fadrozole-treated white birds as well as those with substantial patches of white. In all cases in the present study, and consistent with previous results (Wade and Arnold, 1996, Wade et al., 1996), animals with masculine plumage and no oviduct were determined to be genetic males, whereas animals with feminine plumage and an oviduct were genetic females. Of the remaining fadrozole-treated animals in the second cohort for which genetic sex was not determined, the plumage was distinctly masculine or feminine. In the behavioral analyses involving those individuals, plumage was used as criteria for gender.

**RESULTS**

**Gonads**

Despite adult testosterone treatment, the gonads from all treatment groups appeared histologically comparable to those reported in the previous study in which birds received the same prehatching treatment, but no posthatching hormone manipulation (Wade and Arnold, 1996). That is, in the present study (Figs. 1 and 2) the gonads of all 14 saline-treated males (Figs. 1B and 2C) and all 6 fadrozole-treated males (Fig. 2D) were of normal morphology: voluminous testes containing developed spermatids aligned at the edge of the lumen of seminiferous tubules. All 9 saline-treated females had one ovary on the left side (Fig. 1D), as is typical for birds, and no testicular tissue. Of the genetic females treated with fadrozole, 7 of 9 had an ovotestis on the left (a core of testicular tissue surrounded by ovarian follicles) and a testis on the right (Fig. 1C), consistent with the findings of the previous study. Unlike the previous study, 2 of the fadrozole-treated females were found to have bilateral testes with no detectable ovarian tissue (Fig. 1A). Unfortunately, one of these two animals (not pictured) died before completion of the study and had to be excluded from all statistical analyses and values reported, but brain measurements were ob-
tained (see Discussion). In most cases the testicular tissue in genetic females appeared functional, with developed seminiferous tubules and spermatids, although in 4 of these animals, the right gonad appeared to be more developed and contain more organized layers of maturing sperm than the left (Figs. 2A and 2B). The proportion of testicular tissue present in fadrozole-treated females ranged from 2.6 to 100% (mean ± SE: 66.6 ± 14.5%, or 33.4% ovarian tissue). Testis volume (Fig. 3) was significantly greater in males than in females (one-way ANOVA: fadrozole- and saline-treated males and fadrozole-treated females $F(2,25) = 8.200, P = 0.002$; Fisher's PLSD: each group of males vs fadrozole-treated females, $P < 0.003$), but the greatest amount of testicular tissue found in any individual was in a fadrozole-treated female (26.37 mm³; male maximum = 26.11 mm³). Testicular volume of fadrozole- and saline-treated males did not differ significantly (Fisher's PLSD, $P = 0.306$). Fadrozole-treated females had more testicular tissue than saline-treated females (fadrozole-treated females vs hypothesized mean = 0: $t(7) = 2.321, P = 0.053$).

Behavior

Interesting behavioral effects were found on a number of levels, including between treatment groups, between sexes, and within individuals across testing situations. Song was observed at least once over the course of testing in all 17 fadrozole-treated males, and in 12 of 14 saline-treated males (Fisher’s Exact Test, $P = 0.196$). Eight of 21 fadrozole-treated females also appeared to exhibit some singing behavior. That is, they made throat movements that closely resembled those made by males during song, and often this behavior was directed at the stimulus bird as well as coupled with other typical courtship behaviors such as dancing, beak wiping, and an erect posture. Since these throat movements are also similar to those made while a bird is eating or drinking, this female “singing” behavior was counted only when it was paired with at least one other stereotyped courtship behavior and was not counted if it was done shortly after eating or drinking. All of these “song” bouts in females were inaudible. Additionally, one saline-treated animal was observed

FIG. 1. Gonads from zebra finches treated during embryonic development. (A) Testes from a fadrozole-treated female with no detectable ovarian tissue. (B) Testes from a control male. (C) Ovotestis and testis from a typical fadrozole-treated female. (D) Ovary from a control female. Bar, 1 mm (A,C); 2 mm (B,D).
FIG. 2. Seminiferous tubules from the (A) left ovotestis and (B) right testis of a fadrozole-treated female, (C) testis of a control male, and (D) testis of a fadrozole-treated male. In most fadrozole-treated females, the testicular tissue in the left gonad was less organized and less mature than that in the right gonad (compare A and B). While seven of eight right testes examined in fadrozole females had spermatids, in five of them the layers of maturing germ cells were less organized than those in males (C and D). Bar, 0.3 mm.

to have performed one similar “song” bout on one test. The proportion of birds “singing” in the two groups of females did not differ significantly (Fisher’s Exact Test, $P = 0.210$), but the proportion of both fadrozole- and saline-treated females displaying these behaviors was significantly less than that in both groups of males (Fisher’s Exact Test for each of the four comparisons, $P \leq 0.007$).

While intrigued by the number of birds that appeared to display some elements of masculine courtship, and the fact that they did so repeatedly over the course of the tests, we chose to limit our statistical analysis of the effects of fadrozole treatment on frequency of song displays to males. We made this choice for the following reasons. First, although it looked similar, the behavior displayed by females was not the same as the song displayed by males; it was always inaudible. Therefore, comparisons between the sexes would not involve the same measure. Second, while a low level of inaudible song in untreated female zebra finches has been documented in other experiments (Adkins-Regan and As- PÅ­0.210), but the proportion of both fadrozole- and saline-treated females displaying these behaviors was significantly less than that in control females, and most importantly, the behavior in females was extremely infrequent compared to the males. As stated above, only one saline-treated female produced one bout on one occasion, and fadrozole-treated females produced only an average of 0.5 to 0.9 bouts per testing condition (Figs. 4 and 5).

Number of male song bouts. Fadrozole-treated males sang significantly more bouts than control males ($F(1, 29) = 6.53, P = 0.016$), and there was a significant
FIG. 3. Volume of testicular tissue in male and female zebra finches. Animals were treated either with the aromatase inhibitor fadrozole or with saline. Saline-treated females had no testicular tissue.

effect of the testing situation \( F(2, 58) = 30.62, P < 0.001 \), but no treatment \( \times \) testing situation interaction \( F(2, 58) = 2.56, P = 0.086 \). Before treatment with exogenous testosterone, birds preferred to sing to females rather than to males \( F(1, 29) = 26.98, P < 0.001 \). When presented with a female stimulus bird, more song bouts were observed after subjects were given a testosterone implant rather than before \( F(1, 29) = 11.44, P = 0.002 \) (Fig. 4).

**Number of days on which song was observed.** Fadrozole-treated males also sang on more days than did saline-treated males \( F(1,29) = 6.58, P = 0.016 \), and testing condition influenced the number of days on which the birds sang \( F(2,58) = 30.68, P < 0.001 \), but the interaction between the two variables was not statistically significant \( F(2,58) = 1.58, P = 0.216 \). In tests performed before adult males received testosterone implants, the birds sang on more days to females than to males \( F(1,29) = 19.25, P < 0.001 \). Following testosterone treatment, males sang more to females than before treatment \( F(1,29) = 10.73, P = 0.003 \) (Fig. 5).

**Mounting.** Regardless of their embryonic treatment, females never attempted to mount. Among males, fadrozole treatment did not affect the number of mounts individuals attempted \( F(1,29) = 0.42, P = 0.524 \) or the number of days on which mounts were attempted \( F(1,29) = 0.18, P = 0.677 \), but the testing condition did have an influence (see Figs. 6 and 7). Males attempted to mount only females and not other males, and they mounted females more frequently following testosterone treatment than before (number of mounts: \( F(1,29) = 4.78, P = 0.037 \); days on which at least one mount attempt occurred: \( F(1,29) = 12.98, P = 0.001 \)).

**Receptivity.** Because female stimulus birds never attempted to mount, and male stimulus birds never mounted male test birds, receptivity could be assessed only in tests of female experimental birds paired with male stimulus animals (before test birds received testosterone implants). Receptive behavior (cloacal contact movement) was displayed infrequently: twice by one fadrozole-treated female and once each by another fadrozole-treated female and one saline-treated female.

**Brain**

A significant sex difference was detected for the volumes of RA and HVC and neuron soma sizes in RA and HVC such that the values were consistently larger in males than in females (all \( F(1,34) > 124.559 \), all \( P < 0.001 \)). There was no effect of treatment in any measure (all \( F(1,34) < 0.469 \), all \( P > 0.498 \)), nor were there any sex \( \times \) treatment interactions (all \( F(1,34) < 1.442 \), all \( P > 0.238 \)) (Table 1).
Development of Zebra Finch Behaviors

FIG. 4. Total number of song bouts (mean ± SE) displayed by male and female zebra finches within each of the three test types: untreated test bird paired with a stimulus male, untreated test bird paired with a female, and testosterone-treated test bird paired with a female. All female songs were inaudible.

Syrinx and Oviduct

Sex \((F(1,34) = 20.8, \ P < 0.001)\) had a significant effect on syrinx weight, such that the syrinxes of males were larger than those of females. There was no significant effect of treatment \((F(1,34) = 0.002, \ P = 0.966)\) or sex \(×\) treatment interaction \((F(1,34) = 2.77, \ P = 0.727)\) (Fig. 8).

All fadrozole-treated and saline-treated females had a single left oviduct. Treatment did not have a significant effect on oviduct weight \((t(16) = 1.8, \ P < 0.0982)\), although the oviducts of all females were hypertrophied, due to the adult testosterone treatment.

DISCUSSION

Investigations of the role of steroid hormones in sexual differentiation of brain and behavior in zebra finches have produced paradoxical results. For example, data from several experiments (see Introduction) have suggested that estradiol can both induce masculine development of the song system and prevent masculine development of the copulatory system. Those data have led to the hypotheses that: (1) testosterone secreted by the testes of males is aromatized to estradiol in the brain, and estradiol subsequently masculinizes the morphology of song control nuclei and singing behavior, whereas (2) estradiol secreted by the ovary of females prevents masculine development of copulatory behavior. Previous experiments involving prehatching treatments of zebra finches with fadrozole have suggested that functional testicular tissue is not sufficient to masculinize the structure of the song control nuclei, although inhibiting aromatase activity beginning on embryonic day 5 may have a small, but long-term, effect in preventing masculine development of one region, RA. The present study expands on those results by addressing the following questions:

(1) Does functional testicular tissue induce masculine development of the song system, in particular singing behavior? If Hypothesis 1 is correct, then the fadrozole-treated females in the present experiment should sing and have masculinized song control nuclei. However, genetic females with substantial quantities of functional testicular tissue never produced audible song. While some females did display some elements of courtship behavior...
typical of males, which may indicate motivation to sing, all females appeared incapable of producing the stereotyped masculine vocalizations. Further, the proportion of females displaying those masculine behaviors was not significantly different in fadrozole-treated and control groups. These results suggest that testicular secretions are not sufficient to masculinize the singing behavior.

The presence of testicular tissue also did not masculinize the central and peripheral anatomy of the song system. The morphology of RA and HVC in fadrozole-treated females was comparable to that in control females, which did not have testicular tissue, and the measures were significantly smaller than in both groups of males. The syrinxes of females were also significantly smaller than those of males, and within each sex there was no significant effect of treatment. The results obtained from brain are consistent with the previous experiment in which birds were treated with fadrozole on embryonic day 5 (Wade and Arnold, 1996). However, the data obtained on syrinx weight are somewhat different due to the adult testosterone treatment in the present experiment. In the previous study, on average the syrinxes of fadrozole-treated females were intermediate in size to those of males of both treatment groups and control females, although most were in the range of those of control males. Those results provided evidence that the testicular tissue induced in females with fadrozole-treatment on embryonic day 5 is functional, since the syrinx grows in response to androgen (Luine, Nottebohm, Harding, and McEwen, 1980). The fact the syrinxes in all treatment groups in the present study were larger than those in the previous study are consistent with that idea. However, the fact that the syrinxes of females were significantly smaller than those of males despite adult testosterone treatment suggests an organizational effect. In other avian species estradiol has been shown to prevent masculine syrinx development (reviewed in Adkins-Regan, 1981). However, to date embryonic estrogen treatments (on days 5 or 3) in male zebra finches have not had feminizing effects (Wade, Gong, and Arnold, 1997; Gerhold and Wade, unpublished results). It is possible, though, that estradiol or some other secretion from the ovarian tissue present in most of the fadrozole-treated females acted at some time in development to prevent complete
masculinization of their syrinxes. In any case, similar to the results on singing behavior, functional testicular tissue is not sufficient to masculinize the morphology of either the song control nuclei or the syrinx.

(2) Is embryonic aromatase activity involved in masculinizing the song system? If so, then the small but statistically significant decrease in RA volume seen in the previous study (Wade and Arnold, 1996) should replicate, and one would also expect a decrease in singing behavior in males treated with fadrozole compared to control males. Consistent with results obtained from fadrozole treatments on embryonic day 8 (Wade et al., 1996) and day 3 (Gong and Arnold, 1996), in the present study treatment had no significant effects on RA or HVC volume or on soma sizes within those brain regions. Taken together, the data suggest that inhibiting aromatase activity during periods in the first half of the typically 15-day incubation period (Wade, unpublished results) does not affect masculinization of song system morphology.

Unlike in females in the present experiment, early fadrozole treatment had a marked effect on the production of audible song in males. On average, fadrozole-treated males sang approximately twice as much as control males. The effect appeared to be specific to the song system, since mount attempts were not increased in a similar manner. The reasons for the increase in song are difficult to hypothesize, but this effect of inhibiting estrogen synthesis is similar to a hypermasculinization observed in the morphology of song control nuclei following posthatching anti-estrogen treatment (Mathews, Brenowitz, and Arnold, 1988; Mathews and Arnold, 1990). These results are consistent with the idea that a high degree of estrogen action inhibits song system development. Alternatively, in the case of the prehatch-aromatase inhibition, it is possible that the treatment temporarily increased available testosterone, which could have had masculinizing effects on the syrinx or brain regions involved in the control of song that were not observed with the morphological assessments made. The effect need not be limited to RA and HVC, which are in the motor pathway controlling song. The change in steroid availability may have influenced another center, perhaps one regulating a motivational component to singing behavior. In any case, it is clear that the inhibition of estrogen synthesis beginning on embryonic day 5 does not prevent the development of masculine song. Therefore, similar to the neuroanatomy of the song system, embryonic aromatase activity at
FIG. 7. Number of days (mean ± SE) in which males made a mount attempt, either with or without testosterone treatment. Females never attempted to mount, and males never mounted male stimulus birds.

this stage of development is probably not critical to the masculinization of singing behavior.

(3) Do ovarian secretions prevent masculine development of the song system? In previous studies, all females that had received embryonic treatment with fadrozole possessed both ovarian and testicular tissue. It was therefore possible that, similar to the prevalent theory for the copulatory system, the morphology of their song control nuclei remained feminine due to a factor secreted by the ovarian tissue. In the present study, we were able for the first time to produce two genetic females with developed testes and no detectable ovarian tissue. One bird survived the entire course of the study, and even in this animal RA and HVC volumes and soma sizes fell within the range of control females. The same results were obtained on HVC volume and soma size and RA volume in the other bird, which died just before adult testosterone treatment. RA soma size was slightly larger than the maximum observed in control females (86.6 μm² compared to 52.8 μm²), but the value from this animal was still substantially smaller than the minimum observed in control males (134.0 μm²). Although the sample size is small, the lack of masculinization in genetic females without identifiable ovarian tissue suggests that ovarian secretions do not act to inhibit normal masculine development of the song system.

(4) Is the morphology of the song system directly related to its function? The fact that the females in the present

### TABLE 1

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<th>Brain Measures (mean ± SE) in Adult Zebra Finches Following Embryonic Treatment with the Aromatase Inhibitor Fadrozole or Control Vehicle</th>
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FIG. 8. Syrinx weight (mean ± SE) in male and female zebra finches treated with fadrozole or saline on embryonic day 5. All birds were treated with testosterone in adulthood.

study had smaller syrinxes and song control nuclei, as well as smaller neurons within the brain regions, and did not produce audible song suggests that a certain degree of structural masculinization is required for complete behavioral masculinization. However, the results from males suggest that size and function are not completely parallel. In the present study, males exposed to fadrozole before hatching sang approximately twice as much as control males, despite comparable song system morphology. Previous studies on posthatching aromatase inhibition produced conflicting results. While 2 weeks of fadrozole treatment causes an apparent but not statistically significant increase in singing behavior (Adkins-Regan, Yang, and Mansukhani, 1996), posthatching vorozole treatment results in a 50% decrease in the number of song bouts displayed by male zebra finches (Balthazart et al., 1995). Importantly, neither study found an effect of treatment on song system morphology, similar to the present study. While inconsistent, all of these changes in behavior without concurrent alterations in morphology suggest that the size of brain areas and the size of the neurons within them are not necessarily predictive of functional differences.

(5) Does estrogen, or do other ovarian secretions, prevent the development of masculine copulatory behaviors? No female attempted to mount, despite the presence of testicular tissue. Most of the fadrozole-treated females in the present study possessed ovarian as well as testicular tissue, and secretions from that ovarian tissue may well have prevented the masculine copulatory behavior from developing. It is true that the females with no detectable ovarian tissue failed to mount. However, even in males the frequency of mounting behavior was relatively low (21% control and 29% fadrozole-treated males attempted to mount). We suspect that copulatory behaviors were inhibited because the use of different stimulus animals on each of the 15-min behavioral tests prevented the formation of pair bonds, which occurs over the course of at least 2 to 3 days in zebra finches (Silcox and Evans, 1982). Therefore, while inconclusive, our data are consistent with those from zebra finches and other avian species showing that estradiol inhibits the development of masculine copulatory behavior (Adkins-Regan, 1981; Adkins-Regan and Ascenzi, 1987). Further, the present data are also consistent with the idea that testicular tissue does not induce masculine development of copulatory behavior.

Conclusions

While it is impossible to prove that the secretions of the testicular tissue in females in the present study were comparable to males at all stages in development, the data provide evidence that testicular tissue is not solely responsible for the masculinization of either the song
or the copulatory systems. In addition, data from two animals indicate that ovarian tissue is not necessary for preventing masculinization of the song system in females. The structure and function of the song system are clearly sensitive to steroid hormones. For example: (1) Embryonic fadrozole treatment (present study) and posthatching treatment with anti-estrogens (Mathews, Brenowitz, and Arnold, 1988; Mathews and Arnold, 1990) hypermasculinize the song system; and (2) numerous studies have documented masculinization of the song system in females with posthatching estradiol treatment (Gurney and Konishi, 1980; Gurney, 1981, 1982; Pohl-Apel and Sossinka, 1984; Konishi and Akutagawa, 1988; Simpson and Vicario, 1991a, b). However, several facts provide evidence that gonadal steroids do not normally induce sexual differentiation of the zebra finch song system: (1) Consistent sex differences in plasma steroid levels have not been documented during the period of sexual differentiation (Hutchison, Wingfield, and Hutchison, 1984; Adkins-Regan et al., 1990; Schlenger and Arnold, 1992), although in some cases circulating androgen levels appear to be higher in females (Adkins-Regan et al., 1990); (2) telencephalic aromatase activity in developing zebra finches is not sexually dimorphic (Schlinger and Arnold, 1992; Wade, Schlenger, and Arnold, 1995); (3) castration has little influence on the development of song (Arnold, 1975; Adkins-Regan and Ascenzi, 1990); and (4) functional testicular tissue in genetic females does not induce substantial behavioral or structural masculinization, even in the absence of ovarian tissue (present study, Gong and Arnold, 1996; Wade and Arnold, 1996; Wade et al., 1996). These results suggest that sexual differentiation of the behavior and anatomy of the song system employs mechanisms different than those common to mammalian (in which testicular androgens aromatized to estradiol masculinize) or avian (in which estradiol masculinizes) males (Arnold, 1975; Arnold, 1996). However, several factors provide evidence that gonadal steroids do not normally induce sexual differentiation of the zebra finch song system: (1) Consistent sex differences in plasma steroid levels have not been documented during the period of sexual differentiation (Hutchison, Wingfield, and Hutchison, 1984; Adkins-Regan et al., 1990; Schlenger and Arnold, 1992), although in some cases circulating androgen levels appear to be higher in females (Adkins-Regan et al., 1990); (2) telencephalic aromatase activity in developing zebra finches is not sexually dimorphic (Schlinger and Arnold, 1992; Wade, Schlenger, and Arnold, 1995); (3) castration has little influence on the development of song (Arnold, 1975; Adkins-Regan and Ascenzi, 1990); and (4) functional testicular tissue in genetic females does not induce substantial behavioral or structural masculinization, even in the absence of ovarian tissue (present study, Gong and Arnold, 1996; Wade and Arnold, 1996; Wade et al., 1996). These results suggest that sexual differentiation of the behavior and anatomy of the song system employs mechanisms different than those common to mammalian (in which testicular androgens aromatized to estradiol masculinize) or avian (in which estradiol masculinizes) systems. Similar to previous results (Arnold, 1975; Harding, Sheridan, and Walters, 1983), the present data show that androgen in adulthood can strongly influence the frequency of both song and mounting behavior, but it seems unlikely that testicular secretions have a similar influence on the ontogenetic sexual differentiation of these behaviors.

ACKNOWLEDGMENTS

We are grateful to Dr. Rick Deshon for assistance in the statistical analyses of behavioral data, to Renee Dory, Tracey Lee, and Melissa Rader for providing technical assistance, and to Lynnette Gerhold for helping with care of the birds. Fadrozole was a gift from Ciba-Geigy/Novartis. This research was supported by NIH MH55488.

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