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Estrogenic control of reproductive behavior in the male zebra finch

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City University of New York, 1988

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ESTROGENIC CONTROL OF REPRODUCTIVE BEHAVIOR

IN THE MALE ZEBRA FINCH

by

MICHAEL J. WALTERS

A dissertation submitted to the Graduate Faculty in Psychology
in partial fulfillment of the requirements for the degree of
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1988

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

ESTROGENIC CONTROL OF REPRODUCTIVE BEHAVIOR
IN THE MALE ZEBRA FINCH
by
Michael J. Walters

Advisor: Professor Cheryl F. Harding

This study consists of three experiments examining aromatization and reproductive behavior in male zebra finches. In the first experiment, castrated males were treated with androstenedione (AE), an aromatizable androgen, and their sexual and aggressive behavior compared with that of castrates treated with AE plus 1,4,6-androstatriene-3,17-dione (ATD), an aromatization inhibitor. Males treated with AE+ATD showed less courtship activity than AE-treated males, less copulatory behavior, and were unlikely to have nests. Estradiol (E), when given concurrently with AE+ATD, restored levels of courtship and copulation to those observed in AE-treated males. Aggressive behaviors were displayed exclusively by AE- and AE+ATD+E-treated males.

The second experiment examined the relationship between aromatization and two types of vocal behavior: courtship song and 'undirected' song. Castrated males were treated with AE, AE+ATD, or AE+ATD+E. Courtship songs were then recorded from individual males paired with a female, and undirected songs recorded from males grouped together in isolation from females. AE+ATD-treated males exhibited significantly fewer courtship songs than males treated with AE or AE+ATD+E, and they were of longer duration. In contrast, the frequency and duration of undirected songs were not affected by ATD treatment.

The third experiment examined estrogen receptor concentrations in brain regions involved in the control of sexual and vocal behavior. Cytosolic [^{3}H]estrogen binding was measured in castrated males and, to minimize competition from endogenously formed estrogen, in ATD-treated castrates. Specific, high-affinity [^{3}H]estrogen binding was detected in both untreated castrates and castrates treated with ATD. Although ATD treatment had no effect on estrogen receptor levels in hypothalamic-preoptic tissue, ATD-treated males has significantly higher levels of [^{3}H]estrogen binding in three vocal control nuclei: the dorsomedial portion of the intercollicular nucleus (DM), the magnocellular nucleus of the anterior neostriatum (MAN), and Area X. Low levels of estrogen binding were also detected in cytosol from the caudal portion of the ventral hyperstriatum (HVc) and the robust nucleus of the archistriatum (RA) of both untreated and ATD-treated castrates.

These experiments clearly demonstrate the importance of estrogen in the control of reproductive activities in male zebra finches and indicate that aromatization appears necessary for maintaining normal levels of sexual and aggressive behavior.

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CHAPTER 1:

GENERAL INTRODUCTION

Studies of a wide variety of vertebrate species have shown that the repertoire of behaviors associated with reproduction in the male, such as heightened intermale aggression, copulation, and the courtship of females, is dependent on male sex hormones (i.e., androgens) secreted by the testes. In virtually every species which has been examined, castration reduces both the quantity and quality of male reproductive behavior (Leshner, 1978). Since the effects of castration can be reversed by treatment with testosterone, and since testosterone seems to be the predominant androgen secreted by the testes in most species, it was assumed for many years that endogenous testosterone was the hormone regulating reproductive activities in the male. However, it is now fairly well established that many of the behavioral and physiological effects of testosterone are due to its transformation to other hormones. The general schema seems to be that the testes produce testosterone which is then released into the bloodstream and circulated throughout the body. Various hormone-dependent tissues, such as the sexual accessory tissues and specific areas of the brain, selectively retain testosterone, and via endogenous enzyme systems, metabolize it to one or more other hormones.

Several biochemical pathways are involved in the metabolism of testosterone. Two pathways seem to be particularly important for the regulation of male reproductive activities. One is the 5α-reduction pathway, which leads to the formation of another androgen, 5α-dihydrotestosterone (DHT). DHT appears to be the active metabolite in peripheral tissues involved in male sexual function and/or behavior. These include the sexual accessory tissues of mammals, such as the prostate and seminal vesicles (Fang, Anderson and Liao, 1969), the comb of chickens (Balthazart and Hirschburg, 1979), the cloacal gland of Japanese quail (Massa, Davies and Bottoni, 1980), and the syrinx of

songbirds (Lieberburg and Nottebohm, 1979). The second important pathway of testosterone metabolism is the aromatization pathway, in which testosterone is transformed into estradiol, an estrogen. This is the way estrogens are normally synthesized in females, but the importance of this pathway in males was overlooked for many years. Finally, there is the oxidation pathway, whereby testosterone is converted to androstenedione, generally considered to be a relatively weak androgen. Androstenedione may, in turn, be metabolized via 5α-reduction or aromatization to other androgens or to estrogen respectively.

Aromatization and 5α-reduction are both irreversible processes (Martini, 1978). Neither DHT nor estradiol, for example, can be converted back to testosterone. It should also be emphasized that once an androgen is 5α-reduced, all evidence indicates that it cannot be aromatized to an estrogen. Consequently, 5α-reduced androgens, such as DHT, are referred to as nonaromatizable androgens.

Comparative studies indicate that both aromatizing and 5α-reducing enzymes are present in the brains of species of every major vertebrate group. (Callard, 1983). A question of primary importance is which of the many metabolites are behaviorally active and which are merely steps in the degradation of testosterone? Data from those species which have been examined indicate that 5α-reduction and aromatization are both involved in the activation of behavior, but the relative importance of each metabolic pathway varies from species to species. In many species, particular male behaviors, most notably copulation or high-intensity aggressive behaviors, appear to be activated by estrogen (e.g., hamster: Vandenburgh, 1971; rooster: Guhl, 1949; mice: Edwards and Burge, 1971; red deer: Fletcher and Short, 1974; guinea pig: Antliff and Young, 1956). Treatment with DHT also restores some degree of sexual and/or aggressive behavior in castrated male hamsters (Whalen and DeBold, 1971), rabbits (Beyer, de la Torre, Larsson and Perez-Palacios, 1975), some strains of mice (Luttge and Hall, 1973), guinea pigs (Alsum

and Goy, 1974), and rhesus monkeys (Phoenix, 1974a, 1974b). Treatment with aromatizable androgens such as testosterone or androstenedione, which can be converted to both estrogens and 5α-reduced androgens, leads to consistent restoration of normal behavior of castrated males of all species. This, together with findings that concurrent treatment with both 5α-reduced androgen and estrogen is more effective than treatment with either hormone alone (Beyer et al., 1975; Foote, Draddy, Briete and Oltenacu, 1977; Harding, Sheridan and Walters, 1983; Joshi and Raeside, 1973), has led to the hypothesis that androgen and estrogen must interact at the neural level to induce the full pattern of male reproductive behavior in most species of animals (Martini, 1982; Perez-Palacios, Larsson and Beyer, 1975).

What follows are a series of experiments examining the role of estrogens in the control of reproductive behavior in the male zebra finch *(Poephila guttata)*, a passerine songbird. In many respects, the zebra finch is an ideal subject for the study of hormonal and neuroendocrine mechanisms underlying reproductive behavior. Male zebra finches exhibit an extensive range of hormone-dependent activities which are associated with reproduction. These include complex visual and vocal displays which are used over an extended period of courtship to attract females. Male zebra finches also share nestbuilding and incubation duties with the female and exhibit a high degree of parental care. Studying such a wide variety of behaviors is advantageous to the behavioral endocrinologist, for it allows the investigator to determine (i) if several functionally related behaviors are controlled by the same hormonal mechanisms, and (ii) whether the hormonal control of a particular behavior depends on the social context in which it is used. For example, are different behaviors commonly exhibited by zebra finches in aggressive interactions (e.g., beak-fencing, pecking, plucking, chasing) all dependent on the aromatization of androgens, or can aromatization activate one behavior without affecting the others? Does singing in non-sexual contexts require the aromatization of androgens, or is aromatization

necessary to activate singing only in the context of courtship?

What makes the zebra finch particularly valuable for the study of the hormonal control of behavior is its neural vocal control system. Vocalizations in male zebra finches are mediated by an interconnected system of discrete brain nuclei originally described in the canary . Four of these structures, nucleus interface (NIf), the caudal portion of the ventral hyperstriatum (HVc), the robust nucleus of the archistriatum (RA), and the tracheosyringeal branch of the hypoglossal nerve (nXIIts), are part of a direct efferent pathway linking the telencephalon to the muscles of the syrinx, the avian vocal organ (McCasland, 1987; Nottebohm, Stokes & Leonard, 1976). Other areas of the song control system, Area X of the parolfactory lobe, the magnocellular nucleus of the anterior neostriatium (MAN), the dorsomedial portion of the intercollicular nucleus (DM), and nucleus Uva, are connected to RA or HVc (Nottebohm & Kelley, 1982; Kelley & Nottebohm, 1979; Ryan & Arnold, 1981). Lesion and multi-unit recording studies indicate that these latter areas are not part of a direct pathway responsible for song production (McCasland, 1987; Nottebohm, 1980). Five of these song control areas are hormone-sensitive: MAN, HVc, DM, RA and nXIIts show heavy labelling following injection of [^{3}H]testosterone (Arnold, 1980; Arnold, Nottebohm and Pfaff, 1976; Arnold and Saltiel, 1979). MAN, DM HVc, RA, nXIIts and Area X are also larger in males than in females (Gurney, 1982; Nottebohm and Arnold, 1976), who do not sing, and this dimorphism in nuclear volume has been shown to be dependent upon the hormonal milieu during early development (Gurney, 1982; Gurney and Konishi, 1980). Thus, anatomical studies suggest that steroid hormones play an important role in both the differentiation and function of brain regions involved in the control of vocalizations.

This hormone-sensitive vocal control system of the male zebra finch is of particular value because it is the only clearly delineated, hormone-sensitive system in the brain with a clearly delineated behavioral function. These nuclei control vocalizations and, apparently, nothing else. This presents the investigator with the unique opportunity to relate molecular

mechanisms of hormone action in the brain (e.g., interactions with binding proteins) to a single group of behaviors. The actions of steroid hormones in other areas of the brain, such as the hypothalamus, are not so easily related to behavior because of the multitude of hormone-dependent processes mediated by such areas. The proposed research should therefore increase our understanding of how estrogens act on the brain to modulate behavior.

CHAPTER 2:

THE EFFECTS OF AN AROMATIZATION INHIBITOR ON THE REPRODUCTIVE BEHAVIOR OF MALE ZEBRA FINCHES

ABSTRACT

Recent evidence indicates that aromatizable androgens are more effective than nonaromatizable androgens in restoring normal levels of sexual behavior in castrated male zebra finches *(Poephila guttata).* To determine whether the efficacy of treatment with aromatizable androgens is due to their conversion to estrogens, castrated male zebra finches were treated with androstenedione (AE), an aromatizable androgen, and their sexual and aggressive behavior compared to that of castrates treated with AE plus 1,4,6-androstatriene-3,17-dione (ATD), an aromatization inhibitor. Males treated with AE+ATD showed less courtship activity than AE-treated males, less copulatory behavior, and were unlikely to have nests. Estradiol (E), when given concurrently with AE+ATD, reversed the inhibitory effects of ATD and restored levels of courtship and copulation to those observed in AE-treated males. Only AE- and AE+ATD+E-treated males displayed aggressive behaviors, but the frequency of of such behaviors was so low that there were no significant differences across groups. These data affirm the importance of estrogen in the control of reproductive activities in male zebra finches and indicate that aromatization may be an obligatory step for maintaining normal levels of sexual and aggressive behavior.

INTRODUCTION

It has long been assumed that testosterone (T), the predominant androgen secreted by the testes, was the hormone controlling reproductive activities in male vertebrates. In virtually every species that has been examined, castration reduces both the quantity and quality of male reproductive behavior, and the effects of castration can be reversed by treatment with T (Leshner, 1978). However, recent research indicates that the conversion (i.e., aromatization) of T to estrogenic metabolites may be an essential step in activating reproductive behavior in many species. Several lines of evidence support this view (for reviews see Harding, 1986a, 1986b; Martini, 1978; Whalen, Yahr and Luttge, 1985). First, studies which have compared the ability of various androgens to elicit sexual behavior in castrated males have found that aromatizable androgens are generally more effective than nonaromatizable androgens in maintaining normal patterns of sexual activity (e.g., Parrot, 1974, 1975; Whalen and DeBold, 1974). Second, brain regions implicated in the control of male behaviors are capable of aromatizing androgens both *in vitro* and *in vivo* (e.g., Lieberburg and McEwen,1975; Selmanoff, Brodkin, Weiner and Siiteri, 1977; Steimer and Hutchison, 1985). Third, systemic or intracerebral administration of anti-estrogens or aromatization inhibitors has been shown to inhibit T-induced sexual behaviors in castated males (e.g., Adkins and Nock, 1976; Christensen and Clemens, 1975; Morali, Larsson and Beyer, 1977).

Recent evidence indicates that the aromatizable androgens T and androstenedione (AE) are superior to the nonaromatizable androgens dihydrotestosterone (DHT) and androsterone (AN) in restoring normal levels of courtship and copulation in castrated male zebra finches *(Poephila guttata)* (Harding, Sheridan and Walters, 1983). These findings suggest that aromatization may be a requisite step for the activation of reproductive behavior in this species. However, one cannot exclude the possibility that the efficacy of treatment with aromatizable androgens may be due to their conversion to some other

undetermined behaviorally active androgenic metabolite, or to some special efficacy of these hormones themselves.

In the present experiment, we used a potent aromatization inhibitor to determine whether the behavioral effects of aromatizable androgens in zebra finches are, in fact, due to their transformation into estrogens. If aromatization is indeed necessary for the activation of reproductive behavior, then birds treated with aromatizable androgen plus aromatization inhibitor should exhibit significantly less behavior than birds treated with with aromatizable androgen alone, and the addition of estradiol (E) to this treatment should restore behavior to normal levels.

METHODS

Animals

All animal rooms were temperature-controlled (24 $\pm$ 2° C) with a 14:10 light:dark cycle. The relative humidity was kept above 50% by console himidifiers, since breeding in this species normally occurs under humid condiditons. Birds were given a commercial finch mix supplemented with a vitamin-mineral preparation (8 in 1, Pet Products) plus water, grit, and cuttlebone *ad libitum*. Fresh orange and greens were provided daily. Zebra finches were obtained from our breeding colony at the American Museum of Natural History. Subjects and stimulus females were at least 120 days of age (doa) with normal plumage patterns. All males were sexually inexperienced prior to testing (males and females were separated before sexual maturity at 60 doa and housed in isosexual groups in holding aviaries).

Castration and hormone treatment

Subjects were bilaterally castrated under Metofane anesthesia (Pitman-Moore) as described in Harding et al. (1983) with one modification. Both testes were removed through an incision in the bird's right side. Following removal of the right testis, the membrane and fatty tissue attaching the vena cava to the dorsal body wall were teased apart

to reveal the left testis and allow its removal. Approximately three weeks following castration, experimental males were randomly assigned to one of three treatment groups. One group was implanted with silastic capsules containing AE (Sigma), an aromatizable androgen which, in previous studies, elicited the highest levels of reproductive behavior in this species of any hormone tested. A second group of castrates received implants containing AE in combination with implants containing 1,4,6-androstatriene-3,17-dione (ATD; Steraloids), an aromatization inhibitor. ATD competitively blocks the *in vitro* conversion of aromatizable androgens into estrogenic metabolites in placental and neural tissue (Alexandre and Balthazart, 1987; Lieberburg, Wallach and McEwen,1977; Schwarzel, Kruggel and Brodie, 1973). The third group of castrates received AE+ATD in combination with estradiol (E; Sigma). The latter treatment was included to ensure that any behavioral deficits following ATD treatment were specific to its inhibition of aromatization. Since these birds received estradiol in combination with ATD, they should not exhibit any deficits in behavior unless the ATD exerted some nonspecific pharmacological effect. AE implants contained 5 mm of packed crystalline hormone in Silastic tubing (0.76 mm i.d., 1.65 mm o.d.; Dow-Corning), and E implants contained 2 mm. ATD implants were twice the area of AE implants, containing 10 mm of packed hormone. These dosages were selected on the basis of previous experiments and of data obtained from other species. In most cases, the hormone capsules were implanted subcutaneously in the upper back region. Because of some initial problems involving the loss of capsules, animals used in the final stages of the experiment were implanted intraperitoneally. Subsequent analyses revealed no differences in the behavior of birds whose hormone capsules were implanted subcutaneously and those whose capsules were implanted intraperitoneally.

Experimental design

All experimental treatments were run concurrently in a randomized block design, with

two males of each treatment represented in a single block of subjects. Experimental males were housed in individual flight cages (0.61^3 m) in visual isolation from other birds. Each cage contained a cardboard nest box (12.7^3 cm, with the upper half of the front panel removed to allow easy entrance and exit) centered high on the rear wall of the cage. During the testing period, males were provided with 4 g of nesting material (green yarn) each day in a wire mesh hopper attached to the front of the cage.

Behavioral testing began one week following hormone implantation (i.e., four weeks after castration). The sexual and aggressive behaviors of zebra finches are described in detail by Morris (1954) and Immelmann (1959). Three behavior categories used in the present study, however, merit further explanation. Courtship displays were classified as high-intensity or low-intensity displays. In a low-intensity display, the male simply turns toward the female and sings. In a high-intensity display, the male stands very erect, sleeks the feathers on the top of his head, fluffs out the sexually dimorphic feathers on its cheeks, throat and flanks, and begins to sing to the female. He then hops down the perch towards her, pivoting 180° with each hop. As he advances, he twists his head and tail towards the female and sings continuously. Courtship displays which included at least one of these high-intensity components were classified as high-intensity displays.

Another behavior closely related to courtship is beak-wiping. In executing a beak-wipe, a bird bends down and scrapes each side of its beak against the perch on which it is standing. Males typically exhibit this behavior during bouts of courtship or when a female is first intoduced into the male's cage. However, birds also beak-wipe after feeding in order to remove food remnants from the beak. Beak-wipes which occurred in this latter context were classified and analysed separately from all other occurrences.

An aggressive behavior was scored when a subject attacked, chased, supplanted or plucked another bird. Two other common aggressive behaviors, pecking and

beak-fencing, were not included in the present analysis. Pecking was excluded because there was some difficulty in distinguishing between pecking and attempting to preen another bird. Beak-fencing was excluded from analysis because it was often difficult to decide which bird initiated the behavior.

Behavioral observations were made between the hours of 0900 to 1200 EST. The order of observations was counterbalanced from test to test and randomized across treatments. In tests for sexual behavior, a female was placed in the male's cage and their behavior recorded for the next 15 min on data sheets divided into 15 sec intervals. Males received three of these tests over a three week period, each with a different female. Following the third test for sexual behavior, the female was left in the male's cage for two weeks. To test for aggressive behavior, an experienced breeding male was released into the pair's cage once per week and the behavior of the three birds was observed for 15 min. Males received two such tests while paired with a female.

At the end of behavioral testing, the birds were anesthetized with Metofane and sacrificed. The body cavity of each bird was thoroughly examined under a dissecting microscope for regenerated testicular remnants. Implants were also checked to make sure they were still in place and still contained hormone. Data from birds with remnants or whose implants were missing were discarded.

Statistical analyses

Since variances were not homoscedastic, all data were analyzed by nonparametric statistics (Siegel, 1956). Differences across the three treatment groups were first tested using the Kruskal-Wallis one-way ANOVA (KWA). If significant differences were found, pair-wise comparisons were made using Mann-Whitney U tests (MWU). Behavioral parameters measured on a nominal scale were analyzed by chi-square tests. The Fisher exact probability test was used in cases where chi-square analysis was inappropriate. One-tailed tests of significance were used for all pair-wise comparisons,

since the directions of treatment effects were predicted at the outset of the experiment.

RESULTS

Significant differences were found across hormone treatment groups on four measures of courtship activity: (i) the number of courtship displays per test (KWA, $p < 0.01$), (ii) the number of high-intensity displays per test (KWA, $p < 0.001$), (iii) the latency to the first courtship display (KWA, $p < 0.05$), and (iv) the frequency of beak-wiping (KWA, $p < 0.001$). Pairwise comparisons revealed that males treated with AE+ATD exhibited significantly fewer courtship displays, fewer high-intensity displays, and less beak-wiping activity than AE-treated males, and had significantly longer latencies to begin courting females. (see Figure 2-1 for individual p values). E, when given concurrently with AE+ATD, reversed the inhibitory effects of ATD and restored levels of courtship activity to those observed in AE-treated males.

Although copulation (i.e., cloacal contact) was rarely observed during tests for sexual behavior, the number of attempted copulations (i.e., mounting or attempting to mount a female) differed significantly across the three treatment groups (KWA, $p < 0.02$; see Figure 2-2). AE+ATD-treated males attempted to copulate less frquently than males treated with AE alone (MWU, $p < 0.05$) or males treated with AE+ATD+E (MWU, $p < 0.01$). Actual copulations occurred only among AE-treated males (25%) or males treated with E in combination with AE+ATD (20%). No AE+ATD-treated male successfully copulated with a female. Similarly, females were observed to solicit copulation on only four occasions, and none of these solicitation displays was directed towards males treated with AE+ATD.

Nestbuilding activity was also related to hormone treatment. Figure 2-3 shows the proportion of males in each treatment group who showed evidence of nest construction after being paired with a female for two weeks. Although chi-square analysis could not be performed across the three treatment groups without violating the assumptions

underlying the test, the data clearly show that AE+ATD-treated males were less likely to construct a nest than males given treatments supplying estrogenic metabolites (Fisher, $p < 0.05$). Whereas 41% of AE- and AE+ATD+E-treated males had a nest after being paired with a female for two weeks, no nests were observed in cages belonging to AE+ATD-treated males at the end of this period. Unfortunately, nestbuilding behaviors never occurred during any of the observation periods, so it is not possible to to determine the relative contributions of the male and the female in the construction of these nests. Normally, however, both the male and female participate (Morris, 1954).

The relationship between hormone treatment and the aggressive behaviors examined was unclear. Pecking and beak-fencing were the most common aggressive behaviors exhibited by males in all treatment groups, but these two behaviors were omitted from analysis for reasons outlined in the preceding section. Other aggressive behaviors (i.e., attacking, chasing, supplanting or plucking) occurred relatively infrequently, and thus did not differ significantly across treatment groups. Nonetheless, it should be noted that these behaviors were exhibited exclusively by AE- and AE+ATD+E-treated males (see Figure 2-4). Males treated with AE in combination with ATD never exhibited these behaviors towards either females or intruder males.

A number of other behaviors occurred fairly frequently during tests for sexual behavior, but did not differ significantly across treatment groups. These included approaching, following or grooming the female, feeding, drinking, and grooming behaviors such as preening, scratching, shaking, and beak-wiping after feeding. The behavior of the stimulus females and intruder males also did not differ significantly across treatment groups.

DISCUSSION

The results of this study affirm the importance of estrogen in the control of reproductive behavior in male zebra finches, and indicate that the aromatization of

androgens is probably a requisite step for maintaining normal levels of sexual behavior. Behaviors normally elicited by aromatizable androgens were inhibited by concurrent pharmacological treatment to block the conversion of androgen to estrogen. Males treated with AE in combination with ATD showed significantly less courtship and copulatory behavior than males treated with AE alone. The level of courtship activity exibited by AE+ATD-treated males was equivalent to the level typically displayed by castrated males (Harding et al., 1983). Males treated with AE+ATD also exhibited significantly less nestbuilding activity than males given treatments supplying estrogenic metabolites. Recent evidence indicates that high concentrations of ATD inhibit not only the *in vitro* formation of estrogen in neural tissue, but also that of DHT (Alexandre and Balthazart, 1987). However, two lines of evidence clearly suggest that the behavioral deficits observed with ATD treatment in the present study are specific to its inhibition of aromatization. First, the addition of E to AE+ATD treatment restored levels of courtship and copulation to those observed in both males treated with AE alone and intact males (Harding et al., 1983). Second, treatment with an inhibitor of DHT synthesis does not inhibit AE-induced sexual behavior in castrated males (Harding, unpublished data).

The relationship between aromatization and aggressive behavior is less clear, since aggressive behaviors occurred too infrequently to make conclusive statistical inferences. Zebra finches are highly gregarious and normally exhibit low levles of aggression in the kind of social environment used in this study. Previous research, however, clearly demonstrated that estrogens play an important role in the activation of aggressive behavior in this species. Males given hormone treatments supplying estrogenic metabolites (i.e., E, T, AE or E+DHT) were significantly more likely to peck or chase intruder males than castrated or intact males (Harding, 1983). Moreover, males treated with E alone were just as aggressive as T-treated males and more aggressive than males treated with AE or E+DHT.

Although aromatization appears necessary for the activation of reproductive behavior in male zebra finches, previous studies suggest that estrogenic metabolites are not sufficient to to maintain normal levels of courtship and copulation. E administered to castrated males did not increase levels of sexual behavior above those of untreated castrates (Harding, 1983; Harding et al., 1983). Treatment with E in combination with DHT, however, restored sexual behavior to levels observed in both intact birds and birds treated with aromatizable androgens. These findings clearly suggest that the actions of aromatizable androgens in this species are due to the effects of both estrogenic and androgenic metabolites.

Both androgens and estrogens appear to be important for the activation of male reproductive behaviors in other avian species as well. However, different behaviors appear to be regulated by different metabolites, and there is considerable variation across species in the relative ability of different metabolites to elicit behavior. Treatment with estrogen alone activated copulation in roosters (Guhl, 1949) and Japanese quail (Adkins, Boop, Koutnik, Morris and Pniewski, 1980), and treatment with ATD or an antiestrogen completely blocked T-stimulated copulation in the latter species (Adkins et al., 1980; Adkins and Nock, 1976). Estrogen also activated various aspects of pre-copulatory behavior in male ring doves (Adkins-Regan, 1981; Cheng and Lehrman, 1975; Hutchison 1970, 1971), and high levels of aromatase activity have been detected in brain regions known to be involved in the control of sexual behavior in this species (Steimer and Hutchison, 1980, 1985). Treatment with DHT also reinstated some degree of sexual and aggressive behavior in castrated male ring doves (e.g., Adkins-Regan, 1981) and Japanese quail (e.g., Adkins et al., 1980), although it was usually not as effective as T. Treatment with aromatizable androgens, however, leads to consistent restoration of normal levels of reproductive behavior in all avian species. This, together with findings that concurrent treatment with both 5α-reduced androgens and estrogen is more effective

than treatment with either hormone alone, has led to the suggestion that androgen and estrogen interact to induce the full pattern of male behavior in most species (Martini, 1982; Perez-Palacios, Larsson and Beyer, 1975).

Courtship vocalizations in male zebra finches are mediated by a sexually dimorphic system of discrete brain nuclei linking higher centers of the brain with the muscles of the syrinx, the avian vocal organ (see Bottjer and Arnold, 1986; Nottebohm, 1980). Several studies have demonstrated that estrogen plays an important role in the differentiation of the neural song control system and male-typical behavior. E administerd to female zebra finch chicks during the first few days after hatching masculinized many vocal control nuclei, in terms of both cellular morphology (Gurney, 1981; Gurney and Konishi, 1979; Pohl-Apel, 1985) and later androgen accumulation (Nordeen, Nordeen and Arnold, 1986; Siegel, Akutagawa, Fox, Konishi and Politch, 1986). Females treated in this manner also showed patterns of courtship and vocal behavior in response to adult T treatment which closely resembled those of normal males (Gurney, 1982; Pohl-Apel, 1985). Several recent studies have detected significant quantities of estrogen receptors in the adult male zebra finch brain, both in grossly dissected tissue (Siegel et al., 1986) and in individual vocal control nuclei (Walters, McEwen and Harding, 1988). These findings, together with those of the present study, suggest that estrogen plays an important role in the activation of the vocal control system in adulthood as well as in its differentiation during development.

While the present study demonstrates the importance of aromatization in the male zebra finch, we do not know where aromatizing enzymes are localized in this species. The conversion of androgen to estrogen has been demonstrated in brain tissue from adult males in every major vertebrate group (Callard, 1983; Callard, Petro and Ryan, 1978). Aromatization appears to be greater in hypothalamic and limbic structures than in other brain areas (Naftolin and Ryan, 1975), and specific regions within the hypothalamus have

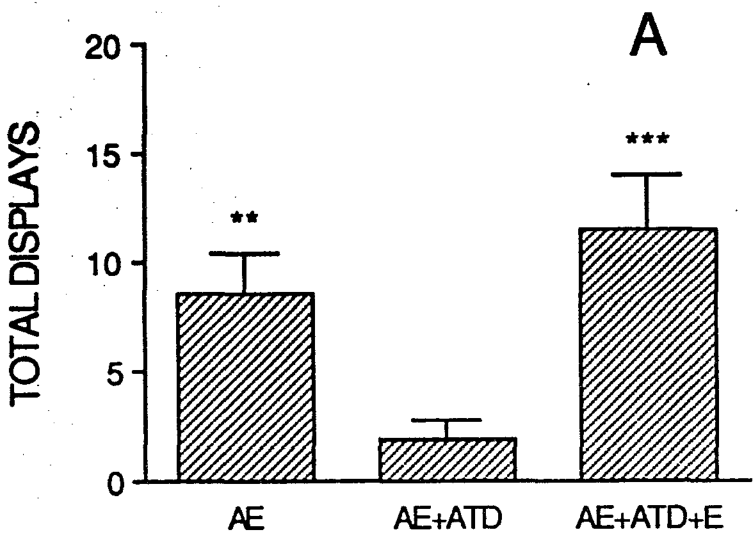
been found to be more efficient than others in converting androgens into estrogens (Selmanoff, Brodkin, Weiner and Siiteri, 1977). However, adult male zebra finches possess exceedingly high levels of E in circulation (Hutchison, Wingfield and Hutchison, 1984). These data strongly suggest that the aromatization of androgens occurs peripherally in this species. On the other hand, the data do not rule out the possibility that aromatization may occur in neural tissue as well, although such a system would seem redundant. We are currently investigating aromatase activity in both neural and peripheral tissue in an effort to resolve this issue.

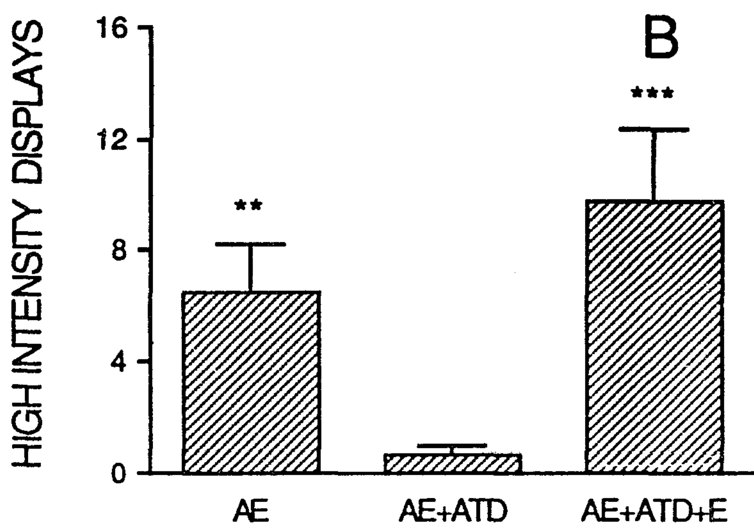
Figure 2-1. The effects of hormone treatment on courtship activity. Panel A: Mean ± SEM courtship displays per test. Panel B: Mean ± SEM high-intensity displays per test. Panel C: Mean ± SEM latency (sec) to begin courting females. Panel D: Mean ± SEM beak-wipes per test. Values marked with asterisks are significantly different from AE+ATD values. See text for abbreviations.

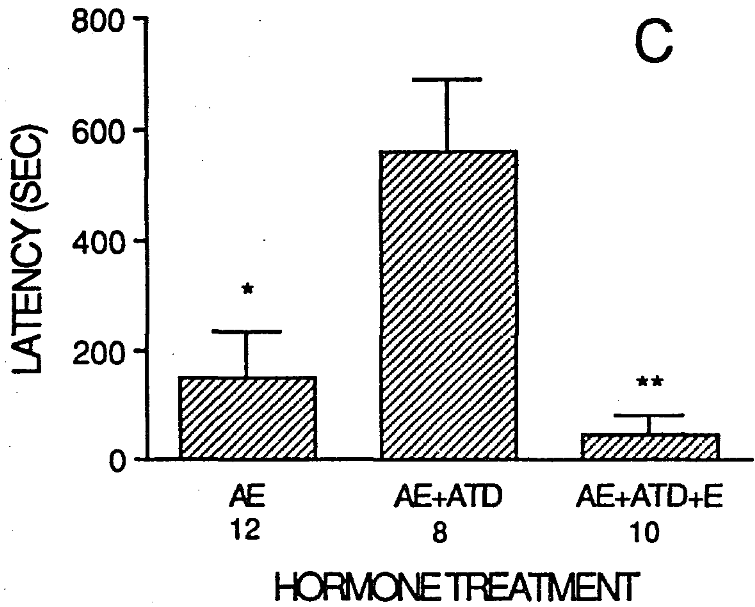
*MWU, $p < 0.05$

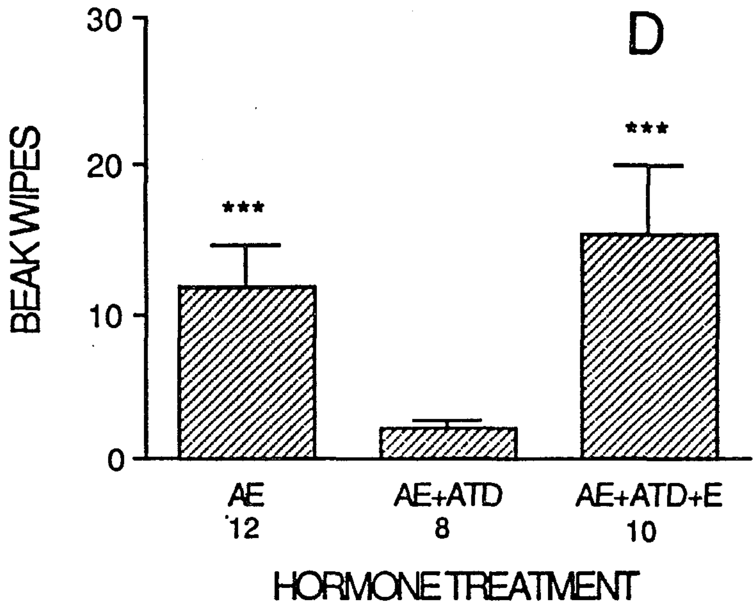
**MWU, $p < 0.01$

***MWU, $p < 0.001$


A
TOTAL DISPLAYS
0
5
10
15
20
**
***
AE
AE+ATD
AE+ATD+E


B
HIGH INTENSITY DISPLAYS
0
4
8
12
16
**
***
AE
AE+ATD
AE+ATD+E


C
LATENCY (SEC)
0
200
400
600
800
*
**
AE
12
AE+ATD
8
AE+ATD+E
10
HORMONE TREATMENT


D
BEAK WIPES
0
10
20
30
***
***
AE
12
AE+ATD
8
AE+ATD+E
10
HORMONE TREATMENT

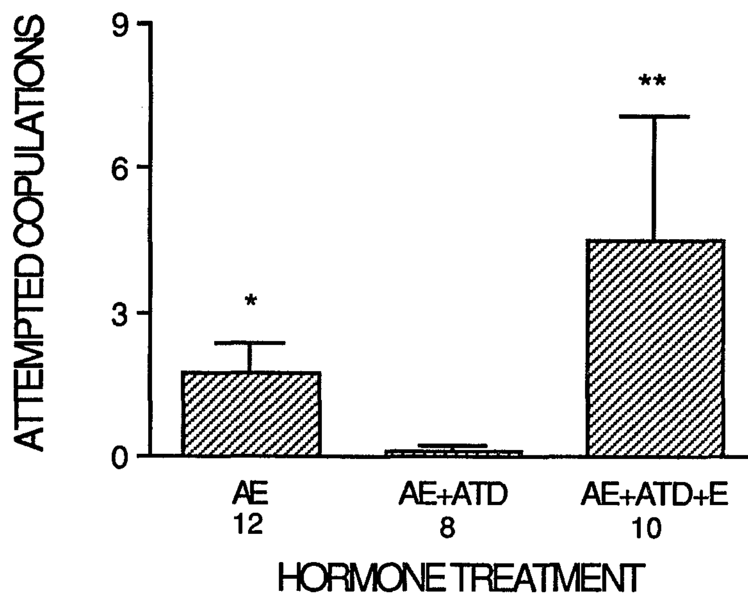


Figure 2-2. Mean ± SEM attempted copulations. Values marked by asterisks are significantly greater than AE+ATD values.

*MWU, $p < 0.05$

**MWU, $p < 0.01$

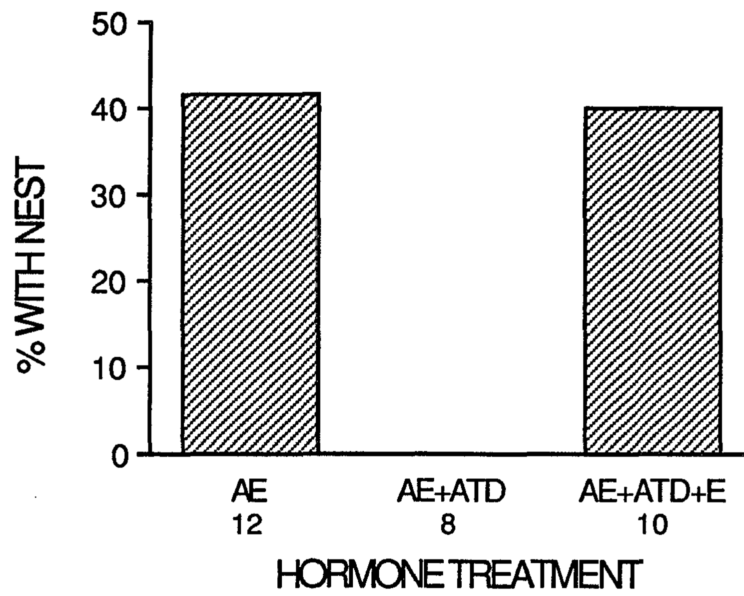


Figure 2-3. Proportion of males in each treatment group showing evidence of nest construction.

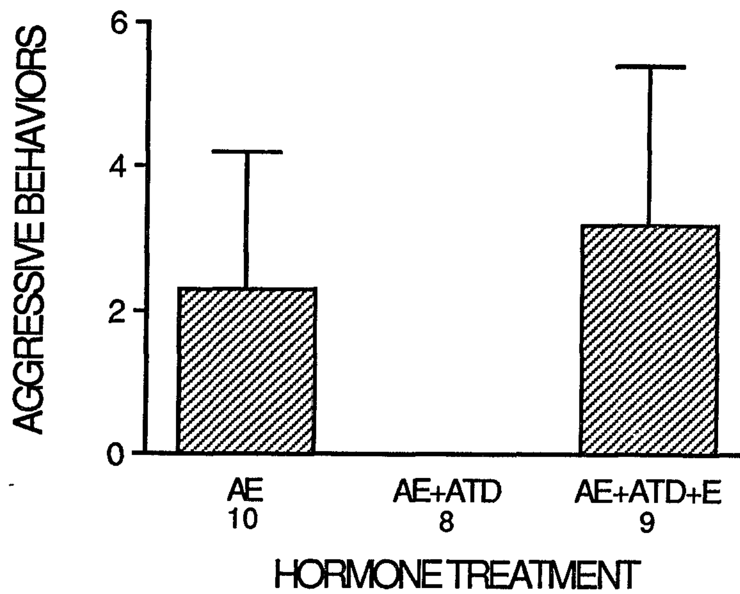


Figure 2-4. Mean ± SEM aggressive behaviors per test. To test for aggressive behavior, an experienced male was released into the subject's cage while it was paired with a female.

CHAPTER 3:
ESTROGENIC CONTROL OF THE MALE ZEBRA FINCH SONG IN DIFFERENT SOCIAL CONTEXTS

ABSTRACT

Recent evidence indicates that the aromatization of androgens to estrogens is necessary for the activation of courtship-related vocalizations in male zebra finches *(Poephila guttata)*. However, although male zebra finches never court females without singing to them, they frequently sing without addressing any object in particular. To determine whether these 'undirected' vocalizations depend on the same hormonal control mechanisms as courtship songs, castrated males were treated with androstenedione (AE), an aromatizable androgen, or AE in combination with 1,4,6-androstatriene-3,17-dione, an aromatization inhibitor which blocks the conversion of aromatizable androgens to estrogenic metabolites. Vocalizations were then recorded in two social contexts: (i) individual males paired with a female, and (ii) males grouped together in isolation from females. AE+ATD-treated males exhibited significantly fewer courtship songs, fewer courtship bouts, longer courtship songs, and longer courtship bouts than males treated with AE alone. In contrast, undirected songs and bouts were not affectd by ATD treatment. These results indicate that estrogens play a more important role in the activation of courtship-related songs than undirected vocalizations.

INTRODUCTION

Male zebra finches *(Poephila guttata)* sing a relatively simple courtship song which, together with specific patterns of body orientation and feather erection, serves as an attractive stimulus for conspecific females. Although males never court females without singing to them, they frequently sing when not courting females. These latter vocalizations, commonly referred to as 'undirected' songs, are sung in a variety of different social contexts: in mixed or monosexual groups, in the area of the nest while visually separated from their mates, and even in complete isolation from other birds. The functional significance of undirected song is unclear. Since zebra finches are non-territorial (Immelmann, 1965), it seems unlikely that undirected songs are important for territorial advertisement and defense. There is some evidence, however, which suggests that the undirected male zebra finch song provides its mate with an acoustic basis of individual recognition that may function to strengthen and/or maintain the pair bond (Miller, 1979).

The undirected songs of the zebra finch share the same basic structure and tonal frequency as courtship songs, although sonographic analyses have revealed subtle bioacoustic differences between these two song types (Sossinka and Böhner, 1980). Courtship songs, for example, contain more introductory notes, are sung faster, and are less variable than undirected songs. Both song types are testosterone-dependent. The quantity of both courtship and undirected songs sung by male zebra finches is linearly correlated with with endogenous testosterone levels in blood plasma (Pröve, 1978). Castrated males also sing both song types much less frequently than gonadally intact birds, and the administration of exogenous testosterone reverses the effects of castration (Pröve, 1974). Undirected songs, however, appear to have a lower threshold for hormonal activation than courtship songs. The frequency of undirected songs decreases more slowly than courtship songs following castration, and can be restored to

pre-castration levels with one half the dose of testosterone required to restore normal levels of courtship song (Pröve, 1974).

The presence of a single testosterone-dependent behavior with seemingly different reproductive functions in male zebra finches presents the behavioral endocrinologist with the unique opprtunity to determine whether the hormonal control of a particular behavior depends on the social context in which it is used. Recent evidence indicates that the conversion of aromatizable androgens (e.g., testosterone, androstenedione) to estrogenic metabolites is necessary for the activation of many of the reproductive behaviors exhibited by male zebra finches (Harding, Sheridan and Walters, 1983; Walters and Harding, 1987). Is the aromatization of androgens a requisite step in the activation of both courtship and undirected song, or are estrogenic metabolites necessary to activate singing only in the context of courtship? To answer this question, castrated male zebra finches were treated with androstenedione (AE), an aromatizable androgen, or AE in combination with 1,4,6-androstatriene-3,17-dione, an aromatization inhibitor which blocks the conversion of aromatizable androgens to estrogenic metabolites. Vocalizations were then recorded in two social contexts: (i) individual males paired with a female, and (ii) males grouped together in isolation from females. If estrogenic metabolites are necessary for the activation of both courtship song and undirected song, then males treated with AE+ATD should sing less than AE-treated males in both social contexts. If aromatization is necessary for the activation of only courtship song, then AE+ATD-treated males should sing less than AE-treated males only in the former context.

METHODS

Sexually naive adult male zebra finches were castrated and randomly assigned to one of three treatment groups. (for complete details of animal maintenance and castration procedures, see Walters and Harding, 1988). One group was implanted with silastic capsules containing androstenedione (AE), an aromatizable androgen which elicits high

levels of sexual behavior in this species. A second group of castrates received AE implants in combination with 1,4,6-androstatriene-3,17-dione (ATD), an aromatization inhibitor which blocks the conversion of aromatizable androgens to estrogenic metabolites. The third group of castrates received AE+ATD in combination with estradiol (E). This latter treatment was included to ensure that any deficits in behavior following ATD treatment were specific to its inhibition of aromatization. Since these birds received estrogen in combination with ATD, they should not exhibit any deficits in vocal behavior unless the ATD exerted some nonspecific pharmacological effect. AE implants contained 5 mm of packed hormone in silastic tubing (0.76 mm i.d., 1.65 mm o.d.), E implants contained 2mm, and ATD implants contained 10 mm.

All hormone treatments were run concurrently with at least two males of each treatment group represented in a single block of 6-8 subjects. Experimental males were initially housed in individual flight cages in visual isolation from other birds. To elicit courtship songs, a stimulus female was introduced into the male's cage for 15 min. All songs were recorded on a Uher 4400 tape recorder and subsequently counted. The behavior of the male was carefully monitored to ensure that the songs being recorded were directed towards the female during bouts of courtship. Males received three of these tests over a three week period, each with a different female. To measure undirected songs, all males comprising a single block of subjects were released together in a small flight room (12.5 m^3) in visual and auditory isolation from other birds. Undirected songs were recorded over a 30 min period on an SSR event recorder (Semiotic Systems) and subsequently counted. Songs directed towards other males were recorded separately and were not included in the data analysis. Recordings were made twice daily for 4-7 days. At the end of the testing period, the birds were anesthetized with Metofane and sacrificed. Data from birds with regenerated testicular remnants or with missing or empty implants were discarded.

The following terms and methods of measurements were used in the analysis. A 'song' was defined as a stereotyped sequence of notes (called 'song units' by Price, 1979 or 'motifs' by Sossinka and Bohner, 1980) preceded by two or more introductory elements. Because of background vocalizations of males in adjacent cages, the introductory notes of courtship songs were often difficult to hear. For this reason, 'song duration' was estimated by measuring the duration of a 'bout' and dividing it by the number of songs it contained. A 'bout' was defined as a series of two or more individual songs that were separated from each other by an interval of less than 5 sec. Normally, songs comprising a bout are separated from each other by 0.4 - 2 sec (Sossinka and Bohner, 1980). A 5 sec criterion was chosen for the present study, however, because it proved to be sufficient time to determine *whether* a new song had begun even if we could not determine precisely *when* it began.

The data were analyzed by 2-way ANOVAs (hormone treatment *x* social context) with repeated measures on one factor (context). If a significant F ratio was found, pairwise comparisons were analyzed by the method of Scheffé (1959) with $\alpha = 0.10$ or less as recommended by Myers (1969, p. 384).

RESULTS

Analysis of variance revealed that the effects of hormone treatment on singing behavior depended on social context. Significant treatment *x* context interactions were observed with song frequency ($F = 5.85$; $df = 2,23$; $p < 0.01$), song duration ($F = 15.4$; $df = 2,17$; $p < 0.01$), bout frequency ($F = 4.3$; $df = 2,23$; $p < 0.05$) and bout duration ($F = 6.4$; $df = 2,17$; $p < 0.01$).

Pairwise comparisons revealed that males treated with AE+ATD exhibited significantly fewer courtship-related songs and bouts than males treated with AE alone or males treated with AE+ATD+E (see Figure 3-1 for individual p values). Whereas all AE+ATD+E-treated males and all but one AE-treated male courted at least one female,

50% of males treated with AE+ATD never courted a female in any of the three tests for sexual behavior. In contrast, the number of undirected songs and bouts were not affected by hormone treatment: males treated with AE+ATD sang no fewer undirected songs than AE or AE+ATD+E-treated males.

Within group comparisons revealed that AE-treated males sang significantly more courtship songs and bouts per 15 min than undirected songs, as did males treated with AE+ATD+E. The number of courtship songs and bouts sung by AE+ATD-treated males, on the other hand, was identical to the number of undirected songs and bouts. All AE+ATD-treated males who failed to court females sang undirected songs when grouped together with other males.

The effects of hormone treatment on the duration of courtship and undirected songs and bouts are shown in Figure 3-2. The duration of courtship songs and bouts of males treated with AE+ATD were significantly longer than those of both AE-treated males and males treated with AE+ATD+E. The duration of undirected songs and bouts was not affected by hormone treatment. Within group comparisons of the duration data indicated that the courtship songs and bouts of both AE-treated and AE+ATD+E-treated males were significantly shorter than undirected songs and bouts. The duration of courtship-related songs and bouts of males treated with AE+ATD were not statistically different from the duration of undirected songs and bouts. It should be noted, however, that five of the eight birds treated with AE+ATD did not sing in one context or the other, leaving only three birds from which to make statistical comparisons.

The effects of hormone treatment on the number of songs sung per bout in the two social contexts are shown in Figure 3-3a. Although AE+ATD-treated males sang fewer courtship songs per bout than either AE- or AE+ATD+E-treated males, analysis of variance revealed only a significant context effect ($F = 28.8$; $df = 1,17$; $p < 0.01$). When averaged across treatment groups, courtship bouts contained significantly more songs

than undirected bouts ($p < 0.01$; see Figure 3-3b).

DISCUSSION

These findings indicate that the *in vivo* conversion of aromatizable androgens to estrogenic metabolites plays a more important role in the activation of courtship-related vocalizations than undirected songs. Treatment with an aromatization inhibitor significantly decreased the frequency and increased the duration of AE-induced courtship songs, but had no effect on either the frequency or duration of undirected vocalizations. Moreover, AE+ATD-treated males who failed to sing courtship songs in tests for sexual behavior displayed no deficits in vocal behavior when grouped with males. Together, these findings strongly suggest that estrogens may affect motivational aspects of vocal behavior and/or the male's sensitivity to relevant stimulus qualities of conspecific females.

Although aromatization appears to be necessary for the activation of courtship song in this species, several lines of evidence indicate that estrogenic metabolites are not sufficient to maintain normal levels courtship song. First, intact males treated with cyproterone acetate, a potent antiandrogen, exhibit fewer courtship songs than untreated controls (Pröve and Immelmann, 1982). Males treated in this manner also exhibit fewer undirected songs. Second, although E administration to castrated males does not increase levels of courtship song above those of untreated castrates, treatment with E in combination with dihydrotestosterone restores courtship songs to levels observed in both intact birds and birds treated with aromatizable androgens (Harding et al., 1983). Third, steroid receptor assays have demonstrated the presence of both androgen- and estrogen-binding proteins in brain regions known to be involved in the control of vocalizations (Harding, Walters and Parsons, 1984; Walters, McEwen and Harding, 1988). These findings clearly suggest that the effects of aromatizable androgens on courtship song are due to the actions of both estrogenic and androgenic metabolites. The fact that undirected songs are inhibited by cyproterone acetate and not by ATD suggests

that androgenic metabolites may be sufficient to maintain normal levels of undirected song in this species.

The intensity differences between courtship songs and undirected songs in male zebra finches are affected not only by endocrine status, but also by the stimulus qualities of the female (Bischof, Böhner and Sossinka, 1981). In fact, the presence of female conspecifics also affect the endocrine status of the male. Males paired with a female have higher testosterone levels than isolated males or males living in monosexual groups, and repeated exposure to an individual female results in increased plasma testosterone levels in isolated males (Pröve, 1978). These findings, together with those of the present study, may explain the different hormonal thresholds for the activation of courtship and undirected song. The low levels of testosterone, such as those observed in zebra finch males in the absence of conspecific females, may provide sufficient androgenic metabolites to maintain baseline levels of low-intensity undirected song, but may not provide sufficient estrogenic metabolites necessary to activate of high rates of courtship song.

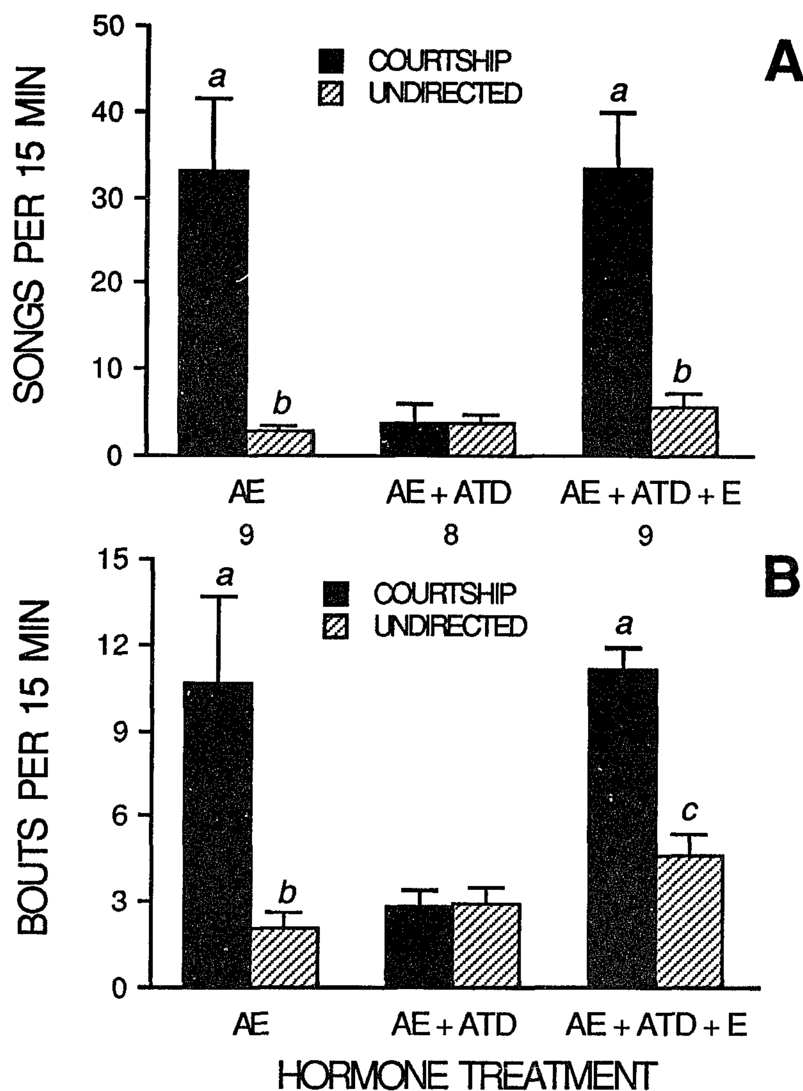


Figure 3-1. The effects of hormone treatment on the frequency of courtship and undirected songs (A) and bouts (B). Courtship songs were recorded from individual males paired with conspecific females, and undirected songs were recorded from the same males grouped together in isolation from females.

a : significantly different from AE+ATD, $p < 0.01$

b : significantly different from courtship song, $p < 0.01$

c : significantly different from courtship song, $p < 0.05$

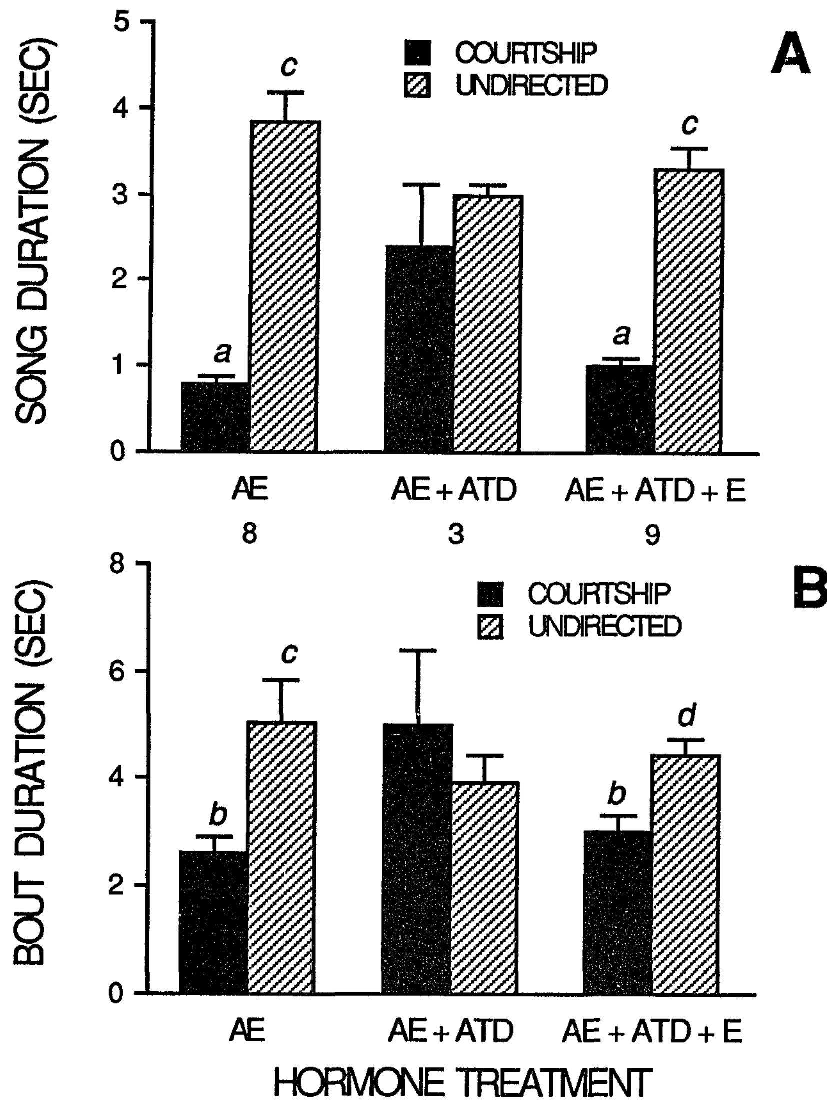


Figure 3-2. The effects of hormone treatment on the duration of courtship and undirected songs (A) and bouts (B).

a : significantly different from AE+ATD, $p < 0.01$

b : significantly different from AE+ATD, $p < 0.05$

c : significantly different from courtship song, $p < 0.01$

d: significantly different from courtship song, $p < 0.10$

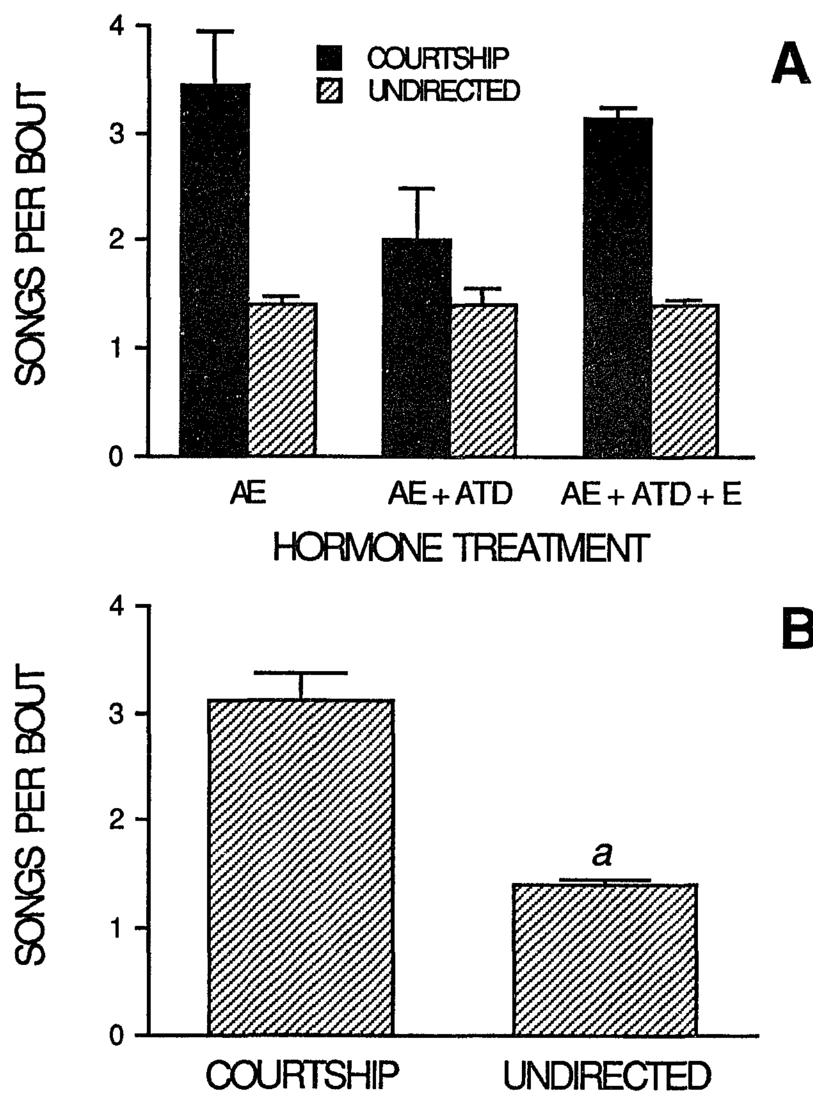


Figure 3-3. The effects of hormone treatment on the number of courtship and undirected songs per bout (A) and the number of courtship and undirected songs per bout averaged across the three treatment groups (B).

a : significantly different from courtship song, $p < 0.01$

CHAPTER 4:
ESTROGEN RECEPTOR LEVELS IN HYPOTHALAMIC AND VOCAL CONTROL NUCLEI IN THE MALE ZEBRA FINCH

ABSTRACT

Estrogens play an important role in the activation and differentiation of vocal behavior in male zebra finches. In the present experiment, we conducted a series of *in vitro* binding assays to quantify estrogen receptor concentrations in individual hypothalamic and vocal control nuclei. Receptor concentrations were measured in cytosol fractions obtained from castrated males and, since adrenalectomy is not a viable possibility in this species, in castrated males treated with 1,4,6-androstatriene- 3,17-dione (ATD), an inhibitor of estrogen synthesis. Specific, high-affinity estrogen binding was detected in both untreated castrates and castrates treated with ATD. Although ATD treatment had no effect on estrogen receptors in hypothalamic-preoptic tissue, ATD-treated males has significantly higher levels of [^{3}H]estrogen binding in three vocal control nuclei: the dorsomedial portion of the intercollicular nucleus (DM), the magnocellular nucleus of the anterior neostriatum (MAN), and Area X. Low levels of estrogen binding were also detected in cytosol from the caudal portions of the ventral hyperstriatum (HVc) and the robust nucleus of the archistriatum (RA) of both untreated and ATD-treated castrates. In most brain regions examined, estrogen receptor levels were lower than androgen receptor levels measured in previous experiments. The presence of both androgen- and estrogen-concentrating neurons in these areas provide compelling evidence for the interaction of androgens and estrogens in the neural control of male vocal behavior in this species.

INTRODUCTION

Male zebra finches *(Poephilia guttata)* sing a relatively simple song which, together with specific patterns of body orientation and feather erection, serves as an attractive stimulus for conspecific females. Many studies have shown this behavior to be androgen-dependent. The number of courtship songs sung by adult zebra finch males, for example, is linearly correlated with endogenous testosterone levels in blood plasma (Pröve, 1978). Castrated adult males sing much less frequently than gonadally intact birds, and administration of exogenous androgen reverses the effect of castration (Arnold,1975; Harding, Sheridan and Walters, 1983; Pröve, 1974). Intact males treated with cyproterone acetate, a potent antiandrogen, also exhibit fewer courtship songs than untreated controls (Pröve and Immelmann, 1982).

Vocalizations in male zebra finches are mediated by an interconnected system of discrete brain nuclei originally described in the canary (see Figure 4-1). Four of these structures, nucleus interface (NIf), the caudal portion of the ventral hyperstriatum (HVc), the robust nucleus of the archistriatum (RA), and the tracheosyringeal branch of the hypoglossal nerve (nXIIts), are part of a direct efferent pathway linking the telencephalon to the muscles of the syrinx, the avian vocal organ (McCasland, 1987; Nottebohm, Stokes and Leonard, 1976). Other areas of the song control system, Area X of the parolfactory lobe, the magnocellular nucleus of the anterior neostriatium (MAN), the dorsomedial portion of the intercollicular nucleus (DM), and nucleus Uva, are monosynaptically connected to RA or HVc (Nottebohm and Kelley, 1982; Kelley and Nottebohm, 1979; Ryan and Arnold, 1981). Six of these song control nuclei are sexually dimorphic. Area X, MAN, HVc, RA, DM and nXIIts are significantly larger in male than in female zebra finches, who do not sing (Gurney, 1982; Nottebohm and Arnold, 1976).

Autoradiographic analyses of the zebra finch brain reveal that five of these nuclei are

sensitive to gonadal steroids. MAN, HVc, RA, DM, and nXIIts accumulate significant quantities of radioactivity following injection of tritiated testosterone into castrated males (Arnold, 1980; Arnold, Nottebohm and Pfaff, 1976; Arnold and Saltiel, 1979). Significant quantities of androgen receptors have also been measured biochemically in grossly dissected neural tissue (Siegel, Akutagawa, Fox, Konishi and Politch, 1986), in individual vocal control nuclei (Harding, Walters and Parsons, 1984), and in the syringeal musculature itself (Lieberburg and Nottebohm, 1979). Males typically exhibit higher levels of neural androgen binding than females (Arnold, 1980; Arnold and Saltiel,1979; Siegel et al., 1986).

These behavioral and anatomical studies clearly establish the importance of androgens in the control of singing in the male zebra finch. However , there is also a growing body of evidence which suggests that estrogens play an important role as well. Aromatizable androgens (e.g., testosterone, androstenedione) are more effective than nonaromatizable androgens (e.g., dihydrotestosterone) in restoring normal levels of courtship song in castrated males, as is treatment with a combination of estradiol and dihydrotestosterone (Harding, 1983; Harding et al., 1983). Recent evidence indicates that androstenedione (AE), the most effective hormone for activating singing in this species, must be converted to estrogenic metabolites in order to exert its effects. Castrates treated with AE plus 1,4,6-androstatriene-3,17-dione (ATD), an aromatization inhibitor, exhibit fewer courtship songs than males treated with AE alone (Walters and Harding, 1988). Estradiol, when given concurrently with AE + ATD, reverses the inhibitory effects of ATD. Intact male zebra finches also possess exceedingly high levels of estradiol in circulation (Hutchison, Wingfield and Hutchison, 1984).

Estrogens also appear to play an important role in the differentiation of the neural song control system. Estradiol administered to female zebra finch chicks during the first

few days after hatching masculinizes many vocal control nuclei, in terms of both cellular morphology (Gurney, 1981; Gurney and Konishi, 1980) and androgen accumulation (Nordeen, Nordeen and Arnold, 1986; Nordeen, Nordeen and Arnold, 1987; Siegel et al., 1986). Females treated in this manner also show patterns of courtship and vocal behavior in response to adult hormone treatment that closely resemble those of the normal male (Gurney, 1982; Pohl-Apel, 1985). Hutchison et al. (1984) report that male zebra fich chicks exhibit a sex-specific surge in circulating estradiol which peaks during the sensitive period for brain differentiation.

In spite of these findings, the adult male zebra finch brain appears to contain very low levels of estrogen-concentrating neurons. Autoradiographic studies reveal that tritiated estradiol concentrates predominantly in the periventricular and infundibular regions of the hypothalamus. No neurons in MAN, HVc, RA or nXIIts appear to concentrate label following injection of tritiated estradiol into castrated males (Arnold, 1979). Biochemical assays have yielded similar results. Using the method of DNA-cellulose chromatography, Siegel et al. (1986) report that estrogen receptor levels are very low in cytosol preparations of forebrain zones which include HVc, RA, MAN, and Area X.

The low levels of estrogen binding observed in these studies may be due to competition from endogenous hormone. Since little is known about adrenal sex steroid secretion in songbirds, it seems possible that estrogens might be synthesized in castrated males via peripheral or central aromatization of adrenal androgens. No autoradiographic studies have been performed with adrenalectomized zebra finches (adrenalectomy is not a viable possibility in these birds), and the males used in the cytosol binding assays conducted by Siegel et al. (1986) were neither adrenalectomized nor castrated.

In the present study, we conducted a series of sensitive *in vitro* binding assays to

quantify estrogen receptor concentrations in individual hypothalamic and vocal control nuclei in the male zebra finch brain. Receptor concentrations were measured in cytosol fractions obtained from castrated males and compared to values obtained when competition from endogenous estrogen was minimized by treatment with ATD.

METHODS

Animals

Adult male zebra finches were obtained from our breeding colony at the American Museum of Natural History (AMNH) or purchased through a local supplier (Canary Bird Farms, Old Bridge, NJ). They were housed in small groups of 2-6 males per cage (0.61 m^3) in visual isolation from other birds. The animal room was temperature controlled ($24 \pm 2^{\circ}$ C) with a 14:10 light:dark cycle. The relative humidity was kept above 50% by console humidifiers, since breeding in this species normally occurs under humid conditions. Birds were castrated under Metofane anaesthesia (Pitman-Moore) as described in Harding et al. (1983). ATD-treated castrates were implanted intraperitoneally with two Silastic tubes (0.76 mm i.d., 1.65 mm o.d.; Corning) packed with 10 mm of ATD (1,4,6-androstatriene-3,17-dione; Steraloids). In previous experiments, this dosage effectively inhibited AE-induced sexual behaviors in castrated males (Walters and Harding, 1988). Animals were transferred from AMNH to Rockefeller University immediately prior to sacrifice. Untreated castrates were sacrificed 1-6 weeks following castration, and ATD-treated castrates were sacrificed 4-6 weeks following hormone implantation.

Saturation assays

Saturation assays were performed to examine the binding capacity of cytosol from the basal hypothalamus-preoptic area (BH-POA) for [2,4,6,7-^{3}H]estradiol (90.4 - 111.0 Ci/mmol; New England Nuclear). Animals were sacrificed by rapid decapitation.

Brains were quickly removed and placed on ice. The BH-POA was excised using the following margins: anterior: tractus mesencephalicus at the level where it forms an inverted V; lateral: 1 mm from the midline; posterior: where the third nerve enters the brain; dorsal: the level of the anterior commisure. Following dissection, tissue samples were maintained at 4° C unless otherwise noted. Tissue from 4-9 zebra finches was pooled and homogenized with a teflon/glass homogenizer in 2 ml TEGD buffer (10 mM Tris-HCl, 1.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol added on the day of use, and pH adjusted to 7.4 with hydrochloric acid). The homogenizer was rinsed with 1.2 ml buffer for a final sample volume of 3.2 ml. The sample was centrifuged at 100,000 x g for 30 min in a Beckman L3-50 ultracentrifuge. Aliquots (100 µl) of the resultant cytosol fraction were incubated with 50 µl of TEGD buffer containing varying concentrations of [^{3}H]estradiol (0.01-2.7 nM). Parallel incubations containing a 100-fold molar excess of unlabeled diethylstilbesterol (DES) were included in all assays to correct for nonspecific binding. Samples were incubated at 4° C for 4-6 hours, conditions which produced maximal binding in preliminary assays.

The [^{3}H]estradiol bound to receptors was separated from free ligand on Sephadex LH-20 minicolumns. The columns were made from 1 ml disposable pipet tips stoppered with a 4 mm glass bead and filled to 1 in from the top of the column with LH-20 swelled in TEG (10 mM Tris-HCl, 1.5 mM EDTA, 10% v/v glycerol, pH 7.4). The columns were first equilibrated with 200 µl TEGD. 15 min after the TEGD wash, 100 µl aliquots of the incubates were applied to the columns, then washed onto the column bed with an additonal 100 µl TEGD. The [^{3}H]estradiol bound to receptors was eluted from the columns 30 min later with 600 µl buffer. 5 ml Betaflour were then added to the eluate and allowed to equilibrate overnight before counting. The protein content of the incubates

was determined by the dye-binding method of Bradford (1976), using 20 μl aliquots of sample. Results, expressed as fmol bound [^{3}H]estradiol/mg protein, were then analyzed by the method of Scatchard (1949).

Specificity assays

The specificity of the receptor was determined by testing the ability of unlabeled estradiol, dihydrotestosterone, progesterone, corticosterone, and ATD to compete with [^{3}H]estradiol for binding sites in BH-POA cytosol. BH-POA from 9 castrated finches were pooled and homogenized in 3.2 ml TEGD followed by a 1.8 ml rinse. Aliquots of cytosol (200 μl) were incubated with 100 μl TEGD containing sufficient [^{3}H]estradiol to make a final concentration of 1 nM. A 100-fold molar excess of unlabeled DES was used to determine nonspecific binding. Parallel incubations contained 1, 10, 100 or 1000 nM unlabeled competitor. Dilutions of unlabeled competitors were made from stock solutions in ethanol so that the ethanol concentration in the incubates never exceeded 1%. Bound hormone was separated from free hormone on LH-20 columns and measured as described for saturation assays. The amount of specific binding of [^{3}H]estradiol in the presence of competing steroid was compared to specific binding in the absence of competing steroid.

Assay of microdissected tissue

Individual nuclei were microdissected according to the method of Palkovits (1973). Chilled 500 μm or 1000 μm stainless steel tubes were used to remove nuclei from 200 μm thick frozen sections of finch brain. The anterior ends of the brains were blocked with a razor blade, mounted onto cryostat chucks with distilled water, and frozen using powdered dry ice. Brains were then transferred to an A/O cryostat and allowed to equilibrate to a temperature of -15° C for at least 20 min before cutting sequential coronal sections. The brains were mounted at such an angle that two sections could be sliced from the rear of the telencephalon before the optic lobes were included in the plane of

section. The sections were quickly thaw-mounted onto glass slides and stored at -40^{0} C overnight before dissection. The location of nuclei was determined using the atlas of Stokes, Leonard, and Nottebohm (1974) and the autoradiographic work of Arnold, Nottebohm and Pfaff (1976) as guides. During dissection, the temperature of the sections was maintained at -15° C with a Cambion thermoelectrically-cooled plate. Three of the vocal control nuclei examined, RA, MAN and Area X, are clearly visible in frozen sections. The positions of HVc, DM, and the two hypothalamic nuclei were determined in reference to other clearly visible landmarks. HVc was taken from the apex of the telencephalic ventricle as it extends caudally from Field L, an auditory relay nucleus. Samples of DM were taken from the portion of the intercollicular nucleus dorsomedial to the nucleus mesencephalicus lateralis, pars doralis. The preoptic sample (POA) was taken from the center of the triangle formed by the tractus septomesencephalicus as it hooks laterally, the midline, and the ventral surface of the hypothalamus. The magnocellular paraventricular sample (PVM) was located with reference to the anterior commissure, angling dorsocaudally from the preoptic area to just ventral to the commissure.

Nuclei from 6 finches were pooled per assay. Pooled nuclei were homogenized in 275 μl TEGD and centrifuged in a Beckman Airfuge at 100,000 *x* g for 30 min. Aliquots of cytosol (100 μl) were added to 50 μl TEGD containing sufficient [^{3}H]estradiol to make a final concentration of 1 nM, then assayed as described for saturation assays.

RESULTS

Scatchard analysis revealed that estrogen binding in BH-POA cytosol from both untreated and ATD-treated castrates was saturable and that only a single, high-affinity component was being measured. The binding capacity (Bmax) of BH-POA cytosol from ATD-treated castrates for [^{3}H]estradiol was identical to that observed in untreated castrates (6.3 $\pm$ 1.0 vs. 6.2 $\pm$ 1.3 fmol/mg of protein respectively). Treatment with ATD

however, produced a slight decrease in the dissociation constant (Kd) of the receptor, from 0.36 ± 0.05 to 0.14 ± 0.01nM, which is consistent with a reduction in the level of an endogenous competitor.

Competition studies revealed that the assay was specific for estrogen receptors (Figure 4-2). Unlabeled estradiol significantly reduced [^{3}H]estradiol binding in BH-POA cytosol, and higher concentrations were more effective than lower ones. Progesterone, corticosterone, dihydrotestosterone, or ATD did not inhibit the binding of [^{3}H]estradiol to any great extent, and increasing concentrations had no effect on the degree of inhibition observed.

Levels of cytosol [^{3}H]estradiol binding in individual nuclei of castrates and castrates treated with ATD are shown in Figure 4-3. Analysis of variance revealed significant brain region ($F = 11.9$; $df = 1,96$; $p < 0.01$), ATD treatment ($F = 4.66$; $df = 6,96$; $p < 0.01$), and region *x* ATD treatment ($F = 2.42$; $df = 6,96$; $p < 0.05$) effects.

In untreated castrates, [^{3}H]estradiol binding was the highest in hypothalamic nuclei, ranging from 8.7 ± 1.7 fmol/mg protein in POA to 9.8 ± 1.8 fmol/mg protein in PVM. Low levels of cytosol estrogen binding were observed in four of the five vocal control nuclei examined: MAN, HVc, RA, and DM. No specific [^{3}H]estradiol binding was ever observed in Area X. The levels of cytosol estrogen binding in these areas were comparable to those obtained under exchange condidtions (Walters and Harding, unpublished observations).

The amount of [^{3}H]estradiol bound in hypothalamic nuclei was unaffected by ATD treatment, as was observed in gross dissections of BH-POA. However, treatment with ATD resulted in significant increases in cytosol estrogen binding in three vocal control nuclei. Levels of bound [^{3}H]estradiol were significantly greater in MAN (Student's t-test, $p < 0.05$) and DM (Student's t-test, $p < 0.05$), and were comparable to those found

in hypothalamic nuclei. Low but significantly greater (Student's t-test, $p < 0.01$) levels of estrogen binding were also observed in Area X. HVc was the only brain region examined in which cytosol estrogen binding was greater in untreated castrates that in ATD-treated birds, but this difference was not significant.

DISCUSSION

A number of recent behavioral studies have demonstrated the importance of estrogens in activating courtship-related vocalizations in male zebra finches (Harding, 1983; Harding et al., 1983; Walters and Harding, 1988). In spite of these findings, autoradiographic studies (Arnold, 1979), cytosol receptor assays (Siegel et al., 1986) and, most recently, immunocytochemical techniques (Gahr, Flügge and Güttinger, 1987), have provided little or no evidence of estrogen-concentrating neurons in the forebrain regions involved in the control of these vocalizations. The results of the present study suggest that the absence of estrogen binding in autoradiographic studies and cytosol receptor assays may be partly due to competition from endogenous estrogen. Treatment with ATD, an inhibitor of estrogen synthesis, resulted in significant increases in cytosolic estrogen receptors in two of the five vocal control areas examined, DM and MAN, and caused estrogen receptors to appear for the first time in a third area, Area X. However, low levels of cytosolic estrogen binding were observed in the forebrain regions of MAN, HVc and RA of untreated castrates as well. These latter findings suggest that discrepancies that exist between the present study and previous autoradiographic and immunocytochemical studies may also be due to the relative sensitivities of the methods employed. Autoradiography and immunocytochemistry are valuable techniques for mapping hormone-sensitive areas of the brain. One of the disadvantages of these techniques, however, is that they may not be able to distinguish uniformly low levels of binding from background labeling. Although *in vitro* binding assays have their

shortcomings, they are generally more sensitive to low receptor concentrations than autoradiography. For example, *in vitro* binding assays have detected low concentrations of neural estrogen (Gerlach, McEwen, Toran-Allerand and Friedman, 1983), progestin (McEwen, Davis, Gerlach, Krey, MacLusky, McGinnis, Parsons and Rainbow, 1983), and glucocorticoid (Meyer, Leveille, de Vellis, Gerlach and McEwen, 1982) receptors in area where autoradiographic analyses have not found evidence of receptors.

Although treatment with ATD resulted in significantly higher receptor concentrations in cytosol from DM, MAN, and Area X, estrogen binding in cytosol from other brain regions was unaffected by ATD treatment. Binding in cytosol prepared from gross dissections of BH-POA was also unaffected by ATD treatment. These data may reflect regional differences in aromatase activity. Comparative studies indicate that the regional distribution of neural aromatizing enzymes is an evolutionarily conservative characteristic, with hypothalamic and limbic structures containing the highest levels of aromatase activity (Callard, 1983). However, songbirds are unique in that their hormone-dependent singing behavior is partly mediated by a relatively recently evolved network of hormone-sensitive forebrain nuclei. The aromatizing capabilities of vocal control nuclei in songbirds remain to be determined. The regional effect of ATD treatment on estrogen binding in cytosol may also reflect regional differences in receptor regulation. In most estrogen receptor systems which have been studied, the administration of a saturating dose of estrogen to hormone-deprived animals results in the dramatic accumulation of occupied receptors in nuclear fractions which is paralleled by a concomitant decrease in binding sites in cytosol fractions. However, Lieberburg, MacLusky and McEwen (1980) found that cytosol receptor levels in rat hypothalamic tissue remained relatively constant after estrogen administration despite a significant increase in occupied nuclear receptors. Tissue from other limbic areas and from pituatary,

on the other hand, exhibited a significant depletion of cytosol estrogen binding sites when nuclear estrogen retention was maximal. Regional differences in the regulation of estrogen receptors were also demonstrated by Clark, MacLusky and Naftolin (1982) in receptor-containing areas of the rat brain. The factors underlying these regional differences in neural steroid receptor regulation remain largely unknown.

In most brain regions examined, the number of cytosol estrogen receptors was lower than the number of androgen receptors measured in previous experiments (Harding et al., 1984; see Figure 4-4). Androgen binding in PVM cytosol was twice as high as estrogen binding, and almost three times greater in POA. Androgen binding was also substantially higher in all vocal control nuclei examined (androgen receptors were not measured in MAN and Area X) except DM, which appears to contain equivalent levels of androgen and estrogen receptors. The presence of both androgen- and estrogen-concentrating neurons in these areas agrees well with behavioral data which indicate that a combination of androgenic and estrogenic steroids is necessary for the activation of courtship song and other sexual behaviors in castrated male zebra finches. Together, these findings provide compelling evidence for the interaction of androgens and estrogens in the neural control of male vocal behavior in this species.

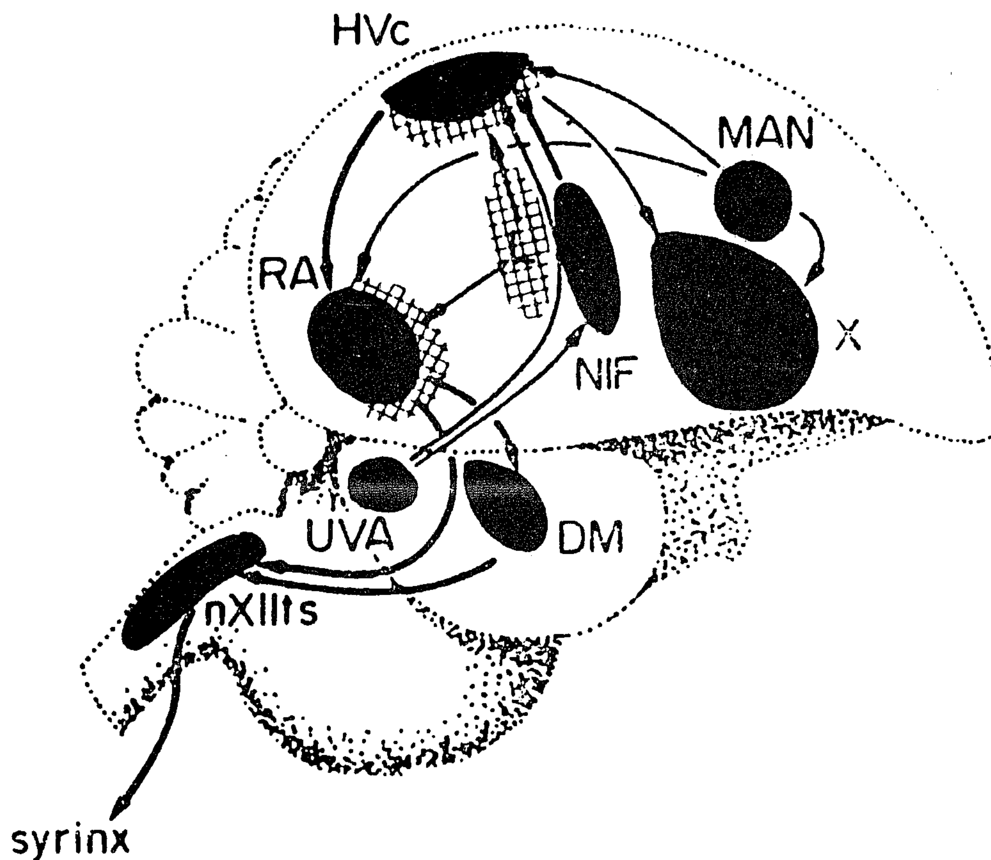


Figure 4-1. Schematic representation of the zebra finch vocal control system. NIf, HVc, RA and nXIIts form a direct descending motor pathway leading to the muscles of the syrinx. Abbreviations: X, Area X; MAN, magnocellular nucleus of the anterior neostriatum; NIf, nucleus interface; DM, dorsomedial nucleus of the intercollicular nucleus; L, Field L; HVc, ventral hyperstriatum, pars caudale; UVA, nucleus Uva; RA, robust nucleus of the archistriatum; nXIIts, nucleus of the tracheosyringeal branch of the hypoglossal nerve. Shaded areas contain auditory neurons. Reproduced from McCasland (1987).

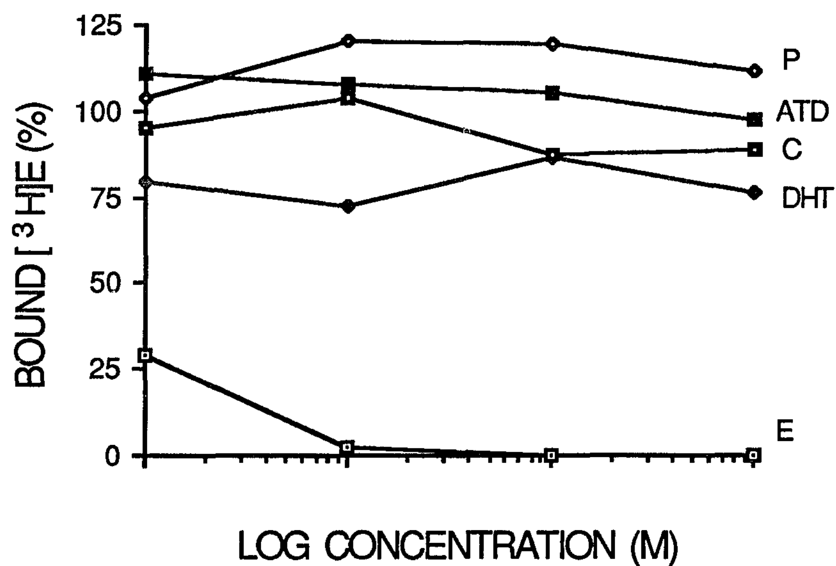


Figure 4-2. Competition by unlabeled steroids for [^{3}H]estradiol (1 nM) in BH-POA cytosol. Data are expressed as a percentage of specific [^{3}H]estradiol bound in the absence of unlabeled competitor. Each data point represents the the mean of 2-4 determinations. E, estradiol; DHT, dihydrotestosterone; P, progesterone; C, corticosterone; ATD, 1,4,6-androstatriene-3,17-dione.

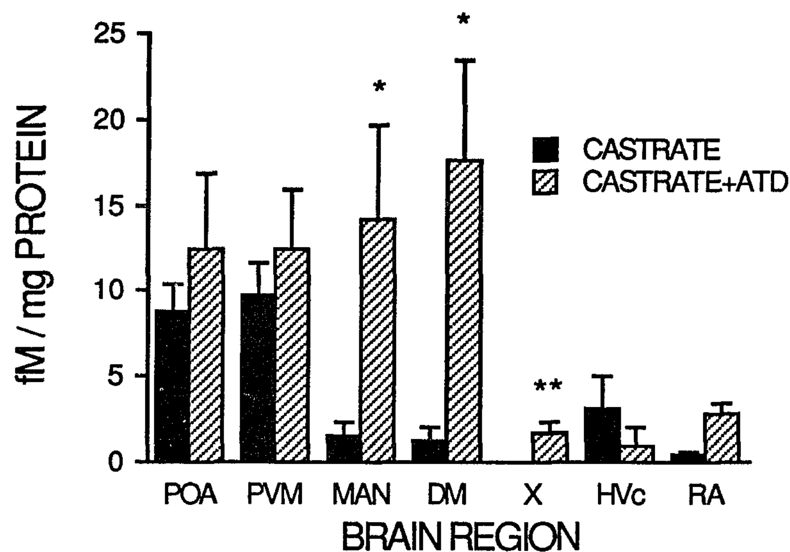


Figure 4-3. Mean ± SEM cytosol [^{3}H]estradiol binding in individual hypothalamic and vocal control nuclei of untreated castrates (n = 8 assays) and castrates treated with ATD (n = 10 assays). Values marked with asterisks are significantly higher than untreated castrate values (Student's t- test, *$p < 0.05$, **$p < 0.01$). See text for abbreviations.

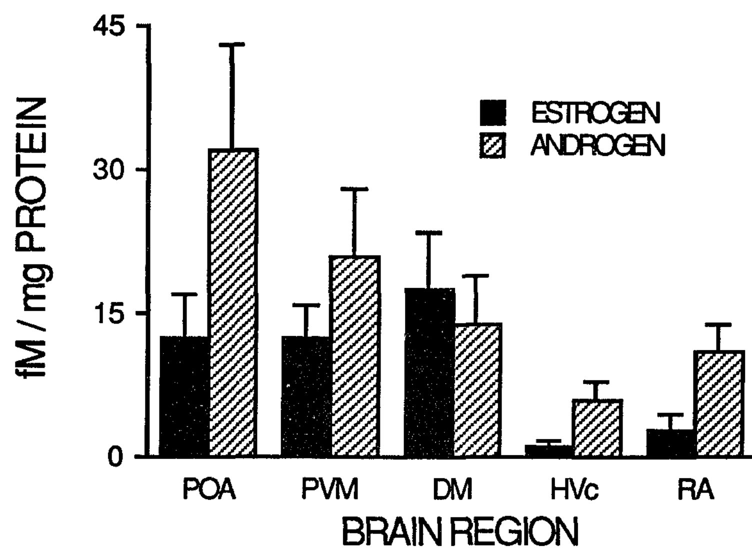


Figure 4-4. A comparison of estrogen and androgen binding levels in hypothalamic and vocal control nuclei. Androgen receptor concentrations were determined in previous experiments with [^{3}H]R1881, a nonmetabolizable synthetic androgen.

APPENDIX

Many actions of steroid hormones are mediated by their interaction with specific, high-affinity intracellular receptor proteins. According to the classical model of steroid hormone action, steroids cross target cell membranes via passive diffusion. Upon entry to the cell, the hormone binds to an unoccupied cytoplasmic receptor protein. This newly-formed hormone-receptor complex is then translocated to the nucleus of the cell where it binds to target cell chromatin. The cell then responds with increased RNA synthesis, followed by an increase in protein synthesis.

The 'translocation' hypothesis is based on evidence obtained from *in vitro* cell fractionation studies, in which various subcellular components of tissue homogenates are separated via differential centrifugation. In hormone-deprived tissues, steroid receptors tend to accumulate in cytosol fractions. Exposure to a saturating dose of hormone *in vivo*, however, results in a dramatic decrease in binding sites in cytosol fractions which is paralleled by an accumulation of occupied receptors in nuclear fractions.

The translocation hypothesis has been strongly challenged in recent years. Several laboratories, using a variety of different experimental protocols, have provided evidence that unoccupied receptors reside predominantly in target cell nuclei *in vivo*, and that the cytosolic localization of receptors *in vitro* may simply represent an artifact of tissue homogenization procedures. Consequently, several alternative models of steroid hormone action have been proposed (reviewed in Walters, 1985) and the subcellular localization of unoccupied steroid receptors *in vivo* remains a matter of debate. Regardless of the controversy, unoccupied receptors do seem to be localized in cytosol *in vitro* when standard tissue homogenization and fractionation procedures are used, while steroid-bound receptors tend to be localized in nuclear fractions.

In the preceding experiment, we conducted a series of *in vitro* binding assays to quantify estrogen receptors in the male zebra finch brain. Because we suspected that

estrogens might be synthesized in castrated males from adrenal androgen, the assays were conducted with cytosol fractions from castrated males treated with an inhibitor of estrogen synthesis. To supplement these studies, we also conducted a series of *in vitro* exchange assays to measure occupied estrogen receptors in cell nuclei from the basal hypothalamus-preoptic area (BH-POA) of castrated males. The results of these nuclear exchange assays revealed that neural estrogen receptors were indeed occupied by endogenous hormone in castrated males. However, subsequent analysis suggested that a substantial portion of these receptors were occupied not by estrogen, but by a stress-related adrenal steroid. These findings, and their possible physiological significance, are discussed below.

METHODS

Adult male zebra finches, obtained from our breeding colony at the American Museum of Natural History (AMNH) or purchased through a local supplier (Canary Bird Farms, Old Bridge, NJ), were castrated 4-6 weeks before sacrifice. They were housed in small groups of 2-6 males per cage (0.61^3 m) in visual isolation from other birds. The animal room was temperature-controlled ($24 \pm 2°$ C) with a 14:10 light:dark cycle. The relative humidity was kept above 50% by console humidifiers, since breeding in this species normally occurs under humid conditions.

Saturation assays were performed to demonstrate the limited capacity of binding sites for [2,4,6,7-^{3}H]estradiol (90.4 -111 Ci/mmol, New England Nuclear) in BH-POA cell nuclei. On the day of assay, animals were transferred from AMNH to Rockefeller University where they were sacrificed by rapid decapitation. Following dissection, tissue samples were maintained at 4° C during all procedures unless otherwise noted (see Harding, Walters and Parsons, 1984 for details of tissue dissection).

BH-POA from 12-18 castrated males were prepared in two equal batches. BH-POA cell nuclei were isolated and purified by sucrose density gradient centrifugation as

described by Zigmond and McEwen (1970). Tissue was homogenized with a Teflon/glass homogenizer in 3 ml NI buffer (1 mM KH_2PO_4, 0.32 M sucrose, 3 mM $MgCl_2$, 0.25% Triton X100, pH 6.5) and centrifuged in a Sorvall RC-2B at 1,500 *x* g for 5 min. After an initial wash in NII buffer (1 mM KH_2PO_4, 0.32 M sucrose, 3 mM $MgCl_2$, pH 6.8), the nuclei were resuspended in 0.4 ml NII, to which 2.1 ml NIII (1 mM KH_2PO_4, 2.39 M sucrose, 1 mM $MgCl_2$, pH 7.0) were added to make a 2 M sucrose solution. This was centrifuged at 25,000 *x* g for 60 min. The pellicles and supernatants were removed, and the sides of the tubes carefully dried.

Nuclear hormone-receptor complexes were extracted according to the method of Roy and McEwen (1977). The nuclei were first dispersed in 700 µl TDB (10 mM Tris-HCl, 1 mM dithiothreitol, 0.5 mM bacitracin, pH 7.6). An equal volume of TDBK.8 (10 mM Tris-HCl, 1 mM dithiothreitol, 0.5 mM bacitracin, 0.8 M KCl, pH 7.6) was then added to make a final KCl concentration of 0.4 M. Samples were vortexed every 5 min, and after 30 min were centrifuged at 25,000 *x* g for 10 min. The supernatant from the two batches of sample were then pooled. Aliquots (200 µl) of the nuclear extracts were incubated in siliconized glass vials with 50 µl TDBK.4 buffer containing varying concentrations (0.05-2 nM) of $[^3H]$estradiol for 5 hr at 25° C. Preliminary experiments indicated that binding was maximal under these conditions. Parallel incubations containing a 500-fold molar excess of unlabeled diethylstilbesterol (DES) were used to correct for nonspecific binding.

Bound $[^3H]$estradiol was separated from free steroid on Sephadex LH-20 minicoloumns. The columns were filled with Sephadex swelled in TK.4 buffer (10 mM Tris-HCl, 0.4 M KCl, pH 7.6). The columns were first washed with 500 µl TDBK.4. After 15 min, 200 µl aliquots of the incubates were applied to the columns, washed in

with 50 µl TDBK.4, and after 30 min eluted with 600 µl TDBK.4 directly into scintillation vials. 5 ml Betafluor (National Diagnostics) was added to the eluate and allowed to equilibrate at room temperature overnight. Samples were counted in a Beckman LS-3801 scintillation counter for 2 *x* 10 min at 58% efficiency. The content of DNA in the nuclear pellets was determined by the method of Burton (1956). Results, expressed as fmol bound [^{3}H]estradiol/mg DNA were then analysed by the method of Scatchard (1949).

Single point assays were used to compare nuclear estrogen binding in intact males, castrated males, and castrated males with various hormone treatments. Hormone-treated subjects were implanted with Silastic tubes (Corning; 0.76 mm i.d., 1.65 mm o.d.) containing androstenedione (AE), estradiol (E), or 1,4,6-androstatriene-3,17-dione (ATD), an aromatization inhibitor. AE implants contained 5 mm of packed hormone, and E implants contained 2 mm. ATD-treated birds received two implants, each containing 10 mm of packed ATD. In previous experiments, this dosage of ATD effectively inhibited AE-induced sexual behaviors in castrated males (Walters and Harding, 1988). AE and E implants were implanted subcutaneously, while those containing ATD were implanted intraperitoneally.

Assays were performed at least four weeks following castration and two weeks following hormone implantation. BH-POA from 8 subjects were pooled for each assay. Tissue from an area of the ventral hyperstriatum (HV) which does not accumulate [^{3}H]testosterone (Arnold, Nottebohm and Pfaff, 1976) served as a negative control. Nuclear hormone-receptor complexes were extracted from isolated purified nuclear pellets as described for saturation assays. Aliquots (500 µl) of the nuclear extracts were incubated with 100 µl TDBK.4 containing sufficient [^{3}H]estradiol to make a final concentration of 1 nM. Parallel incubations containing a 500-fold molar excess of unlabeled DES were used to determine nonspecific binding. Samples were incubated at 25° C for 5 hr, then bound steroid was separated from free steroid on Sephadex LH-20

columns at 4° C and counted as described above.

RESULTS AND DISCUSSION

As expected, substantial levels of occupied estrogen receptors were detected in BH-POA cell nuclei of castrated males. Scatchard analysis revealed that nuclear [^{3}H]estrogen binding was saturable and that only a single, high-affinity component was being measured (Kd = 0.39 $\pm$ 0.07 nM; Bmax = 67.7 $\pm$ 8.8 fM/mg DNA). A representative saturation curve and Scatchard plot are shown in Figure A-1.

Single point values for BH-POA nuclear [^{3}H]estradiol binding under various hormonal conditions are presented in Table A-I. Although the *n*'s were too small to make valid statistical comparisons between treatment groups, Table A-I shows that the level of nuclear binding in untreated castrates was higher than that observed in intact birds. AE- and E-treated castrates possessed the highest levels of nuclear binding, although the variability was considerable. Substantial levels of nuclear [^{3}H]estradiol binding were also observed in castrates treated with ATD. This finding was unexpected and quite surprising; since these birds were systemically treated with an inhibitor of estrogen synthesis, there should be little endogenous estrogen available to occupy nuclear estrogen receptors. Since the level of nuclear [^{3}H] binding was considerably higher in BH-POA than in negative control tissue (HV) in all treatment groups, it seems unlikely that these values are artifactual.

The presence of occupied nuclear estrogen receptors in males which were both castrated and treated with an inhibitor of estrogen synthesis indicates that a steroid is binding to the estrogen receptor which is (i) not testicular, and (ii) not an estrogen. At present, the identity of this hormone is unknown. The hormone does not appear to be corticosterone or ATD itself, since neither of these steroids inhibited [^{3}H]estradiol binding in the competition assays reported in the preceding experiment, even in micromolar concentrations. The binding of this unidentified steroid to neural estrogen receptors

appears to be related to the stress of being transported from our labs at AMNH to Rockefeller University where the animals were sacrificed. The level of nuclear estrogen binding was substantially reduced when birds were quickly removed from their home cages and rapidly decapitated (Figure A-2). This latter finding suggests that the steroid may be of adrenal origin.

The binding of a stress-related adrenal steroid to the estrogen receptor may possibly represent an adaptive mechanism to inhibit reproductive functions under stressful environmental situations. The inhibition of reproductive functions in response to stressful stimuli is a well documented phenomenon that appears to be partly due to a decline in gonadotropin secretion (see Edens, 1983 for a review of the effects of stress on avian reproduction). However, such suppression of reproductive functions generally occur gradually in response to long-term, chronic stress. The results of the present study, on the other hand, seem to suggest an endocrine mechanism whereby reproductive functions can be rapidly attenuated in response to transiently stressful situations. Such a mechanism in birds has been hypothesized by Wingfield (1984) after studying the endocrine responses of male white-crowned sparrows and song sparrows to severe weather conditions. Adult males occasionally abandon their nests (and young) during stormy weather in order to find sufficient food for themselves. As conditions improve, the birds return to their territories and begin a second nesting effort (Elkins, 1983; Morton, Hortsman and Osborne, 1972). In both species, plasma levels of corticosterone are elevated during storms, but plasma levels of LH and testosterone remain unaffected (Wingfield, 1985; Wingfield, Moore and Farner, 1983). On the basis of these observations, Wingfield (1984) hypothesized that elevated levels of corticosterone during stressful situations could "redirect behavior from a reproductive context to one of survival" (p. 593). However, since plasma levels of LH and testosterone remain elevated, the reproductive apparatus is presumably maintained in a functional state,

thereby enabling reproductive activities to resume when environmental conditions improve. The results of the present study clearly suggest a physiological basis for this intriguing hypothesis.

TABLE A-I. Nuclear [^{3}H]estrogen binding in the basal hypothalamic-preoptic area (BH-POA) and ventral hyperstriatum (HV) of the male zebra finches under various hormonal conditions. Purified nuclear extracts were incubated with 1 nM [^{3}H]estradiol. Parallel incubations contained a 500-fold molar excess DES to correct for nonspecific binding.

	BH-POA		HV	
Treatment	*n*	fM/mg DNA	*n*	fM/mg DNA
Intact	4	32.7 ± 4.4	2	0.8 ± 0.8
Castrate	3	51.1 ± 5.9	1	7.2
Castrate + E	3	79.5 ± 29.3	3	2.8 ± 1.5
Castrate + AE	4	80.6 ± 29.5	2	1.6 ± 1.6
Castrate + ATD	5	36.5 ± 15.6	4	7.0 ± 2.3

n = number of assays

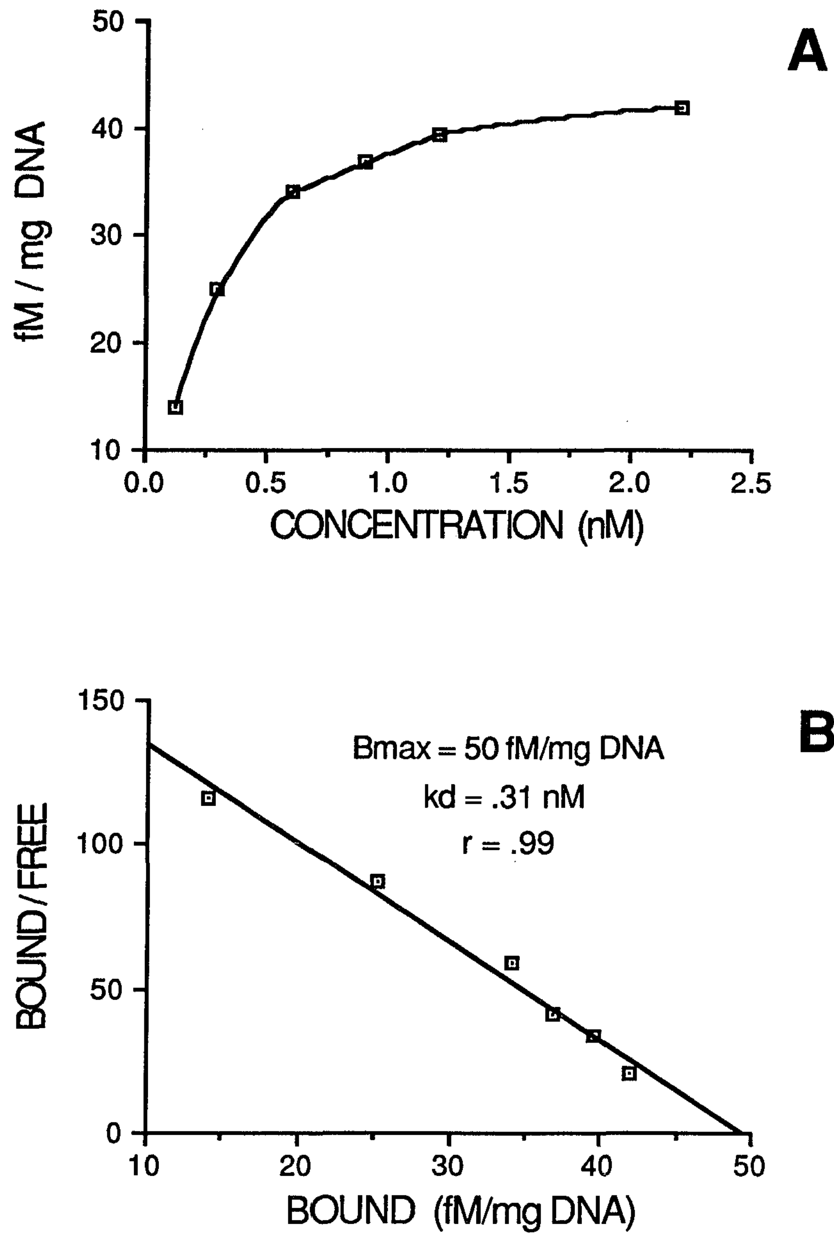


Figure A-1. Representative saturation curve (A) and Scatchard plot (B) of the specific binding of [^{3}H]estradiol in BH-POA cell nuclei of castrated males.

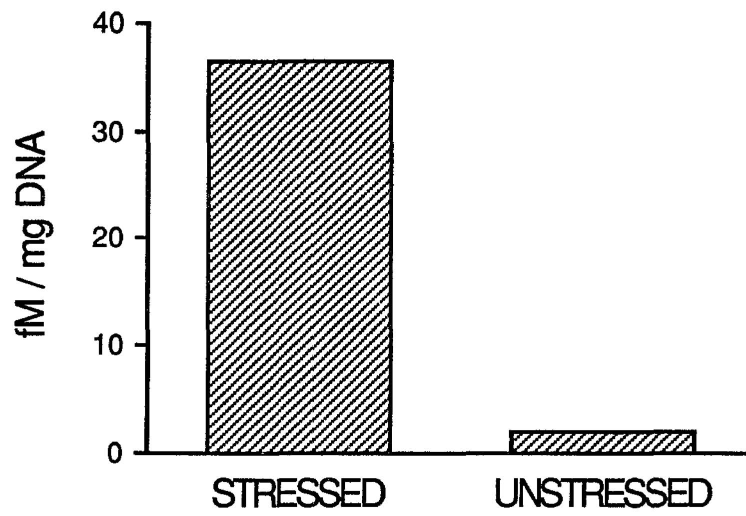


Figure A-2. Specific binding of [^{3}H]estradiol in BH-POA cell nuclei obtained from stressed and unstressed castared males. Stressed animals were transported from our laboratory at AMNH to Rockefeller University before they were sacrificed. Unstressed animals were quickly removed from their home cages and immediately decapitated.

CHAPTER 5:
SUMMARY AND DISCUSSION

The results of these studies clearly establish the importance of estrogen for the activation of male reproductive behavior patterns in this species. The results of the first experiment (Chapter 2) indicated that behaviors normally elicited by androstenedione (AE), an aromatizable androgen, were significantly inhibited by concurrent pharmacological treatment with 1,4,6-androstatriene-3,17-dione (ATD), an aromatization inhibitor which blocks the conversion of aromatizable androgens to estrogenic metabolites. Males treated with AE in combination with ATD exhibited significantly fewer courtship displays, fewer high-intensity displays, and less beak-wiping activity than AE-treated males, and had significantly longer latencies to begin courting females. Estradiol (E), when given concurrently with AE+ATD, reversed the inhibitory effects of ATD and restored levels of courtship activity to those observed in AE-treated males. AE+ATD-treated males attempted to copulate less frequently than males treated with AE alone or males treated with AE+ATD+E, and never successfully copulated with a female. AE+ATD-treated males were also less likely to construct nests than males with treatments supplying estrogenic metabolites. The relationship between aromatization and aggressive behavior, however, was less clear. Although aggressive behaviors (i.e., chasing, plucking, attacking, supplanting) were displayed exclusively by AE- and AE+ATD+E-treated males, the frequency of these behaviors was so low that there were no significant differences across treatment groups. Previous research, however, clearly demonstrates that estrogens play an important role in the activation of aggressive behavior in this species (Harding, 1983).

The second experiment (Chapter 3) examined the estrogenic control of singing behavior in two very different social contexts. Many researchers have assumed that male

zebra finch song is mediated exclusively by testosterone or its androgenic metabolites. The results of the second experiment clearly indicate that estrogens play an important role in the activation of singing behavior, but only in the context of courtship. AE+ATD-treated males exhibited significantly fewer courtship songs, fewer courtship bouts, longer courtship songs, and longer courtship bouts than AE-treated males or males treated with AE+ATD+E. The frequency and duration of undirected songs, on the other hand, were not affected by ATD treatment. Thus, although androgenic metabolites may be sufficient to maintain normal levels of undirected song in this species, estrogenic metabolites are clearly involved in the activation of courtship song. These findings point to the necessity of distinguishing between different types of songs when discussing hormonal mechanisms involved in the control of singing behavior in male zebra finches.

One of the reasons why some researchers believe that estrogens are not involved in the control of male zebra finch song is because autoradiographic, biochemical, and immunocytochemical studies have provided little or no evidence of estrogen-concentrating neurons in the neural vocal control system (Arnold, 1979; Siegel et al., 1986, Gahr et al., 1987). The results of the third experiment (Chapter 4) indicate that the lack of estrogen binding typically observed in the finch vocal control system with these methods may be due to competition from endogenously formed estrogen and/or to the relative inability of these methods to detect relatively low levels of estrogen receptors. Using a sensitive *in vitro* binding assay, substantial levels of estrogen receptors were detected in all vocal control nuclei examined when competition from endogenous estrogen was minimized by treatment with ATD.

The localization of estrogen receptors within the vocal control system indicates that estrogens may act at several different functional levels to affect song production. Low but significant levels of estrogen receptors were observed in RA and HVc, two nuclei which form part of a direct descending motor pathway leading to the muscles of the syrinx, the

avian vocal organ. Estrogen receptors were also detected in three other vocal control nuclei: Area X, MAN, and DM. Although these nuclei are connected to RA or HVc, they do not seem to be part of the direct motor pathway responsible for song production, and their function in adult birds is unknown (McCasland, 1987; Nottebohm et al., 1979). Estrogens may also be involved in sensorimotor mechanisms involved in song production. HVc and RA are each adjacent to shelf-like bands of tissue which receives projections from Field L, an auditory relay nucleus (Kelley and Nottebohm, 1985). HVc itself contains auditory neurons which project to Area X and/or to the hypoglossal motor nerve via RA (Katz and Gurney, 1981; Williams and Nottebohm, 1985). It is believed that these auditory neurons within the vocal control system provide auditory feedback of the bird's own song.

In addition to vocal control nuclei, significant concentrations of estrogen receptors were detected in the two hypothalamic nuclei examined, POA and PVM. Although no one has examined the effects of hypothalamic lesions on male zebra finch behavior, these regions are known to be involved in the control of gonadotropin secretion and sexual and aggressive behavior in a variety of avian and mammalian species. Estrogen binding is typically observed in these regions (Morrell and Pfaff, 1975).

These experiments clearly demonstrate the importance of estrogen in the control of reproductive activities in male zebra finches, and indicate that aromatization may be an obligatory step for maintaining normal levels of sexual and aggressive behavior. There are several lines of evidence, however, which indicate that androgens also play an important role in sexual and vocal behavior. First, behavioral studies indicate that a combination of estrogenic and androgenic metabolites are necessary to restore normal levels of courtship and copulation in castrated males (Harding et al., 1983). Second, intact males treated with an antiandrogen exhibit fewer courtship and undirected songs than untreated controls (Pröve and Immelmann, 1982). Third, androgen-specific binding

proteins have been detected in all hypothalamic and vocal control nuclei known to contain estrogen receptors, except Area X (Arnold, 1979; Harding et al., 1984). Significant levels of androgen receptors have also been detected in the muscles of the syrinx (Lieberburg and Nottebohm, 1979), and androgen-specific changes in the activity of syringeal cholinergic enzymes have been reported (Luine, Harding and Bleisch, 1983). The syrinx does not appear to contain estrogen receptors (Walters and Harding, unpublished observations), and is probably maintained by androgenic metabolites alone. Thus, the hormonal control of sexual behavior in male zebra finches appears to involve the actions of both estrogenic and androgenic metabolites at multiple sites in the CNS and the actions of androgenic metabolites to maintain peripheral tissues involved in male sexual function and/or behavior.

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