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Functional testicular tissue does not masculinize development of the zebra finch song system

(songbird/aromatase/sexual differentiation/estradiol)

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ABSTRACT **Current theories of sexual differentiation maintain that ovarian estrogen prevents masculine development of the copulatory system in birds, whereas estrogen derived from testicular androgens promotes masculine sexual differentiation of neuroanatomy and sexual behavior in mammals. Paradoxically, some data suggest that the neural song system in zebra finches follows the mammalian pattern with estrogenic metabolites of testicular secretions causing masculine development. To test whether the removal of estrogen from males during early development would prevent the development of masculine song systems, zebra finches were treated embryonically with an inhibitor of estrogen synthesis. In addition, this treatment in genetic female zebra finches induced both functional ovarian and testicular tissue to develop, thus allowing the assessment of the direct effects of testicular secretions on song system development. In males, the inhibition of estrogen synthesis before hatching had a small but significant effect in demasculinizing one aspect of the neural song system. In treated females, the song systems remained morphologically feminine. These results suggest that masculinization of the song system is not determined solely by testicular androgens or their estrogenic metabolites.**

Sexual differentiation of avian reproduction has been described by two opposite models. In birds, like most animals, males and females exhibit very different reproductive behaviors. These behaviors include not simply those related to copulation, but in passerine birds, like zebra finches, they also include a courtship song that is typically only sung by males. The brain areas that control the song contain a variety of male-biased sexual dimorphisms, such that they are larger in volume and contain more and larger neurons in males than in females (1, 2). Paradoxically, estrogen is known to have both masculinizing and demasculinizing effects, depending on the species and system investigated. For example, early estrogen treatment of males prevents masculine development of secondary sex structures including the copulatory organ of ducks and the foam gland of Japanese quail (3, 4). Estrogen given to males during a critical period also inhibits the development of masculine copulatory behavior in chickens, quail (5, 6), and zebra finches (7). In contrast, early estrogen treatment of female zebra finches causes the neural masculinization (i.e., increase in volume and neuron size) of brain regions that control song and of the capacity to produce song in adulthood (2, 8). These observations have led to the conflicting theories that (*i*) ovarian estrogen directly inhibits masculine development of peripheral structures and the neural circuit for copulation, and (*ii*) estrogenic metabolites of testicular secretions cause the masculine development of the zebra finch song system.

The latter theory is supported by experiments describing high levels of aromatase (the enzyme that catalyzes the conversion of androgens to estrogens) activity and mRNA in the zebra finch telencephalon, near the sexually dimorphic song control nuclei (9–11). However, other studies do not support that idea. For example, assays of plasma hormone levels (12–14) and brain aromatase activity (15) have failed to detect consistent sex differences during posthatching development. Castration of zebra finches in the second week after hatching has little effect on adult singing behavior (16, 17), and treatment of males with antiestrogens during development causes song nuclei to be hypermasculinized rather than demasculinized (18, 19).

We previously treated birds for the first month after hatching with a potent and specific aromatase inhibitor, fadrozole. Although this drug reduced estrogen synthesis in brain by at least 80% (20), it had no significant effect on the neural song system of birds analyzed at 1 month of age (ref. 21; see also ref. 22). This result and that of a similar experiment using the aromatase inhibitor vorozole (23) suggest that high levels of posthatching estrogen synthesis are not required for sexual differentiation of the zebra finch song system. Therefore, in the present experiment, we treated eggs with fadrozole to test the hypothesis that estrogen synthesis before hatching is responsible for the masculinization of the song system. Because the compound also causes testicular development in genetic female birds (24), this approach allows the evaluation of the role of testicular tissue in the development of a variety of sexually dimorphic characteristics.

METHODS

Zebra finch eggs were injected either with 20 μg of fadrozole in 10 μl saline or with saline alone on day 5 of incubation and returned to the nest. The young hatched and were raised by their parents in aviaries containing at least five normal adults of each sex and their treated young. They then were given an overdose of Equithesin and perfused with saline and phosphate-buffered formalin either as juveniles (31–35 days after hatching) or adults (100–102 days after hatching). Brains and syringes were saved in phosphate-buffered formalin, and gonadal tissue was stored in Bouin's fixative. After postfixing, brains were sectioned frozen at 30 μm and stained with thionin. The volumes of three song control nuclei [robust nucleus of the archistriatum (RA); high vocal center (HVC); area X] were estimated by measuring the area of each nucleus in every third section (using the National Institutes of Health image analysis program, IMAGE), summing these areas, and multiplying by 90 μm, the interval of sampling. Values reported are means of volumes determined from the left and right sides of the brain. An average neuron soma size was

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Abbreviations: RA, robust nucleus of the archistriatum; HVC, high vocal center.

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determined for each individual in RA and HVC using 50 cells (25 on each side of the brain). Cells were randomly chosen for measurement, with the constraints that they had typical neuronal morphology and contained a distinct nucleus and at least one nucleolus. Comparisons were made by two-way ANOVA (sex × treatment), unless they involved only two groups, and in that case were done using a two-tailed *t* test. Gonads were dehydrated, embedded in paraffin, sectioned at 8 μm, and stained with hematoxylin and eosin. The relative amount and development of ovarian and testicular tissue were assessed qualitatively. In addition, we measured the effects of fadrozole on plumage (males have rust colored cheek patches and black and white neck stripes, whereas females are simply gray), syrinx weight (this vocal organ is larger in males than in females), and presence of an oviduct (female birds normally have a single left oviduct).

The genetic sex of all treated birds was determined using a technique developed in the lab of Mizuno (25). Genomic DNA was isolated from a blood sample taken at the time of perfusion. Blood was stored at −80°C in 2% SDS, 50 mM EDTA (pH 8.0), and 100 mM Tris (pH 8.0). A small aliquot of this mixture (less than 300 μl) was then incubated overnight at 50°C in 5 ml of 100 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, 0.5% SDS, and 5% of proteinase K at 20 mg/ml. DNA was extracted from samples using phenol and chloroform (1:1) once and chloroform alone twice. The DNA was then precipitated, rinsed in 70% ethanol, and resuspended in H_2O. DNA (10 μg) was digested with *Eco*RI and run on a 1% agarose gel. Southern blots, each containing lanes of DNA from known males and females as well as from treated animals, were made from the gels and probed with a portion of a cDNA encoding iron response element binding protein, originally isolated from chicken (IREBP; a gift of S. Mizuno, Tohoku University, Japan). Because this gene is located on the Z chromosome, it is more abundant in males (ZZ) than in females (ZW). The amount of hybridization of the probe to the DNA was quantified autoradiographically (^{32}P-labeled probe). To control for the amount of DNA loaded, the blots were stripped and reprobed with a cDNA encoding the immediate early gene ZENK (26), isolated from canary (a gift of D. Clayton, University of Illinois). Several bands were produced in each lane. A ratio was calculated, in absolute optical density units measured on a JAVA image analysis system, of the amount of IREBP/ZENK hybridization for specific bands for each individual. There was no overlap between ratio values for control males and females. Thus, when values for treated animals fell within the range of untreated animals of a given sex (or were more extreme), they were classified as that genetic sex. In a few cases, the ratios for treated animals fell between the male and female ranges. These ambiguous cases were resolved when the samples were reanalyzed.

RESULTS

Gonadal and Genetic Sex. Of the fadrozole-treated animals in this experiment, 15 were determined to be genetic males (5 adult and 10 juvenile) and 14 to be genetic females (7 adult and 7 juvenile). All of the genetic males, both saline- and fadrozole-treated, had bilateral testes of normal morphology. All saline-treated females had a single left ovary, as is typical in birds. In contrast, all genetic females that had been treated with fadrozole had both ovarian and testicular tissue. In juveniles, both ovarian follicles and seminiferous tubules were obvious in every fadrozole-treated female. Typically, both types of tissue were contained in an ovotestis on the left (a core of testicular tissue surrounded by ovarian follicles), and only testicular tissue was present on the right. In adulthood (Figs. 1 and 2), the testes of all fadrozole- and saline-treated males were voluminous and producing sperm. The ovaries of all saline-treated females were also well-developed. In adult females from fadrozole-treated eggs, the development of ovarian and testicular tissues varied (Table 1). Six of seven of these animals had a testis on the right, which varied in size, but contained sperm in five of them. An ovotestis was present on the left of all seven animals. This structure ranged from having numerous large, yolking follicles and a small amount of disorganized medullary (testicular) tissue, to having a few yolking follicles in the cortex and tubules in the medulla containing mature sperm. The testicular tissue of the right gonad in these genetic females appeared more typical of males than that seen in the left ovotestis. That is, in most animals, layers of spermatid development were more distinct, and mature sperm were more often aligned in discrete bundles at the edge of the tubule lumen (Fig. 2).

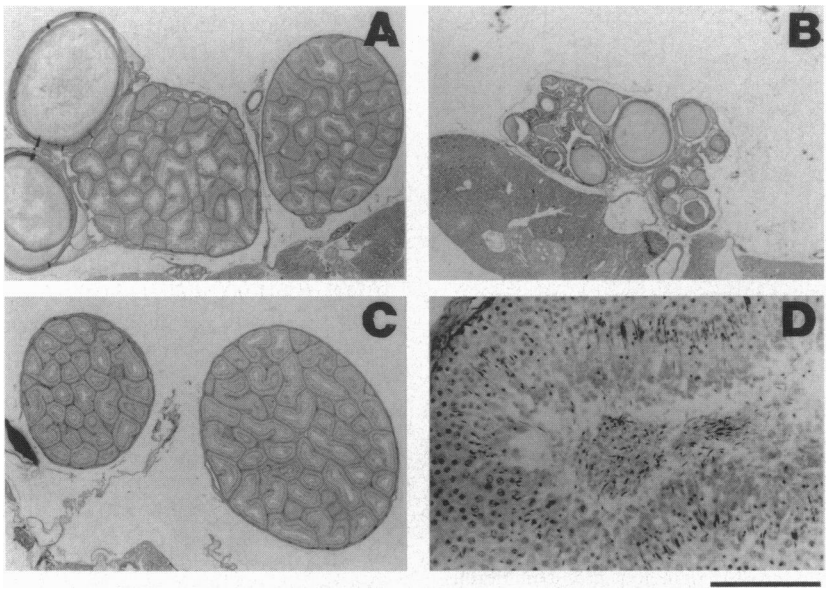

FIG. 1. Gonads in adult animals (paraffin embedded, sectioned at 8 μm, and stained with hematoxylin and eosin). (*A*) Ovotestis and testis in genetic female ("F" in Table 1) treated with fadrozole *in ovo*. Note two developed ovarian follicles present in addition to the large quantity of testicular tissue. (*B*) Ovary in control female. (*C*) Testes in fadrozole-treated male. (*D*) Sperm released into lumen of a seminiferous tubule from the right testis of a genetic female ("C" in Table 1). (*A–C*, bar = 1.3 mm; *D*, bar = 0.1 mm.)

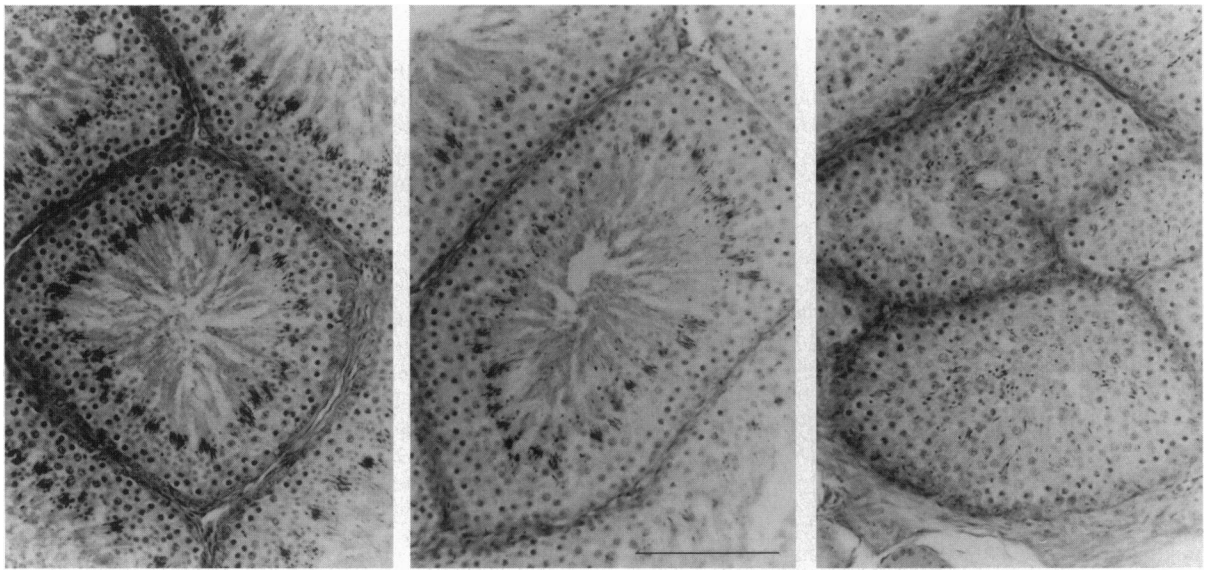

FIG. 2. Seminiferous tubules from three adult zebra finches. (*Left*) From a testis of a saline-treated male. (*Center*) From the right gonad (testis) of a fadrozole-treated female. (*Right*) From the left gonad (ovotestis) of a fadrozole-treated female. In general, the right female testes and male testes were very similar in the cell types present and in their organization; note, for example, the highly organized bundles of darkly staining spermatids in both. The left ovotestes also contained the same testicular cell types, but often lacked the rigid radial cell organization of normal seminiferous tubules. These examples were chosen to illustrate typical patterns of sperm development in the groups, and do not reflect typical sizes of seminiferous tubules. (Bar = 100 μm.)

Syrinx. In general, syrinx weight reflected the degree of testis development (Fig. 3 and Table 1). In juveniles, males had larger syringes than females ($F = 5.47$, $P = 0.03$), but the effects of treatment ($F = 0.44$, $P = 0.51$) and the sex × treatment interaction ($F = 0.12$, $P = 0.73$) were not statistically significant. In adults, males also had larger syringes than females ($F = 25.38$, $P < 0.001$) and the sex × treatment interaction ($F = 5.14$, $P = 0.04$) was statistically significant, since the syringes of fadrozole-treated females were intermediate in size to those of normal males and females. Like juveniles, the effect of treatment ($F = 2.73$, $P = 0.12$) was not significant in adults. When the results in adults were analyzed without the data from the two fadrozole females that did not produce sperm, then all effects were statistically significant (treatment, $F = 7.49$, $P = 0.02$; genetic sex, $F = 25.85$, $p < 0.001$; interaction, $F = 11.94$, $P < 0.004$). In that case, the mean for fadrozole-treated females was 22.8 g.

Table 1. Relationship of amount of testicular tissue to RA and syrinx morphology in fadrozole-treated females

Animal	Gonad	RA volume, mm^3	RA soma size, μm^2	Syrinx, mg
A	α	1 (0.016)	3 (45.9)	1 (14.1)
B	β	4 (0.022)	2 (43.8)	2 (16.2)
C	γ	5 (0.024)	1 (40.6)	3 (17.4)
D	γ	7 (0.032)	7 (76.9)	5 (23.8)
E	γ	3 (0.019)	5 (57.5)	6 (24.4)
F	δ	2 (0.018)	4 (52.5)	7 (26.6)
G	δ	6 (0.031)	6 (61.3)	4 (22.0)
Control female				
Mean ± SEM		0.026 ± 0.005	57.8 ± 3.1	14.4 ± 1.2
Range		0.017–0.041	48.5–61.9	11.1–16.6
Control male				
Mean ± SEM		0.249 ± 0.012	170 ± 11	26.0 ± 1.5
Range		0.211–0.277	140–200	21.2–30.1

Testicular tissue was classified into four categories: α, no identifiable seminiferous tubules, no left gonad (this animal appeared less mature than other embryos at the time its egg was injected); β, bilateral gonads, testicular tissue present on both sides but not well-differentiated; γ, less testicular tissue than in males, but present in both gonads, and producing sperm at least in right gonad; δ, seminiferous tubules, producing sperm, and in quantity similar to males. All fadrozole-treated females had developed ovarian follicles in the left gonad and a single left oviduct. Underlined numbers represent rank in size (degree of masculine development) of each measure (1, smallest; 7, largest). Numbers in parentheses are actual measurements.

Song System Morphology. In contrast to the syrinx, the neural song systems of genetic females with significant amounts of functional testicular tissue were no more masculine than those of females with little or no testicular tissue (Table 1). In animals of both ages, the sex difference in RA soma size (Fig. 4) was statistically significant (juvenile, $F = 164.43$, $P < 0.001$; adult, $F = 337.34$, $P < 0.001$), but the effect of treatment (juvenile, $F = 0.23$, $P = 0.64$; adult, $F = 1.00$, $P = 0.33$) and sex × treatment interaction (juvenile, $F = 0.34$, $P = 0.56$; adult, $F = 2.44$, $P = 0.14$) were not. The same results were found for HVC volume (sex: juvenile, $F = 230.46$, $P < 0.001$; adult, $F = 215.27$, $P < 0.001$; treatment: juvenile, $F = 0.35$, $P = 0.56$; adult, $F = 0.84$, $P = 0.37$; interaction: juvenile, $F < 0.01$, $P = 0.99$; adult, $F = 0.68$, $P = 0.42$) and HVC soma size (only measured in adults; sex, $F = 196.19$, $P < 0.001$; treatment, $F = 0.14$, $P = 0.72$; interaction, $F = 0.41$, $P = 0.53$). Area X cannot normally be seen in females with a Nissl stain, and the same was true for all fadrozole-treated genetic females. In juvenile and adult males, there was no significant difference between fadrozole- and saline-treated animals (juvenile, $t = 0.002$, $P = 1.00$; adult, $t = 0.02$, $P = 0.98$). The only significant effect of treatment detected was in RA volume measured in adults (Fig. 5). In this case, the volume of RA in the adult males from fadrozole-treated eggs was smaller than in control males but still much larger than in females. In juvenile birds, the effect of genetic sex on RA volume was statistically significant ($F = 125.55$, $P < 0.001$), but the effects of treatment ($F = 1.13$, $P = 0.30$) and sex × treatment interaction ($F = 0.001$, $P = 0.98$) were not significant. In adults, all three effects were statistically significant (sex, $F = 463.0$, $P < 0.001$; treatment, $F = 10.43$, $P = 0.005$; interaction, $F = 8.68$, $P = 0.009$). Analyzing the data excluding the two fadrozole-treated females that did not produce sperm does not change the significance of any results obtained from brain.

Plumage and Oviduct. Interestingly, plumage and presence of the oviduct were unaffected by treatment. Males and

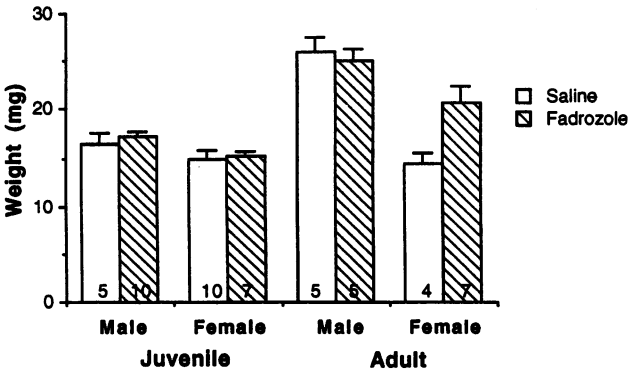

FIG. 3. Syrinx weight (mean + SE) in juvenile and adult animals. At both ages, males had significantly larger syringes than females. In adults, when androgen normally causes a dramatic increase in syrinx size, the syringes of all males grew, as did those of females with testicular tissue.

females from fadrozole-treated eggs had markings comparable to control animals. Regardless of treatment, all adult females had a single oviduct on the left side.

DISCUSSION

Two conclusions about the sexual differentiation of the zebra finch song system can be drawn from these data. Both of these conclusions argue against the theory that the song system is normally masculinized solely by testicular secretions of sex steroids.

1. Substantial amounts of functional testicular tissue do not cause masculine development of the neural song system. Fadrozole treatment on embryonic day 5 induced testicular tissue to develop in genetic females, yet the song systems of these animals were completely feminine. This result implies that testicular secretions are not sufficient to cause masculine development of the neural song system. The feminine nature of the song system in these animals may have one of several underlying causes:

(*i*) The testicular tissue in genetic females failed to secrete sufficient quantities of a masculinizing hormone at the appropriate time in development. Although it would be impossible to prove that the tissue was completely normal in its secretory profile, the present evidence suggests strongly that it was functional, at least in terms of androgen secretion. In several cases, the tissue was present in a quantity similar to normal males, and in most birds, it was producing sperm. Furthermore, in four of these females, the androgen-sensitive syrinx was well within the size range for normal males (Table 1). In the other three fadrozole-treated females, the syrinx was either feminine

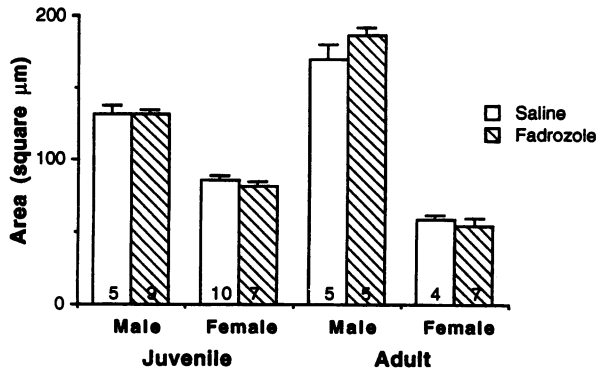

FIG. 4. RA soma size (mean + SE) in juveniles and adults. At both ages, males had larger neurons in RA than females. However, there was no effect of treatment or sex × treatment interaction at either age.

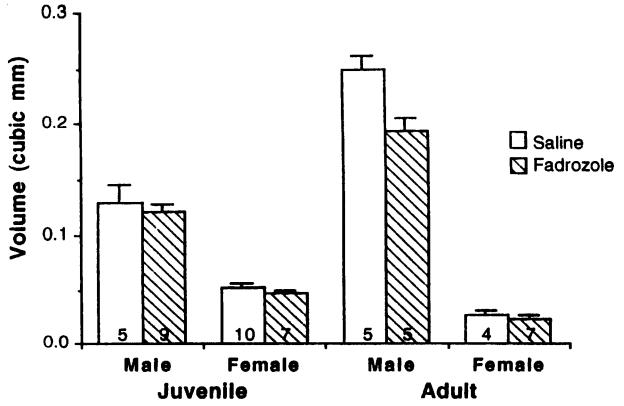

FIG. 5. RA volume (mean + SE) in juvenile and adult animals. The effects of sex, treatment, and sex × treatment interaction were all statistically significant in adults. In juveniles, only the sex difference was significant.

in weight or between control male and female values. In these cases, an ovarian factor may have inhibited masculine syringeal development, as occurs in other avian species (3). Alternatively, the testicular tissue may have secreted less than masculine levels of androgen. However, the average increase in syrinx size in females with testes compared with control females mimics that seen in adult females given high levels of exogenous testosterone (27). These results suggest that the histologically mature testicular tissue (Figs. 1 and 2) found in these genetic females secreted androgens in a manner similar to males.

The presence of a left oviduct in each genetic female with testicular tissue could be interpreted as evidence that secretions of the left ovotestis were not completely normal. That gonad may not have produced Müllerian inhibiting hormone (MIH), which normally causes regression of the oviduct in males. However, this interpretation may not be valid because the ovarian portion of that gonad may have secreted estrogen, which can inhibit MIH-induced regression (28). Moreover, the normal regression of the right oviduct could imply MIH secretion on that side. Whatever the explanation for the left oviduct in fadrozole-treated females, that observation does not bear on the ability of the testicular tissue to secrete testosterone or other sex steroids.

(*ii*) The female testicular tissue secreted androgen but the brain was not masculinized because the conversion of that androgen to estrogen was blocked by the fadrozole injected into the eggs. This scenerio is unlikely because of the minimal demasculinizing effect detected in genetic males that received the same treatment.

(*iii*) The testicular tissue in females did secrete masculinizing factors in a normal manner, but they were counteracted by ovarian secretions that normally inhibit masculine neural development. This intriguing possibility is supported by observations on two females from another study. Those animals were treated with fadrozole in a manner identical to the birds in this study but were housed differently, in small single-sex groups beginning at 60 days of age. These two birds had at least as much testicular tissue and less ovarian tissue than the most masculine female in the present study. In both of these animals, measures of the neural song system were slightly more masculine than those recorded for the fadrozole-treated females in this experiment, but were much closer to female than male values. For example, RA volumes (mm^3) were 0.044 and 0.065 and RA soma sizes (μm^2) were 95.4 and 73.0, respectively (compare with values in Table 1). Thus, testicular tissue in fadrozole-treated females may have had a small masculinizing effect in two cases in which the amount of ovarian tissue was at a minimum. We have not yet been able to produce females

with testicular tissue that are completely lacking ovarian tissue to more critically test the idea of ovarian inhibition of masculine development.

2. The inhibition of aromatase activity around embryonic day 5 has a minimal effect in preventing development of a masculine song system in males. RA volume was slightly, but significantly, decreased in males from fadrozole-treated eggs in this study. This result was found only in adult males, not in juveniles who were nevertheless sexually dimorphic. It suggests that estrogen synthesis *in ovo* could have long-term effects on neural development. However, the small magnitude of the effect and the fact that demasculinization was not found on any of four other anatomical measures suggest that if estrogen synthesis is important in males, it is at a time other than around embryonic day 5.

Possible Mechanisms of Sexual Differentiation. Although the present results strongly suggest that testicular secretions (e.g., testosterone aromatized to estradiol) are not sufficient to cause maculine development of the neural song system, they leave open the precise mechanisms of sexual differentiation. At least two ideas are worth considering. As suggested above, it is possible that some ovarian factor (present in all females, including those from fadrozole-treated eggs) prevents masculine song system development. Alternatively, the male pattern may be genetically determined and develop without the influence of steroid hormones. While the results of the present study are consistent with both of these ideas, it is difficult to reconcile either of them with the robust finding that estrogen treatment of females masculinizes the song system both morphologically and functionally. One possibility is that high doses of estrogen after hatching pharmacologically induce expression of some factor in females that is expressed constitutively in males (or increased by an estrogen-independent mechanism). Candidates for this factor include the neurotrophins, which are known to interact with estrogen (29, 30). A second possibility is that estrogen treatment interferes with normal ovarian function, reducing the secretion of a factor that normally inhibits masculine development of the song system in females. The high doses of estrogen required for song system masculinization in females (8) and the hypermasculinizing effect of anti-estrogens (18, 19) are consistent with this theory.

The present data together with those indicating a lack of sex differences in plasma estrogen levels (12–14) and brain aromatase activity (15), and the lack of a robust demasculinizing effect of inhibiting estrogen in males after hatching (by a variety of techniques; refs. 16–19 and 21–23), all argue against the idea that estrogenic secretions of the testes alone are responsible for the masculinization of the zebra finch song system.

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