EXPERIENTIAL, ACOUSTICAL AND PHYSIOLOGICAL INFLUENCES ON SONG PREFERENCES IN FEMALE ZEBRA FINCHES

by

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Abstract

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Adviser: Professor Cheryl F. Harding

Female songbirds use male songs as an important criterion for mate-selection. Properties of male songs are thought to indicate the male's quality as a potential mate. Song preferences in female zebra finches are reported to be influenced by two factors – early auditory experience and the acoustic nature of males' songs. Additionally, many studies have investigated song preferences of female songbirds by first priming them with estrogen. However, estrogenic influences on song preferences have not been studied. In the first study, we investigated the relative influences of early auditory experiences, acoustic features of songs and estrogen on song responsiveness in female zebra finches. Juvenile female zebra finches were raised without exposure to male songs aside from brief tutoring with three acoustically different song types – simple songs, long-bout songs and complex songs. One group of females was untutored. Their song respensiveness were ascertained under no treatment, estradiol treatment and estrogen-depleted conditions. Our results lead to three conclusions about the relative importance of these three variables in determining song preferences. First, inexperienced female zebra finches appear inherently biased to respond more to complex songs over other song types. Second, song responsiveness in female zebra finches are significantly influenced by their early acoustic experience. Depending on the type of stimuli to which females are exposed, acoustic experience may either reinforce or weaken the inherent responsiveness for complex songs. Third, the strongest song responsiveness are seen in estrogen-treated females. Estrogenic influences on sexual behaviors are often mediated via norepinephrine (NE). In the second study, We used the noradrenergic neurotoxin, N- (2-chloroethyl)- N-ethyl-2-bromobenzylamine hydrochloride (DSP-4) to investigate if estrogenic influences on song responsiveness of female zebra finches are mediated by NE. We tested the responsiveness of adult female zebra finches for three acoustically different songs under three treatment conditions – untreated, estradiol-treated and estradiol + DSP-4-treated. Females only showed a differential responses when treated with estradiol alone, preferring complex songs. Following DSP-4 treatment, this differential responsiveness disappeared. Our results suggest that estrogenic influences on song responsiveness are mediated by NE.

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Dedicated to my parents

There was a time you were anachronistic and everything I didn’t want to be
I was young and bold - Full of ideas and terms. And I thought you had no clue
I was young like an unstoppable train. You watched and steered, and I didn’t even know.
And now, I find more of you in me. I think like you dad and pine like you mom.
Though not in so many words, you made me practical
You taught me dad to always be strong and be ready to face myself.
You taught me to look for weakness on the inside and not out.
You taught me mom to reach for the stars - the glowing ambition
And you dad, that no failure is final
I am dramatic as you mom and gesticulate like you dad.
I thought I knew, but you showed me that unconditional is the love given unconditionally.
Pundits may come and pundits may go.
What kids get from their parents, they may never know.
I am still a kid, and always will be. It’s a luxury, for you are around for me.

Thanks to:

My mentor: Prof. Cheryl F. Harding: For teaching me, guiding me and giving me the confidence in my self.

My Wife, Soma: For sticking with me when even I could not have faced myself

My Sister, Roli: For being there always and supporting me unconditionally

My Son, Naman: For making my life beautiful beyond imagination

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Introduction

Sexual Selection

According to Darwin, traits that increase animals' chances of finding mates tend to be naturally selected and increase in frequency. This process is called sexual selection. Sexual selection occurs, because not all sexually reproducing individuals will be equally successful in acquiring mates. The process of sexual selection is reported to be widespread in the animal kingdom (Andersson, 1994), and is believed to cause certain extreme cases of sexual dimorphisms (Schilthuizen, 2003), like, elongated tail feathers (Andersson, 1982) and bright coloration (Boyce, 1990) in certain avian species; enlarged horns and spikes in the genitalia of some arthropods (Eberhard, 1985). In sexually dimorphic species, reproductive success is typically unequally distributed among males, either because of female choice and/or direct male-male competition (Darwin, 1871 as cited in Andersson, 1994). When females, on the basis of visual, auditory, tactile, or other stimuli, choose which males' sperm they will use for fertilizing their ova, sexual selection by female choice occurs. For sexual selection to occur efficiently, two conditions must be met - 1) Presence of certain traits that vary with the sexual attractiveness of animals, like health (Hamilton & Zuk, 1982), genes (Zahavi, 1975) and territory quality (Hoelzer, 1990); 2) Presence of mechanism/s that can detect the characteristics correlated with these traits and elicit differential mating by conspecifics of the opposite sex. Qualitative and quantitative variations in behavioral and/or morphological traits are linked to reproductive success of males in many species. For example, in red-collared widowbirds (*Euplectes ardens*), conspecific females preferred males with longer tails (Pryke, Andersson, & Lawes, 2001), and in North American tree frogs (*Pseudacris crucifer*), male reproductive success correlated with the males' calling rates (Wells & Bard, 1987). Variability in these sexually-stimulating traits is reported to be indicative of health of males of the species. For example, in greenfinches (*Carduelis chloris*), carotenoid-based plumage coloration influenced female mate preferences and was positively correlated with male immunocompetence and health status (Saks, Ots, & Horak, 2003). Female sedge warblers (*Acrocephalus schoenobaenus*) preferred males with larger song repertoires, which were positively correlated with male immunocompetence (Buchanan, Catchpole, Lewis, & Lodge, 1999). As mentioned earlier, for these traits to elicit preferential sexual attraction, potential mates must possess mechanisms that can detect and differentially respond to these traits. During the course of evolution, females of sexually-selective species have acquired different mechanisms for selecting the appropriate mate(s) (Fisher, 1930). For example, tungara frog (*Physalaemus pustulosus*) females preferred males emitting mating calls with low fundamental frequencies. It turns out that neurons in the internal ears of females had a response bias towards low-frequency components of the mating calls (Ryan, 1980). In females of socially monogamous species, it is important that these mechanisms have a high fidelity, as most of an animal's reproductive capital will potentially be invested in just one individual of the opposite sex. For example, studies indicate that female prairie voles (*Microtus ochrogaster*), a monogamous species, are more selective in their choice of potential mates compared to female montane voles (*M. montanus*), which are polygamous (Young, Wang, & Insel, 1998).

Sexual selection for male songs in female song birds

Many songbirds (order: *Passeriformes*) found in the temperate regions exhibit prominent sexual dimorphisms in singing (Riebel & Slater, 1998). Song is typically the function of males, but there are many exceptions. Females of some species of songbirds, like mockingbirds, cardinals, and black-headed grosbeaks, have songs that are as complex as their respective conspecific males' (Andersson, 1994). Unless otherwise noted, further discussion is limited to songbird species in which singing is a sexually dimorphic behavior. Male songs can influence a female songbird in several ways: attracting her from a distance, priming her into physiological condition for breeding and stimulating her to copulate (Kroodsma & Byers, 1991). Female songbirds use male songs as one of the criteria for mate choice (Catchpole, 1987). Research in several species suggests that female song preferences are influenced by two factors: 1) Songs with certain acoustic characteristics tend to be preferred; and 2) Songs which female songbirds heard as juveniles tend to be preferred.

Influence of acoustic features on female song preferences

Female songbirds exhibit stronger preferences for songs with certain acoustic characteristics. These are commonly called sexually-stimulating songs. Females of many songbird species prefer males with higher song rates. Female song rate preferences were reported in European starlings (*Sturnus vulgaris*, Eens, Pinxten, & Verheyen, 1991), pied flycatchers (*Ficedula hypoleuca*, Alatalo, Glynn, & Lundberg, 1990) and zebra finches (*Taenopygia guttata*, Collins, Hubbard, & Houtman, 1994). Female preferences were also influenced by song bout length (Searcy & Marler, 1981). Female European starlings (Gentner & Hulse, 2000a) and female white-throated sparrows (*Zonotrichia albicollis*, Wasserman & Cigliano, 1991) showed stronger preferences for longer song-bout lengths. Another property of male birds' songs that influences female mate preferences is song complexity. Song complexity may either include number of song types in the repertoire of the animal or variability in the types of syllables a male may sing (Nowicki, Searcy, & Peters, 2002). Female song sparrows (*Melospiza melodia*) showed a higher number of copulation solicitation displays (CSDs) to male songs with *eventual variety* (AAAA followed by BBBBB….) than to *immediate variety* (ABCDABCD…). Vallet and Kreutzer (1995) reported that certain song phrases elicited a higher number of CSDs in female canaries. These phrases included abrupt frequency falls and short silences and were later called 'sexy syllables' by Leitner and Catchpole (2002), who characterized them as more complex two-note structures, with rapid frequency modulations, high repetition rates and wide frequency ranges. Female red-winged blackbirds (*Agelaius phoeniceus*) also preferred male songs played at higher sound pressure levels (Searcy, 1996). These acoustic features putatively convey information either about the males' nutritional state, territory status, genetic make up, health status or willingness to provide parental care. Song output rates were correlated with the nutritional states of male pied flycatchers (Gottlander, 1987). In willow warblers (*Phylloscopus trochilus*), higher song rates preferred by females were correlated with territory quality (Arvidsson & Neergaard, 1991). Nowicki *et* al. (2002) suggested that singing at high output rates or loudly incurs obvious costs to the singer and thus communicates the energy status of the singer to females. Garamszegi *et* al. (2003) and Moller *et* al. (2000) conducted comparative studies on songbirds and reported that song complexity was correlated with male immunocompetence. The nutritional stress hypothesis, proposed by Nowicki *et* al.

(2002), states that investment in song learning and energy expenditure during song production help keep male signaling honest and thus, female preferences should be stronger for song components that require greater ontogenetic investment during learning and expenditure during production.

Influence of early experience on female song preferences

Adult female songbirds exhibit stronger preferences for songs they heard during early post-fledge life. Casey and Baker (1992) reported that female sparrows preferred male conspecifics whose songs' acoustic characteristics were close to those of the auditory environment experienced in their natal area in early life. This pattern of female preferences was preserved into late adulthood even without any subsequent exposure to male songs. Adult female canaries exhibited more CSDs to conspecific songs they heard as juveniles (Depraz, Leboucher, & Kreutzer, 2000; Nagle & Kreutzer, 1997). Reports suggest that exposure during a critical period early in life is essential for the development of song preferences. Female zebra finches preferred their father's song to an unfamiliar one, when given the choice between approaching two speakers (Miller, 1979). Females removed from their fathers before day 25 did not show this preference, but those removed at day 35 did show preferences (Clayton, 1988a). Riebel (2000) reported that adult female zebra finches pecked keys at higher frequencies if key pecks resulted in the playback of songs they heard as juveniles. Preference for a song heard early in life was preserved even after females were exposed to other songs during adulthood.

Song acquisition in songbirds

Since song preferences in female songbirds are influenced by early post-fledge exposure to songs, it can be assumed that females memorize the songs early in life. Song memorization is believed to be the method by which male songbirds learn songs (see Brenowitz, 1997). In males, song learning occurs in two stages. During the first stage, called sensory acquisition, juvenile males simply memorize the song produced by adult/s. In the second stage, called sensorimotor learning, juvenile males start vocalizing their own songs and use auditory feedback to adjust their songs to match the stored template (Nordeen & Nordeen, 1997). The neural circuitry that regulates singing has been extensively studied. Singing is regulated by a system of interconnected brain nuclei (Nottebohm, 1993), called the vocal control system (VCS). The VCS can be divided into an afferent pathway for sensory acquisition (anterior forebrain circuit) and an efferent pathway (posterior forebrain circuit) for song production. The afferent pathway includes projections from the high vocal center (HVC) to Area X of the medial striatum, then to the medial nucleus of the dorsolateral thalamus (DLM), from DLM to the lateral magnocellular nucleus of anterior nidopallium (lMAN). lMAN also projects to the robust nucleus of arcopallium (RA). The anterior forebrain circuit is necessary for normal song development. Damage to lMAN (Bottjer, Miesner, & Arnold, 1984) or area X (Scharff & Nottebohm, 1991) in juvenile zebra finches prevented normal song learning, whereas comparable damage in adulthood doesn’t affect song production. The efferent pathway includes the HVC, which sends projections to RA. RA in turn projects to the tracheosyringeal part of the hypoglossal nucleus (nXIIts), which innervates the syrinx. An insult to any part of the posterior forebrain circuit results in immediate disruption of singing (Nottebohm & Arnold, 1976). HVC also receives projections from the nucleus interfacialis (NIf) and the thalamic nucleus, uvaformis (Uva). Uva is thought to send auditory and proprioceptive feedback to HVC (Nottebohm, 1993). RA also sends indirect projections to nXIIts, via the dorsomedial nucleus of the intercollicular nucleus (DM). Female zebra finches do not sing, because they have smaller VCS nuclei, and area X of the learning loop is missing (Nottebohm & Arnold, 1976). In addition, Simpson and Vicario (1991) reported that in female zebra finches projections from HVC to RA were either absent or minimal. Gurney and Konishi (1980) reported that early post-hatch treatment with estrogen masculanized female VCS nuclei in zebra finches. These masculinized females learned songs and sang during adulthood, if treated with testosterone (Bottjer & Arnold, 1997). Holloway and Clayton (2001) reported that brain-derived estrogens guide the development of the male vocal control system during early development.

There is ample evidence suggesting that both male and female songbirds memorize songs, while only males are able to sing. In different experimental paradigms, both adult males (Adret, 1993; Houx & Ten Cate, 1999) and adult females of different songbird species (Clayton, 1988a; Miller, 1979) showed stronger preferences for the songs they heard as juveniles. The most direct evidence to date regarding similarity in song memorization in both sexes was provided by Riebel (2000) She reported that early exposure to song equally influenced song preferences in both male and female zebra finches. Moreover, in both sexes song memorization is limited by a critical period early in life (Clayton, 1988b; Miller, 1979), the only difference being that the duration of critical period was reported to be shorter in females (Nelson, Marler, Soha, & Fullerton, 1997).

However, data regarding sexual equality in song acquisition are not consonant with the reports of neural sexual dimorphism of VCS. Of late, studies have implicated brain areas outside of the sexually dimorphic parts of the song circuitry in song learning (Bolhuis, Zijlstra, Den Boer-Visser, & Van Der Zee, 2000). Bolhuis *et al.* (2000) looked at the expression of the immediate early genes (IEG), *c-fos* and *egr-1* (ZENK) in the brains of juvenile males. They reported that cells in the caudomedial nidopallium (NCM) showed IEG expression only when stimulated by conspecific songs. Duffy and colleagues (1999) reported that *ZENK* expression in NCM was not sexually dimorphic. Such IEG expression was not seen when birds were stimulated with non-song stimuli or heterospecific songs (also see Mello & Clayton, 1994). More importantly, none of the VCS nuclei exhibited IEG expression only in response to conspecific songs. IEG expression is thought to play a role in consolidation of memories (Davis & Squire, 1984). NCM is a region of the avian telencephalon, which is considered analogous to the mammalian secondary sensory or association cortex (Mello & Clayton, 1994). NCM receives projections from field L, the analog of the mammalian primary auditory cortex and sends projections to the caudomedial mesopallium (CMM), which in turn projects to NIf and HVC (Brainard & Doupe, 2000). Some studies have also implicated *ZENK* expression in the CMM in song discrimination (Avey, Phillmore, & MacDougall-Shackleton, 2005; McKenzie, Hernandez, & MacDougall-Shackleton, 2006; Tomaszycki, Sluzas, Sundberg, Newman, & DeVoogd, 2006). Bolhuis *et* al. (2000) tutored juvenile male zebra finches with adult male songs. The males were subsequently stimulated with their tutors' song and *ZENK* and *FOS* expression were quantified. The strength of *ZENK* and *FOS* expression in the NCM and CMM of male zebra finches was positively correlated with the number of elements the males had copied. This lead Bolhuis *et* al.

(2000) to suggest that the 'sensory memory' of learnt songs must be stored in the NCM and/or CMM.

Hormonal correlates of mate choice

Hormones are well known to influence sexual behaviors, including female mate choice (for review see Adkins-Regan, 1998). In many mammalian species, estrous females prefer intact as opposed to castrated males. Anestrous females show no such preferences (see Johnston, 1979). These studies suggest that the hormonal profiles of females may influence their preferences for potential mates.

Hormones can influence mate choice in at least three different ways (Bateson, 1983). First, hormones can influence the mechanism of sexual imprinting, a process early in development that leads to the establishment of sexual preferences. Second, hormones, acting early in ontogeny, can lead to the development of mechanisms that will be used in the process of mate selection - organizational effects. Third, hormones can lead to fluctuations in sexual behaviors during adulthood by changing the responsiveness of hormone-sensitive structures to environmental stimuli - activational effects.

Clayton and Prove (1989) reported that female Bengalese finches treated with 17 β-estradiol (E_2) were more likely to give CSDs to conspecific rather than heterospecific males' songs. Many wild-caught passerine females also exhibit CSDs when treated with E_2 and stimulated with conspecific songs. Moore (1982) reported that in female white-crowned sparrows, E_2 treatment prolonged CSDs in response to conspecific male songs. E_2-treated female red-winged blackbirds exhibited CSDs to the playback of conspecific male songs, while untreated females did not (Searcy & Cave, 1997). E_2-treated female song sparrows and swamp sparrows showed more CSDs than untreated females (Searcy, Marler, & Peters, 1981). The role of estrogens in eliciting CSDs is however less clear in some other species of songbirds. E_2 treatment had no effect on CSDs in female canaries (Leboucher, Beguin, Mauget, & Kreutzer, 1998) and brown-headed cowbirds (*Molothrus ater*, King & West, 1977), when stimulated with conspecific male songs. These observations lead Searcy and Cave (1997) to suggest that females from species that breed and lay eggs in captivity do not require E_2 treatment to exhibit CSDs in captivity, while females from species that do not breed and lay eggs in captivity do require E_2 treatment to exhibit CSDs in captivity. To clarify the role of estrogen in eliciting CSDs, Leboucher and *et* al. (1998) treated domesticated female canaries with an aromatase inhibitor, fadrozole to decrease estrogen synthesis. They concluded that a threshold level of estrogen was critical to elicit CSDs in female canaries, and once the threshold was reached, higher estrogen levels (measured by RIA) did not elicit a greater number of CSDs to conspecific male song stimuli. Similar conclusions were also drawn by Searcy and Cave (1997), who found that beyond a critical level, higher doses of E_2 failed to elicit a higher number of CSDs in female red-winged blackbirds that heard conspecific male songs. It is however not known if estrogen influences song preferences in female zebra finches.

In mammals, many estrogenic effects on female sexual behaviors have been reported to be modulated by norepinephrine (NE) (for reviews see Etgen, Ansonoff, & Quesada, 2001; Etgen, Chu, Fiber, Karkanias, & Morales, 1999; Fabre-Nys, 1998). NE also appears to play an important role in mediating estrogenic effects on sexual behavior in songbirds. In zebra finches, NE appears to mediate estrogenic activation of courtship singing in males (for review see Harding, 2004a). Estrogen treatment increased NE levels and turnover in brain nuclei that activate singing and sexual behavior in reproductively-active male zebra finches (for review see Harding, 2004a). Additionally, NE depletion through DSP-4 treatment decreased the frequency of song-induced CSDs in female canaries (Appeltants, Del Negro, & Balthazart, 2002) and courtship singing to females in male zebra finches (Barclay, Harding, & Waterman, 1992, 1996).

Rationale and objectives

The above discussion indicates that our knowledge regarding female songbirds' sexual preferences for male songs is incomplete. Although some significant discoveries have been made, certain important questions remain unanswered.

In my doctoral research study, I attempted to address the following issues about song preferences in female zebra finches:

1a. Some studies have reported that female zebra finches prefer songs they heard as juveniles. Other studies reported that female zebra finches prefer songs with certain acoustic features. I investigated the relative influences of acoustic song characteristics and early experience on female zebra finches' song preferences.

1b. Researchers have often primed female songbirds E_2 before testing their song preferences. When one examines studies in which female zebra finches were found to prefer songs they heard early in life, the methodology suggests that females probably had low estrogen levels. In comparison, in studies in which female zebra finches were found to prefer particular acoustic characteristics, females probably had high levels of estrogen. I investigated if song preferences change in female zebra finches depending on their estrogen levels.

2. Studies suggest that estrogenic influences on sexual behaviors are often mediated via norepinephrine. I investigated if estrogenic influences on song preferences in female zebra finches are mediated via norepinephrine.

Study 1: Experiential, Acoustical and Estrogenic Influences on Song Responsiveness in Female Zebra Finches

Introduction

Female songbirds use male songs as an important criterion for mate selection (Catchpole, 1987; Kroodsma & Byers, 1991; Searcy & Andersson, 1986; Tomaszycki & Adkins-Regan, 2005). In zebra finches (*Taenopygia guttata*), songs are used almost exclusively in a sexual context (Neubauer, 1999), and song was reported to be a more important criterion for mate-selection than other characteristics like beak coloration (Collins et al., 1994). Properties of male songs are thought to indicate the male's quality as a potential mate (Andersson, 1994; Gibson & Langen, 1996; Jennions & Petrie, 1997). Two types of factors are reported to influence song preferences in female zebra finches. Some studies reported that female zebra finches preferred songs they heard as juveniles (Clayton, 1988b; Miller, 1979; Riebel, 2000). Others reported that females preferred songs having certain acoustic characteristics like songs sung at faster rates (Collins *et al.*, 1994; Forstmeier, 2004; Forstmeier & Birkhead, 2004), or having longer bout lengths (Clayton & Pröve, 1989; Neubauer, 1999) or having greater song complexity (Clayton & Pröve, 1989).

Female songbirds of other species were also reported to show these two types of song preferences. For example, female white-crowned sparrows (*Zonotrichia leucophrys*, Casey & Baker, 1992) and female canaries (*Serinus canaria*, Depraz *et al.*, 2000; Nagle & Kreutzer, 1997) were reported to prefer songs they heard when they were juveniles. Preferences for acoustic features were also reported in female house finches (*Carpodacus mexicanus*, Nolan & Hill, 2004), European starlings (*Sturnus vulgaris*, Eens et al., 1991; Gentner & Hulse, 2000a), pied flycatchers (*Ficedula hypoleuca*, Alatalo *et al.*, 1990), white-throated sparrows (*Zonotrichia albicollis*, Searcy & Marler, 1981; Wasserman & Cigliano, 1991), song sparrows (*Melospiza melodia*, Searcy & Marler, 1981), and canaries (Leitner & Catchpole, 2002; Vallet & Kreutzer, 1995). To the best of our knowledge, no single study has investigated the relative importance of early experience and acoustic characteristics to song preferences in female songbirds.

As in other vertebrates, estrogens play an important role in activating female sexual behavior in songbirds (Silver, 1989). During normal reproductive cycles, exposure to singing males stimulates estrogen secretion, inducing ovulation and readiness to copulate (Catchpole, 1987; Kroodsma, 1976). Song quality affects the rapidity of reproductive development (Kroodsma, 1976). Studies of female choice have turned this paradigm around and administered estrogen to female songbirds to make it easier to determine their song preferences. In this commonly-used paradigm, females are treated with estrogen, and researchers determine which songs elicit species-specific copulation solicitation displays (CSDs). This paradigm demonstrates the significance of male song for female reproductive behavior, since song playback is often sufficient to elicit CSDs in the absence of other cues. However, females, particularly wild-caught birds, will usually not show CSDs without estrogen treatment (Clayton & Pröve, 1989; Searcy & Cave, 1997; Searcy & Marler, 1981). One obvious question, which has not been investigated, is whether estrogen treatment alters female preferences. There are ample data indicating that circulating gonadal hormones influence mate choice in vertebrates (for reviews see: Adkins-Regan, 1998; Puts, 2006). It is therefore likely that gonadal hormones influence song evaluation in female zebra finches. Interestingly, in studies in which female zebra finches were reported to prefer songs having certain acoustic characteristics, females probably had high circulating estrogen levels, either because of experimental manipulations or social environment. Clayton & Pröve (1989) treated female zebra finches with estradiol prior to testing and reported that females tended to prefer longer more complex songs. Tomaszycki & Adkins-Regan (2005), Frostriemer & Birkhead (2004) and Collins *et al.* (1994) also reported female zebra finches preferred songs having certain acoustic characteristics. They had housed their females in a mixed-sex environment, where females were probably exposed to male songs before testing. Evidence suggests that song exposure increases estrogen levels in female songbirds. Tchernichovski *et* al. (1998) reported that female zebra finches visually and acoustically or acoustically exposed to male zebra finches had increased fecal estrogen levels. Fecal estrogen levels were found to be positively correlated with circulating estrogen levels in several avian species. (Bishop & Hall, 1991; Cockrem & Rounce, 1994; Lee, Whaling, Lasley, & Marler, 1995). Exposure to songs also stimulated the hypothalamo-pituitary-gonadal (HPG) axis in female canaries and female song sparrows (Bentley, Spar, MacDougall-Shackleton, Hahn, & Ball, 2000). In studies that reported female preferences for songs heard during development (Clayton, 1988a; Miller, 1979; Riebel, 2000), females were maintained in a same-sex environment prior to testing which should lead to lower estrogen levels. It is possible that in the studies showing effects of early experience, the preferences exhibited by female zebra finches were not sexual in nature, but merely indicating a social preference for familiar conspecifics.

The current study was conducted to better understand the interaction between experiential and acoustical influences on conspecific song responsiveness in female zebra finches, and the role their estrogen levels might play in this process. Two experiments were run. In the first, we drastically limited song exposure and tutored juvenile female zebra finches with one of three acoustically different song types. A fourth group was untutored. All females were raised in acoustic isolation from adult male vocalizations, and their behavioral responses to the three song types were tested under three conditions – untreated, estradiol-treated and ATD-treated. A second experiment was conducted to help put these data in perspective. In this experiment, experienced breeding females were separated from their mates and the female's behavioral responses were quantified when stimulated by her mate, a male from a different breeding pair, and an unpaired female.

Experiment 1: Materials and methods

Subjects

Subjects were 44 female zebra finches bred in our colony. Breeding pairs were kept in individual cages. Each wire-mesh cage measured 56 X 56 X 56 cm with a 13 X 12 X 12.5 cm nest box centered high on the rear wall. Adults were fed a vitamin-supplemented commercial finch seed mix, grit, water and cuttlebone *ad libitum*. This diet was supplemented with hard-boiled eggs, fresh greens and oranges. Animal rooms were temperature controlled (24° C + 2°C), with over 50% relative humidity and with a 14:10 light: dark cycle for optimum breeding. Nests were checked daily, and each chick was marked upon hatching. Since zebra finches have been reported not to memorize any songs they are exposed to before 35 days of age (Clayton, 1988b), juveniles stayed with their parents till the oldest chick in the brood was 15 days of age (D15). Broods along with their mothers were then moved to a separate room that housed only mothers and chicks. Each brood was housed in a separate cage within the room. When the middle chick in a given brood was 25 days old, the mother was removed and returned to the breeding room. Male fledglings were removed from the room for use in other experiments when they were approximately 30 days old, and females were assigned to an experimental group. All animal care and experimental procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of Hunter College.

Tutoring

The 44 juvenile female zebra finches were assigned to 4 groups, using a randomized block design. Three groups of birds were tutored with male zebra finch songs. Birds were tutored for 10 days from about D30 to D40 with 40 repetitions of a song/day in two sessions, in an operant response training paradigm developed by Tchernichovski et al (1999). Tutored females were tutored with one of the three song types described below. One group (6 birds) of females was not tutored. They were simply exposed to the tutoring chambers for the same period of time. After tutoring/exposure, females were moved back to the chicks and females-only room, where they remained isolated from male vocalizations for the duration of the study, except during behavioral testing.

Songs

We followed Sossinka & Bohner’s (1980) and Price’s (1979) descriptions of typical zebra finch courtship songs. Typically, each song begins with *introductory notes*, followed by repeated sequences of a relatively rigid succession of *elements* or *syllables*, forming *motifs* or *song units*. A song bout was defined as a sequence of song units, preceded by introductory notes. Normally, in songs directed at females, each bout has about 5.4 song units, preceded by an average of 6.3 introductory notes (Sossinka & Bohner, 1980).

All songs were recorded from males in our colony. Courtship songs were recorded from male zebra finches placed inside sound-attenuated chambers using high-fidelity microphones (Earthworks® SRO) and Sound Analysis Pro© software (Tchernichovski & Mitra, 2004) on Dell Optiplex® Pentium-4® computers. Based upon their acoustic characteristics, songs were divided into three categories:

Prototypical songs (P): Prototypical songs met the following criteria: 1) 6 ± 2 introductory notes. 2) 4-5 song units/bout. 3) Average inter-song unit similarity (as measured by the Sound Analysis Pro© software) was > 90%. 5) Each song unit had all note types (clicks, highs, stacks, downsweeps). Example: AAAAA BCDE BCDE BCDE BCDE ('A' depicts the introductory note and other letters depict a syllable each). There were three exemplars of prototypical songs. Thirteen females were assigned to be tutored by the one of the exemplars using a randomized-block design.

Long-bout songs (L): Long-bout songs were the same as the prototypical songs in all regards except that the number of syllables in a song unit was greater and the number of song units in a bout was > 7. We had to add some song units to the bouts produced by males in our colony to create these songs. Acoustic and temporal characteristics of these songs were in the normal range of zebra finch songs identified by Sossinka & Bohner (1980). Example: AAAAA ABCDEFG BCDEFG BCDEFG BCDEFG BCDEFG BCDEFG BCDEFG BCDEFG. There were three exemplars of long-bout songs. Twelve females were assigned to be tutored by one of the exemplars using a randomized-block design.

Complex songs (C): Song complexity may has typically been defined either include by the number of song types in the repertoire of males or variability in the types of syllables males sing (Nowicki *et al.*, 2002). In the present study, complex songs had a larger number of unique syllables and also had acoustically different song units. For complex songs, the inter song-unit-similarity was < 77%, and their bout lengths were similar to those of long-bout songs. We had to add some song units to the bouts produced by males in our colony to create these songs. Acoustic and temporal characteristics of these songs were in the normal range of zebra finch songs identified by Sossinka & Bohner (1980). Example: AAAAAA ABCDEF GHCDEF BCDEF ICDEF BCDEF GHCDEF BCDEF ICDEF. There were 3 exemplars of complex songs. Thirteen females were assigned to be tutored by one of the exemplars using a randomized -block design.

Songs were filtered, edited and amplified using GoldWave® (Version 5.14. www.goldwave.com) software. Filtering was performed to remove any background noise. Additional editing was performed to match inter-syllable intervals and inter-song-unit intervals of different songs. In some cases, additional introductory notes were added. Following editing, songs were transferred to magnetic tape cassettes (TDK Audio Cassette, Standard Size, Normal Bias, 60 Minutes). Nine songs, 3 of each type, were obtained in this manner. Each female was tutored with only one song of one type. Each song was matched with a song of each of the other two types based on song similarity. Females were then tested with the song with which they had been tutored and the two related songs of the other two song types. Untutored females were randomly block-assigned to the song groups for behavioral testing.

Behavioral testing

After tutoring, females were housed 4 to a cage in the cages described above and remained in acoustic isolation from males. At about D89, each female was housed singly in a standard cage. Following acclimation, the female was removed, the cage was moved to a behavioral testing room and fitted with a speaker on top of the cage and a male model centered on a perch directly under the speaker. The female was subsequently released back into her cage and allowed to acclimate to the testing room for 30 minutes. The behavioral testing room had a white-noise generator. A flowing water sound was also played continuously. White noise and flowing water sounds were played back to minimize extraneous background noises. The sound pressure level of each was 60 dB in the center of the cage. The white noise generator was placed on one side about 2 feet from the center of the cage, and water sounds were played back from about 10 feet from the center of the cage on the opposite side. Songs were played-back at the average sound pressure level of 72dB in the center of the cage, with a range of 67-74 db for each song. Three Marantz® cassette players (model 201) were used to play the songs.

During testing, each song was played back for 5 minutes, followed by 2 minutes of silence, followed by the playback of the next song type. Each testing session had two trials, so that during each testing session, the females were exposed to each song twice for 5 minutes each. The order of playback of the different songs during the trials and across the birds was counterbalanced. During both playback and the silent periods, the females' behaviors (including shakes, orienting towards the speaker, long-calls, beak-wipes etc.) were scored by an observer (AV) sitting in front of the testing cage. After testing, females were given hormone or drug implants and tested again seven days later. Between tests, females were returned to the female and chick housing room.

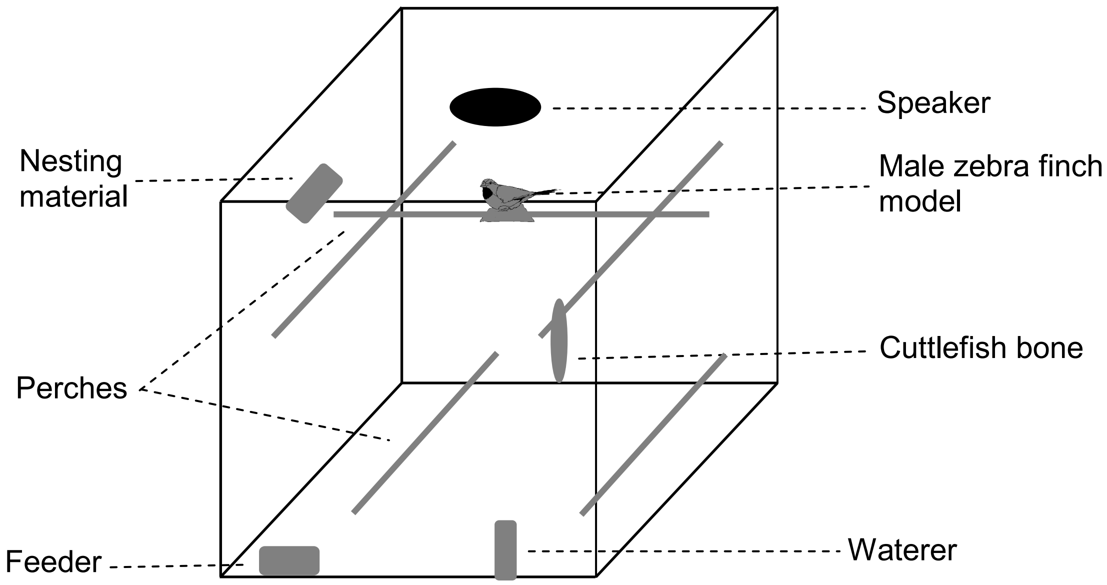

Figure 1: Schematic illustration of the experimental test cages used to quantify song responsiveness in female zebra finches. Songs were broadcast from a speaker on top of the cage. A model of an adult male zebra finch was placed below the speaker inside the cage. The left wall of the cage was opaque, and the other walls, the floor and the roof were made of 1 in. X 0.5 in. stainless steel mesh. The floor of the cage was covered with brown tech-board paper. The observer sat 7 feet in front of the cage.

Hormone and drug treatments

Exposure to songs increases estrogen levels in female zebra finches (Tchernichovski *et al.*, 1998). Since females were housed in isolation from songs for over 50 days before testing, it was assumed that estrogen levels in untreated females should be low. After the first behavioral test, each female was sequentially treated with the hormone 17 β-estradiol (E_2) to raise estrogen levels and an aromatase inhibitor ATD (1,4,6-androstatriene-3,17-dione) - that inhibits the synthesis of estrogen to lower estrogen levels further. We used ATD rather than ovariectomizing females, because evidence suggests that female zebra finches have significant levels of brain-derived estrogen (Saldanha, Popper, Micevych, & Schlinger, 1998; Schlinger & Arnold, 1993), and ovariectomy would not have decreased brain-derived estrogen. Half the birds received E_2 first and half the birds received ATD first. We followed Walters & Harding's (1988) methodology. E_2 was administered via silastic implants (0.76 mm i.d., 1.65 mm o.d.; Fisher Scientific) containing 2 mm packed hormone and ATD via 2 silastic implants (0.76 mm i.d., 1.65 mm o.d.; Fisher Scientific) each containing 5 mm of packed drug. Harding *et* al. (1983) treated male zebra finches with the same size E_2 implants, and reported that E_2 treatment increased female-typical CSDs in male zebra finches tested with females. They also reported that this amount of E_2 was also sufficient to activate some male courtship behaviors. Schlinger & Arnold (1993) reported that silastic implants containing 10 mm of ATD significantly reduced estrogen levels in female zebra finches. Adult estrogen levels were not found to be sexually dimorphic in zebra finches (Adkins-Regan, Abdelnabi, Mobarak, & Ottinger, 1990), indicating that similar doses of E_2 should be sufficient for activating behavior in both males and females. Moreover, results of one of the experiments in the present study indicate that the dose of E_2 used in the present study increased the frequency of CSDs by over 300% over the levels in untreated females, indicating that the dose of E_2 used in this study was effective in activating females' sexual behaviors.

At least seven days after each hormone and/or drug treatment, females were again tested for their responses to songs. After the behavioral test, the hormone/drug implants were removed and the other hormone/drug implanted. This also confirmed the presence of hormone/drug implants. After the third behavioral tests, females were again checked for the presence of implants.

Behavioral analysis

During each behavioral test, the frequencies of several behaviors during each play-back were scored. A preliminary analysis was done to identify behaviors that were differentially exhibited to the different song types. There were several behaviors (like short calls, perch changes, etc.) that were exhibited at very high frequencies by most birds under all treatment and stimulus conditions. These behaviors were omitted from further analysis. There were other behaviors (like copulation solicitation displays) that were exhibited by very few birds. These were also omitted from further analysis. Three behaviors – shaking, orientating towards the speaker and long calls were exhibited at high differential frequencies to different songs under the three treatment conditions. Shakes were high frequency side-to-side movements of the upper body, immediately preceded by fluffing the feathers. Regardless of how many times the animal moved its upper body, the number of shakes was defined by the initial fluff. Looking up towards the speaker was defined as orienting. Long calls are characteristic loud distance calls that zebra finches produce when they are visually isolated from other birds (Zann, 1996).

During the silent phase between tests, females typically sat in a corner on one of the upper perches. Playbacks of particular songs typically resulted in a rapid change in females' behavior. As soon as the song playbacks started, females first shook. Shakes were quickly followed by a series of long calls and orienting responses. Long calls and orienting responses were exhibited either simultaneously or in quick succession. The frequency and latency of behaviors depended upon the songs being played. As the playbacks progressed, some females maintained the successive or simultaneous exhibition of orienting and long calls, while others didn't. For most females, the frequency of shaking was lower than those of orienting and long calls. Typically a greater frequency of shakes was observed at the beginning of playbacks following the silent periods. Females that shook less also oriented less and emitted fewer long calls.

Data analysis

For each behavioral test, the frequencies of each of these three behaviors in response to each song type for the two trials were summed. The proportional frequency of behavior to each song type compared to the frequency to all three song types was calculated. To avoid division of/by zero, if any of the three behaviors were not exhibited during each trial, a frequency of 0.1 was assigned for the behavior. The effects of early acoustic experience, song-type and hormone/drug treatment on these proportional frequencies were analyzed using mixed-model 3-way ANOVAs (acoustic experience X test song-type X hormone/drug treatment) followed by planned comparisons. Tutoring was the between-subject factor and song types and treatment were within-subject factors. P-values < 0.05 were considered statistically significant. To further examine the effects of song types, acoustic experiences and hormone/drug treatment, the proportional frequencies of behaviors in each treatment condition for each of the tutored and untutored groups were analyzed using a series of 1-way ANOVAs, followed by multiple comparisons when the ANOVA was significant.

In some of the statistical analyses reported below, the assumptions of *sphericity* were violated. Sphericity is an assumption about the nature of covariance in a repeated measures design. Sphericity is to repeated-measures designs, what homogeneity of variance is to between-subjects designs. If sphericity is violated, several corrections can be made, each of which results in the loss of degrees of freedom, with a concomitant loss in the confidence level. Lower-bound epsilon correction is one of the most conservative estimates of confidence levels if sphericity is violated. While we report the data using lower-bound epsilon corrections, all sphericity violations disappeared if the smaller group of untutored females was not included in the analyses. Large intra-group variation in the behavior of the untutored group was the major contributor towards sphericity violations. This was true for all the indicated violations mentioned below (Analyses not shown). Removing the untutored group from the overall analyses did not affect significant differences between the other groups.

Results: Experiment 1

<u>Results of acoustic experiences X test song type X treatment</u>

Depending upon early experiences, under different treatment conditions, females shook ($F_{3, 39}$ = p = 0.044; corrected for sphericity), oriented ($F_{12, 156}$ = 2.694; p = 0.003) and emitted long calls ($F_{3, 39}$ = 3.399; p = 0.027; corrected for sphericity) at differential frequencies to the different song types. This indicated that all the three factors studied, interacted to influences females' relative behavioral responses to the three song types.

Under different endocrine conditions, females shook ($F_{1, 39}$ = 4.527; p = 0.040; corrected for sphericity) and emitted long calls ($F_{1, 39}$ = 4.723; p = 0.036; corrected for sphericity) at different frequencies to the different song types. This indicated that endocrine status significantly affected the frequencies of long calls and shakes to the different song stimuli.

Females with different acoustic experiences shook ($F_{12, 156} = 8.053$; $p < 0.001$) and emitted long calls ($F_{3, 39} = 5.079$; $p = 0.005$; corrected for sphericity) at different frequencies to the different song types. This indicated that different acoustic experiences had different effects on relative behavioral responses to the different song types.

Over all females responded differentially to the different song types. Females shook ($F_{2, 78} = 17.167$; $p < 0.001$), oriented ($F_{1, 39} = 36.048$; $p < 0.001$; corrected for sphericity) and emitted long calls ($F_{1, 39} = 14.323$; $p < 0.001$; corrected for sphericity) at significantly differently frequencies when stimulated with the three song types. Pair-wise comparisons (Bonferroni's) revealed that: Females shook more when stimulated with complex songs than when stimulated with prototypical songs ($p = 0.005$) or long-bout songs ($p < 0.001$); Females shook more when stimulated with prototypical songs than when stimulated with long-bout songs ($p = 0.0012$); Females oriented more when stimulated with complex songs than when stimulated with prototypical songs ($p = 0.023$) or long-bout songs ($p = 0.023$); Females emitted more long calls when stimulated with complex songs than when stimulated with prototypical songs ($p = 0.02$) or long-bout songs ($p < 0.001$);.

To further ascertain the effect of early experience and treatment on females' behavioral responses to the different song types, data were analyzed using a series of one-way ANOVAs. Through these one-way ANOVAs, we separately analyzed the behavioral responses of females with different early acoustic experiences to the different song types under the different treatment conditions.

Results of 1-way ANOVAs

Untutored females

Untreated females shook ($F_{1, 5} = 14.863$; $p = 0.012$; corrected for sphericity) and oriented ($F_{2, 10} = 13.409$; $p = 0.001$) at different frequencies to the different song types. The frequencies of long calls did not differ across song types in untreated females ($F_{1, 5} = 4.079$; $p = 0.082$; corrected for sphericity). Pair-wise comparisons (Bonferroni's) revealed that: Females shook more to prototypical songs than long-bout songs ($p = 0.009$); Females shook more to complex songs than long-bout songs ($p = 0.0023$); Females oriented more to complex songs than long-bout songs ($p = 0.011$). The difference between the mean frequency of shakes to complex songs and that to long-bout songs is greater than the difference between the mean frequency of shakes to prototypical songs and long-bout songs. Yet, the difference in the mean frequency of shakes to prototypical songs and long-bout songs is more statistically significant than the difference in the mean frequency of shakes to complex songs and prototypical songs. This results fromfrom differences in the responses of individual females in the repeated-measures design. Under E_2 treatment females did not respond differentially to the different song types. After ATD treatment, females shook differentially to the different song types ($F_{1, 5} = 113.761$; $p < 0.001$; corrected for sphericity). Pair-wise comparisons (Bonferroni's) revealed that females shook more to complex songs than long-bout songs ($p < 0.001$), and females shook more to prototypical songs than long-bout songs ($p < 0.001$). These results are summarized in Figure 2.

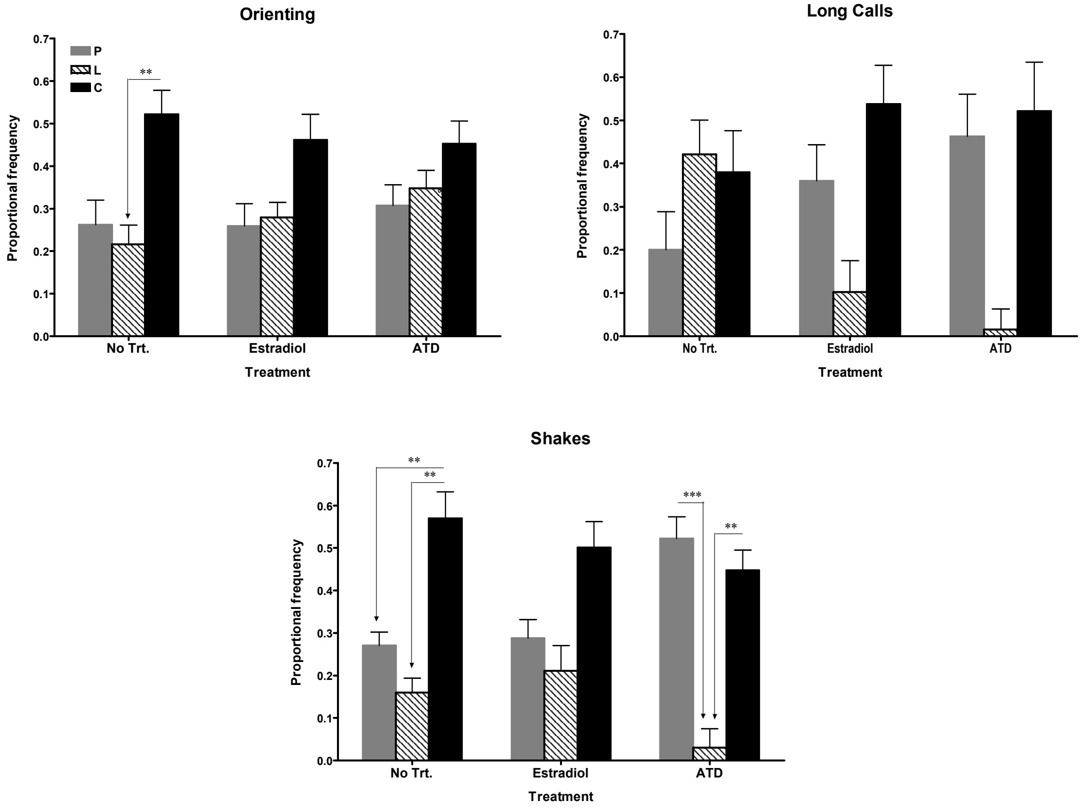

Figure 2: Proportional frequencies of orienting, long calls and shaking when untutored females were stimulated with the three different song types under the three hormone conditions. Values are plotted as means ± SEM. The bar pattern shows to which song type female was responding. ** = $p < 0.01$; *** = $p < 0.0001$

Females tutored with prototypical songs (P-tutored)

When untreated, females in this group oriented differentially to the different song types ($F_{2, 24} = 4.575$; $p = 0.021$), but pair-wise comparisons (Bonferroni's) did not reveal any significant differences in the frequency of orienting to the different song types. No significant effects were found following E_2 or ATD treatment. These results are summarized in Figure 3.

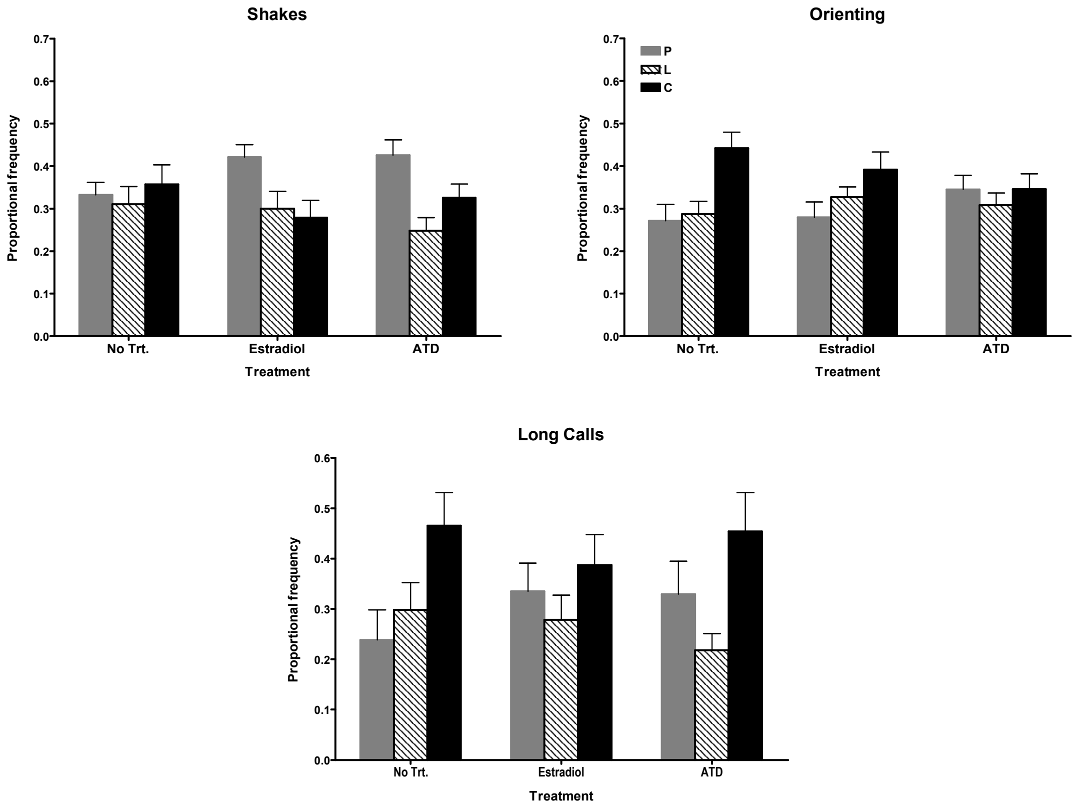

Figure 3: Proportional frequencies of orienting, long calls and shaking when females tutored with prototypical songs were stimulated with the three different song types under the three hormone conditions. Values are plotted as means ± SEM. The bar pattern shows to which song type female was responding.

Females tutored with long-bout songs (L-tutored)

When untreated, females in this group did not respond differentially to the different song types. Under E_2 treatment, females shook ($F_{2, 24} = 4.153$; $p < 0.04$) and oriented ($F_{2, 24} = 5.047$; $p < 0.018$) at different frequencies to the different song types. Pair-wise comparisons (Bonferroni's) revealed that: females shook more to complex songs than prototypical songs ($p = 0.025$). Though the overall ANOVA was significant, the post-hoc analysis did not reveal difference in orienting to the different song types -

females tended to orient at non-significantly higher frequencies to complex songs than long-bout songs ($p = 0.06$). After ATD treatment, females did not respond differentially to the different song types. These results are summarized in Figure 4.

Females tutored with complex songs (C-tutored)

Untreated females did not differentially respond to the different song types. Under E_2 treatment, females shook ($F_{1, 12} = 22.720$; $p < 0.001$; corrected for sphericity) oriented ($F_{1, 11} = 34.891$; $p < 0.001$; corrected for sphericity) and emitted long calls ($F_{1, 11} = 52.338$; $p < 0.001$; corrected for sphericity) at different frequencies to the different song types. Pair-wise comparisons (Bonferroni's) revealed that: Females shook more to complex songs than prototypical songs ($p = 0.001$) and long-bout songs ($p = 0.001$); Females shook more to long-bout songs than prototypical songs ($p = 0.04$); Females oriented more to complex songs than prototypical songs ($p < 0.001$) and long-bout songs ($p = 0.002$). Females oriented more to long-bout songs than prototypical songs ($p < 0.001$). Females emitted more long calls to complex songs than to prototypical songs ($p < 0.001$) and long-bout songs ($p < 0.001$). After ATD treatment, females oriented ($F_{2, 22} = 3.528$; $p = 0.047$) and emitted long calls ($F_{2, 22} = 7.460$; $p = 0.03$) at different frequencies to the different song types. Pair-wise comparisons with Bonferroni's adjustment revealed that: Females oriented more to complex songs than long-bout songs ($p = 0.019$). Females emitted more long calls to complex songs than prototypical songs ($p = 0.05$) and long-bout songs ($p = 0.02$). These results are summarized in Figure 5.

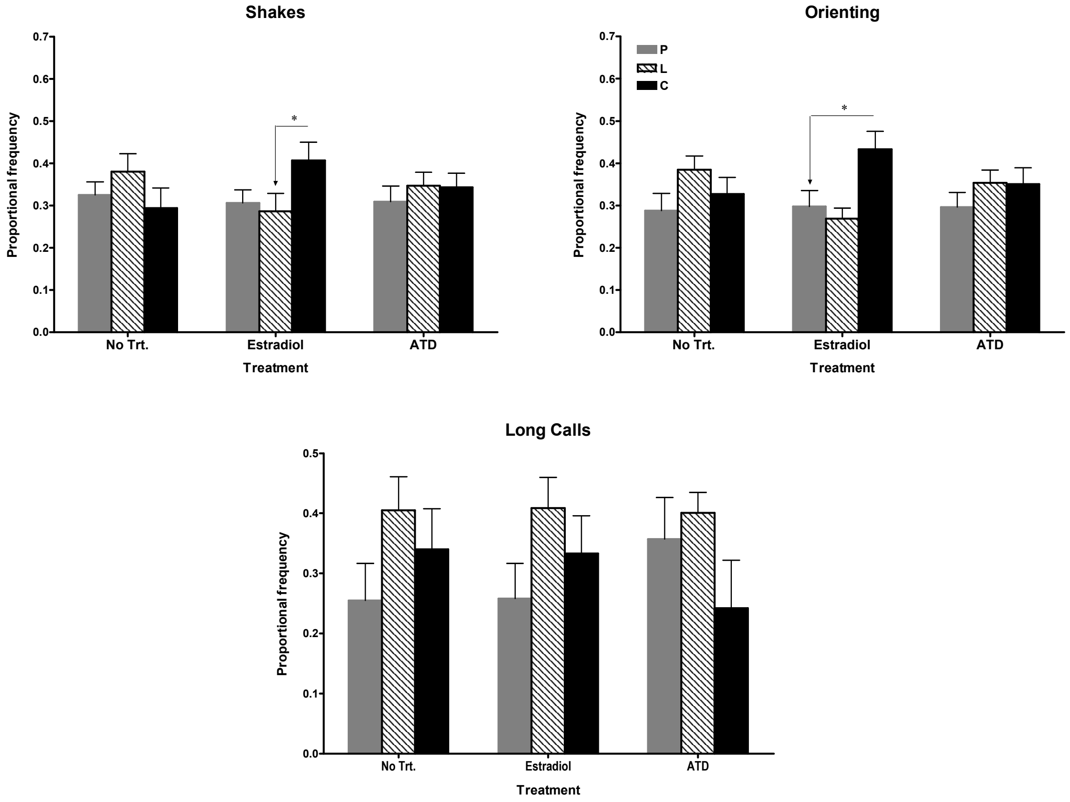

Figure 4: Proportional frequencies of orienting, long calls and shaking when females tutored with long-bout songs were stimulated with the three different song types under the three hormone conditions. Values are plotted as means ± SEM. The bar pattern shows to which song type female was responding. * = $p < 0.05$

The order of E_2 and ATD treatment did not affect females' behavioral responses

The order of E_2 and ATD treatments did not affect females' behavioral responses to the different song types Whether females received E_2 or ATD first or second did not affect the frequencies of shakes, ($F_{1,41} = 0.607$; $p = 0.441$), orienting ($F_{1,41} = 0.054$; $p = 0.817$) or long calls ($F_{1,41} = 0.536$; $p = 0.449$) in response to the different song types.

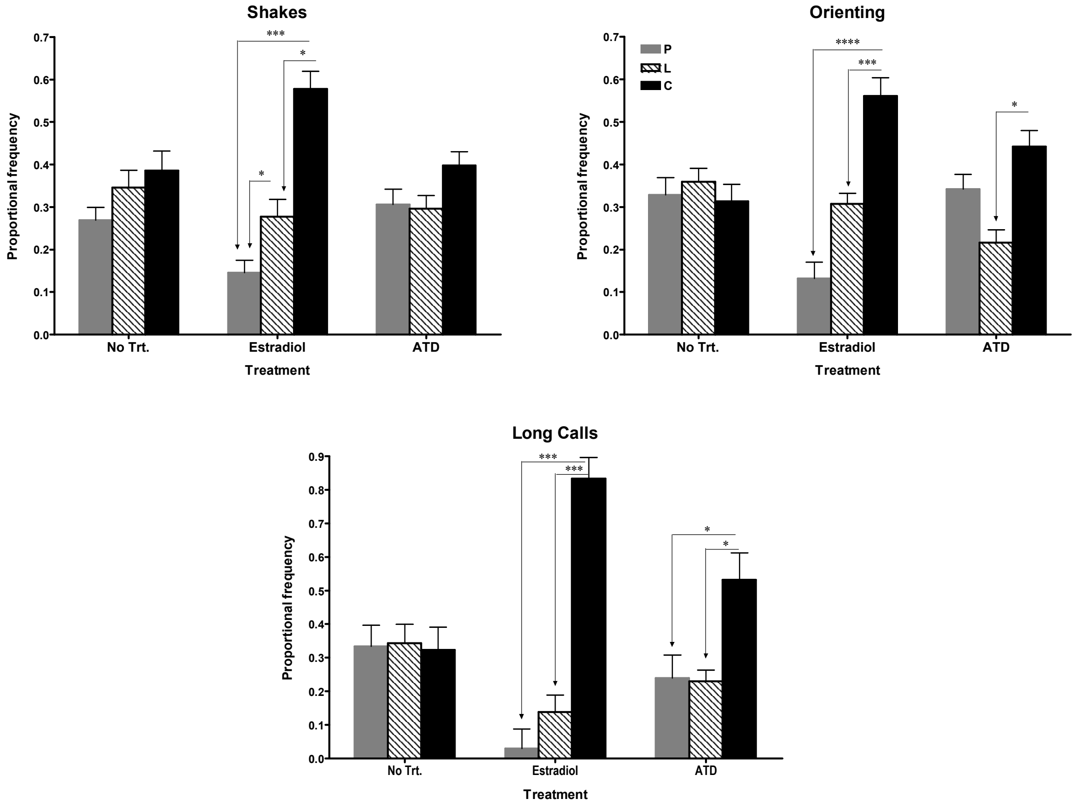

Figure 5: Proportional frequencies of orienting, long calls and shaking when females tutored with complex songs were stimulated with the three different song types under the three hormone conditions. Values are plotted as means ± SEM. The bar pattern shows to which song type female was responding. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Experiment 2: Materials and methods

This experiment was conducted to test the internal validity of the earlier experiment. In Experiment 1, we quantified the proportional frequencies of behavioral responses to the different song types. In Experiment 2, we wanted to see if the same behaviors were differentially exhibited by normally-raised, breeding females to live birds of different sexual salience. Every attempt was made to match the housing and behavioral testing conditions of the females in this experiment to those of females in the first experiment. Unfortunately, conclusions from this experiment were limited by the small number of birds available for testing.

Subjects

Females from six breeding pairs were used in this experiment. All breeding pairs had successfully produced at least one clutch of chicks. Breeding pairs were separated, and females were moved to the female and chicks-only room while males stayed in same-sex cages in a mixed-sex room. Females remained in isolation from males for two weeks before the start of behavioral testing. Each female was stimulated by three different conspecifics: by her own mate, by the mate of a different female, and by an adult female. To increase sexual motivation, stimulus males were implanted with androstenedione (Harding *et al.*, 1983) one week before the first behavioral test. Each female was tested under two treatment conditions, untreated and E_2-treated. Details of E_2 treatment were the same as in Experiment 1. All animal care and experimental procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of Hunter College.

Behavioral testing

Two cages were used – one on top of the other. Cages were stacked so that animals had visual and acoustical contact with each other (See Fig. 6). Between 5 – 7 days prior to the tests, each experimental animal was acclimated to the experimental set-up for two 4 hour periods. Each stimulus animal was acclimated for about 30 minutes immediately prior to the tests. At the beginning of the tests, the female was introduced into the lower cage. About ten minutes later, a stimulus animal was introduced into the upper cage for 5 minutes, and behaviors of the subjects were scored. The order of the presentation of the stimulus animals was counter-balanced across females. After the first set of behavioral tests, each subject was implanted with E_2 and tested again after 7 days of treatment.

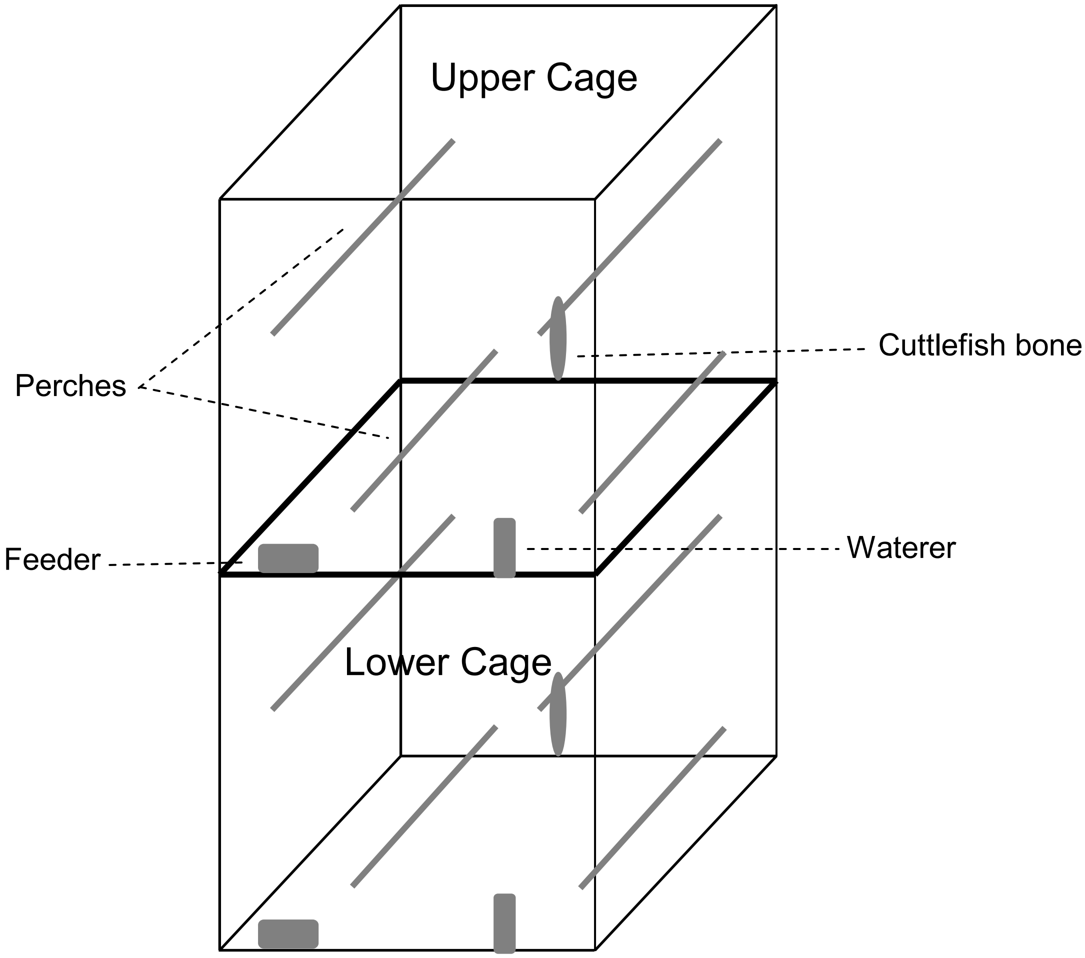

Figure 6: Schematic illustration of the experimental test cages used to quantify the behavioral responses of female zebra finches stimulated with live conspecifics. The left wall of the cage was opaque, and the other walls, the floor and the roof were made of 1 in. X 1 in. stainless steel mesh. The floor of the upper cage was left uncovered, so that the animals could remain in visual contact with each other. Experimental females were placed in the bottom cage and stimulus animals were placed in the upper cage. Experimental females and the stimulus animals were in visual and acoustic contact for the duration of the tests. The observer sat 7 feet in front of the cage.

Data analysis

A preliminary analysis of the data was performed, and only those behaviors that were exhibited at differential frequencies in response to the different stimuli were considered for further analysis. Three behaviors were differentially exhibited in response to the three different live stimuli – shaking, orienting, and CSDs. For each behavioral test, the relative frequency of behavior elicited by each stimulus compared to the frequency elicited by all three stimuli was calculated (e.g. frequency of behavior elicited by own mate /sum of frequency of behavior elicited by all three stimuli). It was a 3 X 2 repeated-measures design, with the type of stimulus animals and the hormone treatment conditions being the two independent variables. Data were analyzed using repeated-measures 2-way ANOVAs. P-values < 0.05 were considered statistically significant.

Results: Experiment 2

Females also tended to shake ($F_{2, 10} = 3.079$; p = 0.091) and exhibit CSDs ($F_{2, 10} = 2.861$; p = 0.104) at non-significantly different frequencies to the different live stimuli. Females oriented ($F_{2, 10} = 4.248$; p = 0.046) differently to the different live stimuli. E_2 treatment did not affect behavior. Multiple comparisons with no adjustments revealed that: Females shook at non-significantly greater frequencies when stimulated by their mates than when stimulated by females (p = 0.056); Females oriented at non-significantly greater frequencies when stimulated by their mates than when stimulated by other males (p = 0.052) or females (p = 0.080); Females exhibited CSDs at non-significantly greater frequencies when stimulated by their mates than when stimulated by females (p = 0.109). These results are summarized in Figure 7.

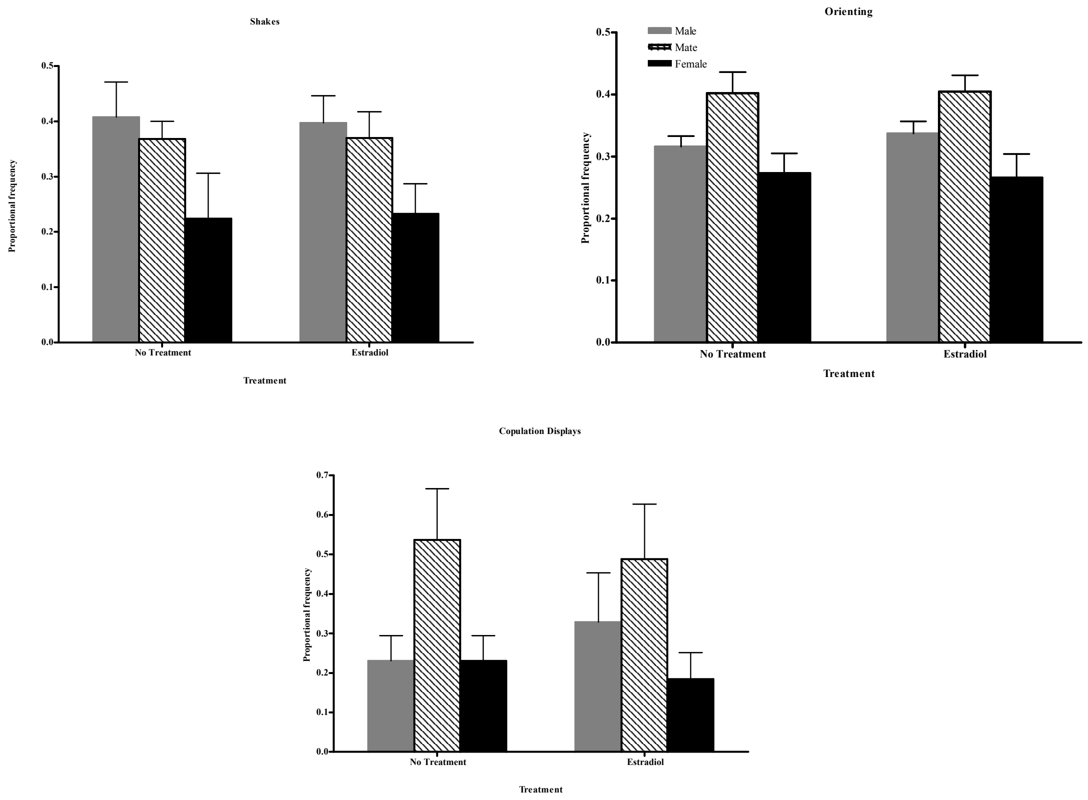

Figure 7: Proportional frequencies of shaking, orienting, and CSDs when females were stimulated with three different live stimuli, a female, a male or their mates, under two hormone conditions, untreated or treated with estradiol. Values are plotted as means + SEM. Bar patterns illustrate the type of stimulus with which the females were tested.

Discussion

To the best of our knowledge, the current study is the first to investigate the relative importance of early experiential and acoustical factors on song responsiveness in the same female songbirds. It is also the first study to investigate if estrogen influences how female zebra finches respond to different song types.

Different early experiences differentially influence female's responses to songs

We found that untutored females responded more to the complex songs, indicating that females are inherently predisposed to respond more to complex songs. Intra-group variability in the behavioral responses of the untutored females was very high. This may be attributable to their extreme acoustic isolation during early development and/or to the small sample size of 6. Although it is not obvious from the proportional frequencies, untutored females emitted long calls at much lower frequencies than other females, perhaps because of their extreme lack of early acoustic and/or social experience.

Females tutored with complex songs, and to a lesser extent, females tutored with long-bout songs responded more to complex songs. However, females tutored with prototypical songs did not respond differentially to the different song types. This indicates that early experience with complex songs strengthened females' behavioral responses to complex songs relative to the untutored females, and early experience with the other two song types appeared to weaken their behavioral responses to complex songs compared the untutored females.

Behavioral responses of these females cannot be attributed to their abnormal upbringing. In another study (Vyas, Harding, McGowan, Snare, & Bogdan, Submitted), we stimulated normally reared females with the same three song types, and found that those females also exhibited the same behavioral responses more to complex songs. Additionally, Experiment 2 of this study showed that normally reared females exhibited two of the same behaviors – shaking and orienting to sexually salient live stimuli.

Estrogen selectively accentuates females' behavioral responses to songs

Estrogen greatly influenced the relative behavioral responses of the tutored birds to the different song types. Under untreated conditions, none of the tutored females responded differentially to the different song types. E_2 treatment selectively accentuated females' behavioral responses to complex songs. Under E_2 treatment, C-tutored females responded more to complex songs followed by long-bout songs. Under E_2 treatment, L-tutored females also responded more to complex songs. ATD treatment greatly diminished differential responsiveness to the three song types by L-tutored females, but C-tutored females maintained, albeit at lower levels, their higher responses to complex songs.

The lack of estrogenic effects in untutored birds is intriguing. They responded more to complex songs under all treatment conditions. Their song responsiveness appeared less susceptible to hormone manipulations. When tested under the untreated condition, it was their first experience with songs and they might have been very excited regardless of the lack of hormones. While not significant, under ATD treatment, they did respond relatively less to complex songs, compared to the E_2 trials. One reason why the untutored females reacted differently might be that tutoring causes estrogen release, strengthening preferences for the tutored song. This preference then reappears when estrogen levels rise in adulthood. Untutored females don't show this effect, because their first exposure to song was during testing, and they were exposed to all three song types with their first exposure.

All the females in the current study were tested first in the untreated condition. Subsequently, females were sequentially tested after E_2 and ATD treatments. Half the females received E_2 first, half received ATD first. It is extremely unlikely that the results of the current study are attributable to the order of treatments: 1) Statistical analyses revealed that the order of E_2 and the ATD treatment did not significantly affect females' behavioral responses to the songs. 2) Relative frequencies of behaviors increased and/or decreased with the type of treatment, rather than the serial position of treatment. 3) In a different study (Vyas, Harding, McGowan *et al.*, Submitted), we sequentially tested normally-reared adult female zebra finches' responses to the same three song types under three treatment conditions – no treatment, E_2 treatment, and N- (2-chloroethyl)- N-ethyl-2-bromobenzylamine hydrochloride (DSP-4) treatment. DSP-4 is a noradrenergic neurotoxin which causes striking long-term damage to noradrenergic neurons. Thus, in a within-bird design, it had to be given last. We found that the relative frequencies of shaking, orienting and long calls to complex songs increased after E_2 treatment and decreased after DSP-4 treatment. If there had been order effects in these two studies, the relative frequencies of behavioral responses should have been more similar across tests.

Not all complex songs may be attractive to females

In Experiment 1, we found that females responded maximally to complex songs. Song complexity is often defined as variability in songs (for review see Nowicki & Searcy, 2005). It was suggested that males can develop variable songs by either copying variable songs from their fathers, or by copying from multiple tutors (for review see Williams, 2004). However, song variability can also result from abnormal juvenile acoustic environment (for review see Williams, 2004) and low circulating sex hormones (for review see Brenowitz, 2004). Juvenile male zebra finches raised in acoustic isolation from adult males (isolates) tend to develop songs that are not only variable, but also tend to have atypical note structure and organization (Williams, Kilander, & Sotanski, 1993). Their songs also have fewer distinct phrases and inconsistent frequency modulations (Eales, 1987; Price, 1979). Isolates were reported to have lower reproductive success than tutored males (Williams *et al.*, 1993), indicating that female zebra finches tend not to prefer this type of song variability. Complex songs used in the current study did not have the acoustic characteristics of those sung by isolated males. Male songbirds tend to sing more variable songs when sex hormone levels are low (for review see Brenowitz, 2004). However, in zebra finches, low hormone levels caused by either castration (Harding *et al.*, 1983) or ATD treatment (Walters & Harding, 1988) cause males to sing fewer female-directed songs. ATD treatment also resulted in songs sung at slower rates, with fewer songs in a bout (Walters & Harding, 1988). In other words, the few female-directed songs ATD-treated males sang had the characteristics of undirected songs (Walters & Harding, 1988). Evidence suggests that female zebra finches tend to prefer males singing complex, long-bout songs (Collins *et al.*, 1994). In the present study, complex songs also had longer bout lengths, and song rates of the three song types were comparable. Thus, it is likely that complex songs in our study were representatives of songs produced by males with normal upbringing and normal to high hormone levels.

Significance of behavioral responses to songs

In Experiment 1, during the silent phases between tests and those between songs, females typically sat in a corner on one of the upper perches. Females typically exhibited active behaviors only during the playback phase of tests. The latency and the frequency of behaviors varied dramatically depending upon the particular songs. For some songs, females sat almost silent and still. Whereas for other(s), as soon as playback of the song started, females first shook, then made a series of long calls and orienting responses. This differential responsiveness to the different songs became even clearer in the second trial – it seemed as if females sat ‘waiting’ for specific songs. During testing sessions, the observer (A.V.) could easily tell which songs were ‘boring’ or ‘exciting’ for the females. It became clear to us that females were differentially aroused by the different songs. The ‘meaning’ of these behaviors is poorly understood. Our interpretation is that females shook, called, and oriented when songs caught their attention. We found that females shook, called, and oriented differentially to the different song types, indicating that different songs recruited attentional processes differentially. It is well known that attention is differentially recruited by stimuli of different subjective saliencies, and animals tend to preferentially attend to stimuli that are subjectively important to them. This suggests that different songs were differentially important to the females.

Zann (1996) reported that zebra finches emit long calls to communicate with conspecifics they cannot see. Females emitted long calls at high frequencies during the trials, indicating that females could ascertain that songs were not coming from the male model. This was perhaps because, despite females’ efforts, the model did not ‘interact’ with them. Different frequencies of long calls indicates differential efforts by females to communicate with the ‘males’ singing different songs. Nagle *et* al. (2002) stimulated adult female canaries with four different songs – hetrospecific male songs, conspecific songs of males from the same strain, conspecific songs of males from a different canary strain, and conspecific songs of males from the same strain containing so-called ‘sexy syllables’ (Leitner & Catchpole, 2002). They (Nagle *et al.*, 2002) reported that females called differentially in response to the different songs. Females called most in response to songs containing the ‘sexy syllables’. The authors suggested that call responses of female canaries like CSDs indicate females’ song preferences.

In Experiment 2, we stimulated females with three live stimuli of putatively different sexual salience. Female zebra finches convey their readiness to copulate by assuming a characteristic posture – the CSD. We found that females exhibited CSDs at different frequencies to the different live stimuli, confirming the different sexual salience of the stimuli. We also found that stimuli that elicited more CSDs also elicited more shaking and orienting responses. However, none of the behaviors were exhibited at statistically significantly differential frequencies, and the differences in responses to the mate and unfamiliar male were very small. Two factors might account for the weakness of these data: 1) Breeders were initially force-paired with their respective mates and were not given the choice of selecting a subjectively-attractive member of the opposite sex. Nevertheless, paired birds did have a history of successful copulation with each other, as evidenced by their success in producing chicks. 2) Because we were in the process of closing down our breeding colony, we had a relatively small sample size of 6 animals.

Results from Experiment 1 indicate that females differentially attended to the different songs, suggesting that different songs might have different importance for the females. Female songbirds use male songs as one of the main criteria for mate-selection (Searcy & Yasukawa, 1996; Catchpole, 1987). This suggests to us that differential responsiveness to the different songs indicates differential song preferences. Female zebra finches do not readily exhibit CSDs when stimulated with songs alone. Studies therefore have used other behavioral paradigms like operant responses (Clayton, 1988b; Riebel, 2000) or the speaker approach paradigm (Clayton & Pröve, 1989; Forstmeier, 2004; Miller, 1979; Neubauer, 1999) to measure females' song preferences. Data from Experiment 2 indicate that live stimuli that elicited more CSDs also tended to elicit more shaking and orienting responses. This further suggests that differential frequencies of behaviors in Experiment 1 indicates differential song preferences.

Acoustic nature of songs is important for female mate-choice

Female songbirds use male songs as one of the main criteria for mate-selection (Searcy & Yasukawa, 1996; Catchpole, 1987). Properties of male songs tend to indicate a male's quality as a potential mate (e.g. Andersson, 1994; Gibson & Langen, 1996; Jennions & Petrie, 1997). For example, Garamszegi *et al.* (2003) and Moller *et al.* (2000) conducted comparative studies and reported that song complexity was correlated with male immunocompetence. Nowicki *et* al (1998) proposed the "nutritional stress hypothesis" and Nowicki and Searcy (2004) and Buchanan *et al.* (2003) proposed the "developmental stress hypothesis". According to these hypotheses, the acoustic quality of songs is an indicator of the developmental stress experienced by males. A direct prediction of these hypotheses is that juvenile male songbirds exposed to nutritional or other forms of stress should not be able to learn and/or sing certain song types. Over the years, a robust body of empirical evidence has accumulated that suggests that developmentally-stressed males are less likely to produce complex and/or long-bout songs (for review see Nowicki & Searcy, 2005). This suggests that complex and long-bout songs are 'superior' songs, and female songbirds should be biased to prefer these songs over shorter, simpler songs. Our results support the above hypotheses – females in our study responded most strongly to complex songs over the other song types.

Both, early experiences and acoustic nature of songs may influence song preferences

Earlier studies suggested that song preferences in female zebra finches are influenced by two factors – the acoustic nature of songs (Clayton & Pröve, 1989; Collins *et al.*, 1994; Forstmeier, 2004; Forstmeier & Birkhead, 2004; Neubauer, 1999) and early experience (Clayton, 1988b; Miller, 1979; Riebel, 2000). We found that that females seem to be inherently biased to prefer complex songs over other song types – as exemplified by the untutored females' greater responsiveness to complex songs. However, our data also indicate that early experience can enhance or interfere with the inherent preference – Early experience with complex songs, enhanced females' responsiveness for this song type, while early experience with long-bout or prototypical songs weakened the responsiveness to complex songs. This suggests that early exposure to 'inferior' songs interferes with females' ability to either discriminate between or prefer 'superior' songs. Similar findings were reported by Lauay *et al.* (2004). They suggested that "female's preference for songs with more notes and spectral complexity is learned during early exposure to adult males that produce such songs." However, our study indicates that preferences for certain song types are not learned per se, rather early experience with only 'inferior' songs interferes with females' ability to prefer certain 'superior' song types. Additionally, it seems that early experience with superior songs also enables females to differentially 'rank' songs – as exemplified by the C-tutored females' greatest responsiveness to complex songs and their greater responsiveness to long-bout songs compared to prototypical songs.

It is possible that song preferences in female zebra finches might be susceptible to experience-dependent plasticity even during adulthood, and that the female will switch their preferences for 'superior' songs if they are repeatedly exposed to them. It has been reported that in female mountain white-crowned sparrows (*Zonotrichia leucophrys oriantha*, MacDougall-Shackleton, MacDougall-Shackleton, & Hahn, 2001) and female cowbirds (King, West, & White, 2003) song preferences are influenced by auditory experiences during adulthood. In our study exposure to songs was limited to testing sessions, therefore, more research needs to be done to answer that question.

Estrogen may increase mate selectivity in female zebra finches

The effects of estrogen manipulation were very interesting. Untreated tutored females did not respond differentially to any song types. E_2 treatment elicited differential responses to the different song types. Little research has been conducted on the effect of sex hormones on mate selectivity in songbirds. Female zebra finches typically form pair-bonds very early in life (Zann, 1996). Their estrogen levels at this point are not known, and the effects of estrogen on pairing decisions are not well understood. Perhaps the best support for estrogenic influences on mating-decisions in female songbirds comes from data on extra-pair copulation (EPC). Many species of songbirds form socially stable pair-bonds. However, it has been reported females in these apparently stable pair-bonds indulge in EPCs. Data from female songbirds indicate that females indulge in EPCs only with males that appear to be more attractive than their mates. For example, zebra finches were reported to indulge in EPCs with males that wore more attractive leg bands (Burley, Enstrom, & Chitwood, 1994), and female sedge warblers (*Acrocephalus schoenobaenus*, Marshall, Buchanan, & Catchpole, 2007), female black-capped chickadees (*Poecile atricapilla*, Mennill, Ratcliffe, & Boag, 2002; Otter, Ratcliffe, Michaud, & Boag, 1998), female dusky warblers (*Phylloscopus fuscatus*, Forstmeier, Kempenaers, Meyer, & Leisler, 2002) chose males for EPCs based upon males' superior songs. In songbirds, most EPCs occur when females are fertile (Birkhead & Moller, 1992), and in songbirds female fertility is dependent upon high estrogen levels (Silver, 1989). These data suggest that females are especially tuned to the quality of potential mates when their estrogen levels are high.

We found that E_2 treatment selectively accentuated the behavioral responses of females, indicating that estrogen increases song selectivity in female zebra finches. We therefore believe that *sexual* preferences of female zebra finches can only be ascertained when the circulating estrogen levels are optimal. Song preferences ascertained with low estrogen levels might not indicate *sexual* preferences for the songs, but might indicate certain other kinds of preferences. For instance, studies in which female zebra finches were reported to prefer tutored songs over the untutored ones (Clayton, 1988b; Miller, 1979; Riebel, 2000) might simply indicate preferences for familiarity over novelty - perhaps a tendency to associate with relatives or flock-mates.

Conclusions

Based upon the results of our study, three conclusions can be drawn about the relative influences of early experience, acoustic factors and estrogen on song preferences in female zebra finches. First, inexperienced female zebra finches are inherently biased to respond to complex songs over other song types. Second, song responsiveness in female zebra finches is strongly affected by early acoustic experience. Depending on the type of stimuli to which females are exposed, acoustic experience may either reinforce or weaken the initial responsiveness to complex songs. Third, estrogen greatly influences and selectively accentuates song responsiveness in female zebra finches.

Study 2: Noradrenergic Mediation of Estrogenic Influences on Song Preferences in Female Zebra Finches

Introduction

Female songbirds use male songs as an important criterion for mate-selection (e.g. Catchpole, 1987; Kroodsma & Byers, 1991; Searcy & Andersson, 1986; Tomaszycki & Adkins-Regan, 2005). Properties of male songs are thought to indicate the male's quality as a potential mate (e.g. Andersson, 1994; Gibson & Langen, 1996; Jennions & Petrie, 1997). Song processing and mate-choice in female songbirds have been proposed as an excellent model for investigating the processing of complex acoustic communication signals (Gentner & Margoliash, 2002; Theunissen & Shaevitz, 2006). Many studies have investigated and increased our understanding of various developmental, experiential, acoustical, and physiological factors that influence song preferences in female songbirds (for review see Gentner & Margoliash, 2002; Nowicki & Searcy, 2004; Theunissen & Shaevitz, 2006). However, certain important gaps remain – for example the extent to which estrogen influences song preferences and the mechanisms through which it acts are not well understood.

As in other female vertebrates, estrogens play an important role in activating female sexual behavior in songbirds (Silver, 1989). During normal reproductive cycles, exposure to singing males stimulates estrogen secretion, inducing ovulation and readiness to copulate (Catchpole, 1987; Kroodsma, 1976). Song quality affects the rapidity of reproductive development (Kroodsma, 1976). Studies of mate choice have turned this paradigm around and administered estrogen to female songbirds to make it easier to determine their song preferences. In this commonly-used paradigm, females are treated with estrogen, and researchers determine which songs elicit species-specific copulation solicitation displays (CSDs). One obvious question, which had not been investigated, is whether estrogen treatment alters female preferences. Recently, we conducted a study (Vyas, Harding, Borg, & Bogdan, Submitted) investigating the preferences of female zebra finches for three acoustically different song types. To ascertain the effects of estrogen, female preferences for the three song types were tested under three hormone conditions – untreated-, estradiol (E_2)-treated and 1-4-6-androstatrien-3,17-dione (ATD)-treated (an aromatization inhibitor used to deplete estrogen levels). We found that when treated with E_2, females preferred complex songs followed by long-bout songs. Untreated females did not exhibit any song preferences, and ATD treatment reduced or eliminated song preferences.

Our study clearly showed that estrogen influenced song preferences in female zebra finches. In mammals, many estrogenic effects on female sexual behaviors have been reported to be mediated by norepinephrine (NE) (for reviews see Etgen *et al.*, 2001; Etgen *et al.*, 1999; Fabre-Nys, 1998). NE also appears to play an important role in mediating estrogenic effects on sexual behavior in songbirds. In zebra finches, NE appears to mediate estrogenic activation of courtship singing in males (for review see Harding, 2004a). Estrogen treatment increased NE levels and turnover in brain nuclei that activate singing and sexual behavior in reproductively-active male zebra finches (for review see Harding, 2004a). Additionally, NE depletion through DSP-4 treatment decreased the frequency of song-induced copulation solicitation displays (CSD) in female canaries (Appeltants *et al.*, 2002) and courtship singing to females in male zebra finches (Barclay *et al.*, 1992, 1996).

The current study investigated if estrogenic influences on song preferences in female zebra finches are mediated by NE. The preferences of adult females for the three different song types were tested under no treatment, under E_2 treatment, and under E_2 treatment following DSP-4 treatment. We found that estrogenic effects on song preferences in female zebra finches appear to be mediated by NE.

Methods

Subjects

The subjects were 12 adult female zebra finches obtained from Canary Bird Farm (Old Bridge, New Jersey). Birds were housed in a large isosexual aviary in a mixed-sex room until needed. All animal rooms were temperature controlled (24 ± 2°C) with a 14:10 light: dark cycle. The relative humidity was maintained over 50% to keep birds in breeding condition. Birds were fed a vitamin-supplemented commercial finch seed mix, grit, and water *ad libitum*, supplemented with fresh greens and oranges. In addition, experimental birds received hardboiled eggs from their first neurotoxin treatment until the end of the experiment. Birds were moved from the aviary to a room containing only females and chicks under a month old at least 1 month prior to testing, and housed in groups of four in cages (56 x 56 x 56 cm) isolating them from the vocalizations of adult males until the experiment concluded. All animal care and experimental procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of Hunter College.

Songs

We followed Sossinka and Bohner's (1980) and Price's (1979) descriptions of typical zebra finch courtship songs. Typically, each song begins with *introductory notes*, followed by repeated sequences of a relatively rigid succession of *elements* or *syllables*, called *motifs* or *song units*. A song bout is defined as a sequence of song units, preceded by introductory notes. Normally, in songs directed at females, each bout has approximately 5.4 song units, preceded by an average of 6.3 introductory notes (Sossinka & Bohner, 1980).

All songs were recorded from males in our colony. Courtship songs were recorded from male zebra finches placed inside sound-attenuated chambers using high fidelity microphones (Earthworks® SRO) and Sound Analysis Pro© software (http://ofer.sci.ccny.cuny.edu/html/sound_analysis.html) (Tchernichovski & Mitra, 2004) on Dell Optiplex® Pentium-4® computers. Based upon the acoustic characteristics, songs were divided into three categories:

Prototypical songs (P): Prototypical songs met the following criteria: 1) 6 ± 2 introductory notes. 2) 4-5 song units/bout. 3) Average inter-song-unit similarity was > 90% (as measured by Sound Analysis Pro© software. 5) Each song unit had all note types (clicks, highs, stacks, downsweeps). Example: AAAAA BCDE BCDE BCDE BCDE (‘A’ depicts the introductory note and other letters depict a syllable each).

Long-bout songs (L): Long-bout songs were the same as prototypical songs in all regards except that the number of syllables in a song unit was greater and the number of song units in a bout was > 7. We had to add some song units to the songs produced by males in our colony to create these songs. Example: AAAAA ABCDEFG BCDEFG BCDEFG BCDEFG BCDEFG BCDEFG BCDEFG BCDEFG.

Complex songs (C): Song complexity is usually defined by the number of song types in the repertoire of males or by the variability in the types of syllables males sing (Nowicki *et al.*, 2002). In the present study, complex songs had both a larger number of unique syllables and also had acoustically different song units. For complex songs, the inter song-unit-similarity was < 77%, and their bout lengths were similar to those of long-bout songs. We had to add some song units to the bouts produced by males in our colony to create these songs. Example: AAAAAA ABCDEF GHCDEF BCDEF ICDEF BCDEF GHCDEF BCDEF ICDEF.

Songs were filtered, edited and amplified using GoldWave® (Version 5.14. www.goldwave.com) software. Filtering was performed to remove any background noise. Additional editing was performed to match inter-syllable intervals and inter-song unit intervals of different songs. In some cases, additional introductory notes were added. Following editing, songs were transferred to magnetic tape cassettes (TDK Audio Cassette, Standard Size, Normal Bias, 60 Minutes). Nine songs, 3 of each type, were obtained in this manner. Songs were classified into three groups based on song similarity, with each group having one song of each type. Songs within each group were about 50% similar to each other. Females were assigned to be tested with a specific song group using a randomized block design and tested with the songs in that group only.

Behavioral testing

One day prior to each behavioral test, each female was housed singly in a standard cage. Following this acclimation, the female was removed and her cage was moved to a behavioral testing room and fitted with a speaker on top of the cage and a male model on a perch positioned directly under the speaker (See Fig. 1). The female was subsequently released back into her cage and allowed to acclimate to the testing room for 30 minutes. The behavioral testing room had a generator playing white-noise. A flowing water sound was also continuously played. These sounds were played to minimize interference from extraneous background noises from outside the room. The sound pressure level of each was 60 dB in the center of the cage. Each female was tested with the three songs in her song group. Each song group had one prototypical song, one long-bout song and one complex song. Songs were played-back at the average sound pressure level of 72dB in the center of the cage, with a range of 67-74 db for each song. Three Marantz® cassette players (model 201) were used to present the songs.

During testing, each song was played back for 5 minutes, followed by 2 minutes of silence, followed by the playback of the next song type. Each 5 minute block of playback was divided into five 1-minute intervals. Each 1-minute interval had one song bout, plus silence. Each testing session had two trials, so that during each testing session the females were exposed to playback of each song twice for 5 minutes each. The order of song playbacks during trials and across birds was counterbalanced. During the playback and the silent periods, the females' behaviors (including shaking, orienting towards the speaker, long-calls, beak-wipes etc.) were scored by an observer sitting in front of the testing cage. After the first test, females were given E_2 implants and tested again seven days later. After the second test, females were treated twice with DSP-4 to deplete NE. Seven days after the second DSP-4 treatment, females were tested a third time. Between tests, females were returned to a room where they were maintained in isolation from adult male vocalizations.

Hormone and drug treatments

After the first behavioral test, each female received a subcutaneous silastic implant (0.76 mm i.d., 1.65 mm o.d.; Fisher Scientific, Houston, Texas) containing 2 mm packed E_2 following Walters & Harding's (1988) methodology. In an earlier study (Vyas, Harding, Borg *et al.*, Submitted), we found that the same dose of E_2 increased the frequency of copulation displays to live males in female zebra finches by more than 300% over the untreated levels. After about 7 days of hormone treatment, birds were again tested for their responses to the songs. After the second behavioral test, the birds were treated with the noradrenergic neurotoxin, DSP-4 following Waterman and Harding's (Submitted) methodology. Noradrenergic and serotonergic axon terminals tend to reuptake DSP-4 (Jonsson, 1980). The drug is subsequently retrogradely transported back to the cell body, killing the neuron (Jonsson, 1980). To protect serotonergic neurons, DSP-4 injections were preceded by injections of zimelidine dihydrochloride (Jonsson, Hallman, & Sundstrom, 1982). Zimelidine dihydrochloride (20 μg/0.01 ml saline/gm body weight) and DSP-4 (50 μg/0.01 ml saline/gm body weight) (generous gifts of Trevor Archer, Astra Pharmaceuticals, Ltd) were injected intraperitonially on alternate sides, superio-laterally to the cloacal opening. Drugs were put into solution immediately prior to injections. Administration of DSP-4 occurred within 10 minutes of drug preparation to prevent the formation of the non-toxic aziridinium derivative (Lookingland, Chapin, McKay, & Moore, 1986). In order to maximize NE depletion, a second series of injections was administered 10 - 12 days after the initial treatment. About seven days after the second round of injections, females' song preferences were tested.

Data analysis

During each behavioral test, the frequencies of several behaviors exhibited during playbacks were scored. A preliminary analysis was done to identify behaviors that were differentially exhibited to the different song types. There were several behaviors (like short calls, perch-changes, etc.) that were exhibited at very high frequencies by most birds under all treatment and stimulus conditions. These behaviors were omitted from further analysis. Other behaviors (like copulation solicitation displays) were exhibited by very few birds. These were also omitted from further analysis. Three behaviors, shaking, orientating towards the speaker and long calls were exhibited at high differential frequencies to the different songs. Shakes are high frequency, side-to-side movements of the upper body and are immediately preceded by fluffing. Looking up towards the speaker was defined as orienting. Long calls are loud distance calls that zebra finches produce when they are visually isolated from other birds and are an attempt to get back in contact (Zann, 1996). For each behavioral test, the frequency of each of these three behaviors in response to each song type for the two trials were summed. The proportional frequency of each behavior to each song type compared to the frequency across all tests to all three song types was calculated. To avoid division of/by zero, if any behavior was not exhibited during a trial, a frequency of 0.1 was assigned for that behavior. We used proportional frequencies rather than absolute frequencies for two reasons: 1) We wanted to compare females' relative responses to the different song types. 2) Using proportional responses helped to reduce the effects of intra-group variability. Two females died after the DSP-4 injections, therefore the reported data are for 10 females. The proportional frequencies of behaviors were analyzed for the effects of song type and treatment using repeated measures 2-way ANOVAs (song type X treatment) followed by planned comparisons. To further examine the effects of song types and treatment, the proportional frequencies of behaviors under each treatment condition were analyzed using a series of

1-way ANOVAs, followed by multiple comparisons. P-values < 0.05 were considered significant.

In some of the statistical analyses reported below, the assumptions of *sphericity* were violated. Sphericity is an assumption about the nature of covariance in a repeated measures design. Sphericity is to repeated-measures designs, what homogeneity of variance is to between-subjects designs. If sphericity is violated, several corrections can be made, each of which results in the loss of degrees of freedom, with a concomitant lowering of the confidence level. We used lower-bound epsilon correction, one of the most conservative estimates of confidence levels, if sphericity was violated.

Results

Females shook differentially ($F_{2, 18} = 9.436$; $p = 0.002$), oriented differentially ($F_{2, 18} = 7.468$; $p = 0.004$) and emitted long calls differentially ($F_{2, 18} = 3.758$; $p = 0.05$) to the three song types. Pair-wise comparisons, with Bonferroni's adjustments revealed that: Females shook more when stimulated with complex songs than when stimulated with prototypical songs ($p = 0.014$); Females oriented more when stimulated with complex songs than when stimulated with prototypical songs ($p = 0.016$). Despite the significant ANOVA result, post-hoc comparisons did not reveal any significant differences in long calls in response to the different song types.

Effect of song types and treatment on behavioral responses

When untreated, females did not respond differentially to the different song types. Under E_2 treatment, females shook differentially ($F_{1, 9} = 7.545$; $p = 0.023$; corrected for sphericity), oriented differentially ($F_{2, 18} = 12.048$; $p < 0.001$) and emitted long calls differentially ($F_{2, 18} = 6.063$; $p = 0.01$) to the three song types. Pair-wise comparisons with Bonferroni's adjustment revealed that under E_2 treatment: Females shook more to complex songs than prototypical songs ($p = 0.041$); Females oriented more to complex songs than prototypical songs ($p = 0.008$) and long-bout songs ($p = 0.018$); Females emitted more long calls to complex songs than to long-bout songs ($p = 0.028$). Following DSP-4 treatment, E_2 treated females did not behave differentially to the three song types. These results are summarized in Figure 8.

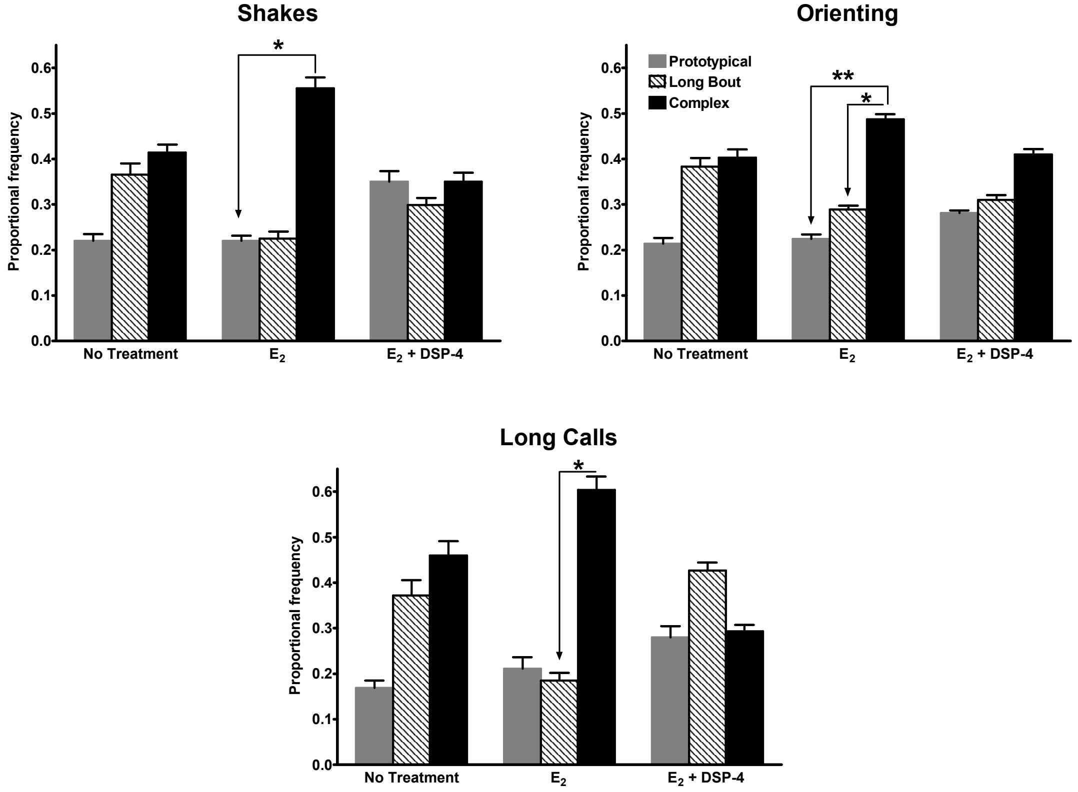

Figure 8: Proportional frequencies of shaking, orienting and long calls when females were stimulated with the three song types under the three treatment conditions, untreated, estradiol-treated (E_2) and E_2 + DSP-4-treated. Values are plotted as means + SEM. * = $p < 0.05$. ** = $p < 0.005$.

Discussion

The current study investigated if estrogenic effects on song preferences in female zebra finches are mediated by NE. This study replicated our earlier findings that E_2 is a critical factor motivating females to show strong song preferences. As predicted, NE appears to play a critical role in mediating the song preferences activated by E_2.

Female zebra finches convey their readiness to copulate by assuming a characteristic posture commonly called the copulation solicitation display (CSD). Perhaps the most convincing measure of sexual preference is a differential frequency of CSDs by females in response to different stimuli. However, female zebra finches do not readily exhibit CSDs when stimulated with songs alone. Studies therefore have used other behavioral paradigms like operant responses (Clayton, 1988b; Riebel, 2000) or the speaker approach paradigm (Clayton & Pröve, 1989; Forstmeier, 2004; Miller, 1979; Neubauer, 1999) to measure females' song preferences. In an earlier study (Vyas, Harding, Borg *et al.*, Submitted), we found that shaking, orienting and long calls are good indicators of song preferences in female zebra finches.

Our results support those of earlier studies (Clayton & Pröve, 1989; Vyas, Harding, Borg *et al.*, Submitted) demonstrating that female zebra finches prefer complex songs over other song types. Female songbirds use male songs as one of the main criteria for mate-selection (Catchpole, 1987). Researchers have argued that properties of male songs tend to indicate a male's quality as a potential mate (e.g. Andersson, 1994; Gibson & Langen, 1996; Jennions & Petrie, 1997). For example, Garamszegi *et al.* (2003) and Moller *et al.* (2000) conducted comparative studies on songbirds and reported that song complexity is correlated with male immunocompetence. Nowicki *et* al. (1998) proposed the "nutritional stress hypothesis" and Nowicki and Searcy (2004) and Buchanan *et al.* (2003) proposed the "developmental stress hypothesis". One prediction of these hypotheses is that complex and long-bout songs are 'superior' songs because they are more difficult to learn and produce, and female songbirds should prefer these songs over shorter, simpler songs (for review see Nowicki & Searcy, 2005). Our results support these hypotheses –females clearly preferred complex songs over the other song types. The results of the present study also confirm results from our earlier study (Vyas, Harding, Borg *et al.*, Submitted), in which it was found that estrogen reveals and selectively accentuates song preferences in female zebra finches.

In the present study, although all the birds received drugs in the same order, changes in differential responses are unlikely to be due to repeated testing. In our earlier study (Vyas, Harding, Borg *et al.*, Submitted), females were first tested without treatment, then were sequentially treated with E_2 and an aromatase inhibitor, ATD. Half the females received E_2 first, while the others received ATD first. Following each treatment, females' song preferences were tested using the same songs and the same testing paradigm used in the current study. As in the current experiment, females' preferences were tested 3 times, once after each treatment. Relative frequencies of behavioral responses were found to be dependent on the treatment, not on the treatment order.

It is important to point out that decreases in relative behavioral responses following DSP-4 treatment cannot be attributed to generalized deficits in motor activity or malaise. Following several days of recovery after the DSP-4 treatments, females appeared to behave normally. The DSP-4-treated females ate and drank as well as the untreated birds. We did not directly measure general activity, but we did not observe any differences in the time or effort it took to catch DSP-4 treated females. Other studies have also reported that DSP-4 treatment in songbirds does not cause any obvious long-term feeding or motor deficits (Appeltants *et al.*, 2002; Barclay *et al.*, 1992; Riters & Pawlisch, 2007).

The survival and reproductive success of animals depend upon their ability to discriminate between and selectively attend to salient stimuli and behave in adaptive ways. Stimulus salience depends upon a combination of dynamic internal and external factors. NE is one of the primary neurotransmitters involved in the selection and processing of sensory signals (for review see Berridge & Waterhouse, 2003; Hurley, Devilbiss, & Waterhouse, 2004). Noradrenergic projections from the LoC have been shown to influence selective attention in many ways, including influencing arousal and increasing signal-to-noise ratio (for review see Hurley *et al.*, 2004). Noradrenergic projections from the LoC were shown to be involved in responding to salient information in awake and behaving cats (Rasmussen & Jacobs, 1986; Rasmussen, Morilak, & Jacobs, 1986), rats and monkeys (Aston-Jones, Chiang, & Alexinsky, 1991) etc. Since DSP-4 treatment preferentially disrupts projections from the LoC and ultimately can destroy noradrenergic cells in the LoC (e.g. Jonsson, 1980; Kitt & Brauth, 1986), one would expect DSP-4 treatment to adversely affect discrimination and attention to salient stimuli. For the most part, behavioral studies have supported this expectation.

Few studies have investigated the effects of DSP-4 treatment on behavioral responses in songbirds. Additionally, every laboratory using DSP-4 tends to have its own administration protocol. Differences in administration methodology as well as species differences can lead to differences in the extent of NE depletion and the particular areas affected (Waterman & Harding, Submitted). Appeltants et al (2002) superimposed varying intensities of either non-stimulating songs (NS) or white noise (WN) on sexually-stimulating songs. NS and WN were considered to be distracters. They tested the preferences of DSP-4-treated and control females for the two kinds of auditory stimuli and reported that the greater the intensity of either of the distracters, the greater the decrease in the number and intensity of CSDs. They further reported that the decrease in

CSDs was much greater in the DSP-4-treated than the control females. They also found that the decrease in CSDs was much greater when NS were superimposed over stimulating songs than when WN was superimposed over the songs. They attributed the greater decrease in the number and intensity of CSDs in the DSP-4-treated birds to attentional deficits caused by NE depletion. These results suggest that the presence of white-noise and water sounds in our study might have contributed to the large decrease in relative responses to the different songs in DSP-4-treated birds. NE also appears to focus the attention of androgen-treated males on females. Barclay et al (1992; 1996) reported DSP-4 treatment decreased female-induced singing and sexual behavior in male zebra finches. The decrease in female-directed behavior appeared to be caused by neurotoxin-induced attentional deficits. Once DSP-4-treated males began interacting with females, their behavior appeared fairly normal. They just had significantly longer latencies before they started to court.

While the above studies reported DSP-4 induced decreases in responses to sexual stimuli, one recent study (Riters & Pawlisch, 2007) tested responses of female starlings treated with E_2 and DSP-4 and reached the opposite conclusion. They reported that E_2 + DSP-4-treated female starlings approached nest-boxes playing male courtship songs at greater frequencies than control females. They concluded that NE played an inhibitory role in appetitive responses to male courtship songs. There are a few probable reasons for the discrepancy in results between this study and the others: First, the effects of DSP-4 vary across species (See discussion in Waterman & Harding, Submitted). Second, we injected DSP-4 twice, while Riters and Pawlish (2007) injected the same dose of DSP-4 once. Barclay *et al.* (1992) reported that a single systemic dose of DSP-4 failed to significantly deplete central NE levels in finches as it does in rats. NE levels in zebra finch brains are about ten times higher than those found in comparable brain areas in rats (Barclay & Harding, 1988). This may account for the inability of a single treatment with the standard rat dosage to significantly deplete telencephalic NE levels. Third, Riters and Pawlish (2007) did not pretreat the females with zimelidine. Depending upon species and zimelidine pretreatment, DSP-4 targets different neuronal populations (discussed in Waterman & Harding, Submitted). Fourth, Riters and Pawlisch (2007) conducted behavioral testing for DSP-4 effects 3 days after treatment, while we conducted our tests about 17-19 days after the first treatment. In male zebra finches, our DSP-4-double treatment regime caused a 60% loss of noradrenergic LoC cells and an 80% loss of ventral subcoeruleus cells by 20 days after the first treatment (Waterman & Harding, Submitted). While one would expect NE levels to be affected by three days after treatment, noradrenergic cells most likely were not.

Castelino and Ball (2005) systemically treated male zebra finches with the same dose of DSP-4 once and reported that DSP-4 treatment resulted in significant decreases in the immunolabeling for dopamine β hydroxylase (DBH) and tyrosine hydoxylase (TH) in the LoC. TH is the rate-limiting enzyme involved in the synthesis of catecholamines. DBH is involved in the synthesis of NE from dopamine (DA). DA is the precursor for NE. Results of these studies (Castelino & Ball, 2005; Waterman & Harding, Submitted) suggest that NE function in our females was profoundly disrupted at the time of testing. Castelino and Ball (2005) reported that DSP-4 treatment does not significantly influence TH and DBH immunolabeling in the DA synthesizing nuclei of male zebra finches, including the ventral tegmentum (VTA) and substantia nigra. Barclay *et* al. (1992)

reported that DSP-4 treatment does not influence DA levels in the VCN of male zebra finches. These studies indicate that results of the current study are unlikely to be caused by DSP-4 effects on DA neurons.

Most of the data cited above come from studies conducted on male zebra finches. Studies suggest that there is prominent sexual dimorphism in the noradrenergic sensitivity and innervation in songbirds. However, most of these differences were limited to sexually dimorphic areas in the vocal control system implicated in either in song production or song learning (Appeltants, Ball, & Balthazart, 2001; Riters & Ball, 2002). Singing is sexually dimorphic in zebra finches – only males learn to sing. No sexual dimorphism has been reported in nuclei involved in attentional mechanisms or sensory processing. Therefore, it is unlikely that the effects of DSP-4 on stimulus discrimination and attention are sexually dimorphic.

Jarvis *et* al. (Jarvis, Scharff, Grossman, Ramos, & Nottebohm, 1998) reported that singing-driven genomic responses in the vocal control nuclei (VCN) of zebra finches varies with the social context in which songs are sung. Castelino and Ball (2005) treated male zebra finches once with DSP-4 and reported that DSP-4 treatment eliminated differential singing-driven genomic response in one VCN, area X. This finding was particularly interesting, because Area X is part of the avian basal ganglia and has an extremely strong dopaminergic innervation (for review see Doupe, Perkel, Reiner, & Stern, 2005), with much higher levels of DA than NE (Barclay & Harding, 1988, 1990). Finding that NE, not DA, mediated the effects of social context on gene expression in Area X was quite surprising, but a clear indication that NE is involved in neural processing of social-salient information in zebra finches.

From the studies cited above (Appeltants *et al.*, 2002; Barclay *et al.*, 1992, 1996), and the well-documented effects of NE on selective attention (for review see Berridge & Waterhouse, 2003; Hurley *et al.*, 2004) it seems likely that in the present study DSP-4 treatment affected females' ability to discriminate between the different song types because of attentional deficits. However, data from other vertebrate groups suggest that NE may also have additional effects on sensory processing. In mammals, noradrenergic projections influenced sensory processing by affecting mechanisms in addition to attention, e.g., modulating receptive field properties of the afferent sensory neurons**,** influencing feature extraction and influencing sensory representation in the response properties of neuronal ensembles (for review see Berridge & Waterhouse, 2003; Hurley *et al.*, 2004).

The schematic in Fig. 9 depicts the auditory processing system of songbirds. The auditory areas, nucleus mesencephalicus lateralis, pars dorsalis (MLd), nucleus ovoidalis (OV), field L and the caudomedial nidopallium (NCM) receive noradrenergic innervation (Mello, Pinaud, & Ribeiro, 1998). MLd, OV and NCM + field L are avian homologs of the inferior colliculus, median geniculate nucleus and auditory cortex respectively. Data from electrophysiological studies in male zebra finches demonstrated that NE modulated auditory responses of some forebrain auditory nuclei (e.g. Cardin & Schmidt, 2004a; Cardin & Schmidt, 2004b).

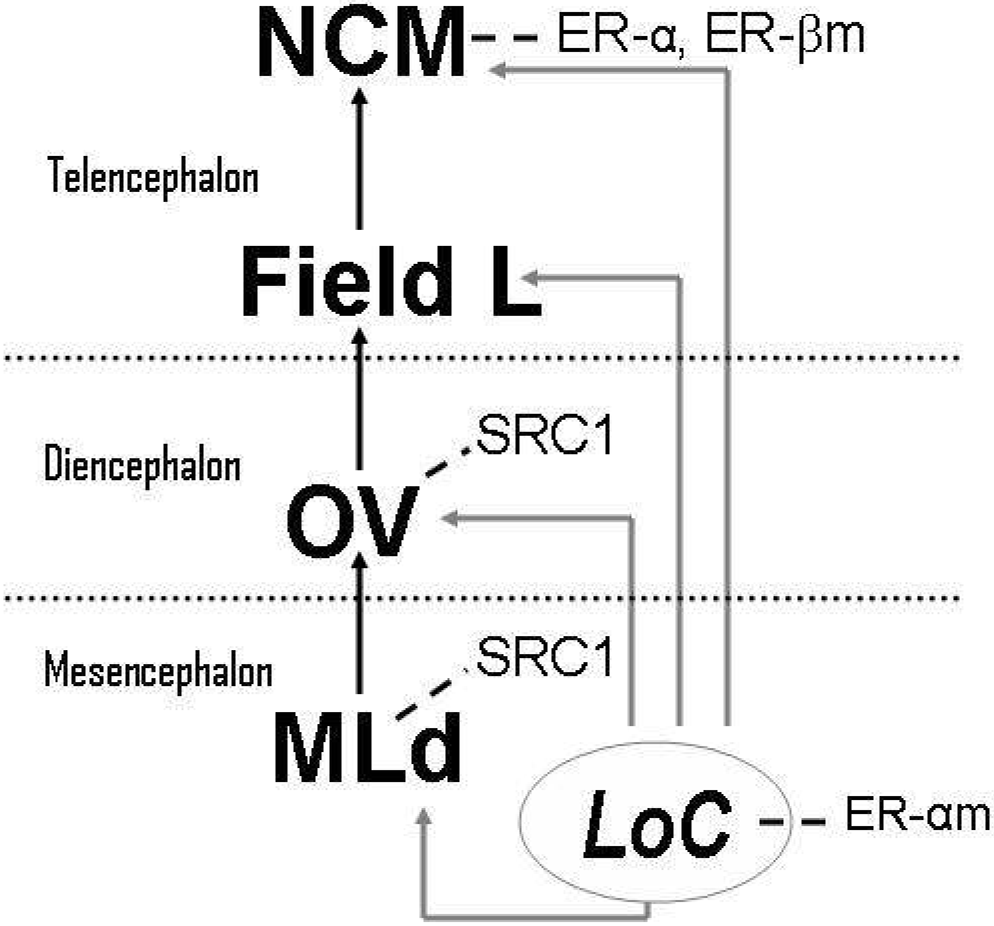

Figure 9: Schematic representation the auditory pathway of songbirds, with a depiction of some of the major auditory nuclei. MLd = Nucleus mesencephalicus lateralis, pars dorsalis. Ov = Nucleus ovoidalis. NCM = Caudomedial nidopalium. LoC = Locus coeruleus. SRC 1 = Presence of steroid receptor coactivator-1 has been reported. ER-α = Presence of estrogen receptor- α has been reported. ER-αm = Presence of estrogen receptor- α mRNA has been reported. ER-βm = Presence of estrogen receptor- β mRNA has been reported. Solid black arrows depict efferent projections from the auditory nuclei (MLd, OV and Field L). Gray arrows depict putative noradrenergic projections from the LoC.

In rats, DSP-4 has been shown to specifically target the LoC and its projections, sparing the hypothamus (Fritschy & Grzanna, 1989; Zaczek, Fritschy, Culp, De Souza, & Grzanna, 1990). Waterman and Harding (Submitted) showed that the treatment regimen used in the current study caused a striking decreases in noradrenergic cells in the LoC and subcoeruleus and in the noradrenergic innervation of many of their targets including the one auditory nucleus they examined (MLd) .

Sexual behaviors in female vertebrates are known to be modulated by noradrenergic influences on hypothalamic circuits that coordinate sexual behavior and hormone secretion (for review see Etgen *et al.*, 2001; Etgen *et al.*, 1999; Fabre-Nys, 1998). However, systemic treatment with DSP-4 has never been found to affect hypothalamic NE in zebra finches (Barclay *et al.*, 1992; Waterman & Harding, Submitted). Therefore, it is unlikely that results of the present study can be attributed to the effects of DSP-4 on behavioral processes mediated by the hypothalamus.

There is evidence for possible estrogenic modulation of auditory processing in songbirds. Schlinger (1997b) reported the presence of steroid receptor coactivator-1, (SRC-1) in the neurons of MLd, Ov and NCM of female canaries. SRC-1 mediates the action of several nuclear receptors, including estrogen receptors (Charlier, Balthazart, & Ball, 2003). However, the presence of SRC-1 does not directly indicate estrogen sensitivity. Saldanha and Coomaralingam (2005) reported the presence of estrogen receptor α (ER α) in the NCM of male and female zebra finches. Bernard *et al.* (1999) reported the presence of estrogen receptor β (ERβ) mRNA in the NCM of male and female European starlings. It was also reported that NCM in zebra finches (Saldanha & Coomaralingam, 2005) and canaries (Schlinger, 1997a) had high levels of aromatase, indicating possible brain-derived estrogenic influences on auditory processing. Maney *et al.* (2006) provided evidence for estrogenic effects on auditory processing and reported that E_2 treatment influenced the auditory selectivity of song-induced genomic responses in midbrain and forebrain auditory nuclei in female white-throated sparrows *(Zonotrichia albicollis)*.

Results from the current study, combined with data from studies of the neurochemical neuroanatomy of songbirds suggests that NE might also mediate estrogenic influences on auditory processing in songbirds. Evidence suggests that estrogen influences noradrenergic function. LoC neurons in male canaries express ER α mRNA (Maney, Bernard, & Ball, 2001) and NE levels in the VCN of zebra finches are strongly modulated by estrogen (Barclay & Harding, 1990).

The mechanisms by which NE mediates estrogenic influences on auditory processing are not clear. However, extensive research has been done on NE mediation of estrogenic influences on mammalian sexual behavior (for review see Etgen *et al.*, 2001; Etgen *et al.*, 1999; Fabre-Nys, 1998). The mammalian ventomedial hypothalamus (VMH), which is well known to coordinate female sexual behavior receives extensive noradrenergic inputs from the noradrenergic tegmental nuclei. Both the tegmental nuclei and the VMH are estrogen sensitive. In the tegmentum, estrogen was reported to facilitate the release of NE. In the VMH, gonadal steroids appeared to locally modulate both excitatory and inhibitory presynaptic inputs onto NE terminals. Additionally, estrogen can influence the number and sensitivity of noradrenergic receptors (for review see Etgen *et al.*, 2001; Etgen *et al.*, 1999; Fabre-Nys, 1998). If indeed there are similarities between estrogenic and noradrenergic interactions between the VTA → hypothalamic connections and LoC → auditory nuclei connections, we can speculate that estrogen acts on the brain-stem noradrenergic nuclei to influence the production and transmission of NE, and on the auditory nuclei to influence the release of NE or the number and/or the affinity of noradrenergic receptors, or the reuptake of NE.

In our earlier study (Vyas, Harding, Borg *et al.*, Submitted), we found that juvenile females raised in acoustic isolation from male song tended to prefer complex songs over simple songs, suggesting that female zebra finches are inherently predisposed to prefer complex song types. However, in testing song preferences of birds tutored with different song types, we also found that early auditory experiences can interfere with and/or accentuate the initial predisposition. Additionally, there is evidence that song preferences in female songbirds may be changed by adult auditory experience (King *et al.*, 2003; MacDougall-Shackleton *et al.*, 2001). It therefore seems that female songbirds tend to inherit a *template* of preferred song type, and this template is susceptible to modification depending on experience both during development and in adulthood. Results from this and our previous study (Vyas, Harding, Borg *et al.*, Submitted) suggest that with the induction of reproductive state (i.e., increased Estrogen levels), behavioral responses to songs that match the template are selectively accentuated. It appears that such selective accentuation of behavioral responses is possibly dependent upon NE-mediated estrogenic influences at multiple levels of auditory processing. This issue deserves further study.

Discussion and Conclusions

In various studies, female zebra finches were reported to prefer either songs they heard as juveniles or songs having certain acoustic characteristics (Nowicki & Searcy, 2004). To clarify the relative influences of early experiential and acoustical factors, we drastically limited female zebra finches' auditory experience and tutored them for ten days with one of three acoustically different songs. A fourth group was untutored. During adulthood, untutored females, females tutored with complex songs, and females tutored with long-bout songs preferred complex songs over the other song types. Contingent upon auditory experience, E_2 treatment either revealed and/or selectively accentuated females' song preferences. Song preferences of untutored females were not affected by estrogen treatment. In the second study, we found that adult females with unknown developmental histories, but with extensive auditory experience also preferred complex songs over the other song types. E_2 treatment revealed song preferences in these females too. We also found that estrogenic influences on song preferences might be mediated by NE.

Tinbergen (1963) in his seminal work suggested that in order to understand a behavior, we must investigate it along four different dimensions: function, phylogeny, ontogeny and causation. Here I attempt to address 'song preferences in female songbirds' along these four lines of enquiry.

Function and phylogeny of song preferences

In zebra finches, males sing, females don't. Song was reported to be one of the important criteria that females use to evaluate potential mates (for review see Nowicki & Searcy, 2004). In addition to songs, female zebra finches were also reported to base mating decisions on other phenotypic characteristics like beak color (Burley & Coopersmith, 1987), leg-band color (Burley, 1986), chest-plumage symmetry (Swaddle & Cuthill, 1994) etc. However, song was reported to be more important than symmetry in plumage (Feil & Curio, 1993) or beak color (Collins *et al.*, 1994). Interestingly, presence of attractive songs was reported to be positively correlated with other preferred physical features in zebra finches (Houtman, 1990)

Studies have suggested that the acoustic qualities of song indicate a male's quality as a potential mate. The acoustic features of song putatively convey information either about the males' nutritional state, territory status, genetic make up, health status or potential help in parental care. Song output rates were correlated with nutritional state in male pied flycatchers (Gottlander, 1987). In willow warblers (*Phylloscopus trochilus*), higher song rates preferred by females were correlated with males' territory quality (Arvidsson & Neergaard, 1991). In gray catbirds *(Dumetella carolinensis),* song rates were positively correlated with male parental care (Dolby *et al.*, 2005). Buchanan and Catchpole (2000) reported that song-repertoire sizes were correlated with parental care in sedge warblers. Gil *et* al. (1999) reported that female zebra finches mated with attractive males deposited higher amounts of testosterone and 5α-dihydrotestosterone in their eggs. The amount of maternally-derived testosterone in the egg was reported to be positively correlated with critical developmental indicators in male canaries (Schwabl, 1993, 1996). Cotton *et* al (2005) conducted a 20-year study on song sparrows, and reported that males having larger song repertoires were preferred more by females. These males also produced more chicks, and these offspring themselves produced more descendants. These studies indicate that song indeed serves as a good indicator of the quality of males.

However, benefits accrued by female zebra finches mated with males singing preferred song types have not been investigated.

For a signal to act as a reliable indicator of the quality of potential mates, the signal must be honest. In many species, signaling animals sometimes exaggerate subjectively attractive traits. For example, in three-spined sticklebacks, (*Gasterosteus aculeatus)*, females chose males mainly based on the intensity of the males' red breeding coloration, which indicates lipid content in the male body (Candolin, 1999). However, Candolin (1999) reported that in certain situations, even males in extremely poor condition are able to express high intensities of red coloration. Based upon many other similar observations, it was suggested that such signals can be adaptive to the receiver only if there are mechanisms that guarantee the accuracy or honesty of the signals (Grafen, 1990; Zahavi, 1975). It has since been reported that most signals that are preferred by members of the opposite sex, also tend to engender significant costs to the animals producing them. For example, in many avian species, females prefer highly ornate males. However, the same exaggerated ornamentation that makes males attractive to females, also make them more conspicuous to predators (Andersson, 1994).

Evidence regarding the metabolic costs of producing songs with preferred acoustic features are not conclusive. Thomas *et* al (2003) reported that European robins (*Erithacus rubecula)* gained less body mass when they sang more, and suggested that this was because of a decrease in time birds spent foraging. Singing at faster rates was reported to incur significant metabolic costs to the singer (Davies & Lundberg, 1984; Gottlander, 1987). However, costs involved in producing other preferred song characteristics are not thought to be significant enough to make these traits honest

(Nowicki & Searcy, 2005). Oberweger and Goller (2001) performed a comparative study on three species of songbirds, and reported that there were relatively small changes in oxygen consumption with changes in acoustic characteristics of songs. Nowicki *et* al. (1998) proposed the "nutritional stress hypothesis" and Nowicki and Searcy (2004) and Buchanan *et al.* (2003) proposed the "developmental stress hypothesis". According to these hypotheses, acoustic qualities of songs are an indicator of the developmental stress experienced by males. A direct prediction of these hypotheses is that juvenile male songbirds exposed to nutritional or other forms of stress before or during the period of song learning should not be able to learn and/or sing certain song types. Over the years, a robust body of empirical evidence has accumulated that suggests that developmentally-stressed males are less likely to produce songs with preferred acoustic characteristics (for review see Nowicki & Searcy, 2005). However, Gil *et* al. (2006) manipulated brood sizes, and reported that food shortage created because of increased brood sizes did not influence the accuracy of song copying by juvenile male zebra finches. It is however unclear if these nutritionally-challenged males would be able to learn acoustically complex songs.

Many studies (for review see Nowicki & Searcy, 2004) including ours have found that female songbirds prefer acoustically complex songs. According to Williams (2004), one of the sources of variability in songs is the inability of males to accurately copy their tutors' songs. It is however not known whether females will prefer variability generated due to inaccurate copying, and whether these songs advertise fitness to females.

Ontogeny of song preferences

Genetic influences on mate preferences in songbirds vary with species. Slagsvold *et* al. (2002) cross-fostered female great tits *(Parus major)*, blue tits *(Parus caeruleus)* and pied flycatchers with each other. They reported that the effect of cross-fostering on mate choice was greatest in blue tits, followed by great tits. Pied flycatchers' mate choices were not affected by cross-fostering. In our study, untutored females preferred complex songs over the other song types, indicating that some basic song preferences might be inherited.

However, data from cross-fostering experiments conducted on female zebra finches suggest that early experiences normally play an important role in mate choice. Sonnemann and Sjölander (1977) reported that female zebra finches cross-fostered to Bengalese finches *(Lonchura striata)* preferred Bengalese finch males over conspecific males. Female zebra finches of subspecies *guttata* when raised by zebra finches of subspecies *castanotis* tended to prefer similarly cross-fostered conspecific males. However, cross-fostered *guttata* females also preferred *guttata* males over the males of the cross-fostering subspecies (Clayton, 1990). These studies indicate that song preferences in female zebra finches are influenced by both genetic as well as experiential factors.

Our study suggests that females are inherently predisposed to respond preferentially to complex songs. However, we also found that early auditory experiences may also interfere-with or strengthen inherent preferences for complex songs. Additionally, there is evidence that song preferences in female songbirds may be changed by adult auditory experience (King *et al.*, 2003; MacDougall-Shackleton *et al.*, 2001). It therefore seems that female songbirds tend to inherit a *template* of preferred song type, and this template is susceptible to modification depending on experience both during development and in adulthood. Perhaps these are mechanisms meant to ensure maximal reproductive stimulation of females by the ‘best’ available males.

The physiology of song preferences

Neural correlates

Studies on neural correlates of song preferences in both female and male songbirds have focused on four brain areas: two nuclei of the sexually dimorphic VCS, HVC and lMAN and two areas outside the sexually dimorphic VCS, CMM and NCM. The HVC ‘shelf’, that projects to HVC receives auditory input from field L (Vates, Broome, Mello, & Nottebohm, 1996). Neurons of HVC but not field L responded selectively to birds’ own songs (Margoliash, 1986) Female canaries with intact HVCs show copulation displays to conspecific songs only. Brenowitz (1991) reported that in canaries, bilateral lesions of a portion of the HVC resulted in copulation solicitations to heterospecific song as well as conspecific songs, suggesting that HVC is critical for conspecific song perception in females. Del Negro *et* al. (2000) conducted an electrophysiological study in female canaries, and reported that HVC neurons responded differentially to the presence and absence of sexually-stimulating song phrases. Leitner and Catchpole (2002) reported that female canaries that showed more CSDs to sexually-stimulating songs had bigger HVCs. These studies suggest a role for HVC in determining song preferences in female canaries. However, in a study conducted by MacDougall-Shackleton and colleagues (1998), HVC lesions did not disrupt the display of song preferences in zebra finch females, but lesions of CMM did. Gentner and colleagues(2000b) also reported that HVC lesions did not disrupt the display of song preferences in female starlings. Gentner and colleagues (2000) suggested that HVC may not be a locus for conspecific song discrimination, and that differential elicitation of CSDs by different song stimuli may be because of differential habituation to some songs. In other words, CSD response to particular songs underwent selective habituation.

lMAN is another VCN structure implicated in mediating song preferences in female songbirds. Based upon Brenowitz's (1991) study, Hamilton & colleagues (1997) hypothesized that HVC lesion may reduce the input lMAN receives from other VCS nuclei, and therefore lMAN may be involved in conspecific song discrimination and influence song preferences. They reported that female cowbirds that show more CSDs to sexually-stimulating songs, have bigger lMANs. As previously cited, Leitner and Catchpole (2002) reported that the size of HVC not lMAN was positively correlated with the number of CSDs displayed by female canaries to sexually-stimulating songs. Thus, the role of lMAN in mediating song preferences in female songbirds remains unclear.

Another brain area implicated in mediating song preferences in female song birds is the NCM. The anatomical connections of NCM make it a good candidate as a locus for song discrimination. NCM receives auditory inputs from field L which does not respond differentially to songs and sends outputs to HVC which does respond differentially. . The role of HVC in song discrimination is unclear, and it is likely that differential responses in HVC to songs result because of inputs from NCM via CMM. Genomic and electrophysiological activity in NCM neurons were reported to differentiate between different conspecific songs, in males as well as females. Mello and colleagues (1992) conducted experiments on adult male zebra finches and canaries, and reported that expression of *ZENK* in NCM was lowest in birds exposed to silence, higher to heterospecific songs and highest to conspecific songs. This study indicates that genomic activity in NCM can differentiate between conspecific and heterospecifc songs. Stripling and colleagues (1997) exposed adult male zebra finches to different conspecific and heterospecific songs and recorded electrophysiological responses of NCM neurons. They concluded that "each song generates a unique signature of dynamic electrophysiological activity across a population of neurons within NCM," and that "NCM acts as a processing center for the momentary representation of complex auditory information."… (Stripling et. al., 1997). As mentioned previously, Female songbirds prefer songs with certain acoustic features like complexity, longer song-bout length, higher sound pressure levels etc. Gentner and colleagues (2001) presented female European starlings with songs of three song-bout lengths, and reported that *ZENK* expression in NCM was positively correlated with conspecific song-bout lengths. It has been reported that in female zebra finches (Bailey, Rosebush, & Wade, 2002), and female European starlings (Sockman, Gentner, & Ball, 2002) *ZENK* expression in NCM was positively correlated with conspecific male song-bout lengths. Eda-Fujiwara and colleagues (2003) reported that complex conspecific songs induced higher *ZENK* expression in the NCM of female budgerigars (*Melopsittacus undulatus*). These reports indicate that there is a relationship between ZENK induction in NCM and the salience of auditory stimuli. To the best of our knowledge, lesion studies on NCM haven't been conducted. It will be interesting to see the effect of NCM lesions on the attractiveness of conspecific songs in female songbirds.

Gamma-aminobutyric acid (GABA) is one of the main inhibitory neurotransmitters in the vertebrate brain. Pinaud *et* al. (2004) reported that the auditory nuclei, Field L, NCM and CMM in zebra finches contain a high number of GABAergic cells. Auditory nuclei, MLd and OV in zebra finches were also reported to contain GABAergic cells (Pinaud & Mello, 2007). Pinaud *et* al. (2004) also reported that song-induced genomic responses in the telencephalic auditory nuclei of zebra finches are influenced by GABAergic activity. These findings suggest that song processing in female zebra finches is influenced by GABA.

Hormonal correlates

As in other vertebrates, estrogens play an important role in activating female sexual behavior in songbirds (Silver, 1989). One obvious question, which had not been investigated, is whether estrogen treatment alters female preferences. There are ample data indicating that circulating gonadal hormones influence mate choice in vertebrates (for review see: Adkins-Regan, 1998; Puts, 2006). It is therefore likely that gonadal hormones influence song evaluation in female zebra finches.

Interestingly, in studies in which female zebra finches were reported to prefer songs having certain acoustic characteristics, females probably had high circulating estrogen levels, either because of experimental manipulations or social environment. Clayton & Pröve (1989) treated female zebra finches with estradiol prior to testing and reported that females tended to prefer longer more complex songs. Tomaszycki & Adkins-Regan (2005), Frostriemer & Birkhead (2004) and Collins *et al.* (1994) also reported female zebra finches preferred songs having certain acoustic characteristics. They had housed their females in a mixed-sex environment, where females were probably exposed to male songs before testing. Exposure to songs increases estrogen levels in female zebra finches (Clayton & Pröve, 1989; Tchernichovski *et al.*, 1998) and female canaries (Bentley *et al.*, 2000). In studies that reported females preferred songs heard during development (Clayton, 1988a; Miller, 1979; Riebel, 2000), females were maintained in a same-sex environment prior to testing which should lead to lower estrogen levels. It is possible that in the studies showing effects of early experience, the preferences exhibited by female zebra finches were not sexual in nature, but merely indicating a preference for familiarity over novelty.

In both of our studies, we found that estrogen tends to reveal and/or accentuate song preferences. In the first study, the effects of estrogen were dependent upon females' early auditory experiences. The lack of estrogenic effects in untutored birds is intriguing. They responded preferentially to complex songs under all hormone treatment conditions. Their song responsiveness seemed to be less susceptible to hormone manipulations. One reason why the untutored females reacted differently might be that tutoring causes estrogen release, strengthening preferences for the tutored song. This preference then reappears when estrogen levels rise in adulthood. Untutored females don't show this effect, because their first exposure to song was during testing, and they were exposed to all three song types with their first exposure.

Female zebra finches typically form pair-bonds very early in life (Zann, 1996). Their estrogen levels at this point are not known, and the effect of estrogen on pairing decisions are not well understood. Tomaszycki *et al.* (2006) treated unpaired adult female zebra finches with ATD plus flutamide (an antiandrogen), and reported that sex steroids had little effect on the likelihood that a male or female will be chosen as a mate by a bird of the opposite sex. However, in the same study, they (Tomaszycki, Banerjee *et al.*, 2006) also reported that ATD plus flutamide-treated females were much more likely to pair with other females than the control females, which suggests that estrogen availability does affect mate choice selections.

Perhaps the best support for estrogenic influences on mating-decisions in female songbirds comes from data on extra-pair copulation (EPC). Many species of songbirds form socially stable pairbonds. However, it has been reported females in these apparently stable pair bonds indulge in EPCs. Data from female songbirds indicate that females indulge in EPCs only with males that appear to be more attractive than their mates. For example, zebra finches were reported to indulge in EPCs with males that wore more attractive leg bands (Burley *et al.*, 1994), and female sedge warblers (*Acrocephalus schoenobaenus*, Marshall *et al.*, 2007)**,** female black-capped chickadees (*Poecile atricapilla*, Mennill *et al.*, 2002; Otter *et al.*, 1998), female dusky warblers (*Phylloscopus fuscatus*, Forstmeier *et al.*, 2002) chose males for EPCs based upon the quality of males' songs. In songbirds, most EPCs occur when females are fertile (Birkhead & Moller, 1992), and in songbirds female fertility is dependent upon high estrogen levels (Silver, 1989). These data suggest that females are especially tuned to the quality of potential mates when their estrogen levels are high.

Not much research has been conducted on the effect of sex-hormones on mate selectivity. We found that E_2 treatment increased song selectivity in females, and selectively accentuated their preferences primarily for complex songs. Lynch *et al.* (2006) reported that female tungara frogs *(Physalaemus pustulosus)* tend to become less selective when treated with human chorionic gonadotropin (HCG). They (Lynch et al., 2006) attributed this increase in sexual permissiveness to HCG-induced increase in estrogen levels. This difference in estrogenic effects on mate selectivity can be attributed to differences in mating systems. Adkins-Regan (1998) suggested that mate selectivity is constrained by temporal factors in species with explosive breeding systems. However, zebra finches are socially monogamous. Females potentially invest their entire reproductive capital in one male. Therefore, it might be an evolutionarily sound strategy that when they get ready to mate, they become more selective rather than less.

Neuroendocrinology of song preferences

It has been suggested that steroid hormones influence sexual behaviors, including processing of sexual stimuli via two mechanisms (Ball, Riters, & Balthazart, 2002): 1) Acting directly on brain regions that mediate sexual behaviors. 2) Acting indirectly (i.e., trans-synaptically) by acting on certain cells that then project to brain areas mediating sexual behaviors.

One of the obvious mechanisms by which estrogen can influence female sexual behaviors, including song preferences is by acting on behavioral processes mediated by the hypothalamus (for review see Silver, 1989). However, there is ample evidence from neurochemical and functional neuroanatomy for possible estrogenic modulation of auditory processing in songbirds. Nuclei along the auditory pathway of songbirds were reported to be estrogen sensitive (Bernard *et al.*, 1999; Charlier *et al.*, 2003; Saldanha & Coomaralingam, 2005; Schlinger, 1997a, 1997b). Maney *et al.* (2006) provided evidence for estrogenic effects on auditory processing and reported that E_2 treatment increased the auditory selectivity of song-induced genomic responses in the midbrain and forebrain auditory nuclei of female white-throated sparrows.

Estrogenic effects on neural processes are often mediated by the monoamine neurotransmitters – dopamine (DA), norepinephrine and serotonin (5HT) (for review see

Fabre-Nys, 1998). Dopaminergic and serotonergic influences on song preferences in female songbirds haven't been investigated. The neurotransmitter, NE is one of the primary neurotransmitters involved in the selection and processing of sensory signals (for review see Berridge & Waterhouse, 2003; Hurley *et al.*, 2004). Noradrenergic projections from the LoC are reported to influence selective attention in many ways, including influencing arousal and increasing signal-to-noise ratio (for review see Hurley *et al.*, 2004). Multiple auditory nuclei were reported to exhibit both direct estrogenic (Bernard *et al.*, 1999; Saldanha & Coomaralingam, 2005; Schlinger, 1997b) and noradrenergic (Barclay & Harding, 1988; Harding, Barclay, & Waterman, 1998; Mello *et al.*, 1998) sensitivity. Additionally, the noradrenergic nucleus, LoC which is thought to have extensive projections to auditory areas was reported to be estrogen sensitive (Mello *et al.*, 1998). It is therefore likely that some estrogenic influences on auditory processing are mediated by NE.

Our results suggest that estrogenic influences on song preferences in female zebra finches might be mediated by NE. In our experiment, we found that DSP-4 treatment abolished differential behavioral responses in estrogen-treated female zebra finches. This suggests that NE mediates behavioral responses to songs. Sexual behaviors in many female vertebrates are known to be modulated by noradrenergic influences on hypothalamic circuits that coordinate sexual behavior and hormone secretion (for review see Etgen *et al.*, 2001; Etgen *et al.*, 1999; Fabre-Nys, 1998). However, Waterman and Harding (submitted) found that the DSP-4-treatment protocol used in the current study did not affect the noradrenergic innervation of hypothalamic areas. Therefore, it is unlikely that results of our study can be attributed to the effects of DSP-4 on behavioral processes mediated by the hypothalamus. Evidence from neurochemical neuroanatomy suggest that NE might mediate estrogenic influences on song processing.

Song preferences in female songbirds as a model system

Historically, progress in Neuroscience has been a function of identification and development of model systems. The large neurons of giant squid made it possible for scientists to investigate and understand basic neurophysiological processes. Research on aplysia greatly furthered our understanding of the molecular mechanisms of memory. 'Song preferences in female songbirds' similarly has been proposed as an excellent model for investigating the processing of complex acoustic communication signals (Gentner & Margoliash, 2002; Theunissen & Shaevitz, 2006). The model is ecologically salient – female songbirds depend upon song processing for their reproductive success. There is striking individual variation in songs of males of most species, and songs can be quite complex (Williams, 2004). Accurate processing of songs is even more important in socially monogamous species like zebra finches, European starlings etc.

Brain areas involved in auditory processing are perhaps better mapped-out and investigated than in most non-avian vertebrate species. In song birds, auditory processing and/or song-induced behavioral responses seem to be influenced by a variety of neurochemicals, like estrogen (Maney *et al.*, 2006), NE (Cardin & Schmidt, 2004a, 2004b; Harding, 2004b), GABA (Pinaud, Fortes, Lovell, & Mello, 2006; Pinaud & Mello, 2007; Pinaud *et al.*, 2004), and cannabiniods (Whitney, Soderstrom, & Johnson, 2003). The songbird auditory system of songbirds provides a unique model to investigate how various neurochemicals contribute and interact to modulate auditory processing. For example, our study suggests that NE mediates estrogenic influences on song processing;

Barclay and Harding (1988; 1990) reported that estrogen modulates catecholaminergic influences in auditory nuclei of zebra finches; Pinaud *et* al.(2006) suggested that estrogen might influence GABAergic modulation of auditory processing in zebra finches.

Many studies, including mine, have reported that song preferences in female songbirds are influenced by early experience. Additionally, there is evidence that song preferences in female songbirds may be changed by adult auditory experience (King *et al.*, 2003; MacDougall-Shackleton *et al.*, 2001). Vignal *et* al. (2005) reported that genomic responses to songs in auditory nuclei are influenced by social context in male zebra finches. Auditory processing in songbirds provides a unique model to investigate neural bases of experience dependent plasticity in sensory processing, both during development and adulthood.

Unlike mammalian brains, areas mediating perception in songbirds appear to be discreet and well defined, and therefore our understanding of avian functional neuroanatomy has advanced very rapidly, often leading to similar advances in the study of mammalian brains. Overall, ‘song preferences in female songbirds’ provides a unique ecologically salient model to investigate how animals process complex auditory signals.

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