

Early Administration of 17β -Estradiol Partially Masculinizes Song Control Regions and α_2 -Adrenergic Receptor Distribution in European Starlings (*Sturnus vulgaris*)

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The vocal control system in many songbird species is a sexually dimorphic neural circuit that mediates learning and production of song. The mechanism by which this system is sexually differentiated has been investigated in only one species, the zebra finch (*Taeniopygia guttata*). Estradiol may be involved in the sexual differentiation of this system, as female zebra finches treated with estradiol as nestlings develop a male-like song system; however, blocking estradiol action in embryonic and nestling male zebra finches does not demasculinize the song system. Therefore, the role of estradiol in song system development is unclear. The role of estradiol in song system sexual differentiation was assessed in European starlings (*Sturnus vulgaris*). This species is of potential interest because it is less extreme in the degree of sexual dimorphism of the song system and song behavior than zebra finches. While in the field, starling nestlings were implanted with 500 μg of estradiol at 3 days of age. These birds were brought into the laboratory at Day 11 and hand-reared. In females, estradiol produces significant increases in the volumes of song control regions defined by Nissl stain, as well as by autoradiography for α_2 -adrenergic receptors; however, these estradiol-treated females have song systems that more closely resemble those of control females than control males. Estradiol-treated males exhibit significant hypermasculinization at 210 days of age, but this effect is transient and hypermasculinization is no longer evident at Day 345. The role of estradiol in sexual differentiation of the neural circuit mediating song behavior remains enigmatic. © 1996 Academic Press

Over the past 35 years, an extensive amount of research has focused on understanding the endocrine regulation of sexual differentiation of the vertebrate brain (see Goy and McEwen, 1980; Arnold and Gorski, 1984;

Breedlove, 1992, for reviews). In a number of sexually dimorphic neural structures, such as the medial preoptic nucleus (POM) of quail, the spinal nucleus of the bulbocavernosus (SNB) and the sexually dimorphic nucleus of the medial preoptic area (SDN-POA) in rats, the endocrine regulation of sexual differentiation is now well understood (Arnold and Gorski, 1984; Breedlove, 1992; Panzica, Viglietti-Panzica, and Balthazart, 1996). In many cases, 17β -estradiol (E_2) has been demonstrated to play a key role during early development in organizing sex differences in the brain.

Many songbirds exhibit sex differences in both song behavior and a discrete circuit of interconnected brain nuclei that has been implicated in the learning and production of song (for a description of the song system, see Fig. 1). Zebra finches exhibit extreme sexual dimorphisms in song behavior (i.e., only males sing) as well as song system cytoarchitecture (Arnold, 1975; Nottebohm and Arnold, 1976). The endocrine basis of sexual differentiation of the zebra finch vocal control system has been studied in some detail; however, the possible role played by sex steroid hormones in the endocrine control of the sexual differentiation of the vocal control system is still poorly understood (Adkins-Regan, 1987; Arnold and Schlinger, 1993; Balthazart and Ball, 1995).

Initial studies implicated an estrogen as the endogenous factor that regulates the sexual differentiation of the song system in zebra finches. Estradiol administered for 2 weeks immediately after hatching causes the masculine development of song nuclei in females (Gurney and Konishi, 1980). If these same females are administered androgen as adults, they exhibit singing behavior that is qualitatively similar to the song of male zebra finches (Gurney and Konishi, 1980); however, the administration of exogenous androgen in adulthood is

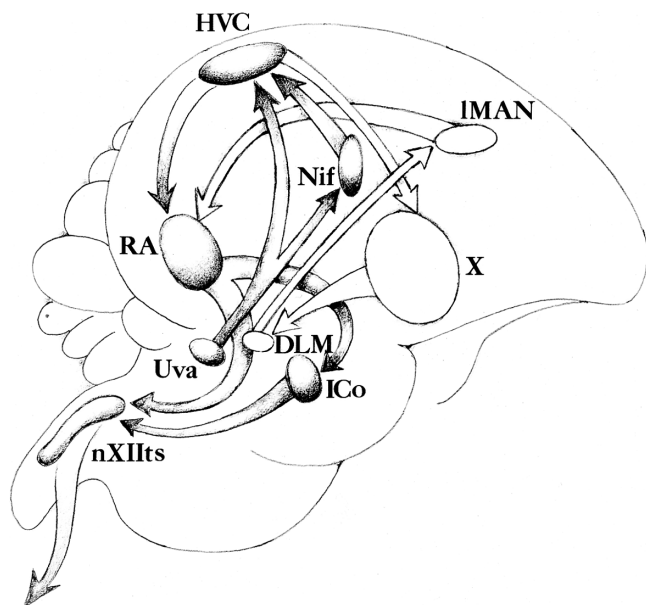


FIG. 1. Schematic representation of a "generic" songbird brain illustrating the sexually dimorphic network of nuclei that controls the acquisition and production of birdsong. The motor pathway necessary for song production includes the HVC to RA to nXIIIts projection. The HVC to X to DLM to IMAN to RA forebrain pathway possesses auditory characteristics and is important for song learning. HVC, high vocal center; RA, robustus archistriatalis; IMAN, lateral part of the nucleus magnocellularis of the anterior neostriatum; X, area X; DLM, medial part of the dorsolateral nucleus of the anterior thalamus; ICo, nucleus intercollicularis (see Bottjer, Halsema, Brown, and Meisner, 1989; Nottebohm and Arnold, 1976; Nottebohm, Kelly, and Paton, 1982; Nottebohm, Stokes and Leonard, 1976; Okuhata and Saito, 1987; Scharff and Nottebohm, 1991; Sohrabji, Nordeen, and Nordeen, 1990).

not necessary for early E_2 -treated females to produce learned vocalizations (Simpson and Vicario, 1991a). That early E_2 treatment of female zebra finches leads to masculinization of some song system characteristics has since been confirmed in a number of investigations (e.g., Grisham and Arnold, 1994; Grisham, Mathews, and Arnold, 1994; Hermann and Arnold, 1991; Nordeen, Nordeen, and Arnold, 1987; Pohl-Apel, 1985; Simpson and Vicario, 1991b).

Several complications are associated with the notion that early endogenous E_2 masculinizes the song control system in zebra finches (for reviews, see Adkins-Regan, 1987; Arnold and Schlinger, 1993; Balthazart and Ball, 1995). First, studies of gallinaceous birds such as the Japanese quail (*Coturnix japonica*) suggest that endogenous E_2 demasculinizes the neural substrate mediating sex differences in crowing and copulatory behavior (see Adkins-Regan, 1987; Balthazart and Ball, 1995). In contrast, when E_2 is administered to male zebra finch nest-

lings during the period when E_2 has the potential to masculinize the female song system, these males exhibit feminized copulatory behavior, but sing normally (Adkins-Regan and Ascenzi, 1987). Thus, it is unclear how a single endocrine environment could produce opposite effects on two male-typical behaviors, singing and copulation. It is possible that sexual differentiation of the song system occurs during a sensitive period that does not coincide with the sensitive period for organization of male reproductive behavior; however, attempts to delineate such circumscribed periods of E_2 sensitivity have been unsuccessful (Adkins-Regan, Mansukhani, Seiwert, and Thompson, 1994).

A second complication concerns the extent to which males and females differ in their exposure to E_2 during early development. Unlike most other species in which sexual differentiation of brain morphology has been studied, consistent sex differences in circulating sex steroids have not been detected in developing zebra finches. Three independent studies have assessed E_2 and testosterone (T) titers in developing male and female zebra finches (Hutchison, Wingfield, and Hutchison, 1984; Adkins-Regan, Abdelnabi, Mobarak, and Ottinger, 1990; Schlinger and Arnold, 1992a). These studies have yielded conflicting results, but it appears that male and female zebra finches are not exposed to different levels of sex steroids in the peripheral circulation during early postnatal development. It is, however, possible that there are sex differences in the hormonal milieu during the embryonic stage of development when plasma sex steroid titers have yet to be assessed.

Plasma concentrations of sex steroids in the general circulation may not accurately reflect the availability of these sex steroids at target tissues within the central nervous system. It is possible that sex differences in E_2 exposure occur due to local aromatization of androgen to estrogen within the brain. Unlike most vertebrate taxa in which the concentration of brain aromatase is high in limbic system structures but low in telencephalic regions, aromatase activity is distributed throughout the zebra finch telencephalon and is significantly higher than telencephalic aromatase levels in mammalian species (Arnold and Schlinger, 1993; Schlinger and Arnold, 1991; Vokel, Pröve, and Balthazart, 1990). Aromatase activity is also detectable in homogenates of diencephalon or telencephalon taken from the brains of hatchling zebra finches (Schlinger and Arnold, 1991, 1992b). Much of the E_2 present in the plasma of male zebra finches is produced by the aromatization of T to E_2 in the brain, and it appears that the brain is the major source of estradiol production in male zebra finches (Schlinger and Arnold, 1992a; Ar-

nold and Schlinger, 1993). Sex differences in brain aromatase activity could produce sex differences in estradiol availability at potential brain target areas such as the song control nuclei; however, no sex differences in brain aromatase activity have been detected during early development (Schlinger and Arnold, 1991).

A third complication associated with the notion that early endogenous E_2 masculinizes the song control system in zebra finches is that all attempts to block masculinization of the male zebra finch song system by inhibiting E_2 production or E_2 action during ontogeny have been unsuccessful. Administration of antiestrogens, which block estrogen receptors, or aromatase inhibitors, which inhibit E_2 production in the brain, as well as early gonadectomy, all fail to inhibit masculinization of the song system in male zebra finches (Adkins-Regan and Ascenzi, 1990; Adkins-Regan *et al.*, 1990; Balthazart, Absil, Fiasse, and Ball, 1994; Mathews and Arnold, 1990; Mathews, Brenowitz, and Arnold, 1988; Wade and Arnold, 1994, 1996; Wade, Schlinger, Hodges, and Arnold, 1994). Despite extensive research, it is still unclear whether E_2 or any other sex steroid acts to promote normal sexual differentiation of the zebra finch song system.

Thus far all efforts aimed at describing and understanding sexual differentiation of the vocal control system in songbirds have used the zebra finch as a model system; however, there are approximately 4500 species of living songbirds (nearly 50% of all bird species, Bock and Farrand, 1980) and it appears that all of them possess the neural circuit that mediates vocal learning and production (Ulinski and Margoliash, 1990). Extensive species variation exists but there are large sex differences in the song control systems of many species besides the zebra finch (see Arnold, Bottjer, Brenowitz, Nordeen, and Nordeen, 1986; Ball, Casto, and Bernard, 1994, for reviews). Unfortunately, although the zebra finch has been the most intensively studied species, it may not be representative of songbirds in general. Based on the approximately one dozen species that have been studied to date from several different families, zebra finches are extreme in several regards. First, unlike all other songbirds studied to date, zebra finches exhibit qualitative, as opposed to the more typical quantitative, sex differences in their vocal control circuit. For example, one key song control nucleus, area X, is not even visible in female zebra finches and a major projection in the vocal production pathway (from HVC to RA) is completely absent in female zebra finches (see Arnold, 1992, for a review). Second, female zebra finches are never observed to sing, even after T administration in adulthood, whereas in many other

songbird species females do sing though less often than males, and T administration in adulthood will greatly increase their rate of singing (e.g., Hausberger and Black, 1991; Kern and King, 1972; Kriner and Schwabl, 1991; Nottebohm, 1980). It is not yet possible to quantify precisely how many songbird species exhibit the zebra finch pattern of such extreme sex differences, as opposed to a more moderate pattern observed in other species, because so few species have been investigated at the neurobiological level in sufficient detail. It is, however, clear that there is extensive species diversity among songbirds in the degree to which there are sex differences in brain and behavior and the pattern exhibited by zebra finches may represent one extreme of a continuum rather than the mode. Therefore, investigating the mechanisms mediating sexual differentiation in other songbird species is critical for a complete understanding of songbird sexual differentiation, in particular, and vertebrate sexual differentiation, in general.

European starlings (*Sturnus vulgaris*) are an excellent species in which to study early hormonal regulation of song system sexual differentiation. Starlings breed seasonally in the wild and nest readily in nest boxes easily accessible to researchers. Because starling young are altricial, like all passerines, early hormone manipulations can be performed on nestlings in the wild. At 11 days of age starlings can be brought into the laboratory and easily hand-reared (Böhner, Chaiken, Ball, and Marler, 1990; and personal observation). Starlings are less dimorphic in both brain and behavior than zebra finches, and exhibit moderate sex differences in the volume of many song control regions (Bernard, Casto, and Ball, 1993). In addition, a number of histochemical markers that clearly delineate the boundaries of nuclei in the starling song system have been described (e.g., Bernard *et al.*, 1993; Bernard and Ball, 1995). The use of these markers in defining the boundaries of brain nuclei can enhance one's ability to measure these structures in young birds. Many of these histochemical markers can also provide information concerning the extent to which the early hormonal environment influences sexually dimorphic neurochemical systems within the song control circuit (Ball *et al.*, 1994).

This experiment investigates the effects of early E_2 exposure on the sexual differentiation of the vocal control system in European starlings. In particular we test the hypothesis that early E_2 administration masculinizes the song system of starlings. Song control nucleus volumes are assessed both by standard histological techniques (i.e., Nissl staining) and by the distribution of α_2 -adrenergic receptors in the song system as defined by *in vitro* receptor autoradiography. The high density

of α_2 -adrenergic receptors in the song system, in comparison to surrounding neural tissue, delineates the boundaries of certain song control nuclei that are ambiguous in Nissl-stained material, and also provides information regarding possible effects of E_2 on the development of the sexually dimorphic noradrenergic projections within the song system.

METHODS

The starlings used in this experiment were hand-reared in captivity after Posthatching Day 11, to ensure that the development of the experimental subjects could be properly observed. It is impossible to reliably follow and recapture individually marked juvenile starlings in the wild in numbers sufficient to conduct experimental research; however, the rearing environment was designed to mimic the natural progression of the major environmental and social stimuli that occur in the wild. Free-living juvenile starlings fledge at approximately 21 days of age. On fledging, juveniles are still dependent on their parents for food for as long as 2 weeks. On becoming independent, juvenile starlings tend to disperse and form juvenile and mixed juvenile/adult flocks during the summer months, and roost communally in the winter months (Feare, 1984). With this sequence in mind, housing conditions, the transition from hand rearing to independence, as well as photoperiod were manipulated to correspond to potentially significant natural variation, whenever possible.

Rearing and Early Hormonal Treatment of Animals

The juvenile starlings used in this experiment were the progeny of free-living birds breeding in nest boxes at the Johns Hopkins University farm in Parkton, Maryland. Nest boxes were monitored daily to determine the exact hatch date of individual nestlings. All nestlings collected for this study hatched between May 4 and May 13, 1994, and thus were the offspring of adults that had successful first clutches during the 1994 breeding season. Because hatching within clutches is asynchronous in starlings, birds that hatched a day earlier or later than their siblings were marked on their body feathers with dye for later identification. Seven clutches of nestlings were assigned to each of the following three treatment groups: E_2 implant group, empty implant group, and no implant group. During the first week of development, all of the nestlings in three clutches died (one from each treatment group), leaving six clutches

of nestlings in each treatment group. Mortality of entire clutches due to predation or abandonment by parents is not uncommon in starlings.

Estradiol was delivered continuously to nestlings in the E_2 treatment group during early development by subcutaneously implanting estradiol ropes which slowly release estradiol into the peripheral circulation. Estradiol ropes were made by mixing 1.1 g Silastic adhesive (Dow Corning, Midland, MI) and 0.1 g estradiol (E-8875, Sigma, St. Louis, MO) and then extruding the mixture through a syringe onto glass microscope slides to form ropes. After air-drying for 24 hr, the ropes were then cut to the appropriate length (10 mm) and weight (6 mg, 500 μ g of E_2). An 11:1 ratio of Silastic to E_2 has been used previously to administer masculinizing doses of E_2 to female nestling zebra finches (Nordeen, Nordeen, and Arnold, 1986). Because starlings are significantly larger than zebra finches during early development and when fully grown, we used ropes that were 10 times as long and weighed 10 times as much as those previously used in experiments with zebra finches. Control Silastic ropes were made in a similar fashion; however, E_2 was not added to the Silastic adhesive.

At 3 days of age (day of hatching = Day 1), nestlings were weighed with a spring-loaded scale (Pesola, Switzerland), and were administered one of the three treatments. Nestlings assigned to the E_2 implant or control implant treatment groups were implanted with one E_2 rope or one control rope, respectively. Silastic ropes were incubated in physiological saline at 37°C overnight prior to implantation and were implanted subcutaneously over the left pectoral muscle. Nestlings were returned to their nest boxes after implantation. On Day 8 nestlings were weighed again to assess potential effects of early E_2 exposure on early weight gain. When the majority of nestlings in a clutch were 11 days old, all nestlings were removed from the nest box, sprayed with a flea and mite spray (Scalex, Gimborn-Rich Health, Atlanta, GA) to remove external parasites common to wild birds, banded with numbered aluminum leg bands (National Band and Tag, Newport, KY) for identification, and brought into the laboratory where they were hand-reared until independence. At 11 days of age, juvenile starlings are fully feathered and a clutch of at least three juveniles is capable of thermoregulation when housed in nest bowls at room temperature (Ricklefs, 1979b; and personal observations).

Initially, nestlings were fed with a 35-ml catheter-tip syringe six times daily. After 24 days of age, the number of feedings per day was gradually reduced, as the birds demonstrated their ability to maintain body weight with fewer feeding sessions. The hand-rearing formula

was adapted from Lanyon (1979) and consisted of a mixture of ground commercial poultry feed (Start and Grow, Purina Mills Inc., St. Louis, MO), strained carrots, tofu, whole raw eggs (shell included), wheat germ, and water. Sulfaquinoxaline (0.04%) was added to the mixture daily as treatment for intestinal parasites, such as coccidia, which are common in free-living nestlings (personal observations). Between 25 and 35 days of age, birds were weaned off of the hand-rearing mixture onto an ad libitum softbill food mixture consisting of poultry feed, raisins, frozen peas, and chopped burger-style dog food. Ad libitum drinking and bathing water was also available during this period. By Day 60 all birds had been placed on a diet consisting solely of ad libitum poultry feed and drinking water. Birds were weighed and their wing chord was measured daily from days 11 to 24 to determine if physical development of hand-reared starlings is similar to previously described development in the field.

Hand-reared birds were raised in an aviary which also housed adult male and female starlings. Most of the adult male starlings sang in captivity. The aviary was maintained on a 16:8 (L:D) photoperiod until September 25, 1994 (approximate age = 140 days) at which time the light schedule was switched to 11:13 (L:D). This change in photoperiod coincided with the date at which the natural photoperiod in Maryland falls below 12 hr of light per day. On March 25, 1995 (approximate age = 325 days) the photoperiod was switched back to 16:8 (L:D); this change in photoperiod was exactly 6 months after the change from 16:8 to 11:13 (L:D). On arrival at the laboratory, nestlings were initially housed in small cages (51 × 42 × 23 cm) with their siblings. At approximately 30 days of age, juveniles were transferred into larger cages (92 × 47 × 48 cm) that housed two or three clutches.

Five males and five females chosen at random from each treatment group were killed and their brains extracted at 210 days of age, prior to the onset of measurable gonadal development and marked increases in plasma levels of sex steroid hormones associated with the onset of reproduction. In addition, five males and five females from each treatment group were killed and their brains extracted 20 days after transfer to the 16:8 (L:D) photoperiod (approximate age = 345 days; range, 343–356 days; from hereon referred to as 345-day-old birds). At this time birds were judged to have appreciable levels of circulating sex steroids based on beak coloration and gonadal condition. In starlings, beak coloration is an androgen-sensitive characteristic (Witschi and Miller, 1938). When androgens are circulating, the beak turns yellow (color changes progressively from

the base of the beak out toward the tip). When levels of circulating T fall, the beak turns black. The use of beak coloration to assess androgen exposure is a very sensitive measure, such that wild-caught adult female starlings in which plasma androgen levels are sometimes undetectable by radioimmunoassay (RIA) still show seasonal changes in beak coloration (Ball and Wingfield, 1987). Prior to brain extraction, beak color was scored from 0 (completely black) to 3 (completely yellow) to give some indication of exposure to androgens (see Ball and Wingfield, 1987). Scores between 0 and 3 reflect intermediate levels of yellow or black. For example, a beak that is one-third yellow is scored 1 and two-thirds yellow is 2.

Syrinx mass in songbirds has also been shown to be regulated by androgens (Luine, Nottebohm, Harding, and McEwen, 1980; Johnson and Bottjer, 1995a; Bernard, 1995). Therefore, the syrinx of each bird was dissected and weighted immediately after the brain was removed. In addition to these two androgen-dependent measures, the presence of depleted Silastic implants that the birds received as nestlings was confirmed, and body mass, brain mass, left testis volume (in males), and largest ovarian follicle diameter (in females) were also assessed in all birds killed at 210 and 345 days. In addition, in birds killed at Day 345, a blood sample was collected from all birds, and paired testis mass was assessed in males.

Tissue Processing and Volumetric Reconstruction of Song Control Nuclei

Nissl staining. Birds were killed by rapid decapitation. Brains were quickly removed and frozen on pulverized dry ice. Frozen brains were sectioned in the coronal plane on a cryostat and sections (thickness = 25 μ m) were collected throughout the entire telencephalon. Every section was thaw-mounted on a gelatin-coated microscope slide. Every fifth section was then fixed in a 4% paraformaldehyde solution for 5 min, washed in phosphate-buffered saline, dehydrated in increasing concentrations of ethanol, air-dried, and then Nissl-stained with thionin. The remaining sections that were not Nissl-stained were desiccated and stored at -30°C until processed for autoradiography. The volumes of four song control regions [area X, HVC, and the medial and lateral portions of the magnocellular nucleus of the anterior neostriatum (mMAN, and lMAN, respectively)] and two additional brain regions [spiriformis medialis (SpM) and the left caudal forebrain] were reconstructed from the Nissl-stained tissue. The lateral border of robustus archistriatalis (RA) was

difficult to discern in Nissl-stained tissue at both 210 and 345 days (see Fig. 2C), and Nissl-stained sections were not used to reconstruct RA volume.

Slides containing the regions to be reconstructed were identified with the aid of the canary brain atlas (Stokes, Leonard, and Nottebohm, 1974). Slides were placed on a Chroma Pro 45 light box (Byers Photographic Equipment Company, Portland, OR) that projected an image to a video camera (Model XC-77, Sony, Japan) interfaced via a Data Translation Quick Capture digitizing board (Model DT2255, Marlboro, MA) to an Apple Macintosh IICI microcomputer (Cupertino, CA). Digitized images were analyzed with an image analysis software program (Image version 1.41, Wayne Rasband, NIH, Bethesda, MD). Area measures were obtained bilaterally from the rostral to caudal extent of the region of interest by tracing the perimeter of the region on the captured image displayed on the computer monitor. A micrometer was placed on the light box before analyses commenced and a known distance was measured to scale pixels to millimeters. Thus, the computer calculated area measures in units of square millimeters.

To derive an estimate of volume, each area measurement was multiplied by the sampling interval (125 μm). The area \times distance products were summed to arrive at two estimates (left and right) of the unilateral volume of a nucleus. The two unilateral volume estimates were then averaged to yield a single estimate of volume for a particular song region in an individual bird. Left caudal forebrain volume was determined using the procedure of Nottebohm (1981). Briefly, the entire perimeter of the left telencephalon was traced in the brain section in which HVC exhibited its largest cross-sectional area. The perimeter of the left telencephalon was traced and the volume of the left telencephalon in that section was then calculated. Similarly, volume was calculated in two additional brain sections, one located 250 μm rostral and one located 250 μm caudal to the section with the largest cross-sectional HVC area. These three volumes were then summed to yield an estimate of caudal telencephalic volume. This measure was used to determine if song control nucleus volume was influenced independently of telencephalic volume.

In some cases a brain section was damaged or could not be measured because of technical problems associated with tissue sectioning or staining. In these cases, the mean of the measured areas from the sections immediately caudal and immediately rostral was substituted for the missing area measure. If two or more consecutive sections containing a particular region of interest were unmeasurable or if the caudalmost or

rostralmost section could not be measured, volume reconstruction was not performed. The number of animals used to derive mean volume estimates for each nucleus is indicated in Table 1. In some birds only a single unilateral estimate of volume could be computed for a particular region. In these cases the unilateral volume was used as the estimate of nucleus volume for that particular bird. This procedure is justified because in no case have we been able to assess significant hemispheric differences in the volume of any song control region in starlings.

Autoradiography for α_2 -adrenergic receptors.

Brain sections were processed for *in vitro* receptor autoradiography for α_2 -adrenergic receptors using a protocol adapted from Ball, Nock, McEwen, and Balthazart (1989), which specifically labels α_2 -adrenergic receptors in the avian brain. During the assay, slides containing sections through mMAN, lMAN, area X, HVC, and RA from birds in the E₂ implant and no implant groups were removed from the freezer and fan-dried for 30 min. Every fifth section through a song control region was processed for α_2 -adrenergic receptor autoradiography to determine total binding. In addition, six sections, each of which was adjacent to one of the sections chosen for determination of total binding, were used to determine nonspecific binding.

Once dry, all sections were preincubated in 50 mM Tris-HCl buffer (pH 7.7) + 0.1% paraformaldehyde at room temperature for 30 min to wash endogenous ligand from receptors. Slides used to determine total binding were transferred to slide mailers containing 8 ml of 50 mM Tris-HCl buffer (pH 7.7) + 2.5 nmol *p*-[³H]aminoclonidine (PAC, specific activity = 40.3 or 52.1 Ci/mmol, New England Nuclear, Boston, MA), an α_2 -adrenergic agonist. Slides used to determine nonspecific binding were incubated in 8 ml of a similar medium, containing 2.5 nM [³H]PAC + 10 μM phentolamine mesylate (RBI, Natick, MA), an α_2 -adrenergic antagonist which acted as a cold competitor. All slides were incubated at room temperature for 1 hr. Two 5-min washes in 4°C 50 mM Tris-HCl (pH 7.7) + 0.1% paraformaldehyde then washed unbound ligand from the sections. A final dip in 4°C distilled water removed excess buffer salts. Slides were then fan-dried. When dry, the slides were placed in X-ray cassettes and exposed to tritium-sensitive Hyperfilm (Amersham Corp., Arlington Heights, IL) for 42 days, after which the film was developed for 5 min in Kodak D-19 developer and fixed for 2 min in Kodak rapid fixer.

Analysis of autoradiograms. Autoradiograms were analyzed with the aid of the computerized image analysis system described above. Autoradiograms were

TABLE 1
Nissl-defined Nucleus Volumes: Brain Mass Ratios (\pm SEM) in 210- and 345-day-old Male and Female Starlings

	Males						Females					
	Day 210			Day 345			Day 210			Day 345		
	Estradiol implant	Empty implant	No implant	Estradiol implant	Empty implant	No implant	Estradiol implant	Empty implant	No implant	Estradiol implant	Empty implant	No implant
X	3.728 (± 0.071) n = 4	3.069 (± 0.153) n = 5	3.272 (± 0.121) n = 5	3.389 (± 0.191) n = 5	3.049 (± 0.139) n = 4	3.313 (± 0.079) n = 5	2.018 (± 0.137) n = 5	1.580 (± 0.145) n = 5	1.791 (± 0.181) n = 5	1.838 (± 0.096) n = 5	1.501 (± 0.169) n = 4	1.684 (± 0.141) n = 5
HVC	1.607 (± 0.068) n = 4	1.380 (± 0.042) n = 5	1.300 (± 0.037) n = 5	1.616 (± 0.128) n = 4	1.717 (± 0.031) n = 3	1.638 (± 0.052) n = 4	0.986 (± 0.112) n = 5	0.821 (± 0.079) n = 4	0.738 (± 0.084) n = 5	1.043 (± 0.076) n = 5	0.828 (± 0.039) n = 3	0.890 (± 0.097) n = 4
IMAN	0.179 (± 0.006) n = 4	0.188 (± 0.018) n = 5	0.154 (± 0.008) n = 5	0.145 (± 0.015) n = 4	0.158 (± 0.013) n = 5	0.127 (± 0.011) n = 5	0.107 (± 0.007) n = 5	0.150 (± 0.030) n = 5	0.102 (± 0.015) n = 5	0.111 (± 0.011) n = 3	0.090 (± 0.010) n = 3	0.122 (± 0.012) n = 5
mMAN	0.175 (± 0.013) n = 4	0.169 (± 0.017) n = 5	0.138 (± 0.012) n = 5	0.141 (± 0.013) n = 5	0.130 (± 0.020) n = 5	0.125 (± 0.017) n = 5	0.092 (± 0.011) n = 5	0.088 (± 0.004) n = 5	0.095 (± 0.011) n = 4	0.078 (± 0.002) n = 5	0.084 (± 0.006) n = 4	0.075 (± 0.004) n = 5
Caudal forebrain	19.947 (± 1.589) n = 4	20.144 (± 0.721) n = 5	19.794 (± 0.836) n = 5	20.080 (± 0.692) n = 5	22.556 (± 0.691) n = 5	21.574 (± 1.031) n = 5	20.454 (± 0.757) n = 5	21.718 (± 0.473) n = 5	21.035 (± 0.614) n = 5	23.196 (± 0.961) n = 5	24.257 (± 0.594) n = 4	23.085 (± 0.347) n = 5
SpM	0.123 (± 0.003) n = 4	0.133 (± 0.007) n = 5	0.144 (± 0.016) n = 4	0.123 (± 0.007) n = 5	0.139 (± 0.009) n = 3	0.146 (± 0.003) n = 5	0.134 (± 0.004) n = 5	0.137 (± 0.003) n = 3	0.121 (± 0.006) n = 4	0.155 (± 0.009) n = 5	0.139 (± 0.005) n = 4	0.151 (± 0.016) n = 5

placed on the light box and images were captured via the video camera. All binding data were determined from film density in areas of interest (see below). Standards containing various concentrations of tritium (American Radiolabeled Chemicals Inc., St. Louis, MO) were co-exposed with brain sections on each piece of film for 42 days and used to convert optical density to femtomoles of bound [³H]PAC per milligram of protein. Mean protein content of brain tissue is approximately 10% of tissue wet weight in quail and songbirds (Ball, Nock, Wingfield, McEwen, and Balthazart, 1990). Thus, specific activities per equivalent wet weight provided by the tritium standards were transformed into specific activities per milligram of protein using a ratio of protein versus wet weight of 10%.

Autoradiograms were analyzed to determine levels of specific binding of [³H]PAC in area X, HVC, RA, IMAN, and mMAN. In addition, because the borders of many of the song control regions were well defined by a high density of [³H]PAC binding in comparison to surrounding structures, volume reconstruction was performed based on autoradiographic definition of area X, HVC, RA, and mMAN. Although IMAN was also defined by a higher α_2 -adrenergic receptor density than the surrounding neostriatum, the region defined by [³H]PAC binding was much larger and extended more caudally than Nissl stain-defined IMAN. In addition, the borders of this larger IMAN-like region were indistinct, thus precluding accurate volume reconstruction (see Fig. 2 for a comparison of song control nucleus definition by Nissl stain and α_2 -adrenergic receptor autoradiography).

Specific binding of [³H]PAC in areas of interest was defined as the density of binding in the absence of phentolamine mesylate (total binding) minus binding in the presence of phentolamine mesylate (nonspecific binding). Optical density measurements of total binding and nonspecific binding, in individual song control nuclei, were obtained by digitizing images of brain sections from autoradiograms and tracing the perimeters of the areas of interest. Then, the computer, in addition to computing the area of the traced region, calculated the average optical density for that area and converted that optical density to femtomoles of [³H]PAC per milligram of protein. To determine mean specific binding for an entire nucleus, the mean nonspecific binding from all images of sections in which the region was measured was subtracted from the mean total binding from all images of sections in which a region was measured and was reported as the mean specific binding of a region in a particular bird. To determine specific binding of [³H]PAC in the IMAN-like region, an area that fell well

within the indistinct borders of the region was traced on four images of brain sections in which total binding was assessed along with adjacent sections processed for nonspecific binding. Specific binding was then calculated as described above.

Blood Sampling and Steroid Assay

Plasma samples were obtained from 345-day-old birds immediately prior to decapitation and brain tissue collection. Circulating levels of plasma T were assessed in unextracted plasma of males using a solid-phase ¹²⁵I RIA (Coat-A-Count Total Testosterone). This assay is highly sensitive (i.e., 0.1 ng/ml) and specific (i.e., cross-reactivity to 5 α -DHT < 3.4% and to 17 β -estradiol is 0.02%). Assays were performed by Beverly Smith at the Hopkins Population Center Radioimmunoassay Laboratory of the Johns Hopkins University. Fifty microliters of plasma was added to test tubes coated with the testosterone antiserum. One microliter of ¹²⁵I-T was added to each tube and tubes were then incubated for 3 hr at 37°C. At the end of the incubation period, bound from free was separated by decanting the tube. The bound was then counted in a gamma counter. The amount of T present in each sample was then determined by comparing the percentage bound to a standard curve with six points ranging from 0.1 to 16 ng/ml. Each plasma sample was assayed in duplicate, and plasma T values were determined in a single RIA.

RESULTS

Early Development

Nestling body mass measurements taken in the field on Posthatching Days 3 and 8 from all birds in the 18 clutches were analyzed using a two-factor (age \times treatment) analysis of variance (ANOVA). As expected, body mass increased significantly between Days 3 and 8 in all groups; however, E₂-treated nestlings exhibited smaller increases in body mass between Days 3 and 8 than the two control groups [significant treatment \times age interaction, $F(2, 67) = 3.727$, $P < 0.05$; Fig. 3a].

Daily body mass and wing cord measures recorded during the first 2 weeks of hand rearing from all hand-reared birds were analyzed using mixed model ANOVAs. Males and females exhibited similar growth curves for both body mass and wing cord length (Fig. 3b and c); however, male juveniles

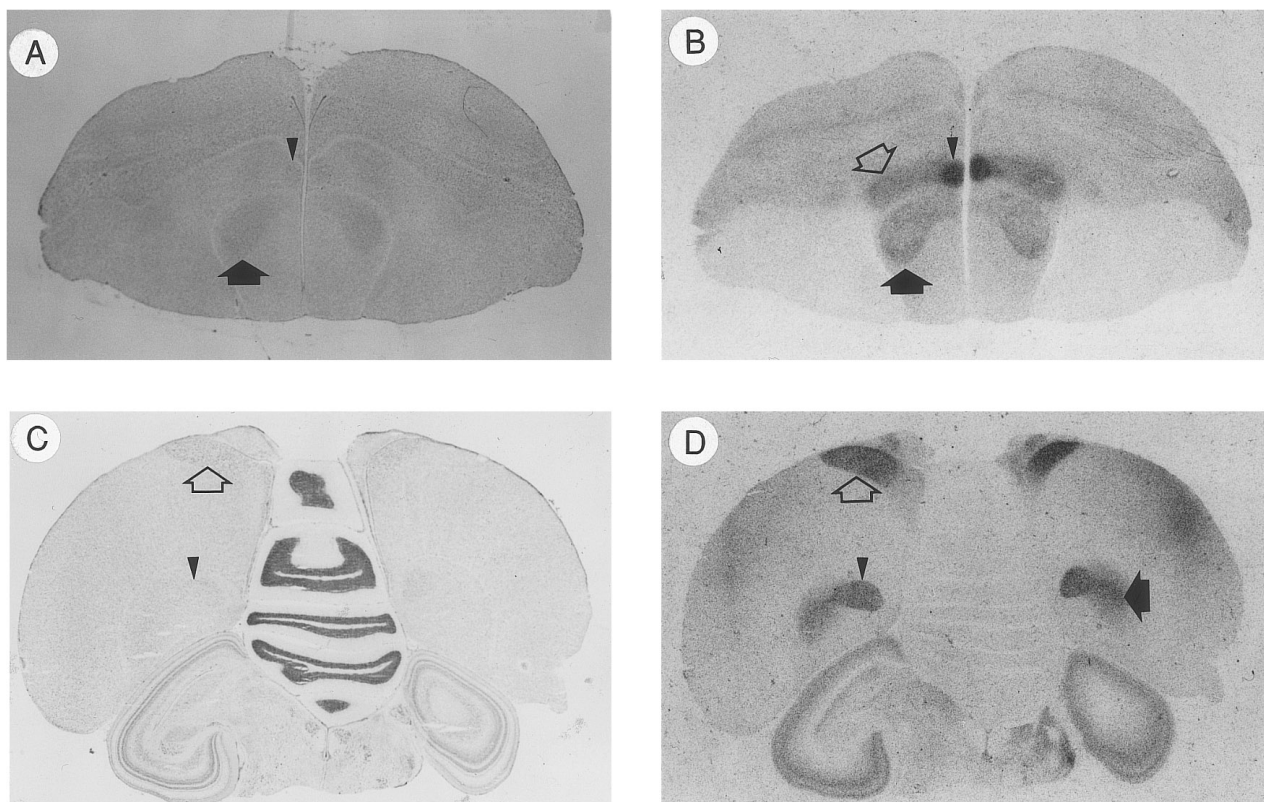


FIG. 2. Photomicrographs comparing the delineation of song control nucleus borders by Nissl-staining and α_2 -adrenergic receptor autoradiography. (A) Nissl-stained coronal brain section from a 210-day-old E_2 -treated female containing area X (black arrow) and mMAN (black triangle). Note that IMAN is not visible in this section. (B) Autoradiogram from a 210-day-old E_2 -treated female illustrating [3H]PAC binding in area X (black arrow), mMAN (black triangle), and IMAN (open arrow). (C) Nissl-stained coronal brain section from a 210-day-old E_2 -treated male containing HVC (open arrow) and RA (black triangle). In many birds the lateral borders of Nissl-defined RA could not be discerned. (D) Autoradiogram from a 210-day-old E_2 -treated male illustrating [3H]PAC binding in HVC (open arrow) and RA (black triangle). Note the crescent-shaped region of high binding lateral to RA (black arrow). This may be the archistriatum pars dorsalis (Ad) which is part of a neural pathway that travels parallel to the anterior forebrain pathway described in Fig. 1 (see Johnson and Bottjer, 1995b). It is the border between RA and Ad that is difficult to discern in Nissl-stained tissue. Scale bar equals 5 mm.

weighed significantly more than females throughout the first 2 weeks of hand rearing [$F(1, 66) = 14.328$, $P < 0.05$]. Although early hormone treatment did not significantly influence body mass during hand rearing, E_2 -treated animals exhibited significantly shorter wing chord lengths throughout the 2-week period in which measures were taken [$F(2, 66) = 3.461$, $P < 0.05$].

Day 210 and 345 Measures

Body mass, brain mass, and syringeal mass, taken at the time of brain extraction on Days 210 and 345, were analyzed using three-factor (sex \times age \times treatment) ANOVAs. In instances where the empty implant and no implant controls did not differ, data from these two

groups were combined to form a single control group for further statistical analyses.

Body mass did not vary significantly between Days 210 and 345; however, control males weighed significantly more than control females at both ages ($P < 0.05$). Early E_2 treatment abolished sex differences in body mass on Days 210 and 345 [age \times treatment interaction, $F(2, 47) = 4.453$, $P < 0.05$; Fig. 4a]. That is, E_2 -treated males exhibited significantly reduced body mass compared with control males at Days 210 and 345 ($P < 0.05$) and did not differ significantly from age-matched females.

Although early E_2 treatment decreased male body mass at Days 210 and 345, E_2 treatment did not significantly affect brain mass at either age. Male brains weighed more than female brains at both 210 and 345

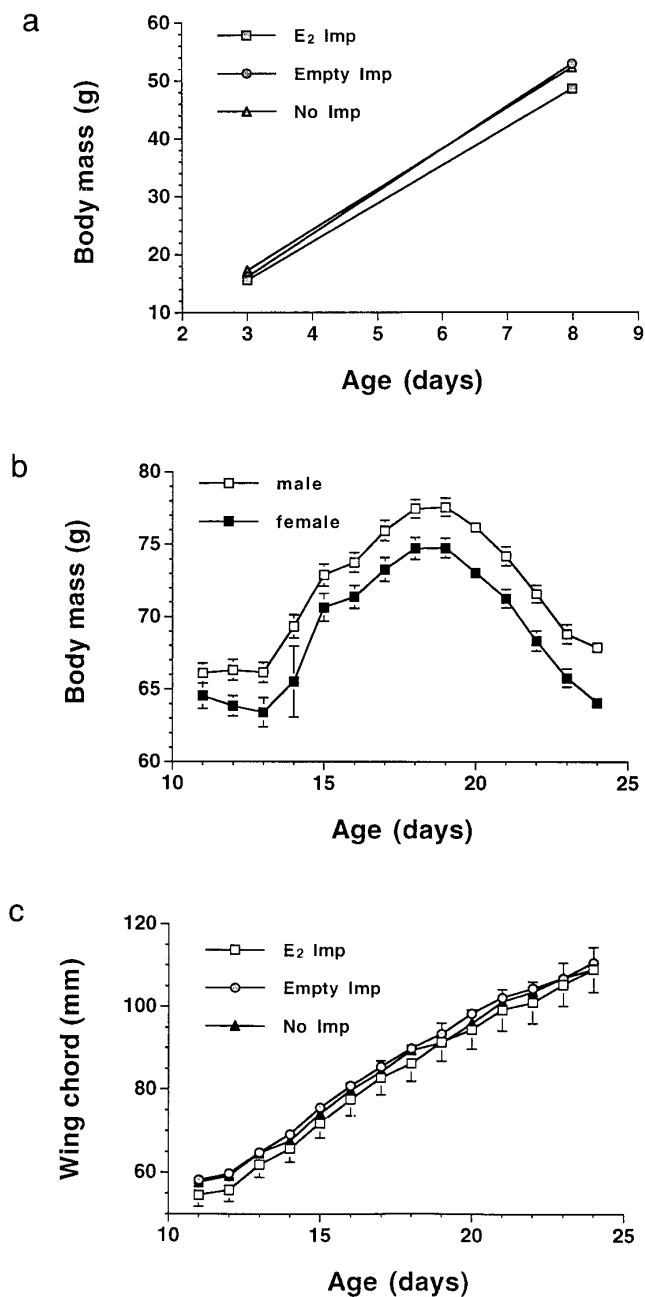


FIG. 3. Influence of early hormonal treatment and hand rearing on juvenile starling development. (a) Mean body mass (\pm SEM) assessed in the field on Posthatching Days 3 and 8. The sex of nestlings at these ages was unknown. Juveniles in the E₂-treated group ($n = 23$) had significantly lower Day 8 body mass than juveniles in the empty implant ($n = 25$) and no implant ($n = 22$) control groups. (b) Mean body mass (\pm SEM) measured on Days 11–24 in juvenile males ($n = 42$) and females ($n = 28$). Males weighed significantly more than females throughout this period. (c) Mean wing chord (\pm SEM) of juvenile starlings measured on Days 11–24. E₂-treated juveniles had significantly shorter wing chord lengths than juveniles in the empty implant and no

days [$F(1, 46) = 66.347, P < 0.05$], and both males and females exhibited significant decreases in brain mass between Days 210 and 345 [$F(1, 46) = 10.872, P < 0.05$; Fig. 4b].

Syringeal mass was also unaffected by early hormone treatment, but was significantly greater in males than females [$F(1, 47) = 256.058, P < 0.05$; Fig. 4c]. Although males, but not females, tended to exhibit increased syringeal mass between Days 210 and 345, the increase was not statistically significant.

Volume measures of the left testis of 210- and 345-day-old males were compared using a two-factor (age \times treatment) ANOVA. As expected, 345-day-old males had significantly larger testes than 210-day-old males in all treatment groups ($P < 0.05$). Left testis volume in males did not differ between treatment groups on Day 210; however, at Day 345 left testis volume in E₂-treated and empty implant control males was significantly smaller than in unimplanted controls [age \times treatment interaction, $F(2, 24) = 5.447, P < 0.05$; Fig. 5a]. In addition to reductions in testis volume, paired testis mass (corrected for differences in body mass) on Day 345 was also significantly smaller in E₂-treated males than in unimplanted males [$F(2, 12) = 4.625, P < 0.05$; Fig. 5b].

Beak coloration changed significantly from no detectable yellow coloration at Day 210 to at least some yellow coloration by Day 345. Males exhibited more yellow beak coloration than females at Day 345 [significant sex \times age interaction, $F(1, 47) = 7.108, P < 0.05$; Fig. 5c].

Plasma testosterone levels of Day 345 males, assessed by RIA, were log transformed and analyzed by a one-way ANOVA. Treatment groups did not differ significantly ($P > 0.05$); however, more than half of the males displayed undetectable T levels. In the birds that did display detectable T, the trend was for birds in the no implant control group to have higher T levels than empty implant control and E₂-treated birds (Fig. 5d).

Measures of largest ovarian follicle diameter in females on Days 210 and 345 were unaffected by early E₂ treatment. In all treatment groups the largest ovarian follicle diameter on Day 210 was too small to measure accurately (<0.5 mm). On Day 345, diameter of the largest ovarian follicle was large enough to measure in all groups, and the mean follicle diam-

implant control groups. In these figures, some of the bars indicating SEM are obscured by the symbols representing the mean.

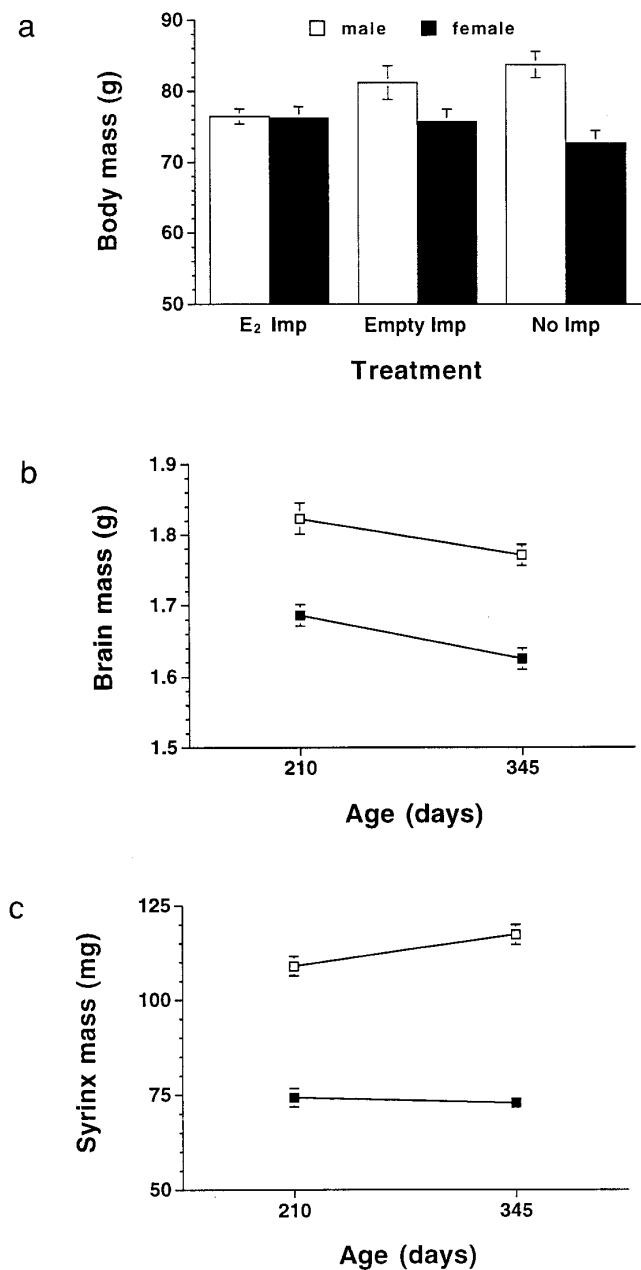


FIG. 4. (a) Effect of early hormone treatment on body mass (means of 210- and 345-day-old males and females \pm SEM). Characteristic sex differences in body mass at Days 210 and 345 were abolished by early E₂ treatment. (b) Mean brain mass (\pm SEM) measured on Day 210 or 345. Males have significantly heavier brains than females at both ages, and both male and female brains decrease in mass between Days 210 and 345. (c) Mean syrinx mass (\pm SEM) measured on Day 210 or 345. Males have greater syrinx mass than females at both ages.

eter (E₂ implant = 2.45 mm, empty implant = 1.65 mm, no implant = 1.83 mm) did not significantly differ between treatment groups ($P > 0.05$).

Volume Reconstruction of Brain Regions in Nissl-Stained Tissue

In general, E₂-treated males and females exhibited increases in the volume of some song control regions; however, for all song nuclei in which volume was assessed males displayed significantly larger volumes than females regardless of early hormone treatment. Because brain mass varied consistently across age and sex, all volume measures were transformed into volume:brain mass ratios prior to statistical analyses. The mean brain nucleus volume:brain mass ratios for each treatment group are listed in Table 1. Although these ratios were used in all statistical analyses, the figures presented here depict actual mean volumes *not* mean volume:brain mass ratios. As described previously, the volumes of all six brain regions could not be measured in all birds. The numbers of birds that contributed to the volume reconstruction of each particular nucleus are presented in Table 1. For each brain region that was reconstructed, volume:brain mass ratios were analyzed using three-factor (sex \times age \times treatment) ANOVAs. Post hoc comparisons were made using Newman-Keuls tests.

The volumes of area X and HVC were significantly larger in males than in females at both ages and in all treatment groups ($P < 0.05$). E₂-treated females exhibited significant increases in volume in area X (17% increase) and HVC (25% increase) compared with controls ($P < 0.05$; Figs. 6c and d). Female area X and HVC volumes from each treatment group did not differ significantly between Days 210 and 345 ($P > 0.05$). Similarly, E₂-treated males exhibited significant increases in volume in area X (11% increase) compared with controls ($P < 0.05$), and area X volumes from each treatment group did not differ between Days 210 and 345 ($P > 0.05$; Fig. 6c). Also like females, HVC volume was significantly increased (20%) in E₂-treated males at Day 210; however, at Day 345 male HVC volume did not differ between treatment groups because of continued growth of HVC after Day 210 in control but not E₂-treated males ($P > 0.05$; Fig. 6d).

Volume measures of both IMAN and mMAN were also larger in males than in females at both ages and in all treatment groups ($P < 0.05$). The volumes of both of these song control regions decreased significantly between Days 210 and 345 (Fig. 6a and b), and were not significantly influenced by early hormonal treatment.

In contrast to the volumes of the song control regions, the volume of the left caudal forebrain was significantly larger (6–10%, $P < 0.05$) in females than in males. The caudal forebrain also increased significantly in volume

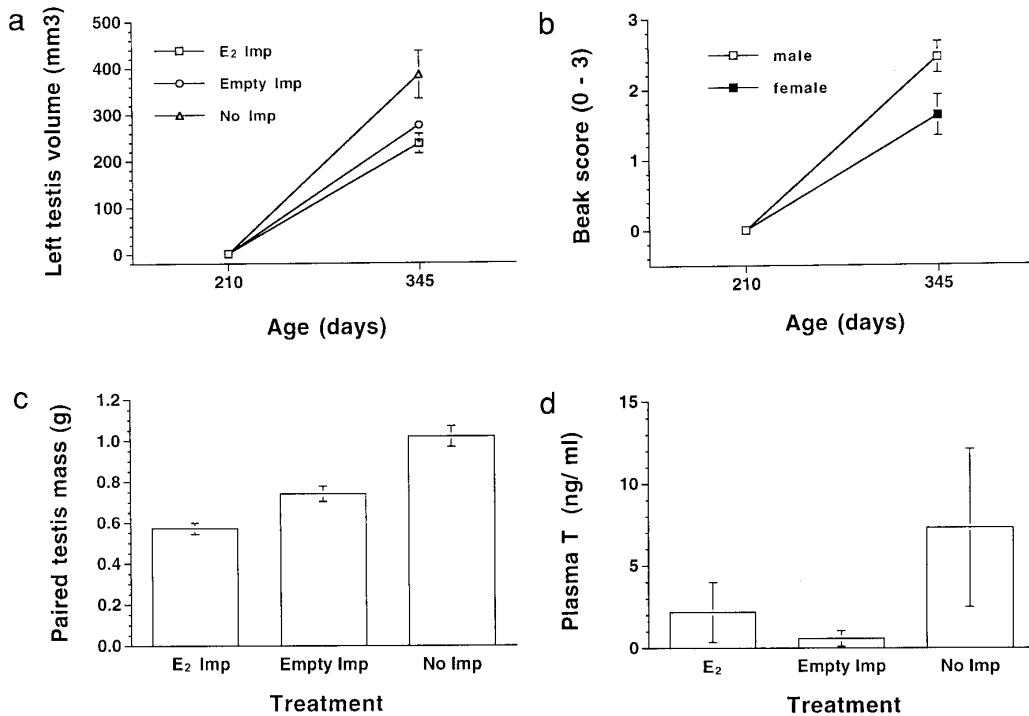


FIG. 5. (a) Mean volume (\pm SEM) of the left testis of males on Day 210 or 345. No between group differences were detected in Day 210 testis volume; however, E₂ and empty implant control males had significantly smaller testes than no implant controls on Day 345. (b) Beak score (\pm SEM) on Day 210 or 345. Completely black = 0 and completely yellow = 3. Early E₂ did not affect beak color, and males had more yellow beak coloration than females on Day 345. (c) Mean paired testis mass (\pm SEM) of males killed on Day 345. When corrected for body mass, testis mass in E₂-treated males was significantly less than in nonimplanted males. (d) Plasma testosterone titers (\pm SEM) of 345-day-old males. T levels were not significantly affected by early hormone treatment.

between Days 210 and 345 ($P < 0.05$), and was not influenced by early hormone treatment ($P > 0.05$). The volume of SpM, a midbrain region that is not associated with song behavior, was not sexually dimorphic, did not vary with age, and was not influenced by early hormone treatment ($P > 0.05$).

Autoradiography-Defined Song Control Nucleus Volume and α_2 -Adrenergic Receptor Density

In general, patterns of song control nucleus volume change associated with age or early hormonal treatment, similar to those delineated by Nissl stain, were also detected when autoradiograms were used to reconstruct nucleus volume. Both area X and mMAN volumes were significantly larger when defined by autoradiography. This difference is most likely not a result of the differential tissue shrinkage or expansion due to processing procedure, as HVC volumes as assessed by both reconstruction procedures did not differ.

Area X volume defined by α_2 -adrenergic receptor au-

toradiography was larger in males than in females [$F(1, 29) = 212.246$, $P < 0.05$]. E₂-treated males and females exhibited significant increases in autoradiography-defined area X volume (21 and 24% increases, respectively) compared with controls on Day 210 but did not differ from controls on Day 345 [treatment \times age interaction, $F(1, 29) = 4.669$, $P < 0.05$; Fig. 7a]. This is likely due to decreases in area X volume in E₂-treated males and females. Autoradiography-defined area X volume was significantly larger (6%) than Nissl-defined X volume in both males and females and in the unimplanted and E₂-implanted groups (paired t test, $P < 0.05$).

HVC volume defined by α_2 -adrenergic receptor autoradiography was larger in males than in females at both ages and in unimplanted and E₂-implanted birds [$F(1, 29) = 81.155$, $P < 0.05$; Fig. 7c]. E₂-treated males and females exhibited significant increases in autoradiography-defined HVC volume compared with controls [$F(1, 29) = 8.723$, $P < 0.05$] on Day 210 (males, 36% increase; females, 15% increase) and on Day 345 (males, 6% increase; females, 20% increase). Although E₂-treated males exhibited no growth in autoradiography-defined

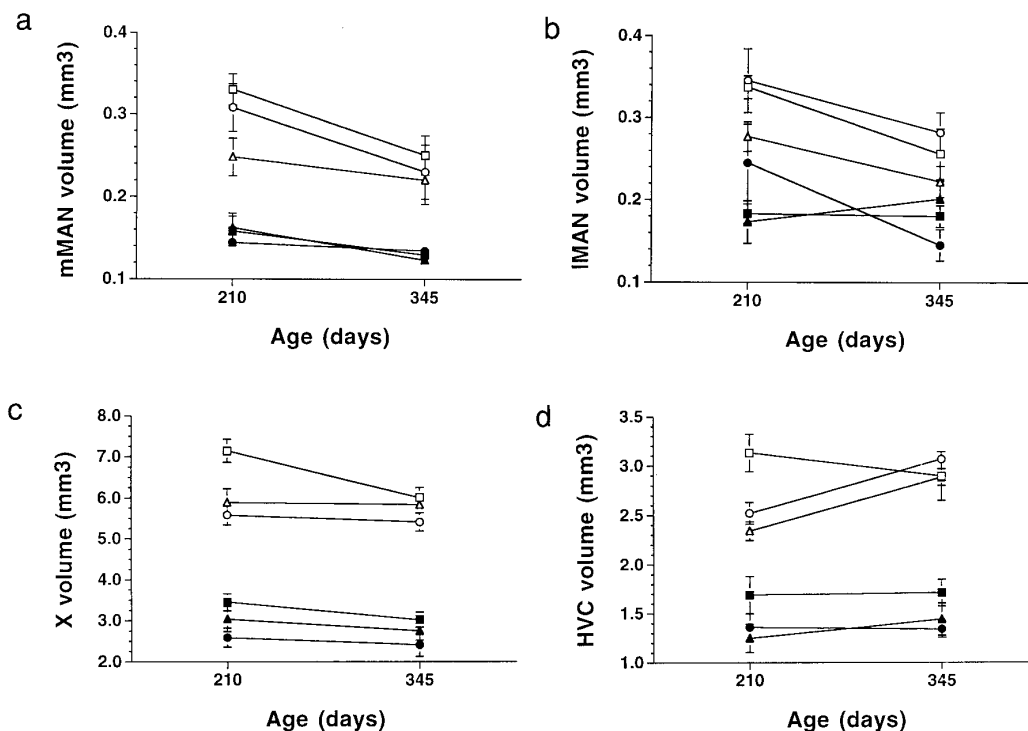


FIG. 6. Mean Nissl-stain defined volumes (\pm SEM) of song control regions in E_2 -implanted (squares), empty implanted (circles), and unimplanted (triangles) males (open symbols) and females (filled symbols). Both mMAN volume (a) and IMAN volume (b) were larger in males than females, decreased significantly with age, and were not significantly influenced by early hormonal treatment. Area X volume (c) was also larger in males than females, and Day 210 and 345 volumes did not differ, except in E_2 -treated males, which exhibited a decrease in volume between Days 210 and 345. Volume was significantly larger in E_2 -treated birds than in controls. HVC volume (d) was also larger in males than in females, did not change with age in females or E_2 -treated males, but did increase between Days 210 and 345 in control males. Volume was larger in E_2 -treated females than in controls on Days 210 and 345 and larger in E_2 -treated males than controls on Day 210; however, E_2 -treated males did not differ significantly from control males on Day 345.

HVC volume between Days 210 and 345, HVC volume in all other groups increased significantly between these ages [$F(1, 29) = 9.086$, $P < 0.05$]. In contrast to area X, Nissl stain and autoradiography-defined HVC volume did not differ significantly ($P > 0.05$).

Autoradiography-defined mMAN volume was larger in males than in females at both ages in both unimplanted and E_2 -implanted birds, and decreased significantly between Days 210 and 345 in males but not in females [significant sex \times age interaction, $F(1, 26) = 5.346$, $P < 0.05$; Fig. 7b]. E_2 -treated birds had significantly larger mMAN volumes than controls at both 210 and 345 days [$F(1, 26) = 9.339$, $P < 0.05$; males, 31 and 34%; females, 64 and 6%, respectively]. The magnitude of the E_2 -induced increase in autoradiography-defined mMAN volume in females on Day 210 is most likely overestimated as volume reconstruction was only possible in two 210-day-old E_2 -treated females that happened to have the smallest Nissl-defined mMAN volumes of the females in that group. As was the case with

area X, autoradiography-defined mMAN volume was significantly greater (45%) than Nissl-defined mMAN volume ($P < 0.05$).

Autoradiography-defined RA volume was larger in males than in females at both ages in both unimplanted and E_2 -implanted birds [$F(1, 29) = 85.197$, $P < 0.05$; Fig. 7d]. E_2 -treated males and females exhibited significant increases in autoradiography-defined RA volume compared with controls [$F(1, 29) = 8.723$, $P < 0.05$] on Day 210 (12 and 25% increases, respectively) and on Day 345 (7 and 21% increases, respectively). Although males and females from both treatment groups exhibited growth in RA volume between Days 210 and 345 (18 and 6%, respectively), this trend was not significant.

The general patterns of specific binding of [3 H]PAC were similar in area X, RA, mMAN, IMAN, and HVC. Males and females exhibited similar levels of specific PAC binding at Day 210, and females exhibited higher binding than males at Day 345, because of increases in [3 H]PAC binding in females but not males. Although

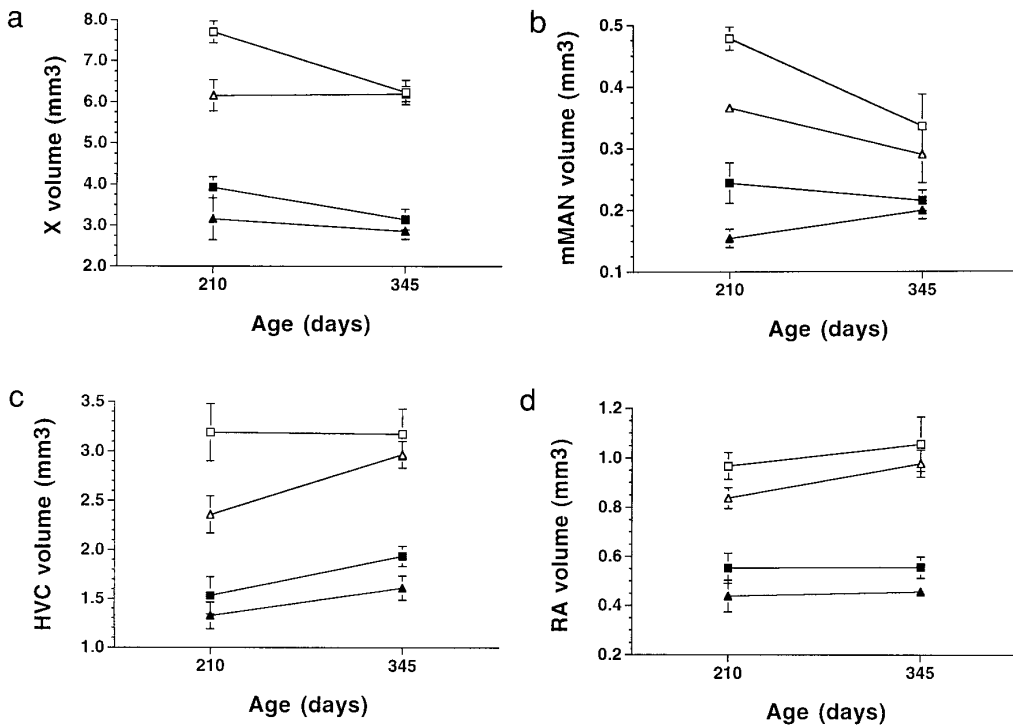


FIG. 7. Mean α_2 -adrenergic receptor autoradiography-defined volumes (\pm SEM) of song control regions in E₂ implanted (square) and unimplanted (triangle) males (open symbols) and females (filled symbols). Area X volume (a) was larger in males than females. E₂-treated males and females exhibited significant increases in autoradiography-defined area X volume compared with controls on Day 210 but did not differ from controls on Day 345 due to decreases in area X volume in E₂-treated males and females between Days 210 and 345. mMAN volume (b) was larger in males than females, decreased significantly with age in males but not females, and was significantly larger in E₂-treated birds than in unimplanted controls. HVC volume (c) was also larger in males than in females. E₂-treated males exhibited no growth in autoradiography-defined HVC volume between Days 210 and 345; HVC volume in all other groups increased significantly between these ages. HVC volume was larger in E₂-treated females than in controls on Days 210 and 345 and larger in E₂-treated males than controls on Day 210; however, E₂-treated males did not differ significantly from control males on Day 345. RA volume (d) was larger in males than in females at both ages in both unimplanted and E₂-implanted birds. E₂-treated males and females exhibited significant increases in autoradiography-defined RA volume compared with controls, and did not exhibit significant changes in RA volume with age.

this same pattern was detected in all five song control regions, the age \times sex interaction was significant only for area X [$F(1, 31) = 5.100, P < 0.05$] and RA [$F(1, 27) = 5.002, P < 0.05$; Fig. 8]. Early hormonal treatment did not significantly alter specific PAC binding within any of the song control regions ($P > 0.05$).

DISCUSSION

Effects of E₂ Administration and Hand Rearing on Early Somatic Development

Juvenile starlings in all three treatment groups exhibited similar patterns of development. The small decrease in Day 8 body mass of E₂-treated nestlings was transient, and by Day 11, E₂-treated birds no longer differed from

controls. The only other detected effect of E₂ administration on early morphological development was a slight decrease in wing chord of males and females. This effect could have been due to estrogenic influences on either of two stages of wing growth: first, the growth of the long bone of the wing and associated flesh, and second, the rapid development of the primary flight feathers, which occurs after Day 15 (Feare, 1984).

Hand-reared birds had growth curves (body mass and wing chord) that were similar to those of naturally developing wild starlings (Feare, 1984; Kessel, 1957; Ricklefs, 1979a; Ricklefs and Peters, 1979). The transient plateau in body mass during the first 3 days of hand rearing is not a feature that is common to natural starling development and may be a result of the dietary change that occurred when birds were transferred from the wild to the laboratory on Day 11. Growth data are

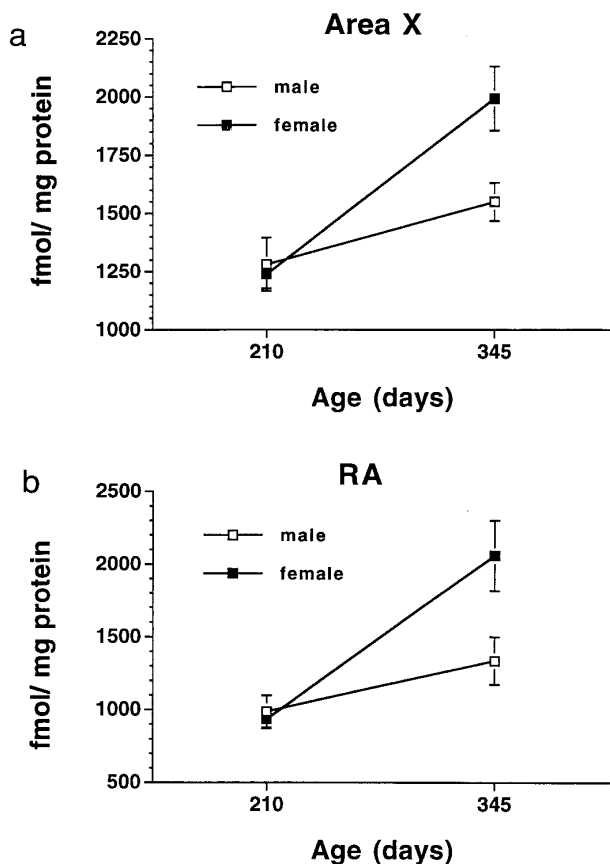


FIG. 8. Mean specific [³H]PAC binding (fmol/mg protein \pm SEM) in area X (a) and RA (b). In both nuclei, females exhibited significantly higher [³H]PAC binding on Day 345 than males. Although similar patterns of binding were detected in IMAN, mMAN, and HVC, Day 345 [³H]PAC binding was not significantly different between males and females.

usually recorded only up until fledging (Day 21) in wild starlings; thus, it is unclear if wild starlings exhibit pronounced decreases in body weight between Days 20 and 24 similar to the hand-reared starlings. However, data from late-fledging nestlings (Kessel, 1957) suggest that weight loss around the time of fledging is not uncommon. Such decreases in body mass at the time of fledging might be expected, as they would most likely facilitate flight in inexperienced birds. The consistent sex difference in body mass between Days 11 and 24 has not been previously reported in starlings. The sex of starling nestlings is not readily apparent and is rarely assessed in studies of nestling development in the wild. Thus, it is unclear if such a sex difference occurs in the wild, or if the sex difference occurs only when birds have access to ad libitum food, as they do during hand rearing.

Gonadal Development and Steroid-Dependent Measures at 210 and 345 Days

The two age groups selected for sampling differed in the degree to which they had been exposed to gonadal steroids associated with the onset of puberty. The starlings sampled at Day 210 had not yet been exposed to the potential activational effects of gonadal hormones as evidenced by the lack of significant gonadal development as well as by the lack of yellow beak coloration. In contrast, 345-day-old birds exhibited marked gonadal development and associated changes in beak coloration, and males from each treatment group displayed detectable levels of T.

Although early E₂ treatment did not significantly alter androgen-dependent beak coloration, syringeal mass, or Day 345 circulating plasma T levels, Day 345 testicular mass and volume as well as body mass on both Days 210 and 345 were reduced in the E₂-treated males. Corresponding effects on gonadal development and body mass were not detected in females. As is the case among most wild birds brought into the laboratory (Farner and Wingfield, 1980), laboratory-housed starlings do not exhibit maximal gonadal steroidogenesis in comparison to free-living starlings (Bernard, 1995), and at least one 345-day-old male from each treatment group exhibited T titers that were undetectable by the RIA. Therefore, it is difficult to ascertain conclusively whether the early E₂ treatment influenced the subsequent testicular steroidogenesis. Most studies of steroid-induced masculinization of the song system have failed to consider potential effects of an altered activational hormonal milieu on song system development, perhaps because the research has focused on zebra finches, which develop so quickly that there is little temporal separation between the ontogeny of the vocal control system and the onset of puberty.

Partial Masculinization of Song Control Nuclei in E₂-Treated Females

Although early E₂ treatment did produce marked enlargement of Nissl stain-defined area X and HVC volume and autoradiography-defined area X, HVC, RA, and mMAN volumes in females, it is important to note that these enlarged volumes were still significantly smaller than those of age-matched control males. As can be seen in Figs. 7 and 8, the volumes of song control nuclei in E₂-treated females are still more similar to those of control females than to those of control males. Unlike data from E₂-treated female zebra finches, which support the notion that E₂ is very important in sexual

differentiation of the song system, these data from starlings further call into question the role of E_2 in the normal masculinization of the songbird vocal control system.

Although plasma E_2 was not assessed in implanted birds in this study, in a subsequent experiment E_2 levels were assessed in juvenile starlings that received identically prepared and administered E_2 implants. In those birds, E_2 was detected in plasma sampled weekly from Day 10 (range, 15–71 pg/ml) through at least Day 45 (range, 12–34 pg/ml) but not through Day 66. Estradiol was not detectable in unimplanted birds (Casto and Ball, unpublished data). Based on these data and the reliable effects of E_2 treatment on early physical development it appears that significant elevation of circulating E_2 did occur in E_2 -implanted birds. It is, however, possible that E_2 levels at target tissues within the brain were not elevated sufficiently to produce full masculinization. It may be impossible to give enough E_2 via peripheral administration to equal potentially high endogenous levels of E_2 produced by aromatization at target tissues within the brain because of the toxic effects associated with high circulating concentrations of E_2 . No sex differences in peripheral levels of circulating T have been detected in starlings between Days 4 and 20 after hatching (Williams, Dawson, Nicholls, and Goldsmith, 1987). Thus, it appears that there are equal levels of the precursor necessary to produce E_2 (i.e., circulating T) and any central sex differences would most likely be due to sex differences in central steroid metabolism or peripheral inactivation.

Based on previous data from zebra finches (Adkins-Regan *et al.*, 1994), the length of E_2 exposure in this study was most likely long enough to encompass an early phase of song system sensitivity to E_2 ; however, E_2 might exert an inhibitory effect on song system growth when exposure occurs after the close of the putative sensitive period. Such a scenario might result in E_2 -treated females that do not exhibit complete masculinization; however, E_2 treatment of female zebra finches after the close of the period of maximal song system sensitivity to the masculinizing effects of E_2 does not result in decreased song system masculinization (Adkins-Regan *et al.*, 1994). Future studies should use shorter E_2 exposure intervals to determine if a circumscribed period of sensitivity to the masculinizing effect of E_2 exists in starlings and if E_2 differentially influences song system development when administered either during or after such a sensitive period.

In most avian and mammalian species studied sex-specific peripheral hormonal signals (rather than sex differences in target tissue metabolism) appear to regu-

late the development of sex differences in the brain. In many mammals and birds the sex-typical peripheral hormone signals necessary for sexual differentiation of the brain, occur during the late embryonic stage of development (Weisz and Ward, 1980; Schumacher, Sulon, and Balthazart, 1988). The timing of our E_2 administration may not have coincided precisely with the entire period of maximal song system sensitivity to the masculinizing effects of E_2 . Although no evidence exists for early sexually dimorphic hormonal milieus in songbirds, plasma steroid titers have not been assessed during the late embryonic stage of development, and thus it is possible that significant sex differences in plasma sex steroids that mediate sexual differentiation of the song system occur prior to hatching. However, embryonic administration of fadrozole to zebra finches in addition to disrupting normal estrogen dependent gonadal development in females, fails to block masculinization of the male song system (Wade and Arnold, 1996), thus calling into question an embryonic period of enhanced song system E_2 sensitivity.

The lack of complete song system masculinization in E_2 -treated females may also be due to sex differences in the availability of some other factor that also influences sexual differentiation of the brain or synergizes with E_2 to effect sexual differentiation. Androgens are obvious candidates for potential factors that may influence sexual differentiation separately from E_2 or by synergizing with E_2 to produce more complete masculinization. Data from zebra finches suggest that 5α -DHT can produce some masculinization of the song system of females in the absence of exogenous E_2 ; however, E_2 appears to be a more potent masculinizing agent (Gurney, 1981; Gurney and Konishi, 1980; Grisham and Arnold, 1994). The effects of androgens administered early in development on song system masculinization should be tested in another species, such as the starling, in which early E_2 administration partially masculinizes the female song system.

The behavioral importance of E_2 -induced song system masculinization was not assessed in this study; however, in female zebra finches early E_2 treatment produces masculinization of the song behavior in adulthood. Such E_2 -treated females exhibit marked masculinization of the song system. For instance, area X, which is not apparent in untreated female zebra finches, is present in females exposed to early E_2 (Gurney, 1982). Additionally, the projection between HVC and RA, which is for the most part absent in normal female zebra finches, is present in E_2 -treated females (Simpson and Vicario, 1991a). Early E_2 exposure might masculinize song behavior in female zebra finches by completing

the circuitry necessary for the production of song. In contrast to female zebra finches, in untreated female starlings area X is visible and a projection from HVC to RA exists (Bernard *et al.*, 1993; Casto and Ball, unpublished data). In addition, female starlings sing in the wild (Witschi and Miller, 1938). Because the gross connectivity necessary for singing exists in untreated female starlings, it is unclear what effect the modest E_2 -induced increases in song control nucleus volume would have on adult singing behavior; however, investigations designed to assess behavioral consequences of early E_2 administration are in progress.

Transient Hypermasculinization of Song Control Regions in E_2 -Treated Males

In control males HVC continues to grow between Days 210 and 345; however, in E_2 -treated males the volume of HVC ceases to increase during the same developmental period. The reason for arrested HVC growth between Days 210 and 345 in E_2 -treated males is unclear. It is possible that there is an as yet undetermined biological constraint on the growth of HVC in first-year birds. In contrast to the pattern of HVC development, autoradiography-defined area X does not increase in volume between Days 210 and 345 in control males; however, in E_2 -treated males, which exhibit hypermasculinized area X volume at Day 210, the volume of area X declines after Day 210, so that by Day 345 area X volume does not differ from that of control males. It is possible that effects of the early hormone treatment on later testicular steroidogenesis, although not statistically significant, were biologically significant and prevented further T-dependent growth in HVC and T-dependent maintenance of area X volume after Day 210. The failure of females to exhibit HVC growth, in addition to slight shrinkage in area X volume during this developmental period, may also be associated with the hormonal milieu they experience during this period (i.e., females experience a high E_2 :T ratio and males experience a low E_2 :T ratio). The behavioral relevance of E_2 -induced hypermasculinization of the song system of male starlings during a circumscribed period of development is not known.

Effects of E_2 Administration on α_2 -Adrenergic Receptor Distribution in the Song System

From the distribution of PAC binding in control and E_2 -treated males and females it appears that E_2 administered early in development can influence catecholaminergic innervation within the song system; however,

early estradiol administration only influences the autoradiographically defined borders of song control nuclei, and does not significantly alter the density of binding within a given nucleus. In contrast, the density of receptor binding within song nuclei appears to be regulated during a different developmental period. Between Days 210 and 345, males and females display different patterns of α_2 -adrenergic receptor density regulation in all song regions that were analyzed. Females exhibit increased density in all song control regions, whereas males display little or no increase in receptor density. The sex difference in receptor regulation may be due to potential activational effects of the different hormonal milieus that males and females experience during this developmental period. Similar increases in α_2 -adrenergic receptor density in response to E_2 administration have been reported in the brains of gonadectomized male and female guinea pigs (Johnson, Nock, McEwen, and Feder, 1985; 1988). An equally plausible explanation is that these patterns of changes in receptor density are behaviorally regulated by sex differences in song learning, song production, or attending to song.

Catecholamines and their synthetic enzyme tyrosine hydroxylase are developmentally regulated in the song system of male zebra finches, and increase substantially during the periods of song system development and of song learning (Soha, Simizu, and Doupe, 1996). The differential regulation of α_2 -adrenergic receptor density in male and female starlings may be related to sex differences in the temporal pattern of song control nucleus development (i.e., in general, males continue to display growth of song control regions after Day 210 but females do not) or sex differences in the ability to learn, produce, or perceive species-specific vocalizations.

CONCLUSIONS

The song control system is a recently derived adaptation in avian evolution, present only in oscine birds (Ball, 1994; Brenowitz, 1991). Therefore, the mechanism by which this system is sexually differentiated could likely differ from other examples of sexual differentiation of neural circuits that mediate other forms of reproductive behavior in nonsongbirds and mammals. The findings that early E_2 administration only partially masculinizes the song system of female starlings and that E_2 treated females have song systems that resemble those of control females more than those of control males suggest that E_2 alone is not the endogenous factor that mediates normal song system sexual differentiation in starlings. Furthermore, although zebra finches

exhibit extreme sexual dimorphism in both brain and behavior, female zebra finches appear to differ quantitatively, but not qualitatively, from female starlings in their song system's response to administration of exogenous E_2 early in life. These results further call into question a role for E_2 in the sexual differentiation of the song control system of songbirds.

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