NEURAL DISCRIMINATION OF COMMUNICATION SIGNALS IN THE AVIAN AUDITORY SYSTEM: ROLES OF SEX, ESTROGENS, AND SOCIAL EXPERIENCE

by

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ABSTRACT OF THE DISSERTATION

Neural Discrimination of Vocal Communication Signals in the Avian Auditory System: Roles of Sex, Estrogens, and Social Experience

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Songs are learned in songbirds, much as speech is acquired in human infants, and serve social and reproductive functions. In most songbirds studied to date, males produce learned vocalizations while females do not and the male brain contains a more developed forebrain vocal control pathway. Males use song both in interactions with other males and in courtship displays to varying degrees in different species. In the zebra finch, the male sings to court females, who do not sing, but use this signal in the process of mate selection. Thus this species provides a model of sexual dimorphism in the use of song. An avian forebrain auditory area, NCM, seems to be specialized to discriminate and remember the unique songs of other individuals, through a process of stimulus-specific adaptation, but sex differences in NCM function have not been

described. The present study compares neurophysiological responses recorded in NCM of adult male and female zebra finches that were either raised in the general aviary by their parents, or reared in individual isolation with an artificial song tutor. Extra-cellular multi-unit recordings were made at multiple sites bilaterally in NCM of awake, restrained adult birds during song playback to assess auditory responses, stimulus-specific adaptation, preference for conspecific song, and song recognition memory for the tutor song as well as songs heard in adulthood. In a subset of these birds, estradiol levels were manipulated pharmacologically to compare the role of this hormone – which is known to be synthesized in the brain - in auditory responses in the two sexes. The results in untreated birds show differences between males and females in adaptation to song playback in NCM, although absolute responses do not differ between the sexes. Estradiol depletion in both males and females interfered with the memory for recently heard songs, although it had no clear effect on the memory for the tutor song heard in development. In addition, in females, but not males, estradiol depletion disrupted the typical neural response bias for conspecific song. These results are consistent with evolving ideas about the dynamic effects of sex steroids on sensory brain function beyond the established role of these hormones in reproductive behavior. The observations further suggest how, in females in the sexually receptive state, estradiol production in addition to its production in the brain itself, may influence the way the songs of potential suitors are discriminated, remembered and compared in the brain, thus contributing to the process of mate selection.

Acknowledgments

First, I would like to thank my advisor for his support and encouragement throughout the past few years. Without it I more than likely would not have stayed on to finish my degree. I would also like to thank my committee members for their guidance. I thank Mimi Phan, who contributed the data from experiments in which untreated animals were compared and on whose work the developmental experiments were based. I am indebted to Dr. Luke Remage-Healey for helping me to FINALLY get a reliable measure of estradiol in zebra finch tissue. I am especially grateful to members of my lab, who have both assisted me in labwork and kept me laughing through the roughest of times, especially Manda Pierce, Utsav Aiya, and Arielle Urman. I consider myself truly lucky to have met Mike Tomasini in these last few years of school. His love, understanding, and patience continue to amaze me and are what I thought one could only wish for in a significant other. Finally, I dedicate this dissertation to my mother, who introduced me to the world of experimental psychology as she was completing her Masters program, taking my brother and me to feed the rats through many a snowstorm. Love you Mom.

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Neural Discrimination of Vocal Communication Signals in the Avian Auditory System: Roles of Sex, Estrogen and Developmental Experience

Chapter 1: Introduction and Background

I: Songbirds as a Model for Auditory Processing of Communication Signals

In many species, individuals communicate with each other through acoustic signals. In order to effectively relay information between conspecifics, these signals must be perceived and discriminated by the listener. In humans, the production and discrimination of communication signals depend on learned processes, shaped by the auditory and social contexts experienced during a sensitive period of development. Songbirds also use a complex system of acoustic signals to communicate, and provide an ideal model in which to study how social experience influences auditory discrimination, because 1) they communicate through vocalizations in a variety of social interactions (Williams, 2008; Zann,1996) 2) males learn their vocalizations through a process of imitation that parallels human speech acquisition (Doupe and Kuhl, 1999) and 3) auditory forebrain areas specialized for processing species-specific sounds have been identified (Chew et al., 1995, 1996b). Songbirds are also a good model system in which hormonal influences on auditory processing can be examined because 1) many of these auditory neurons express estrogen receptors and can rapidly synthesize estrogen from precursors in both male and female songbirds (Saldanha et al., 2011, 2000) 2) Estradiol levels increase within these brain areas during particular social interactions in males (Remage-Healey et al., 2009) 3) Estradiol manipulations alter neural responses to sounds in both sexes

(Remage-Healey et al., 2010; Tremere et al., 2008; Tremere and Pinaud, 2011) and 4) estradiol blockade within the auditory forebrain of songbirds disrupts behavioral discrimination among songs in males (Remage-Healey et al., 2010; Tremere and Pinaud, 2011). This suggests that hormonal changes related to particular social contexts can modulate auditory responses, potentially leading to adaptive changes in behavior.

Behavioral responses to communication signals depend on accurate perception and discrimination of what is being heard, and the auditory system must subserve these processes. Young male songbirds depend on auditory discrimination to identify, memorize and imitate the song of a tutor, which they will use in adulthood as a sexual advertisement to attract potential mates (Nowicki and Searcy, 2004; Spencer et al, 2003; Nowicki et al, 2002). The imitations are not perfect, and each bird sings an essentially unique song. Females in most songbird species that have been studied do not produce a learned song (although females as well as males make a variety of unlearned calls). However, developmental exposure to a tutor-song influences female preferences for songs heard in adulthood that are important in selecting a mate from among courting suitors (Riebel, 2000; Miller, 1979a; Clayton, 1988; Lauay et al, 2003). Females prefer their tutor-song versus a novel male's song (Miller, 1979a), suggesting that they have formed a memory for the tutor-song and can discriminate it from other songs. Females also prefer their mate's song over a novel one (Miller, 1979b; Clayton, 1988), suggesting that they also form memories for songs heard in adulthood. For both sexes, discriminating and remembering the calls and songs of other birds (both male and female) are important in the social life of these gregarious birds (Zann, 1996; Vehrencamp, 2001; O'Lochlen and Beecher, 1999). In addition, call recognition can help

to maintain contact between mated individuals who are visually isolated from each other (Zann, 1996). Behavioral studies have shown that males discriminate male from female calls (Vicario et al., 2001), and that both sexes recognize their mate's call (Vignal et al., 2004; Vignal et al., 2008).

Behavioral responses to communication signals can differ with regard to gender (Vicario et al., 2001; Cynx and Nottebohm, 1992a; Cynx, 1993), hormonal state (Cynx and Nottebohm, 1992b; Leboucher et al., 1998), and degree of developmental exposure to auditory stimuli (Lauay et al, 2003). These factors might also modulate the processing of auditory stimuli, which presumably contributes to observed differences in behavior.

Examining the influences of sex, estradiol, and social experience on the areas that process these communication signals will increase our understanding of auditory processing within a functional network. Previous studies have shown that an auditory area, the caudomedial nidopallium (NCM), in songbirds processes species-specific signals and maintains a neural memory for the songs and calls of other birds in adult birds of both sexes (Chew et al, 1995, 1996a and 1996b; Mello et al, 1992). In addition, NCM expresses a neuronal memory for the tutor-song in adult males reared in isolation with a tutor (Bolhuis et al., 2001; Terpstra et al., 2004; Phan et al., 2006) and its lesioning (Gobes and Bolhuis, 2007) or its disruption with extracellular signal regulated protein kinase (ERK) inhibitors (London and Clayton, 2008) during the sensitive period for song learning impairs song-copying. In adult males, estradiol is required for the behavioral discrimination of the tutor-song from novel songs (Remage-Healey et al., 2010; Tremere and Pinaud, 2011). Behavioral studies have shown that adult females recognize the tutor-

song and mate-song (Miller, 1979a and 1979b), but the auditory processing of these signals in females is less clearly defined. Studies suggest that an additional auditory area, the caudomedial mesopallium (CMM), may be involved in processing songs, especially the tutor-song (Terpstra et al., 2006). Previous work conducted as part of my masters thesis, in which I recorded electrophysiological responses from NCM in artificially-tutored females that were hormonally manipulated in adulthood, suggest that tutor-song memory is present in female NCM and that estradiol manipulation does not influence its expression, but estradiol manipulation does influence the acquisition of new memories for recently heard songs, and the neural discrimination of conspecific versus heterospecific song. Previous studies have shown that estradiol inhibition in male NCM disables the behavioral and neural discrimination of a tutor-song from a novel one (Tremere and Pinaud, 2011), but the influence of estradiol depletion on the neuronal memory for tutor-song and the acquisition of new memories for songs has not been tested. It appears that male and female auditory responses within NCM to playback of songs and calls differ in absolute response magnitudes and adaptation rates (Phan and Vicario, 2010), but these observations have not been directly examined. An examination of brain areas specialized for processing conspecific sounds in both sexes can resolve potential sex differences in auditory processing, and whether estradiol manipulation in male NCM elicits similar effects as found in my earlier study on females administered the same treatment. The following sections provide relevant background information concerning current knowledge of auditory processing in males and females, and the influence of estradiol on the areas that process communication signals.

IIa: Forebrain auditory area NCM is specialized for processing conspecific signals

The ability to discriminate and recognize conspecific songs has been documented in an avian auditory forebrain area called the caudomedial nidopallium (NCM). NCM is an auditory processing area that responds differentially to the individual vocalizations (calls and songs) of other birds (Chew et al., 1996a). As shown in **Figure 1.1**, NCM receives projections from the Field L complex, the avian primary auditory area, and performs higher-order processing of auditory stimuli. NCM may be comparable to

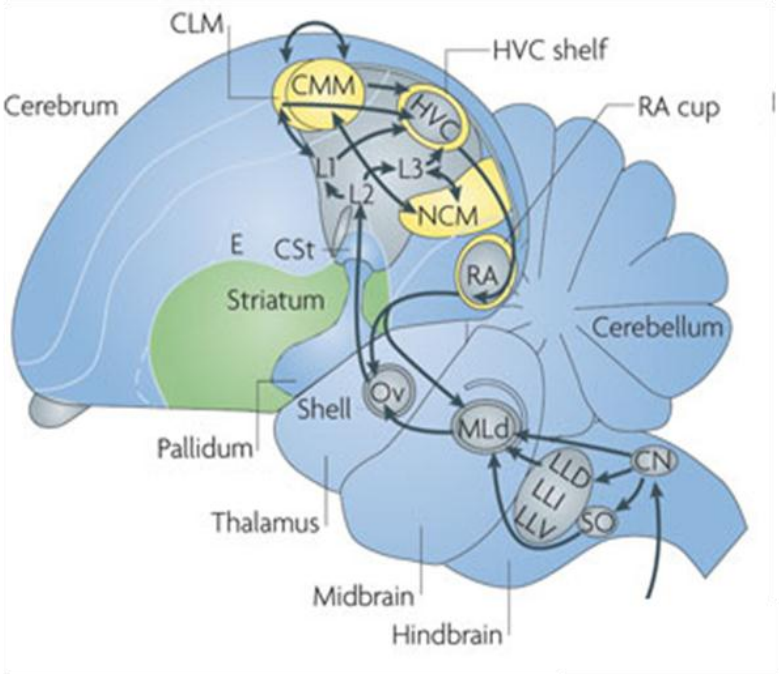

Figure 1.1: Ascending auditory relays within the Avian Brain. Sound information from the peripheral cochlea is transmitted to the cochlear nucleus (CN) within the brainstem and on to MLd , the homolog of mammalian inferior colliculus. Projections from MLd then enter the thalamic Nucleus Ovoidalis (Ov; homolog of mammalian Medial geniculate nucleus). In mammals, these thalamic neurons project to the layered auditory cortex. In the avian system, they project first to the areas of Field L, which sends projections to both NCM and CMM (yellow). Figure from Bolhuis et al, 2010.

superficial layers of A1 in mammals (Karten, 1991; Wang et al., 2010), or to a secondary auditory area. Both electrophysiological and gene-expression studies have characterized NCM response patterns to playback of different sound types. Electrophysiological recordings of NCM neurons have shown that NCM responds more robustly (i.e., higher response amplitude) to conspecific than to heterospecific vocalizations or tones (**Figure 1.2**). A similar pattern is observed in studies that measure the immediate early gene *zenk* (or ZENK, when referring to its protein product; see Mello, 2002), which is expressed in NCM in response to species-specific vocalizations (aka *zif-268;* **Figure 1.3**; Chew et al., 1995, 1996a and b; Mello et al., 1992, 1995) and is implicated as a necessary component in the process of memory acquisition (Clayton, 2000; Mello et al., 2004; Tischmeyer and Grimm, 1999).

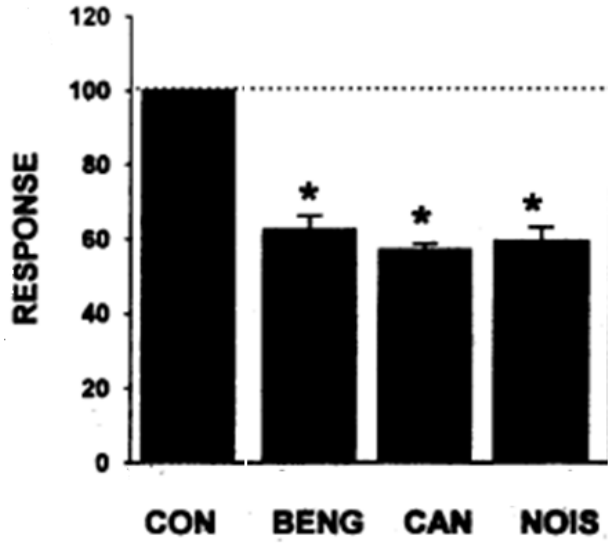

Figure 1.2: Multiunit NCM response strength differs when an animal hears playback of different sound types. Conspecific song (CON: zebra finch, in this case) playback elicits a significantly higher response than playback of either heterospecific song types or white noise. (BENG: bengalese finch, CAN: canary) Chew et al, 1996a.

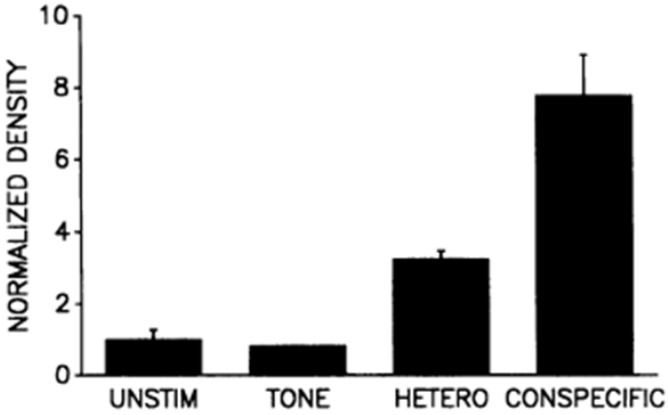

Figure 1.3: Expression of ZENK shows a response pattern similar to electrophysiological measures. Baseline levels of ZENK are low in unstimulated zebra finches but increases following auditory stimulation. Expression is greatest following playback of conspecific song, intermediate after heterospecific song, and near baseline after tone stimuli. Mello, Vicario, and Clayton, 1992.

Neural responses to auditory stimuli recorded in NCM have an intriguing property. When a particular stimulus (e.g. the song of another individual bird) is presented repeatedly, the response amplitude (observed as single- unit responses or multi-unit activity in a population of neurons) decreases with each successive trial in an asymptotic manner to reach a final level for that stimulus (Chew et al., 1995; Stripling et al., 1997). However, when a different, novel stimulus (e.g., the song of a different individual) is played, the response is reinstated (Chew et al., 1995; **Figure 1.4**). The profile of decrease (adaptation) is independent for each individual stimulus, a phenomenon known as stimulus-specific adaptation (SSA). SSA in NCM is long-lasting (Chew et al., 1995, 1996a and b) and provides an index of a song's familiarity that can be interpreted as a form of auditory memory for a song (Chew et al., 1995 and 1996a; Phan et al., 2006). SSA is longer-lasting for conspecific song stimuli (>24h) than for heterospecific stimuli (<6h), a phenomenon that requires ongoing protein synthesis within the adapted neurons to be maintained; if NCM is injected with protein synthesis inhibitors at specific time

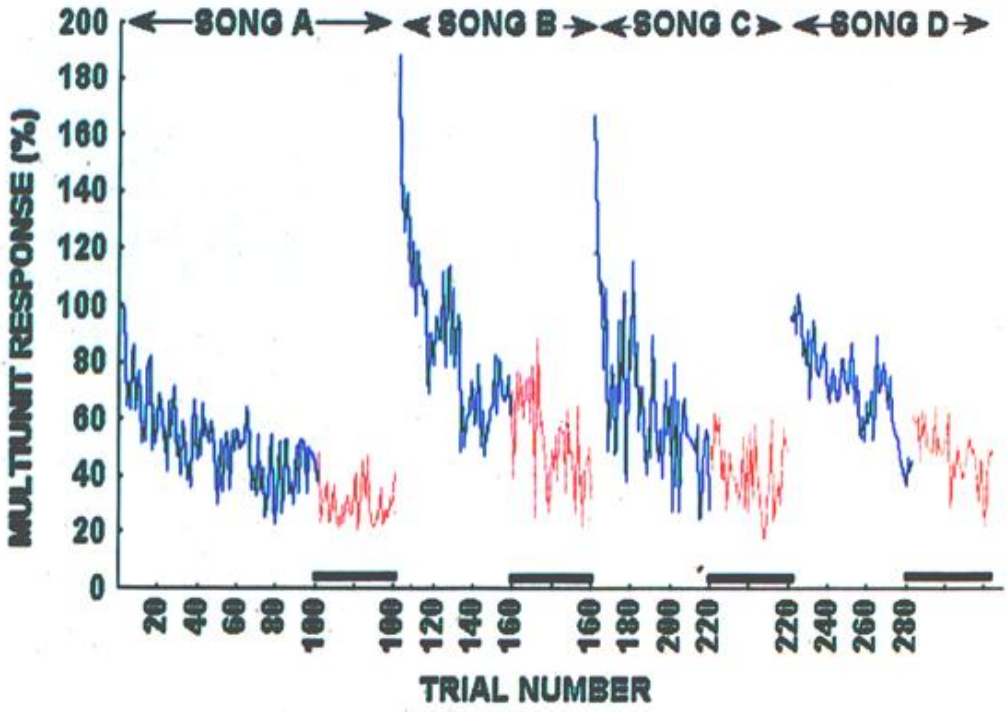

Figure 1.4: MUA responses to four different songs presented sequentially at a single site. All responses have been normalized to the initial responses at song A. Different songs elicited different initial responses but all adapted during training. Immediately after training, songs A-D were tested on trials 281-320, 321-360, 361-400, and 401-440, respectively. The responses collected during testing (red) are plotted consecutively with responses collected during training (blue) to show that the adapted response level was maintained even after training with other songs. Figure from Chew et al, 2005

points following adaptation to a stimulus, the adapted response is lost and the neuronal memory "forgotten" (Chew et al., 1996b). Gene expression studies reveal a similar adaptation phenomenon in response to conspecific song playback: NCM expresses more ZENK protein in response to playback of a novel stimulus than after playback of a familiar one heard 24h earlier (Mello et al., 1995). Thus, NCM maintains an "independent neuronal record of each stimulus" that the bird has heard (Chew et al., 1995) and seems to be specialized for recognizing and remembering familiar conspecific signals.

IIb. NCM is important for song learning in juvenile males, who store a memory for the Tutor-Song

Just as NCM contributes to acquisition of auditory memories in adult birds, it also may participate in forming a memory for the tutor's song in juvenile birds. As previously described, juvenile males learn to imitate the song of their tutor during a sensitive phase of development, and a necessary step in this process is listening to and memorizing the tutor's song. Male NCM appears to store a long-term memory (in the form of SSA) of the tutor song heard in development (Phan et al., 2006) and the strength of this neuronal memory is strongly correlated with the quality of song imitation in each bird. London and Clayton (2008) showed that bilateral blockade of the extracellular signal-regulated kinase (ERK) pathway (which is upstream of and necessary for *zenk* induction; Cheng and Clayton, 2004) in NCM during tutoring impairs learning and results in poor copies of the tutor's song in adult males (reviewed in Hahnloser and Kotowicz, 2010). In adulthood, NCM responds robustly to tutor-song playback. For example, NCM expresses more ZENK in males exposed to playbacks of their tutor's song and these males show a behavioral preference for this song over that of a novel conspecific (Terpstra et al., 2004). Lesions of NCM in adult males suppress this behavioral preference for the tutor-song (Gobes and Bolhuis, 2007). Thus, it appears that NCM plays an essential role in forming, and possibly retrieving, the memory for the tutor-song in males.

IIc. Females have a memory for the tutor-song but may process song differently than males NCM

In females, it appears that NCM processes the memory for the tutor-song, but may require an additional processing area, the caudomedial mesopallium (CMM), which shares reciprocal connections with NCM in males (Vates et al., 1996). CMM also responds selectively to conspecific vocalizations, exhibits SSA (Pierce, Phan, Shukla & Vicario, 2010), and appears to encode altered responses to recent experience with song categories (Sockman et al., 2002) and to conditioned stimuli (Gentner et al., 2004). In males, tutor-song playback elicits relatively robust *zenk* expression in NCM that is correlated with the quality of song-learning (Terpstra et al., 2004). In females, tutor-song playback elicits robust *zenk* expression in CMM but <u>not</u> in NCM (Terpstra et al., 2006; **Figure 1.5**), although the levels within each area were positively correlated in the females that were re-exposed to their father's song. These results suggest that at least

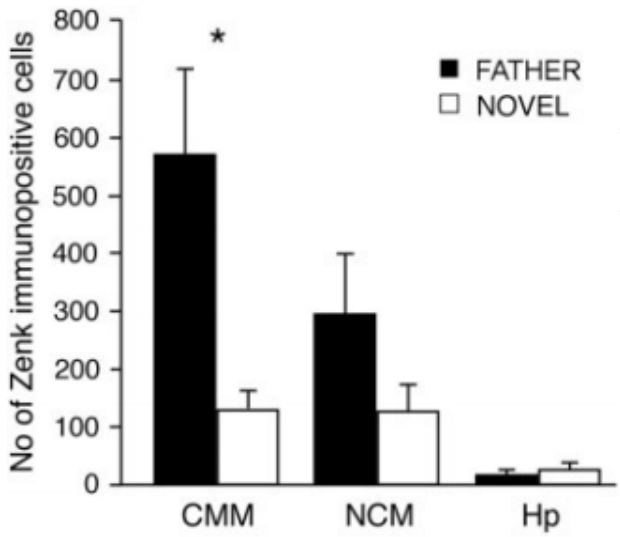

Figure 1.5: Mean number Zenk-immunopositive cells expressed in CMM, NCM and Hippocampus in female zebra finches following playback of Father or a novel zebra finch song. Zenk expression is significantly higher in CMM, but not in NCM, in birds that heard their father's song versus those that heard novel song. The difference between Zenk expression in CMM and NCM in birds exposed to their father's was not significant. Figure from Terpstra et al., 2006.

certain types of auditory stimuli (e.g., the tutor-song) may be processed differently in

males and females, and that female auditory processing may require more than one auditory area. A recent imaging study compared NCM responses of female songbirds raised in acoustic isolation with males raised that were raised in isolation (heard only themselves), box-tutored (heard themselves and an artificial tutor), or reared in an aviary and recorded BOLD fMRI and Event Related Potential (ERPs) responses to playback of stimuli (Maul et al., 2010). The results in males showed that selective responses (i.e., strongest to song and lower to repeated syllables, calls, and tones) typically observed in NCM required developmental exposure to song, since isolated males did not show selectivity for different sounds. Surprisingly, selective responses were observed in females that had been isolated, similar to the males that had been exposed to song during development. These results suggest that this type of song perception within female auditory areas is not as dependent on developmental experience with song, and so female NCM might be innately biased to respond more strongly to conspecific song versus other categories of sounds (Maul et al., 2010). These results are in accordance with those of behavioral studies showing that females raised in isolation respond more (quivers, shakes, orienting) to complex-structured songs over more simple-structured ones, even though they have no prior experience with song (Vyas et al., 2009). If this is the case, then females may have an inherent preference not only for conspecific song, but for complex conspecific songs. Since the behavioral preference for song types in tutored females can be increased or decreased by the birds' tutoring experience, it appears that females may have an innate preference that is then further shaped by their early experiences with song (Riebel et al., 2000). Furthermore, this innate preference may continue to be shaped by more recent experience with songs as adults, since female birds

raised with minimal exposure to male songs do exhibit preferences for particular songs (Riebel, 2000). A similar study (Lauay et al., 2003) showed that females require experience with tutored song in order to prefer it over untutored (isolate; untutored males produce song that is structurally and spectrally simple relative to tutored males) song. All females in this study showed preference for conspecific versus heterospecific song, regardless of rearing environment, supporting the fMRI results of Maul and her colleagues (2010). Though the results of these studies support the idea that females have an innate bias to respond to the songs of their own species that is fine-tuned by experience, and use songs to select a mate (Nowicki and Searcy, 2002), it remains puzzling that adult females perform poorly on tasks designed to test their discrimination ability among communication signals. For example, when adult males and females were trained to discriminate among different conspecific sounds in the same operant task, females learned more slowly to discriminate between the song pairs than did males (Cynx, 1993); females required significantly more trials (approximately twice more) to reach criterion (**Figure 1.6**). These differences may be due to the fact that behavioral relevance of song stimuli differs for males (who learn to imitate a specific song and sing to attract mates) and females (who rely on discrimination among songs of different quality to select a mate). This line of reasoning might also explain the sex difference on a task of call discrimination, in which a series of calls are played to a bird and the number of call responses (i.e., number of calls produced in response to the call stimulus) are recorded (Vicario et al., 2001). Whereas males called twice as much (preference ratio of 2.4) to the calls of females versus those of other males, the females called only slightly

more to female calls (preference ratio of 1.2). When synthetic call stimuli were created along a continuum to represent the different spectral (male and female calls differ in

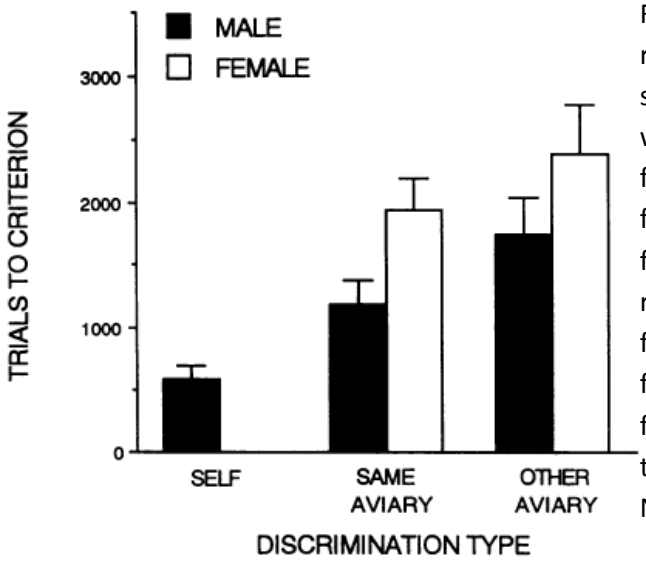

Figure 1.6: Number of trials to reach criterion during training on a song discrimination task. Birds were trained to discriminate self from novel (males only), two birds from the same aviary, or two birds from a different aviary. Males reached criterion in significantly fewer trials than females on the first two tasks, suggesting a familiarity advantage in learning the task. Figure from Cynx and Nottebohm, 1992.

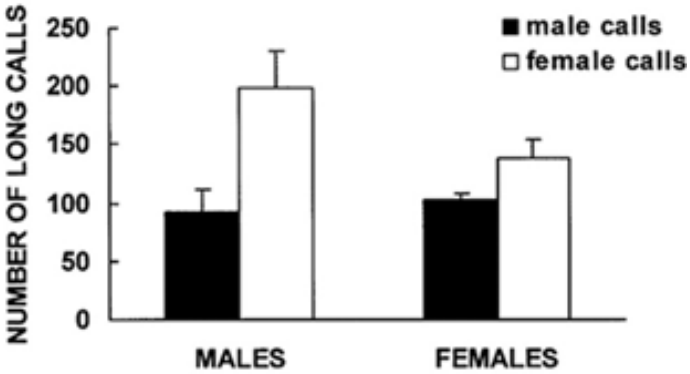

Figure 1.7: Number of response calls made by male and female zebra finches in response to playbacks of male (black bars) and female (white bars) call stimuli. Figure from Vicario et al., 2001.

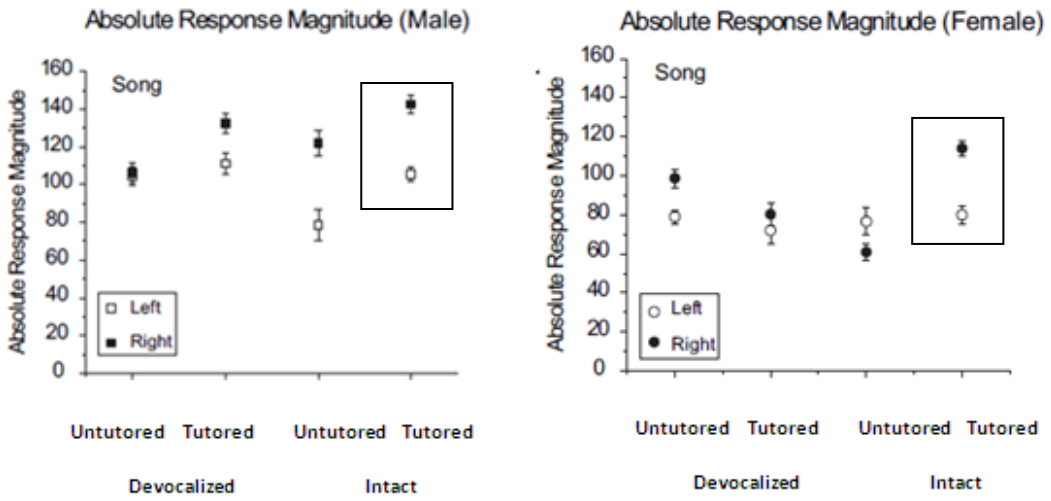

Figure 1.8: NCM Multiunit response amplitudes to novel conspecific song playback in males (left) and females (right) that were raised under different conditions of exposure to song and to their own vocalizations in a study examining lateralization. Birds were either Tutored or Untutored, or underwent devocalization surgery. Intact Tutored birds (box on right side of each figure) were tutored in the same paradigm as those in my previous study in females. Males (left panel) have higher response amplitudes to song playback in NCM than females (right panel) in the same condition. Figure adapted from Phan and Vicario, 2010.

fundamental frequency, and male calls include a frequency modulation) and temporal (female calls are typically longer in duration than male calls) features, similar behavioral results were obtained. Further analysis showed that, whereas males were discriminating the stimulus calls based on both spectral features (fundamental frequency and frequency modulation) and duration, females were discriminating based on duration alone. Whether this reflected motivational factors or differences in discriminative abilities between the sexes remains unclear. Previous observations in box-tutored birds (that appear in the figures from Phan and Vicario (2010) but were not analyzed or commented upon; **Figure 1.8**) suggest that quantitative differences exist between male and female NCM, but this has not been explicitly tested.

IId. Estrogen Modulates Auditory Processing in NCM

Local estradiol in NCM is necessary for behavioral discrimination of song in male songbirds (Remage-Healey et al., 2010; Pinaud and Tremere, 2011). This is consistent with the observation that NCM (in both sexes) expresses high concentrations of estrogen receptors (Gahr, 2001; Saldanha et al., 2000, 2011) and the enzyme aromatase, which converts the androgen testosterone to estradiol, within synaptic terminals of neurons (Schlinger, 1997, 1998; Remage-Healey et al., 2010; Saldanha et al., 2000; Balthazart et al., 1996). It has recently been demonstrated that NCM produces estradiol in response to conspecific song playback in male zebra finches (Remage-Healey et al., 2009). The functional effect of estrogens on NCM processing can be explored by manipulating estradiol or its availability and assessing changes in the known response patterns in NCM.

IId.1. Bias for Conspecific Song

A hallmark feature of NCM is its selectivity for conspecific versus heterospecific songs or synthetic stimuli (Chew et al., 1996a) and this feature appears to be modulated by estradiol. In female white-throated sparrows, suppression of circulating estradiol obtained by manipulating the birds' photoperiod inhibits the classically observed ZENK response bias for conspecific song in NCM (Maney et al., 2006). A similar phenomenon was observed in male black-capped chickadees that were captured during the breeding and non-breeding season and tested for ZENK expression in NCM following song playbacks (Phillmore et al., 2011). In these males, more ZENK was expressed in response to conspecific than heterospecific vocalizations during the breeding season, but

not during the non-breeding season, when testes are regressed and lower concentrations of androgens are available to be converted to estradiol through aromatase (Soma et al, 2003; Wingfield and Hahn, 1994; Soma and Wingfield, 2001).

IId.2. Discrimination among Conspecific Songs

Manipulations of local estradiol availability within NCM also influence the electrophysiological response bias for conspecific songs. In adult male and female zebra finches, direct estradiol injection or retrodialysis into NCM elicited an increase in the firing rate of individual NCM neurons during playback of conspecific songs (Tremere et al., 2009; Remage-Healey et al., 2010). Estradiol injection also induced an increase in the amplitude of NCM multiunit activity (MUA) in response to conspecific stimulus playbacks (Tremere et al., 2009). The electrophysiological changes observed in response to estradiol administration appear to be selective for conspecific song stimuli, since the firing rate of NCM neurons did not increase to playbacks of heterospecific songs or white noise (Remage-Healey et al., 2010). Because estradiol is synthesized in NCM when birds hear conspecific song (males, Remage-Healey et al., 2008; females, Remage-Healey et al., 2012), an increase in firing rate to conspecific song is <u>not</u> observed when Fadrozole is retrodialyzed to inhibit E2 synthesis during song playback. These results suggest that local estradiol is required for increased firing rate to occur to conspecific song.

The selective increase in firing rate or amplitude in MUA recordings may contribute to an animal's ability to discriminate not only conspecific songs from "other" sounds, but also to discriminate among individual conspecific songs. Manipulation of estradiol within NCM has been shown to suppress the male behavioral preference for

particular forms of familiar song within a two-choice playback test, which is frequently used to measure song preferences or recognition in birds (Remage-Healey et al., 2010; Tremere and Pinaud, 2011). During this test, a bird is placed into a cage containing a speaker on either end, each one playing a different stimulus, and an observer records the amount of time the bird spends near each speaker. A bird will spend more time near the speaker emitting the bird's own song (BOS) or the one of his tutor than a speaker emitting a novel song (Holveck and Riebel, 2007), indicating that the bird remembers this song and can discriminate it from other stimuli. However, when estradiol is blocked within NCM during this task, the behavioral preferences for the BOS (Remage-Healey et al., 2010) and the tutor-song (Tremere and Pinaud, 2011) are suppressed. This effect is specific to the blockade, because the behavioral preference is reinstated during subsequent vehicle infusion (Remage-Healey et al., 2010). These results suggest that estradiol in NCM is necessary for the accurate discrimination of at least two forms of familiar song (tutor-song and BOS) from novel ones in males. The same behavioral task was not conducted on female birds, so it is unknown whether local estradiol blockade in NCM would induce a similar deficit in discrimination.

IId.3. Acquisition of Memories for Songs

Blocking estradiol in NCM during novel song playback prevents the expression of the *zenk* gene (Tremere et al., 2009) and may play some role in the neural plasticity that contributes to auditory memories in this area. Because many cellular processes precede and follow this IEG response estradiol blockade in NCM may prevent IEG-associated

events and prevent memory formation of conspecific songs (Tischmeyer and Grimm, 1999; Clayton, 2000).

Direct injections of either Fadrozole (an aromatase inhibitor) or Tamoxifen (an estrogen receptor blocker) into NCM during song playback prevent the normally observed *zenk* induction that occurs in response to conspecific song playback (Tremere et al., 2009). Conversely, estradiol injection into NCM increases ZENK expression in birds exposed to song playback, but it also increases ZENK expression in birds exposed to silence, suggesting that estradiol, which increases in NCM during song playback (Remage-Healey et al., 2009), is sufficient to induce gene-expression and downstream events associated with neural plasticity or memory acquisition. When estradiol synthesis is prevented by injecting ATD, the normally observed induction of *zenk* to hearing song was prevented, suggesting that locally synthesized estradiol within NCM is necessary for the normal gene expression typically observed in untreated birds after hearing song (as observed in Mello et al., 1992). Studies conducted in other labs that measure ZENK expression (Maney et al., 2006; Phillmore et al., 2011) show that the bias for conspecific song versus other categories of sounds in NCM is reduced when circulating estradiol levels are low. Since local estradiol blockade within NCM inhibits the normal induction of *zenk* observed in response to conspecific song playback (Tremere et al., 2009), and *zenk* induction may be necessary for memory acquisition (Clayton, 2000), estradiol may be required in NCM to enable the acquisition of the neuronal memory for songs.

III. Overview of experiments to investigate sex differences, estradiol, and the role of developmental experience in electrophysiological responses to auditory communication signals within NCM:

Taken together, the results of previous studies suggest: 1) that there may be sex differences in the processing of song, 2) that these potential sex differences may occur as the result of developmental experience with song that could be explained by the importance of experience-dependent learning in males and of possibly innate song-dependent choosing in adult females 3) that normal song processing may require estradiol within NCM in both sexes.

In an attempt to further understand the functional modulation of auditory discrimination for salient sounds, I conducted a set of experiments to 1) identify sex differences in the processing and neuronal memory for conspecific songs in NCM of males and females that were reared and tested in similar paradigms (aviary raised, artificially tutored) 2) determine whether estradiol depletion induces differences in the processing of songs and neuronal memory for recently heard songs by manipulating estradiol in adult males and females raised in the aviary and in an artificial box-tutoring paradigm 3) examine Tutor-song memory in box-reared males and females to identify sex differences in processing this memory and whether it is influenced by estradiol depletion in either sex and 4) determine whether the response bias for conspecific songs is altered as a result of estradiol manipulation.

The general methods used for all experiments are described in Chapter 2, and the experiments are described in the chapters that follow.

A note on terminology: I use the term “memory” throughout the text to refer to a differential neuronal response to familiar versus novel songs, as measured by the Familiarity Index (see Methods below). The validity and limits of this operationalized definition will be discussed in Chapter 7.

Chapter 2: General Methods Used for all Experiments

Surgery:

Two days prior to electrophysiology, birds received a partial craniotomy in order to 1) expose the under-skull for later removal prior to electrophysiology and 2)adhere a metal pin onto the rostral portion of the skull in order to stabilize the head during testing. Prior to this procedure, animals are fasted for 30 minutes, then anesthetized with isoflurane (.3 L/min induction, .1L/min maintenance) and placed in a stereotaxic apparatus. Lidocaine (0.04cc, .25%) is injected under the scalp, the feathers above the incision are gently plucked away, and a portion of scalp cut away to expose the underlying skull. A small razor blade is used to cut away the portion of skull that covers the divergence of the sagittal sinus (used as a reference point when placing recording electrodes). After partial craniotomy, the flat portion of a metal pin is adhered to portion of skull just rostral to the opening, using dental cement. The procedure takes 20-30 minutes to perform. Immediately after surgery, the animal is removed from the apparatus, given an intramuscular injection of Metacam (0.04 cc, 5mg/mL) to relieve post-surgical pain, and placed in a cage under a heat lamp for a brief recovery period. Once the animal wakes, it is returned to its cage and given soft nestling food. The bird remains in its cage until the evening before testing. All procedures follow a protocol approved by the Rutgers IACUC.

Memory stimuli:

The evening before testing, birds are acclimated to the testing environment by placing them into a soundproof recording booth (Industrial Acoustics, Bronx, NY). Birds

remain in their cage with *ad libitum* access to food and water. The next morning (8 a.m., 6-8 hours prior to neural recording), a set of auditory "training" stimuli are played to the bird through a speaker in the booth. The training set includes 2 conspecific (zebra finch) and 2 heterospecific (canary) songs, each presented 200 times at a 10-second interstimulus interval (ISI). Heterospecific stimuli are partial canary songs similar in duration to zebra finch songs (approximately 2 seconds). These four stimuli are presented again during neural recording in order to test the neuronal memory of each animal for these songs. The time frame between the initial stimuli presentation and testing lies well within the 48-hour limit of the zebra finch memory capacity for conspecific song and within the 6-8 hour limit for heterospecific song (Chew et al, 1995). Following training, the animal remains in its cage within the sound booth with free access to food and water until the testing procedure begins.

Electrophysiology:

On the afternoon of testing (~1-2 p.m.), the bird is removed from its cage and placed gently into a testing holder designed to minimize movement. The bird's head is stabilized by clamping the pin into an adapter on the stereotaxic apparatus. The brain is exposed by removing a portion of the remaining skull layer. A 30-gauge needle, in combination with microsurgical scissors, is used to cut through the dura, creating a small window above both brain hemispheres near the bifurcation of the mid-sagittal sinus, immediately rostral to the cerebellum. Using a Thomas Eckhorn microdrive, 16 tungsten electrodes are positioned on the surface of the brain. Electrodes are individually lowered into the brain to an initial depth of 500 uM from the surface of the brain, and then

lowered into NCM individually while playing a set of white noise stimuli shaped with the amplitude envelope of zebra finch song from a speaker located 0.5m in front of the bird at 65dB SPL. The physiological responses to these stimuli are amplified (Brownlee Precision, San Jose, CA) and bandpass filtered from 0.5 to 5kHz. After positioning all electrodes in NCM, the neural responses are recorded (Spike2 Software) as the bird listens to four sets of auditory stimuli. These stimulus sets consist of: 1) Memory Set: used to test the memory response as described in previous experiments (Chew et al, 1995, 1996a and b) and includes the 4 songs that the bird heard earlier that morning and 6 novel songs (3 zebra finch songs and 3 canary songs, which are used to test differences in response amplitude to conspecific and heterospecific song 2) Song Set: includes the tutor-song, the bird's own song, the genetic father's song, and eight novel zebra finch songs) 3) Call Set: includes conspecific calls from male and female aviary birds, synthetic calls that systematically vary along the same dimensions as natural calls 4) Tone Set: includes tones of various frequencies (250Hz-5khz in 250Hz increments) and can be used to test tuning of auditory areas. Recording spans approximately two hours with a one-hour set-up and search session. After recording, electrolytic lesions are made (20uA for 12 seconds) at the tips of two electrodes in each hemisphere to verify placement in NCM. The positions of the other electrodes are then reconstructed from the relative placements of all the electrodes.

Analysis of Electrophysiology Data:

Physiological responses obtained during the recording sessions are analyzed to

determine 1) the response amplitude of NCM neurons during stimulus presentation relative to baseline activity 2) the adaptation rate of these driven responses to each stimulus. To determine the response to stimulus presentation relative to baseline activity, the average response to each stimulus is calculated by subtracting the RMS (root mean squared) during the Control window (500 milliseconds before the onset of the stimulus) from the RMS during the Response window (duration of stimulus + 500 milliseconds of silence). Thus, the Response-Control (R-C) is calculated for each trial of each stimulus at each electrode (**Figure 2.1**). To calculate the adaptation rate of NCM responses to each stimulus, a regression is performed on the R-C for trials 6-25 (the linear portion of the adaptation function) for each stimulus presented. The slope of this regression line represents the adaptation rate of NCM neurons to a given stimulus, where a steeper slope (more negative; e.g. -0.8) represents responses that adapt more quickly and a shallower slope (less negative; e.g., -0.3) represents responses that adapt more slowly. Since a novel stimulus elicits faster adaptation from NCM neurons compared to when it is familiar, the relative familiarity of a stimulus may be determined by comparing the adaptation rates of NCM neurons to it in comparison to novel stimuli (**Fig. 2.2**).

Calculating the Familiarity Index (FI):

Following established procedures in the laboratory, each site's relative familiarity to each stimulus was calculated as a Familiarity Index (FI; Phan et al., 2006). The FI for a

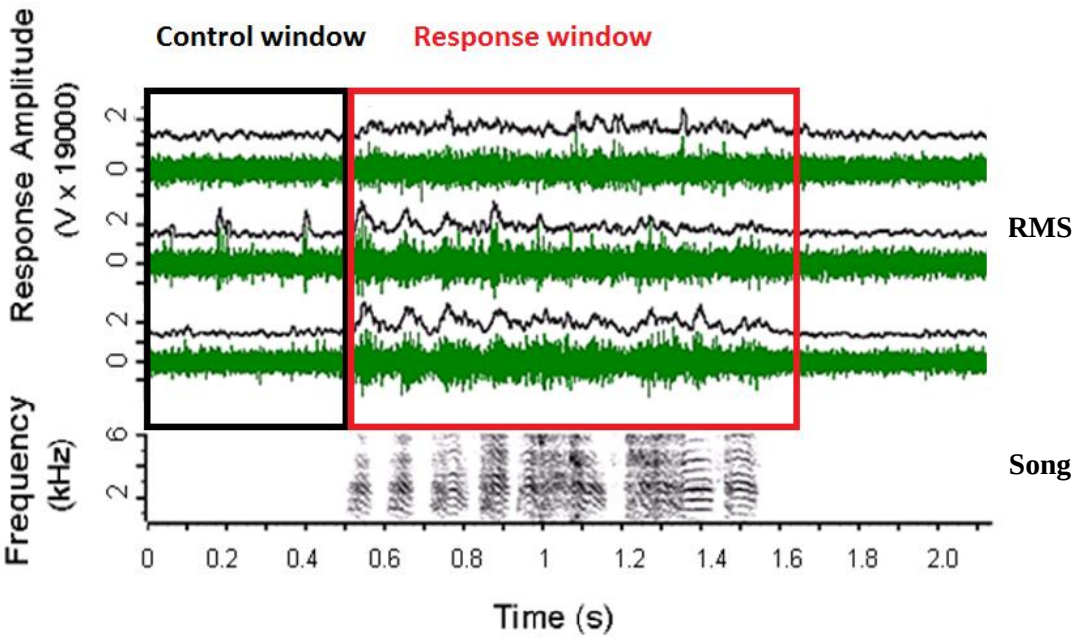

Figure 2.1: Example recording from 3 sites in NCM in response to song playback. To determine the response to stimulus presentation relative to baseline activity, the average response to each stimulus is calculated by subtracting the root mean squared (RMS) calculated over the Control window (black box; 500 milliseconds before the onset of the stimulus) from the RMS over the Response window (red box; duration of stimulus + 500 milliseconds of silence). Thus, the Response-Control (R-C) is calculated for each trial of each stimulus at each electrode.

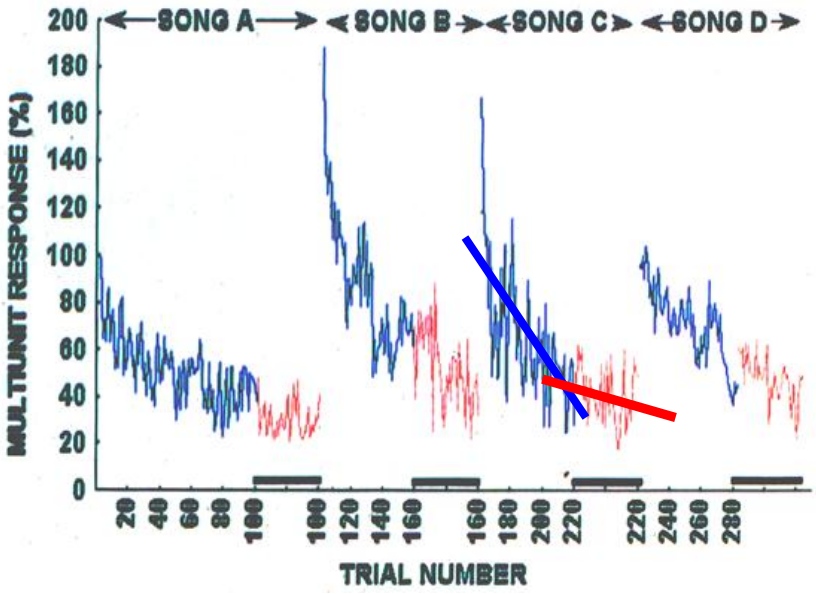

Figure 2.2: Calculating the Familiarity Index. The relative familiarity of a "familiar" (red) stimulus can be determined by dividing its adaptation rate into that of a "novel" (blue) stimulus, which will have a steeper adaptation rate. FIs greater than 1 indicate that the adaptation rate for the test stimulus is more positive than the adaptation rate for novel stimuli. Figure from Chew et al, 1995.

stimulus is the ratio of the average adaptation rate to novel stimuli/average adaptation rate to each familiar stimulus at each site. Because overall responsiveness of NCM neurons and baseline activity levels vary between electrodes, all FI calculations are performed by site to normalize the responses for differing activity level. This index is defined for NCM sites that show adaptation, as measured by a negative slope of the regression between trial number and response size. Non-adapting sites to novel conspecific stimuli (~25%) were excluded. Occasionally, sites that adapt to novel songs show positive slopes to a training song, and thus result in a negative FI; these values were excluded (<10%) from FI comparisons. Example distributions of all FI's and the distribution of included values are presented in **Figures 2.3** and **2.4** for comparison.

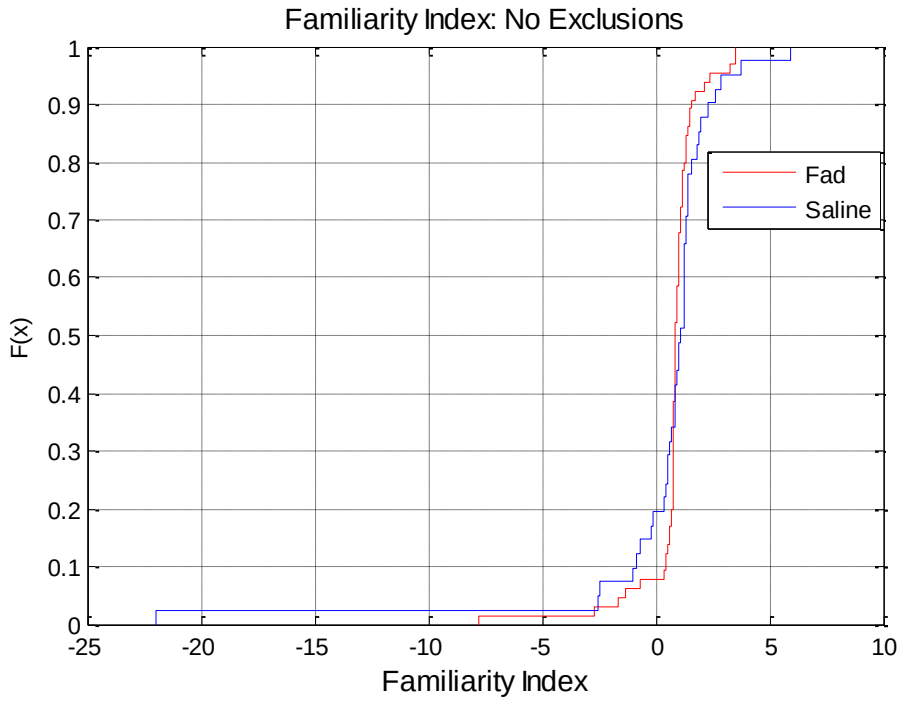

Figure 2.3: Example cumulative frequency distribution (CDF) of Familiarity Indices (FI), exclusion criteria

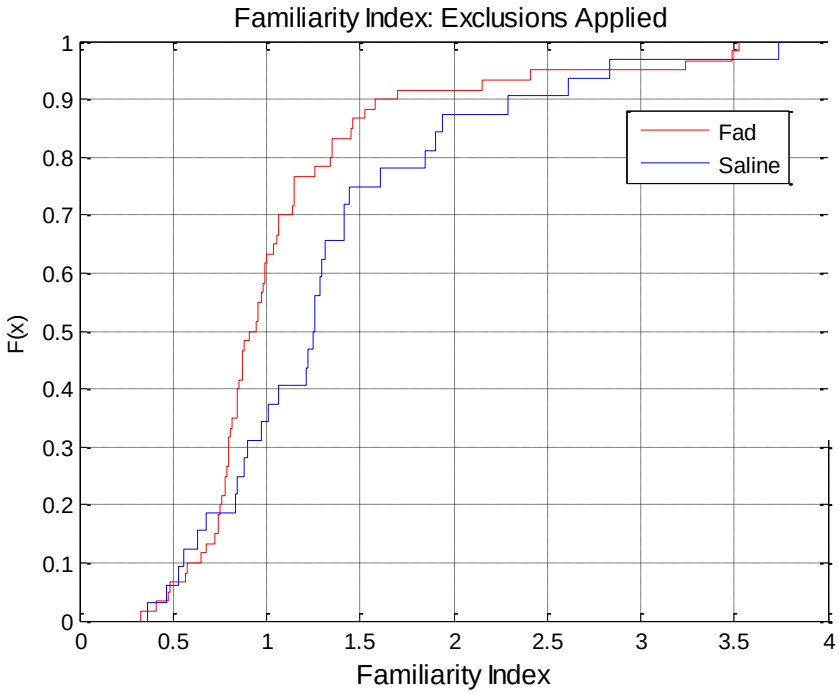

Figure 2.4: The same data presented in Figure 2.3, with the exclusion criteria applied.

Calculating the ConHet Ratio, Response Bias Index (RBI), and Conhet Adaptation Index:

ConHet Ratios were created in order to compare response magnitudes to conspecific versus heterospecific songs at a given site. ConHet was calculated by first averaging the responses for Trials 2 -6 to the novel conspecific songs (CON) and to the novel heterospecific songs (HET) at each site. The ConHet Ratio was calculated by dividing CON by HET (ConHet= CON/HET). For this measure, values greater than 1 indicate that responses to conspecific song are greater than to heterospecific song. In order to correct for differences in general response magnitude among sites, the Response Bias Index (RBI) was created by subtracting HET from CON and then dividing by the average of CONHET (RBI= (CON-HET) / (avg (CON+HET)). An RBI value of zero indicates that there is no bias for either song class. Positive values indicate higher response magnitudes to conspecific than heterospecfic songs, and negative values indicate higher response magnitudes to heterospecific than to conspecific song. RBIs were then compared among experimental groups in order to test for differences in the response bias for conspecific song (**Chapter 4**).

The Conhet Adaptation Index (CI) was created to compare adaptation rates for conspecific and heterospecific song at a given site. CIs were calculated by first averaging the adaptation rates for trials 6-25 to novel conspecific songs (CON) and to novel heterospecific songs (HET) at each site and HET was subtracted from CON (CI=CON-HET). CI values at 0 indicate that adaptation rates are equal for conspecific and heterospecific song. Positive values indicate faster adaptation to conspecific song and negative values indicate faster adaptation to heterospecific song. CIs were then

compared between groups to test for differences in adaptation rates for conspecific versus heterospecific songs (**Chapter 4**).

Data presentation and Statistical Comparisons:

All data are presented as Cumulative Frequency Distributions (CDFs), which enable straightforward comparisons to be drawn between distributions of adaptation rates, response magnitudes, and derived measures such as the FI, RBI, and CI previously described. For statistical comparisons, Kolomogorov-Smirnov (KS) tests were used to determine whether the distributions were significantly different. In some cases, I draw comparisons between multiple pairs of distributions by presenting CDFs, conducting individual KS tests, and then complement these with ANOVA to test for interactions when multiple factors appear to be modulating a measure (e.g., rearing environment and sex).

Box-Tutoring Paradigm:

For experiments testing Box-tutored birds, subjects were bred in our aviary, isolated from male song, and artificially tutored throughout the sensitive period for song learning (**Figure 2.5**). Pairs of adult male and female zebra finches from our aviary were placed into individual family cages to breed. After each clutch of eggs had hatched (10+/-2 days post-hatch), the mother and chicks were placed into a sound isolation box, consisting of an Igloo cooler lined with sound attenuating foam. At 30±2 days post-hatch, zebra finches were placed into individual isolation boxes, and the mothers were returned to the breeding population. Each box is equipped with an overhead fluorescent light set to a 12 hour light: dark cycle and an air-tube connected to a pump (Jemco, Inc.)

to provide ventilation. The finches are supplied with seed (Abba 1900, Elizabeth NJ) and water *ad libitum* throughout the experiment, as well as with daily nestling food (Green 92, Abba, Elizabeth NJ) to encourage healthy development. Prior to 45 days post-hatch, individually housed birds will be sexed by examining their plumage for secondary sex characteristics. Because males begin to produce vocalizations in the form of subsong prior to d45, recordings of all each bird's vocalizations were be checked to verify sex. Males remained in their individual isolation boxes for the duration of the experiment, except when undergoing procedures performed in preparation for testing. Verified females were placed into a separate isolation box and used in a separate experiment.

On day 45, a song "tutor" (a plastic model bird secured atop a wooden perch

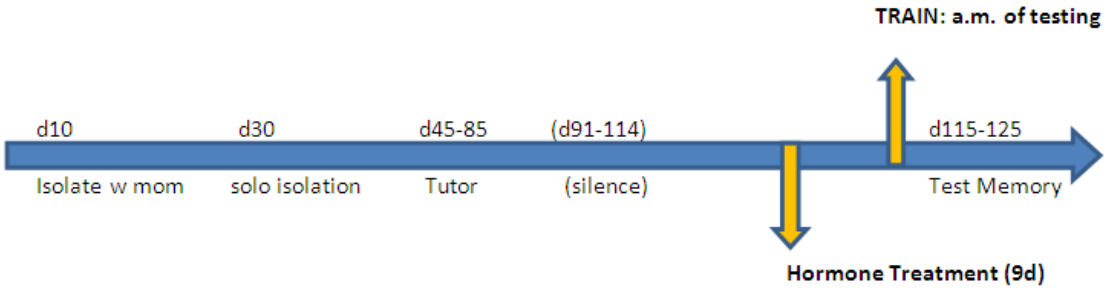

Figure 2.5: Example Box-tutoring experiment. Birds are isolated from adult male song at post-hatch d10, placed into acoustic isolation with their siblings and mothers until they can be individually isolated at d30. They are artificially tutored from d45-85, then remained in silence. Prior to electrophysiological testing, birds receive daily injections of Fadrozole to inhibit estradiol synthesis or saline vehicle. On the morning of testing, birds are "trained" with the Memory stimuli. 6-8 hours later, NCM responses to playbacks of these songs and the Tutor songs are recorded.

placed in front of a speaker) was introduced into the isolation box and the sound playback equipment initialized (Sound Analysis Pro software). By pecking either of two keys (operated via a National Instruments NIDAQ PCI-6503 card) located on the rear wall of the cage, the juvenile bird could elicit tutor-song playback from the speaker a maximum

of twenty times per day (10 times in the morning, and 10 times in the evening). This paradigm has been shown to produce optimal song imitation in young males (Tchernikovski et al, 1998). During this training period, vocalizations were continuously recorded via a microphone (Audio-Technica) located within the cage. The training program was checked daily to ensure that key-pecks elicited song playback and that the computer software was running correctly. At day 85, the tutor-song playback was terminated and the speakers disconnected. Thereafter, the bird remained in isolation until electrophysiological testing (d115-125).

Hormone Treatments:

For experiments involving hormone manipulation, birds were treated with Fadrozole (Novartis Pharmaceuticals, Switzerland) or Estradiol, and control groups received blank implants or injections of saline. For experiments in which Estradiol implants were used: females were randomly assigned to receive Estradiol implants + saline injections, Blank Implants+ Fadrozole injections, or Blank Implants+ saline injections. Treatments began 9 days prior to electrophysiological testing, and the last injection was administered in the evening prior to testing. Validated protocols for estrogen treatment are based on subdermal implants (Harding, 1991), which consist of Silastic tubing (Dow-Corning, NY) filled with 17-beta-estradiol (Steraloids, Newport, RI). Estrogen from these implants may be detected in serum samples between four to five days after implantation and continues to be released for at least 80 days (Maney et al., 2006). The implantation procedure is as follows: Under local anesthetic (Lidocaine

Hydrochloride, 2%), a small incision was made on the dermis of the breast muscle and the implant was placed underneath the skin. The incision was then sealed with cyanoacrylate. The implantation process takes approximately five minutes to perform. After implantation, the first dose of drug or saline was injected into the bird's breast muscle on the side opposite the implant. Fadrozole or saline injections were administered daily for the next 8 days, prior to electrophysiological testing. Validated protocols for Fadrozole treatment in zebra finches require daily injections of 100ug dissolved in 10uL .75% NaCl and has been shown to decrease aromatase activity to 33% after six days of treatment (Wade et al, 1994). In female canaries, daily Fadrozole administration has also been shown to reduce plasma measures of estradiol by 60% and affected egg-laying and CSD to male song (Leboucher et al, 1998). For studies in which estradiol was not administered, birds received the 8 day course of Fadrozole injections, and control animals received saline injections only (birds were not implanted with either estradiol or blank Silastics).

Measuring Estradiol in Brain Tissue:

In order to assess the effects of Fadrozole on brain concentrations of estradiol, a separate group of males (n=3) and females (n=2), that did not undergo electrophysiological recording, were subjected to the same treatment protocol as experimental birds. These birds were sacrificed, and the caudal most portion of the telencephalon containing NCM was dissected out and stored until estradiol assays were performed. With the assistance of the Remage-Healey lab at University of

Massachussetts and according to a standard protocol (Chao et al., 2011). Samples were first homogenized in PBS buffer on ice. Homogenized samples were then extracted in both a liquid phase extraction followed by a slide phase extraction to further remove interfering substances and lipids. During liquid phase extraction, diethyl ether was added to each sample, vortexed for 30 seconds, and then left untouched for 5 minutes so that the organic and aqueous phases would separate. After 5 minutes, each sample was "snap frozen" by suspending the tube in a MeOH/dry ice bath to solidify the aqueous layer, and the unfrozen organic layer was decanted into a new tube. This procedure was repeated twice more on each sample after it had thawed. The collected organic phase was then evaporated under air in a water bath until no liquid remained in the tubes. Each sample was then resuspended in EIA buffer prior to the solid phase extraction procedure. The solid phase extraction procedure involved eluting samples onto C-18 columns and pouring polar solvents down the columns in order to collect the extracted estradiol. Columns were first primed with 100% MeOH followed by distilled water under pressure of a vacuum manifold. Each sample was then loaded onto a column and eluted down the column first with distilled water, then 90% MeOh. After the initial MeOH wash, the final elute was collected, which contained the estradiol fraction. Each sample was again dried under air over a water bath, then stored at -20C until the assay. Samples were equilibrated to room temperature, resuspended in the assay buffer, and vortexed for 30 seconds prior to being assayed according to the ELISA kit instructions. (Cayman Chemical, kit #582251). Fadrozole injected males had lower estradiol levels than saline-injected controls and levels varied between individual males in the saline group (**Figure 2.6**). Estradiol was also lower in the anterior block of telencephalon that was included as

a second sampling region. Only two females were tested in the same assay, and there was considerable variation in saline- and Fadrozole-treated females (one female value excluded in graph).

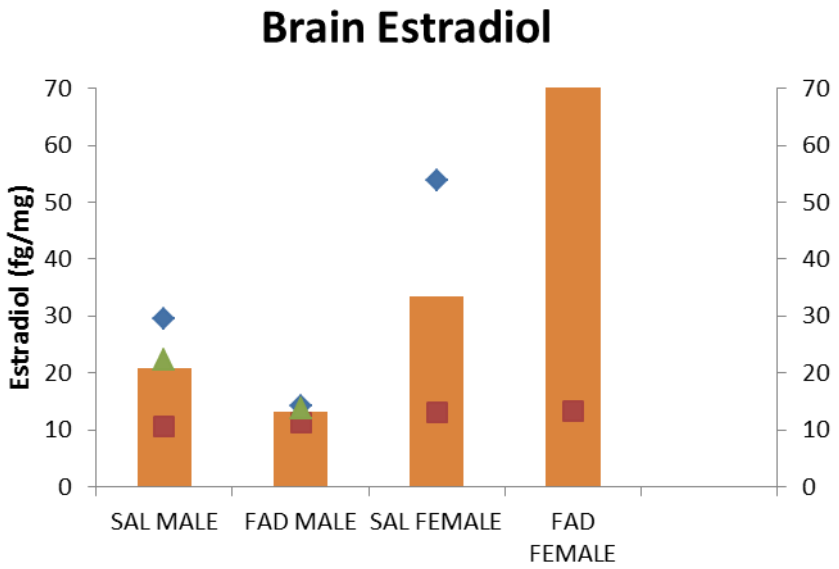

Figure 2.6: Estradiol levels in posterior telencephalon following Fadrozole treatment in males and females (one female in FAD group had value of 289.5, not shown)

In hormonally-treated female box-tutored birds recorded in my previous study, I had attempted to measure estradiol in collaboration with other labs that routinely perform radioimmunoassay (RIA). Originally, I had attempted to measure estradiol in plasma samples from female birds by running these unextracted samples through a standard RIA assay (Diagnostics Systems, Webster, Texas), but observed no differences between the treatment groups using this method. Thereafter, I sent an identical set of samples to a veterinary testing company (Antech Diagnostics, NC) and again gained conflicting results and high estradiol levels among females results that were inconsistent with the treatment groups. I then sought the assistance from the John-Alder lab at Rutgers, where they are familiar with extracting and assaying testosterone from plasma samples. After

several attempts at extraction, the results were not consistent enough to be reliable. These past attempts have yielded problematic results, probably due to high concentrations of lipids (E2 is highly lipophilic) in plasma samples that must be removed prior to the assay procedure and/or to interfering substances that cross-react with the antibody to estradiol. In order to avoid this problem, I subjected another set of animals to the same treatments and collected fecal samples prior to and on the last day of treatments, suspended them in buffer, and sent them to Antech testing facility. These results were promising, in that the Fadrozole birds had lower readings of estradiol than the saline group. Recently, I contacted Luke Remage-Healey (UMass, Amherst) and he assisted me in running the extractions and assay on brain tissue samples, a procedure he had developed at UCLA with Barney Schlinger (Chao et al., 2011). While the data are few at this point, the results show a trend toward Fadrozole lowering estradiol in the caudal telencephalon of male birds, and the data are too variable to make any conclusions in female birds.

Histology:

Approximately 48 hours following recording, animals were killed by an overdose of Nembutal (0.15 cc) administered by intramuscular injection. They then were perfused by intracardial infusion of saline followed by 4% paraformaldehyde. Following decapitation, brains were removed from the skull and refrigerated in paraformaldehyde for at least three days to allow for preservation of tissue. Brains were sliced in 50 uM sagittal sections and mounted on Fisher DuraFrost Slides (Fisher Scientific, Pittsburgh PA). Dried sections were stained with Cresyl-Violet, coverslipped, and visually analyzed for lesions under a microscope. Since NCM is bounded either by ventricular space or by the rostrally-located strip of darkly-staining cells that constitute Area L (**Figure 2.7**), recording sites that lay caudal to Area L verify placement within NCM.

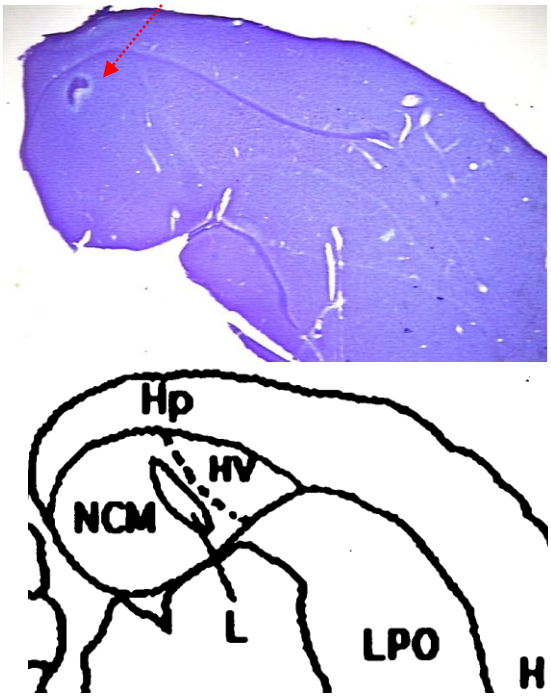

Figure 2.7: Example of a lesion made at a recording site in NCM (red arrow). Lesions were made following electrophysiological testing. Tissue sections were stained with Cresyl-Violet, and examined for electrode placement. NCM is bounded dorsally by ventricular space that separates it from the hippocampus (Hp) and rostrally by the darkly stained cells of Area L. (Chew et al., 1996)

Fadrozole Effects on Vocalizations:

Because I tested for changes in auditory responses that resulted from hormone manipulations, and I wanted to test whether the treatments themselves had induced changes in vocalizations, particularly in males. I recorded the vocalizations of animals that were assayed for brain estradiol levels prior to and during the treatment phase (n=6), in addition to males that were included in the box-tutoring paradigm (n=13). All sound recordings were then filtered using the Sound Analysis Pro feature to extract song vocalizations from noise not related to the animals' vocalizations (cage noise, e.g.). Once files had been filtered, the overall size of the folder and the number of sound files within it were documented for 5 days prior to treatment and 5 days during the treatment period for each bird. A composite measure was created in order to represent the total vocal data of each bird within a given day (number of files per day*total size of folder per day). From these values, a Vocalization Index was calculated to represent the change in vocalizations from pre- to post-treatment (VI= average vocal data post-treatment/ average vocal data post-treatment). VIs less than 1 reflect decreases in singing from baseline and VIs greater than one reflect increases in singing from baseline. VIs varied among individual males. T-tests were conducted to compare VIs between treatment groups. Vis were not significantly different between Fadrozole- and saline-treated males ($p>.05$).

Chapter 3: Response Characteristics and Memory for Conspecific Songs in Untreated Male and Female Zebra Finches

Rationale:

The primary goal of this experiment was to identify potential sex differences in auditory processing of conspecific songs and neuronal memory for recently heard songs. For males, discrimination of individual songs and their features is essential for the song imitation process. The ultimate quality of their song influences their attractiveness to potential mates. For females, exposure to song as juveniles influences their preference for similar songs in adulthood, and she chooses a mate based on song preferences (Riebel, 2000). Both males and females also use auditory discrimination for individual recognition (Miller, 1979a, 1979b; Vignal et al., 2006). However, behavioral studies have shown that female zebra finches perform poorly compared with males on tasks requiring them to discriminate among songs, and so suggest that females might process songs differently than males. Earlier observations from our lab in box-tutored birds suggest that quantitative differences exist between responses in male and female NCM (Phan and Vicario, 2010, see Figure 1.8) but were not analyzed or commented upon in the paper. This experiment will explicitly explore those differences.

To carry out this comparison, I constructed a large dataset that includes NCM recordings from males and females that had been tested in the same experimental paradigm but were reared in two different environments (aviary, box-tutored). This dataset also includes hormonally manipulated males and females that were reared in both conditions. The full set of conditions and treatments is laid out in **Figure 3.1** Hormonal

effects on NCM response characteristics will be described in Chapter 4. Subsequent chapters will describe results on Tutor-song memory (Chapter 5) and preference for conspecific over heterospecific songs (Chapter 6). The present chapter describes results from untreated birds. I examined sex differences in response magnitude, adaptation rates for conspecific song, and the neuronal memory for recently heard songs that were presented as the "training" stimulus set described in the General Methods section (Chap 1). As mentioned previously, the term "memory" within these experiments refers to a differential neuronal response to familiar versus novel songs, as measured by the Familiarity Index. The validity and limits of this operationalized definition will be discussed in Chapter 7.

Question3a: Do untreated males and females exhibit differences in NCM response characteristics to conspecific song?

Question 3b: Does rearing environment influence the response magnitude and adaptation rates for conspecific songs?

Subjects:

From the dataset in Figure 3.1, I tested untreated males and females. All birds heard the "Memory Set" described in the General Methods Section in the morning and then were tested 6-8 hours later for NCM responses to playbacks. This design allowed me not only to test responses to the novel songs presented during the testing session, but also to test

the familiarity of the training songs that were played to the bird 6- 8 hours prior to NCM recording. NCM neural responses were previously shown to have robust recognition memories at this time point (Chew et al, 1996a and 1996b). To test sex differences in NCM, I collapsed rearing conditions to test for overall sex differences, and then compared males and females within each rearing condition.

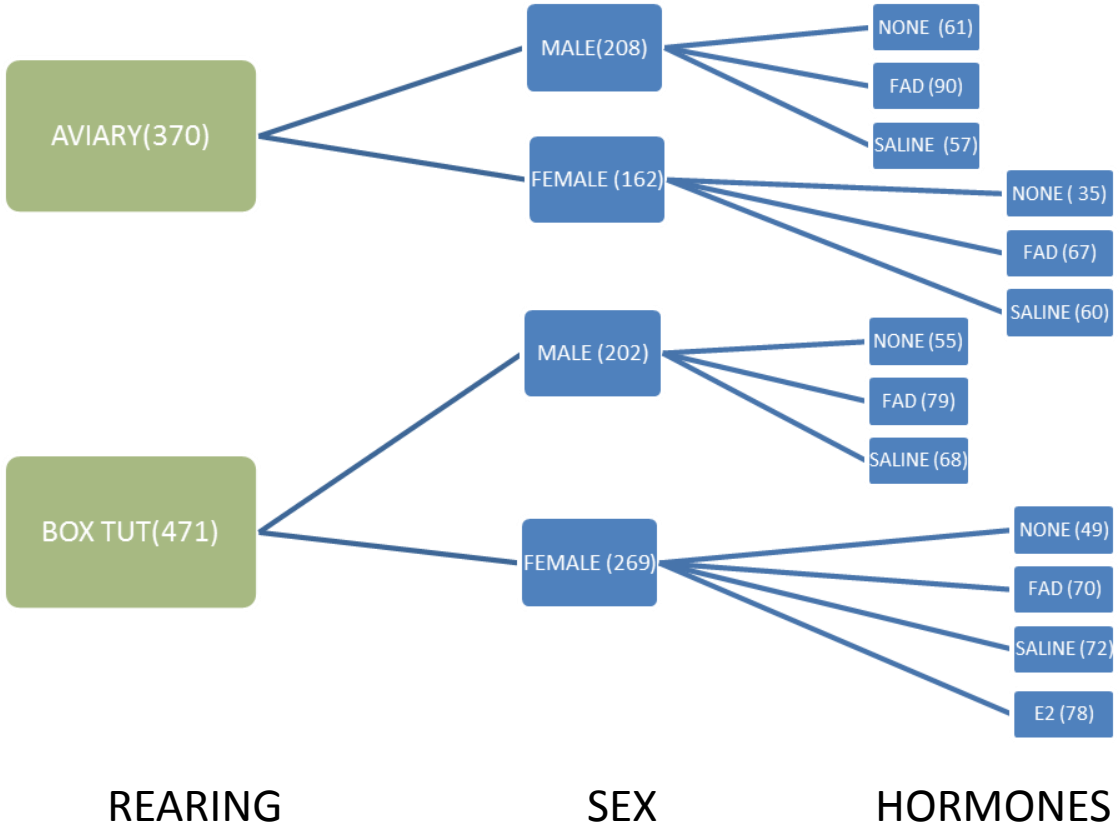

Figure 3.1: Data compiled from previous and current experiments. Aviary birds were housed in the general aviary and had the highest degree of auditory (and social) experience with conspecific vocalizations. Box tutored birds were raised in isolation from male song shortly after hatching and were artificially tutored during the sensitive period for song learning (~d45-85). Isolate birds were raised in isolation and did not undergo tutoring. As adults, some birds received hormone treatments and were isolated for 8d. All birds received playback "training" on the morning of testing, then had NCM recorded 6-8hrs later during the playback "testing" session

Results:

Experimental Question3a: Do untreated males and females exhibit differences in NCM response characteristics to conspecific song?

Absolute Response Magnitudes:

When rearing conditions were collapsed, response magnitudes were not significantly different between untreated males and females (KS, p=.92: **Figure 3.2**). Even when rearing conditions were considered separately, there were no significant differences in response magnitudes between aviary-reared males and females (KS, p=.91; **Figure 3.3**) or between box-tutored males and females (KS, p=.85; **Figure 3.4**).

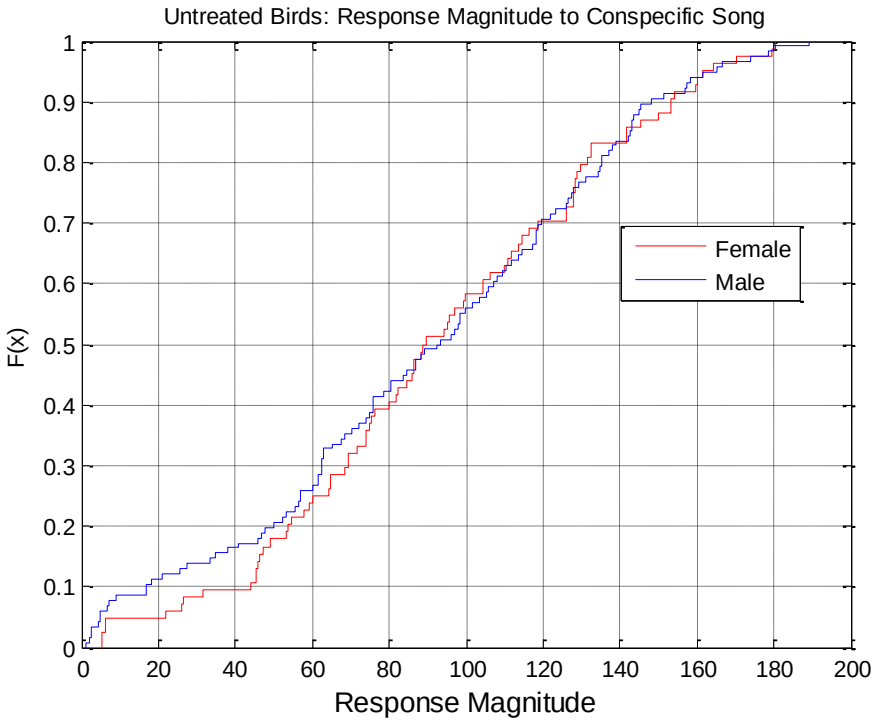

Figure 3.2: Response magnitudes do not differ significantly between untreated males and females, in collapsed housing

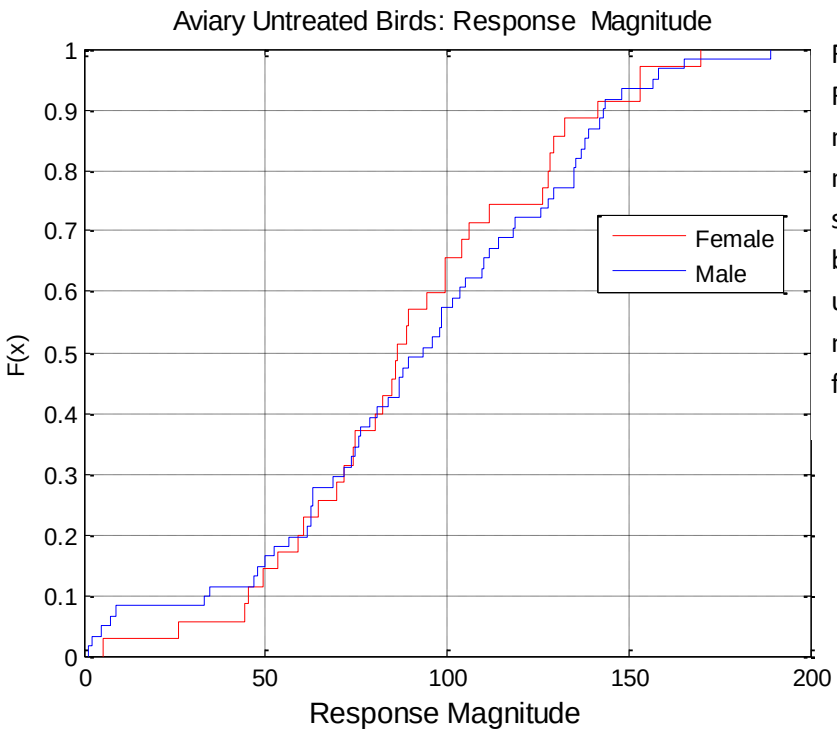

Figure 3.3: Response magnitudes do not differ significantly between untreated aviary males and females.

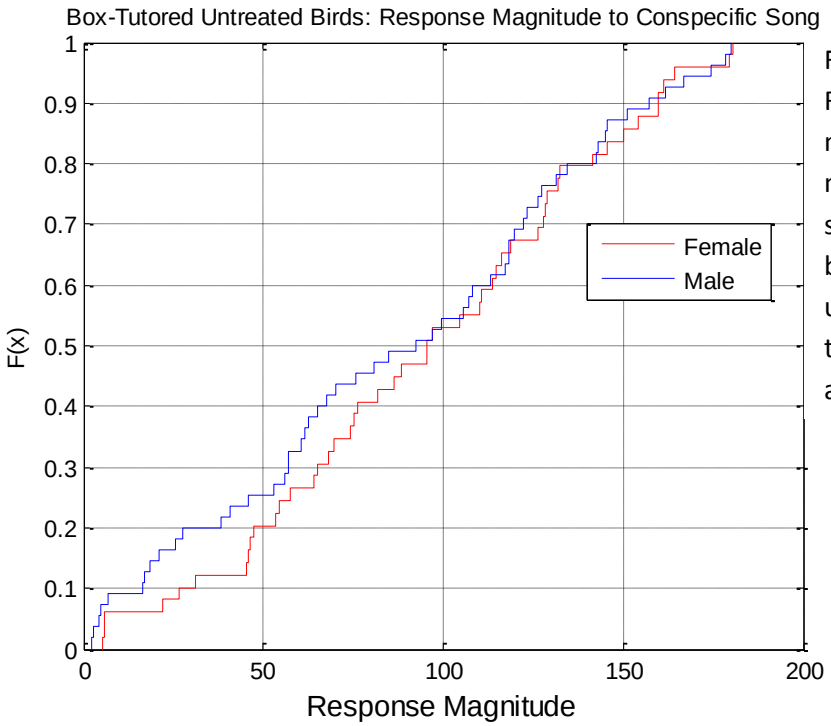

Figure 3.4: Response magnitudes do not differ significantly between untreated box-tutored males and females.

Adaptation Rates:

Taking birds from both housing conditions together, females had significantly lower adaptation rates than males (KS, p=.02; **Figure 3.5**). I then compared the distributions of adaptation rates of males and females reared according to different conditions. The distribution of adaptation rates in aviary-reared females was not different than the distribution for aviary-reared males (KS, p=.48; **Figure 3.6**), but these distributions were significantly different in box-tutored males and females (KS, p=.03; **Figure 3.7**).

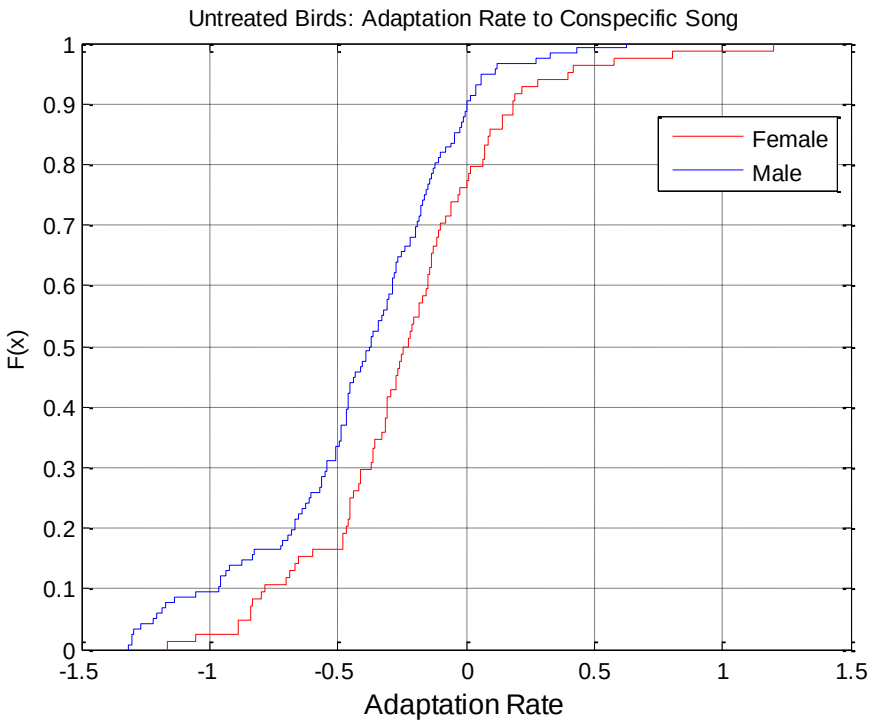

Figure 3.5: Adaptation rates differ significantly between males and females, in collapsed housing condition

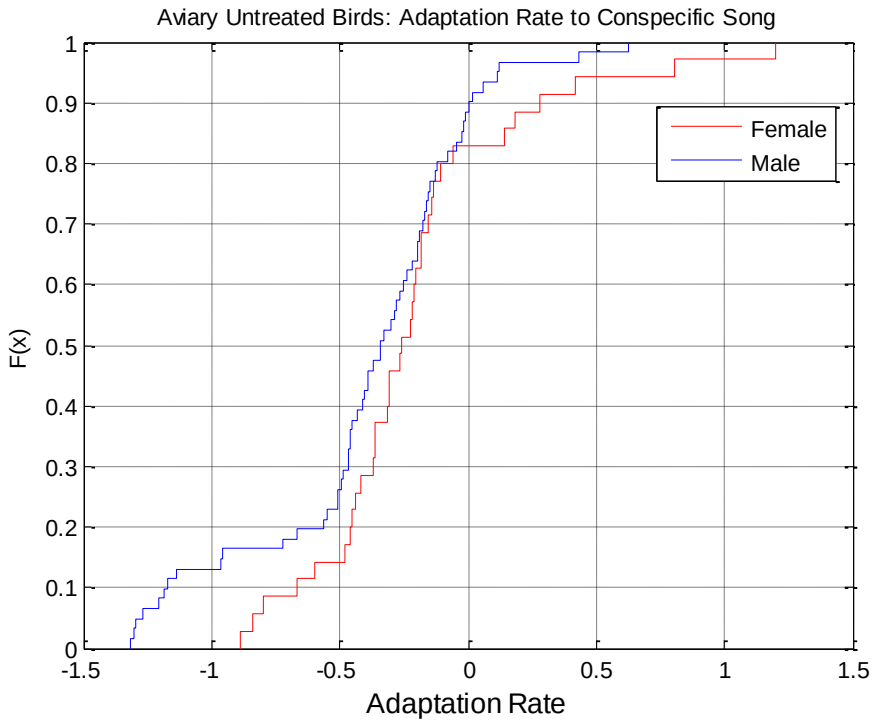

Figure 3.6: Adaptation rates do not differ significantly between males and females that were reared in the aviary

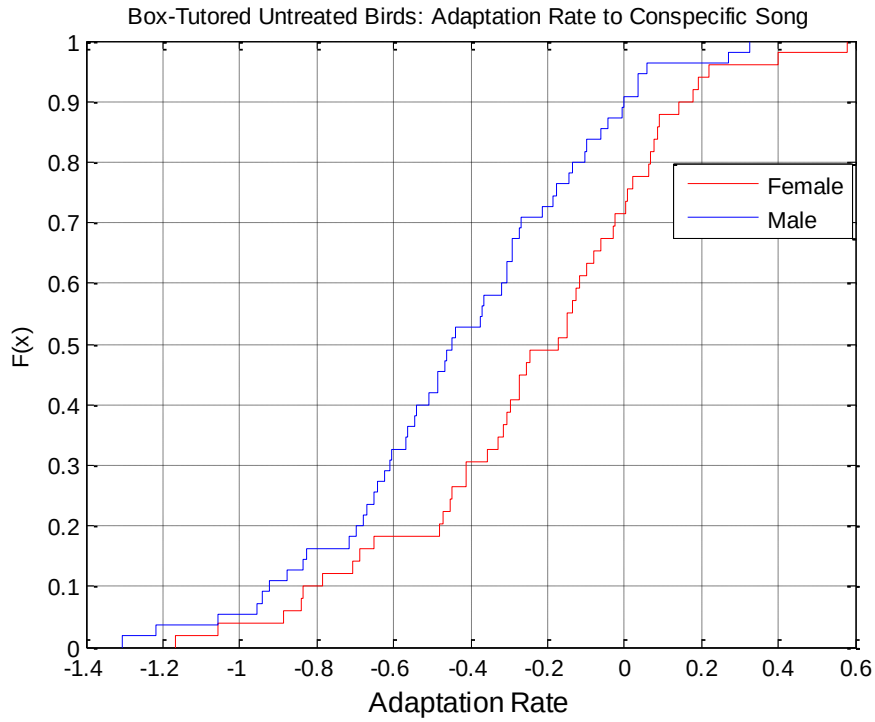

Figure 3.7: Adaptation rates do differ significantly between males and females that were box-tutored.

Memory for Conspecific Song:

To assess memories for recently heard songs, I presented the "training" songs heard 6-8 hours earlier in shuffled sets with completely novel songs in order to calculate Familiarity Indices for the "training" songs (see Methods). For songs that show no recognition memory, the FIs will distribute around 1.0. Both males and females show FIs that are somewhat larger than 1.0, but the distributions for males and females were not significantly different (KS, p=.58; **Figure 3.8**). When I compared the distributions within each rearing condition, I found similar results; in both aviary reared and box-tutored birds, the distributions of FIs were not significantly different for males and females (**Figure 3.9**; KS, p=.57 and **Figure 3.10**; KS, p= .56, respectively).

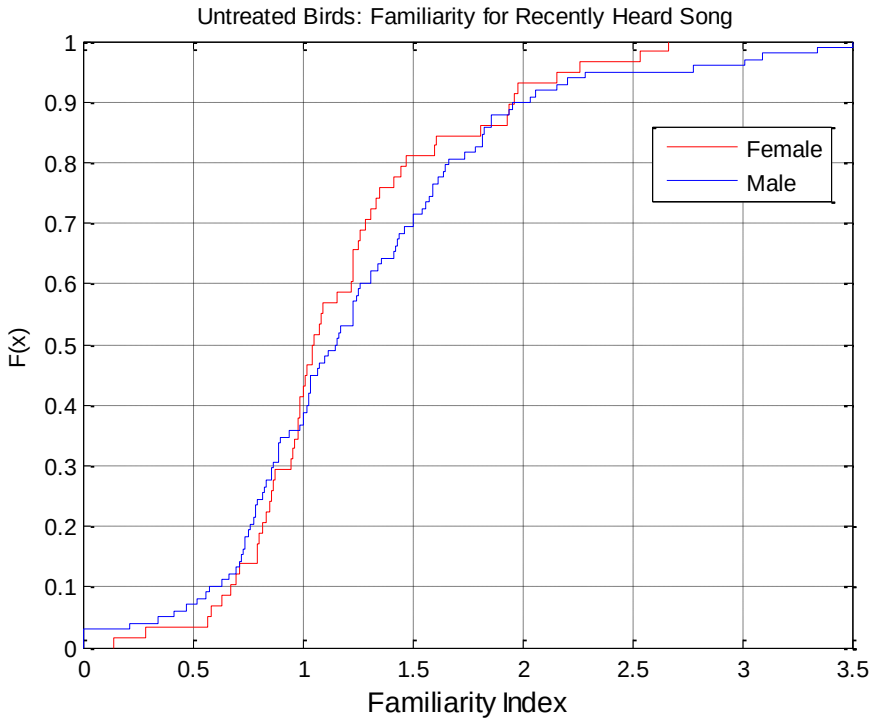

Figure 3.8: Familiarity indices for recently heard song do not differ significantly between males and females, collapsed across rearing conditions.

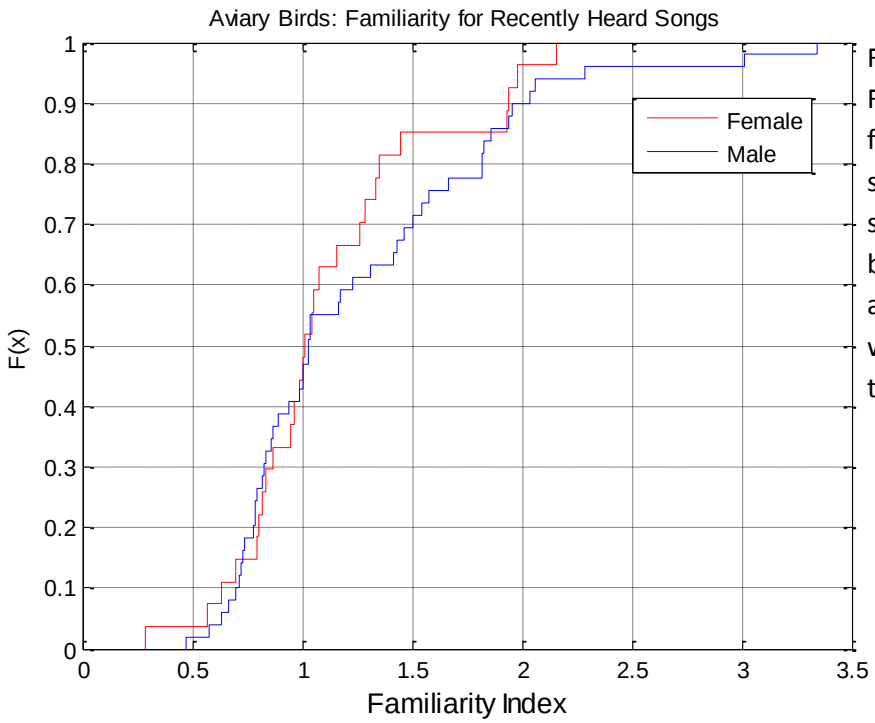

Figure 3.9: Familiarity indices for recently heard song do not differ significantly between males and females that were reared in the aviary.

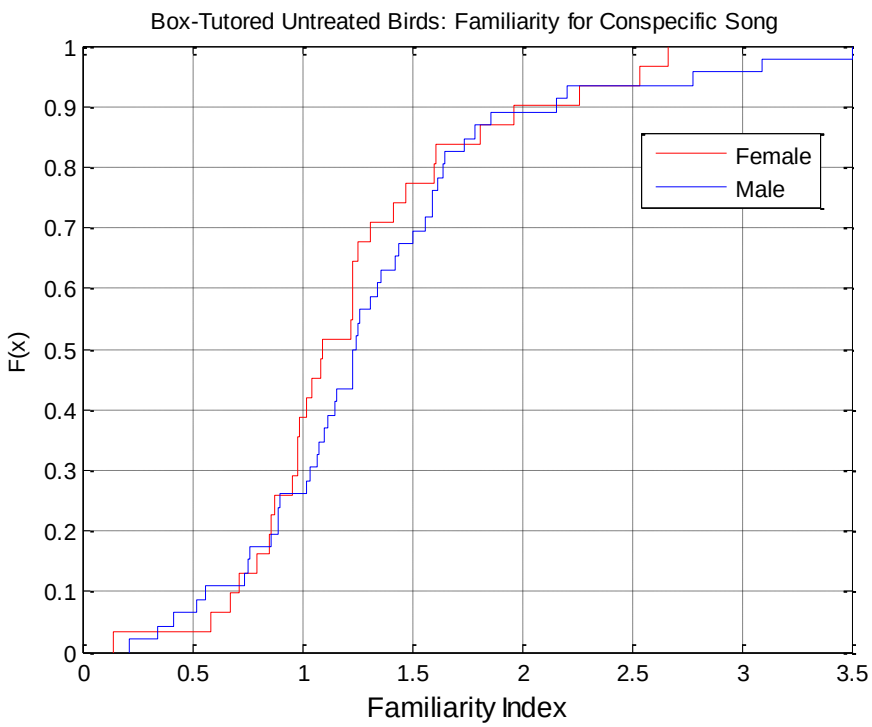

Figure 3.10: Familiarity Indices do not differ significantly between males and females that were box-tutored.

Experimental Question 3b: Does rearing environment influence the response magnitude and adaptation rates for conspecific songs?

Absolute Response Magnitudes, Adaptation Rate, and Memory for Songs

I tested whether rearing condition influenced response magnitudes *within* each sex. For all measures, distributions for males and females were not significantly different for box–tutored and aviary reared birds. Distributions of box-tutored males were not different from those of aviary males on measures of response magnitude (KS, p=.35; **Figure 3.11**) or adaptation rates (KS, p=.33; **Figure 3.12**). Although there was a strong trend for box-tutored males to have higher FIs than aviary males, this difference was not significant (KS, p=.05; **Figure 3.13**). Distributions of box-tutored females were not significantly different from those of aviary females for response magnitude (p=.61), adaptation rate (KS, p=.33) or FI (KS, p=.71).

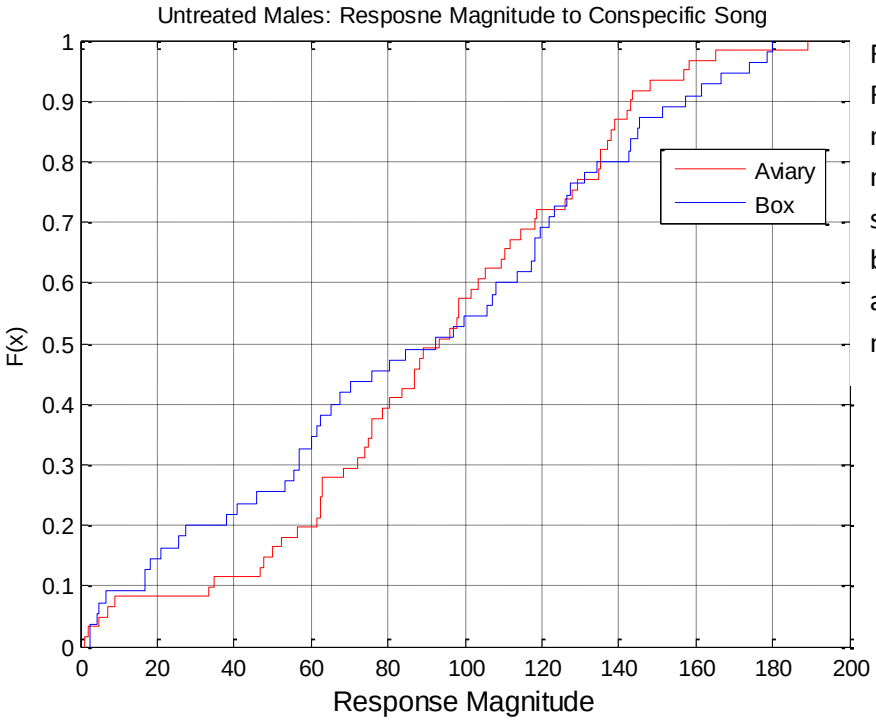

Figure 3.11: Response magnitudes do not differ significantly between aviary and box-tutored males

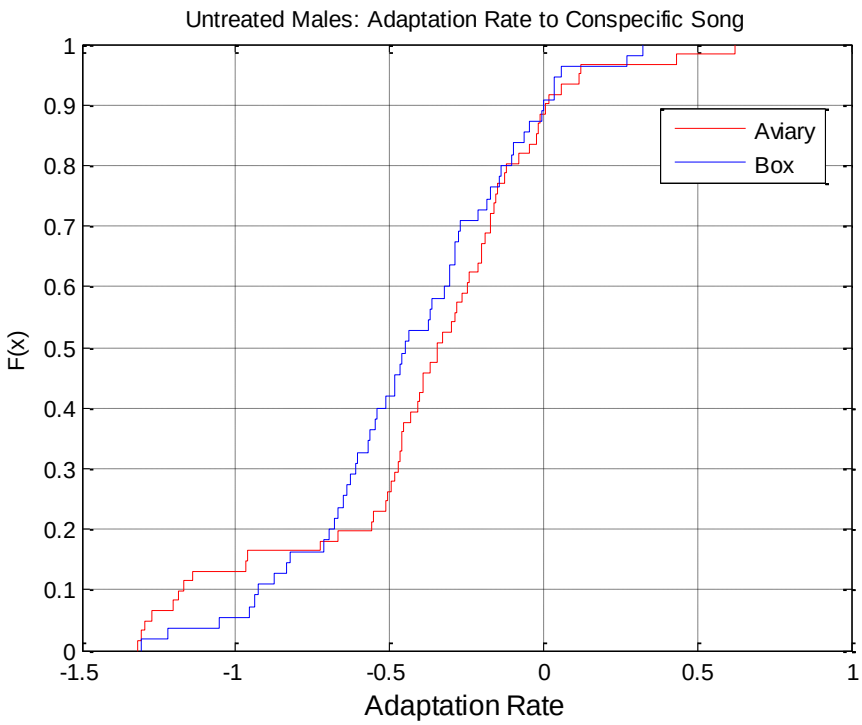

Figure 3.12: Adaptation rates do not differ significantly between aviary and box-tutored males

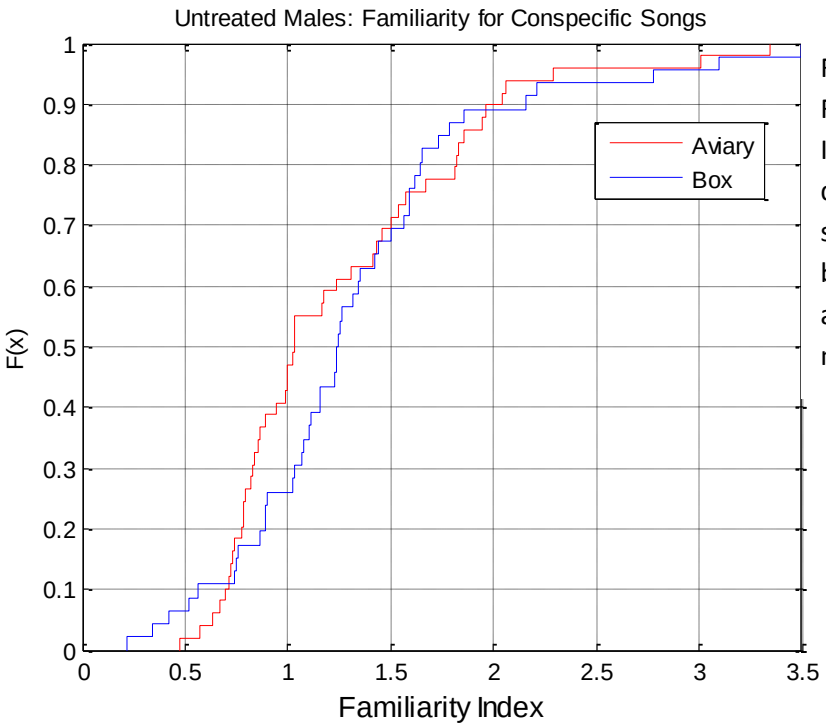

Figure 3.13: Familiarity Indices do not differ significantly between aviary and box-tutored males

I also conducted an ANOVA for each measure, using rearing environment and sex as factors. There was a main effect of sex on adaptation rate ($F(1, 196) = 11.28$, $p < .0001$), as was observed in the distribution differences, but no significant interaction between rearing environment and sex ($p = .97$).

Conclusions:

The results of these analyses suggest that there are underlying sex differences in song processing in NCM, and that these differences are magnified when animals are reared in individual isolation from conspecifics with exposure limited to a single artificial tutor-song during development. Specifically, although response amplitudes were similar for males and females, females exhibited shallower adaptation rates than males did to conspecific songs. This difference was exaggerated in box-tutored birds, who are deprived of the rich social and auditory environment of the aviary, with daily exposure to many conspecific songs. In both sexes, the distributions of adaptation rates were not significantly different between aviary and box-tutored birds. Box-tutored males and females both hear the tutor an equal number of times, but males hear themselves making spectrally-modulated vocalizations in addition to this auditory input. Given that box-tutored males are hearing their own male-typical vocalizations (songs and male long calls) within the box and that females do not sing (they do produce unlearned calls that have little or no spectral modulation), it may be that males have greater experience with acoustic features that differentiate songs. With respect to this experience, any given novel song may be better distinguished - more “novel” - and thus has a high initial response that

adapts rapidly. Females reared only hearing themselves and an artificial tutor song only 20 times a day, may not fully develop the auditory filters needed to hear acoustic details that differentiate songs from each other.

Another possibility suggested by this sex difference is that higher adaptation rates could provide some function in the song-learning process during development or in song production in males, which other areas (e.g. song control areas) may be using to produce matched vocalizations during song-learning (or, in species with territorial song matching, adult song-memorizing of intruder songs). If this were the case, then females as non-singers, who do not engage in vocal learning (even for their calls) would not require the higher adaptation rates because they do not learn to sing. Possibly, greater adaptation rates to novel song could contribute to forming a better memory for the specific features of the song heard. If this were the case, then males would have a better memory with which they could compare their own vocalizations (as juveniles when they are learning to sing or as adult birds that participate in song-matching). If this were the case, then males would form better memory for songs than females do, as a result of them needing to know the specific features of a song before they can adequately copy them. The results of the current analysis suggest that male memories were not higher for the training songs presented prior to testing; FI distributions were not significantly different for males and females. Both males and females have an FI greater than 1, reflecting that the adaptation rates were lower to familiar songs (which were played to the birds prior to testing) and that there was neuronal memory at those sites.

Yet another interpretation is gained one considers the adaptation rate with respect to response magnitude. Because the adaptation rate reflects the decrease in response magnitude over repeated presentations (across trials 6-25) of a song, this means that in males, the responses drop quickly and for females, the responses drop more slowly. In other words, a female may have more sustained responses in NCM when she hears a song compared to a male. This sex difference in the adaptation rate could reflect the different functional role that song plays for males and females in this species. For females, song serves as an "honest" signal of a potential mate's fitness, and so the sustained response to a song may be evidence that a female has sustained attention to a song even as she hears it over and over again. This sustained response potentially contributes to memory acquisition for the songs of potential suitors. Since females will hear these songs sequentially within many different social interactions and across various timepoints, storing a memory for the songs she hears would enable her to compare many males songs against one another and return to the male of her choice (discussed in detail in Chapter 7).

Chapter 4: Effects of Hormone Manipulation on Response Characteristics and Memories for Conspecific Songs in Adult Males and Females

Rationale:

The results of the previous analysis showed a substantial sex difference in the processing of and memory for conspecific songs: box-tutored females showed lower adaptation rates than males to novel songs, but response magnitudes were similar. Recent studies have shown that estradiol in males is required in NCM for the expression of NCM-typical responses to to conspecific song playbacks (i.e. increased firing rate and immediate early gene induction) and for behavioral preference to be exhibited to some forms of familiar songs (Remage-Healey et al., 2008; Tremere et al., 2006; Pinaud et al., 2011). No sex differences were reported in studies in which females have been tested for physiological responses according to the same criteria (Tremere et al., 2006), but gene expression studies have shown that estradiol-deprived females do not discriminate between conspecific songs and frequency-matched tones (Maney et al., 2006). These studies suggest that estradiol may be required in both sexes for NCM to process song normally and thus enable discrimination among sounds. In this experiment, I tested whether estradiol depletion would influence 1) response magnitudes 2) adaptation rates and 3) the memory for recently heard songs in birds that were housed in the general aviary and in the box-tutoring paradigm. The analysis is organized by housing condition and sex, as follows:

Experimental Question 4a: **Does estradiol manipulation influence response magnitude, adaptation rate, and memories for recently heard songs in aviary-reared males and females?**

Experimental Question 4b: **Does estradiol manipulation influence response magnitude, adaptation rate, and memories for recently heard songs in box-tutored males and females?**

Experiment 4 Methods:

Subjects:

Adult birds (>120 days old) from the general aviary were used for this experiment. Aviary birds were housed in single sex cages within a climate controlled room where they can see and hear the vocalizations of other birds. They were maintained on a 12:12 light cycle and were provided access to food and water *ad-libitum*. On the ninth day prior to testing, male (n=10) and female (n=10) birds were removed from the aviary and placed within isolation chambers in order to reduce their level of exposure to conspecific sounds (birds heard their own vocalizations, including learned vocalizations in males) and to parallel the conditions of adult treatments administered in my developmental experiments. During the course of treatment birds were placed into isolation, where they were deprived of auditory exposure with the aviary and where their own vocalizations could be recorded. These recording files were later examined for to assess possible treatment effects on the frequency of songs and calls in males and females.

Hormone Treatments:

Adult male and female zebra finches were randomly assigned to receive either Saline injections (n=10), or the aromatase inhibitor Fadrozole (Novartis Pharmaceuticals, Switzerland) injections (n=10), 8 days prior to electrophysiological testing. Birds received daily injections each afternoon for Days 1-7, and received a final injection on Day 8 on the evening prior to testing. Validated protocols for Fadrozole treatment in zebra finches require daily injections of 100ug dissolved in 10uL of 0.75% NaCl; this dose has been shown to decrease aromatase activity to 33% after six days of treatment (Wade et al, 1994). Following the isolation/treatment period, all training, testing, and data analysis procedures were conducted as described in the General Methods section.

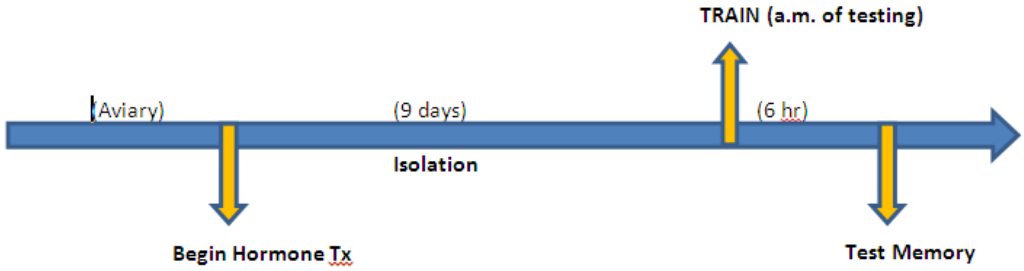

Figure 4.1: Estradiol Depletion and Memory in Aviary Males and Females. Birds were removed from the aviary, administered their first dose of drug or hormone, and placed into isolation boxes, where they will remain in silence until the test day. On the morning of testing, birds undergo playback "training." Six hours later, they are tested for NCM responses to playbacks of these and novel songs to test their short-term memory for the training songs.

Results:

Experimental Question 4a: **Does estradiol manipulation influence response magnitude, adaptation rate, and memories for recently heard songs in aviary-reared males and females?**

Response Magnitudes in Aviary Reared Birds:

Response magnitudes did not differ significantly between Fadrozole- and Saline treated females (KS, p=.8228, **Figure 4.2).** Although Fadrozole-treated males showed a weak trend toward lower response magnitudes, they did not differ significantly from Saline-treated males (KS, p=.1209, **Figure 4.3**). In addition, there were no significant differences in response magnitude between the sexes for saline-treated males and females (KS, p=.434, **Figure 4.4**).

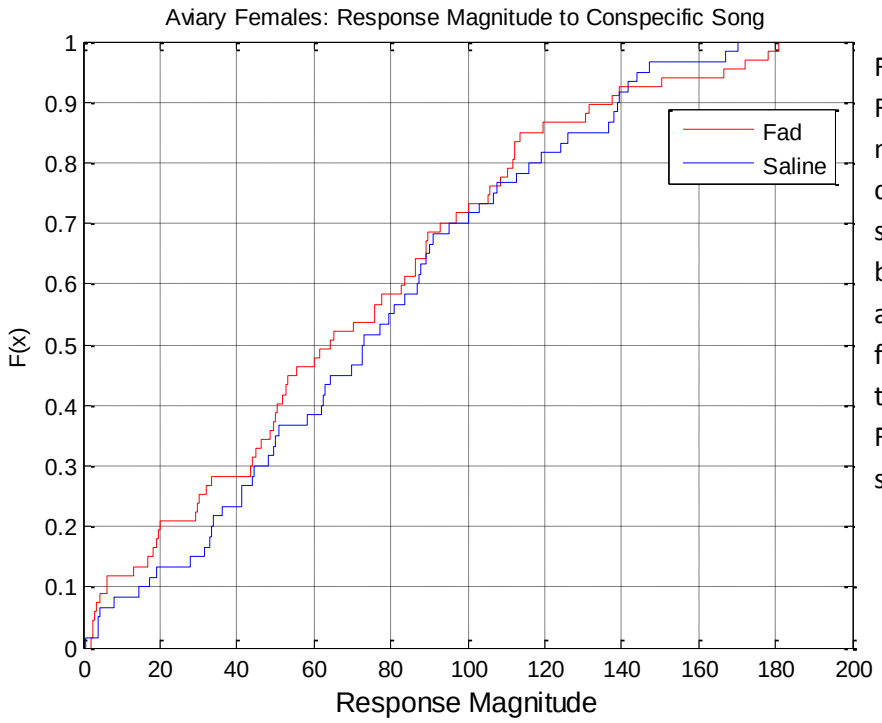

Figure 4.2: Response magnitudes did not differ significantly between aviary-reared females treated with Fadrozole or saline

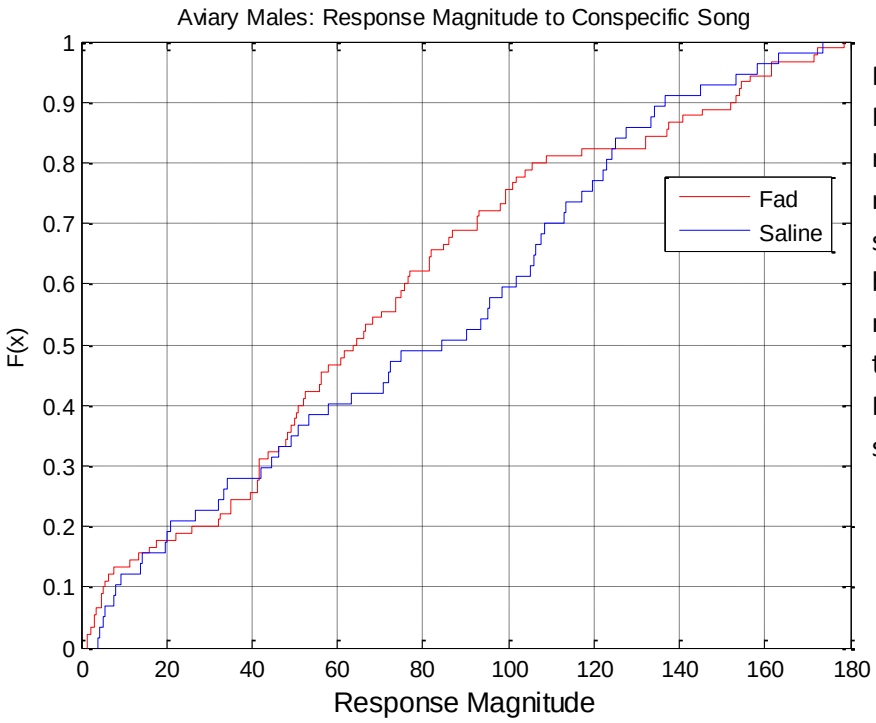

Figure 4.3: Response magnitude did not differ significantly between aviary-reared males treated with Fadrozole or saline

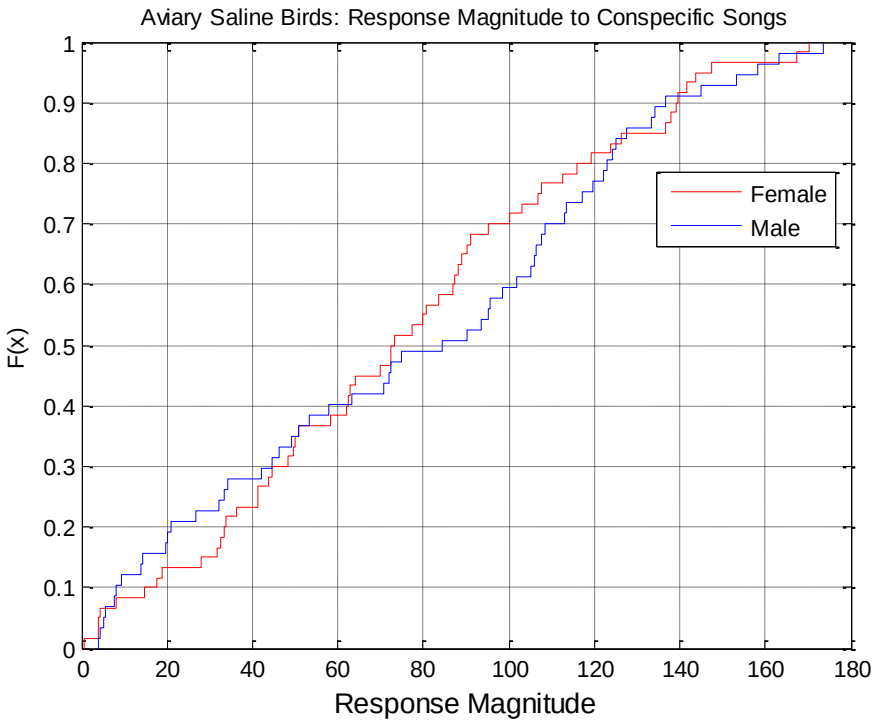

Figure 4.4: Response magnitude did not differ significantly between aviary-reared males and females treated with saline.

Adaptation Rates in Aviary Birds:

Fadrozole treatment did not significantly influence adaptation rates to conspecific song in males (KS, p=.3667, **Figure 4.5**) or in females (KS, p=.6608, **Figure 4.6**). Adaptation rates also did not differ significantly between saline-treated males and females (KS, p=.4557, **Figure 4.7**).

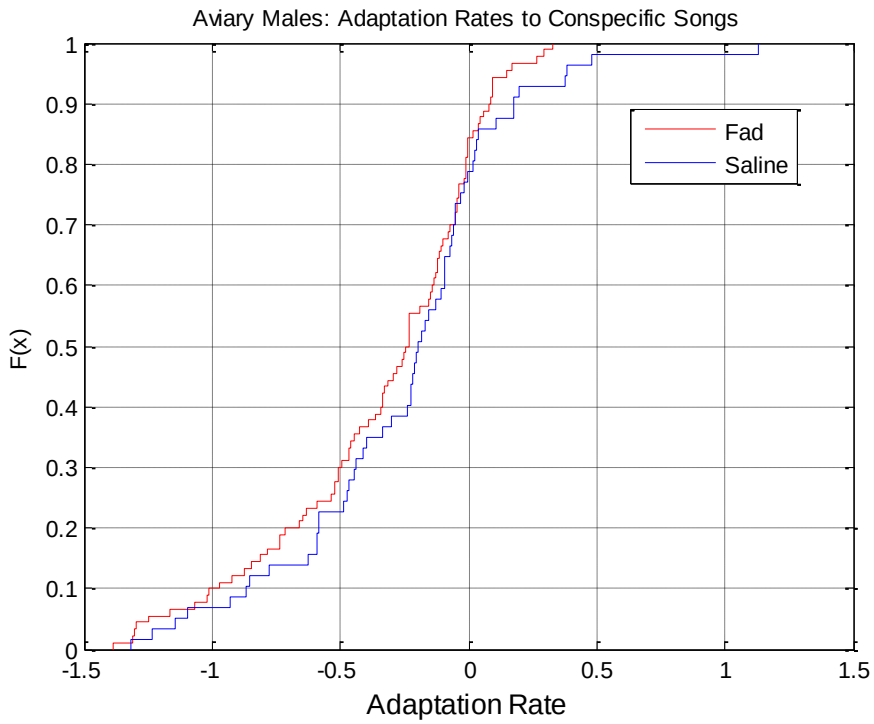

Figure 4.5: Adaptation rates do not differ significantly between Fadrozole and saline-treated males.

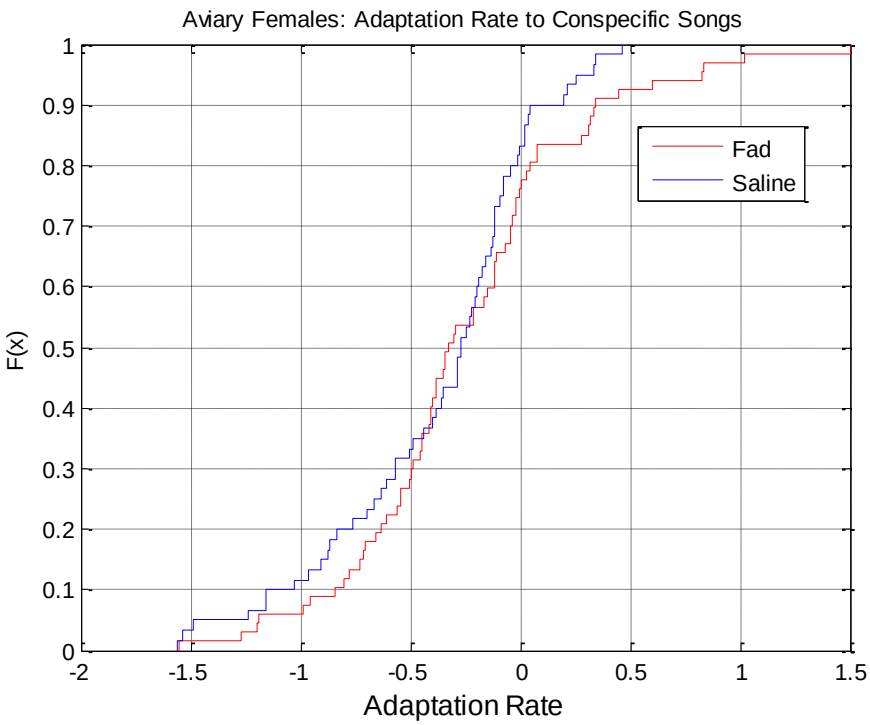

Figure 4.6: Adaptation rates do not differ significantly between Fadrozole- and saline-treated females.

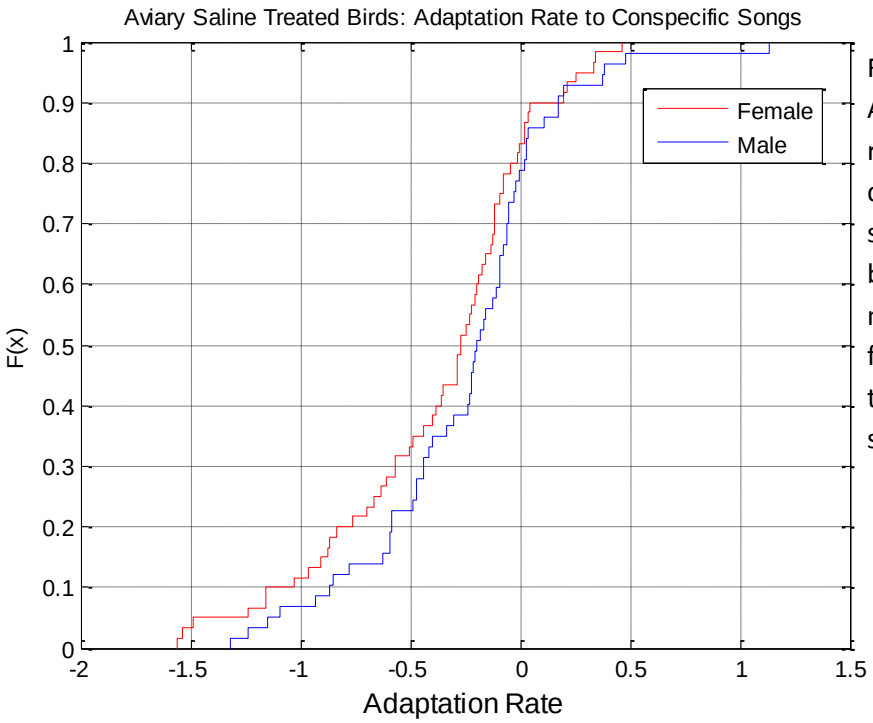

Figure 4.7: Adaptation rates do not differ significantly between males and females treated with saline.

Familiarity for Recently Heard Songs in Aviary Birds:

Fadrozole did not significantly reduce the FIs in females (ks, p=.1779, **Figure 4.8**) but it did decrease FIs significantly in males (KS, p=.006, **Figure 4.9**). It appears that Fadrozole reduces the neuronal memory for the training songs in aviary males, but not females.

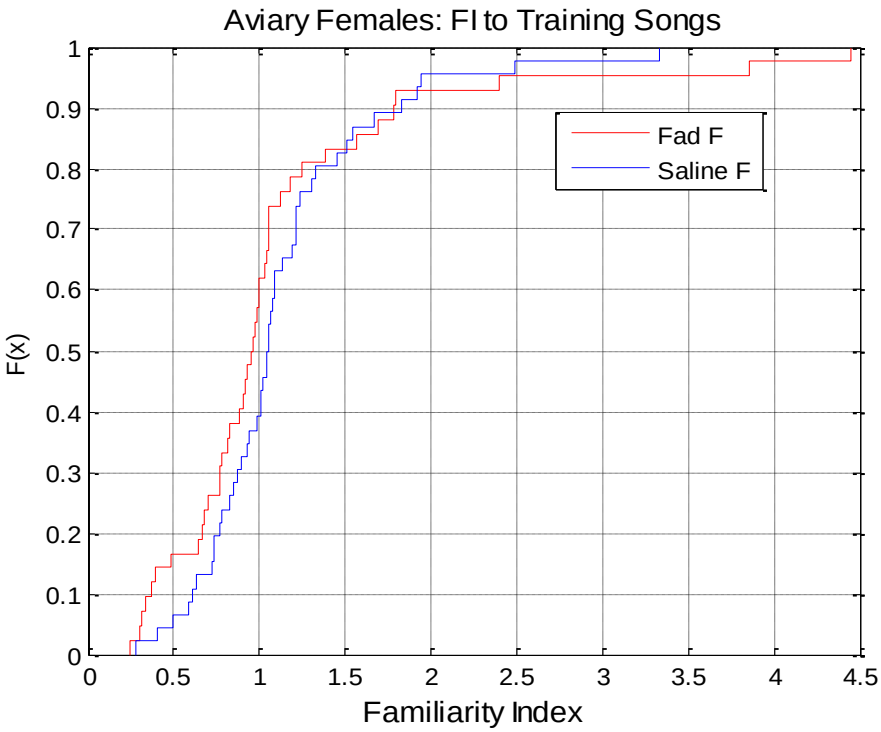

Figure 4.8: Familiarity Indices for recently heard song did not differ significantly between Fadrozole- and saline-treated females reared in the aviary.

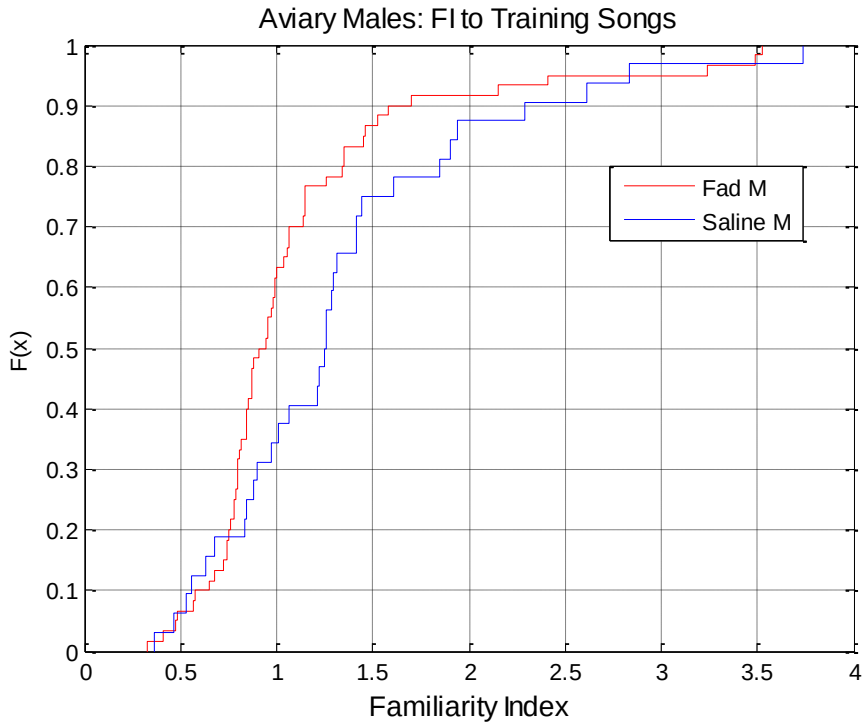

Figure 4.9: Familiarity Indices for recently heard song differed significantly between Fadrozole- and saline-treated males reared in the aviary.

Experimental Question 4b: **Does estradiol manipulation influence response magnitude, adaptation rate, and memories for recently heard songs in box-tutored males and females?**

Response Magnitudes in Box-Tutored Birds:

In contrast to the lack of effect in aviary birds, Fadrozole treatment significantly decreased response magnitudes to song in box-tutored females (KS, p=.0084, **Figure 4.10**). However, Fadrozole treatment did not significantly influence response magnitudes in box-tutored males (KS, p=.2660, **Figure 4.11**). When response magnitudes were compared between saline-treated males and females, the two distributions were not significantly different (KS, p=.1196, **Figure 4.12**), although females showed a trend toward higher response magnitude than males.

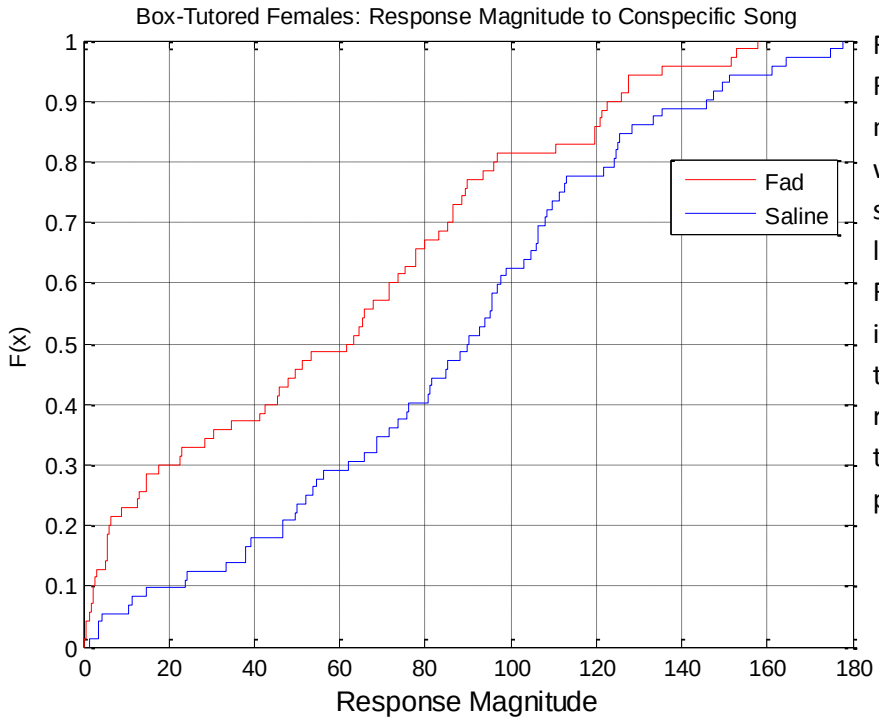

Figure 4.10: Response magnitudes were significantly lower in Fadrozole- than in saline-treated females reared in a box-tutoring paradigm.

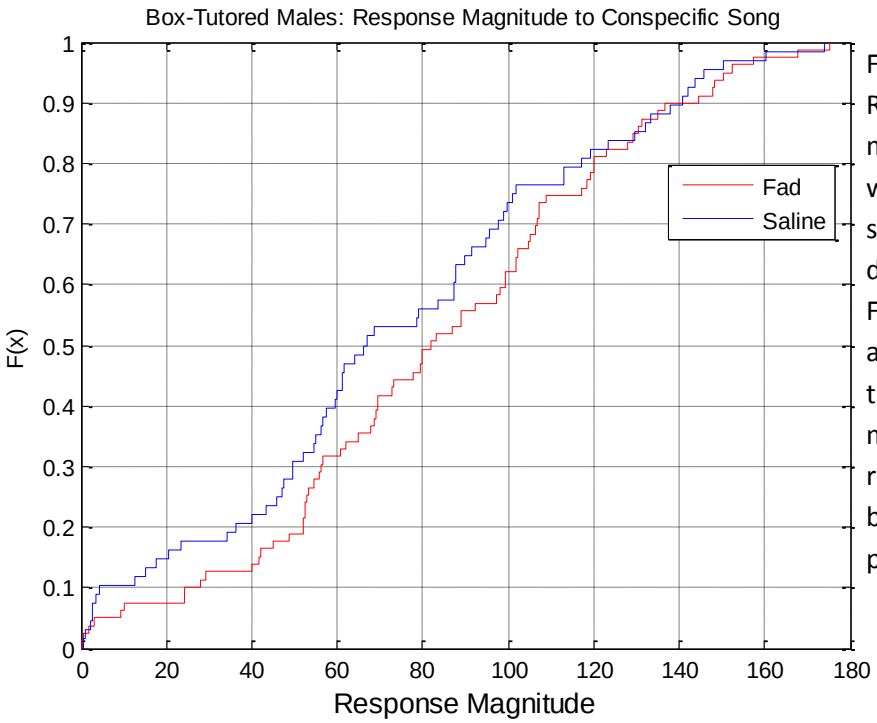

Figure 4.11: Response magnitudes were not significantly different in Fadrozole- and saline-treated males reared in a box-tutoring paradigm.

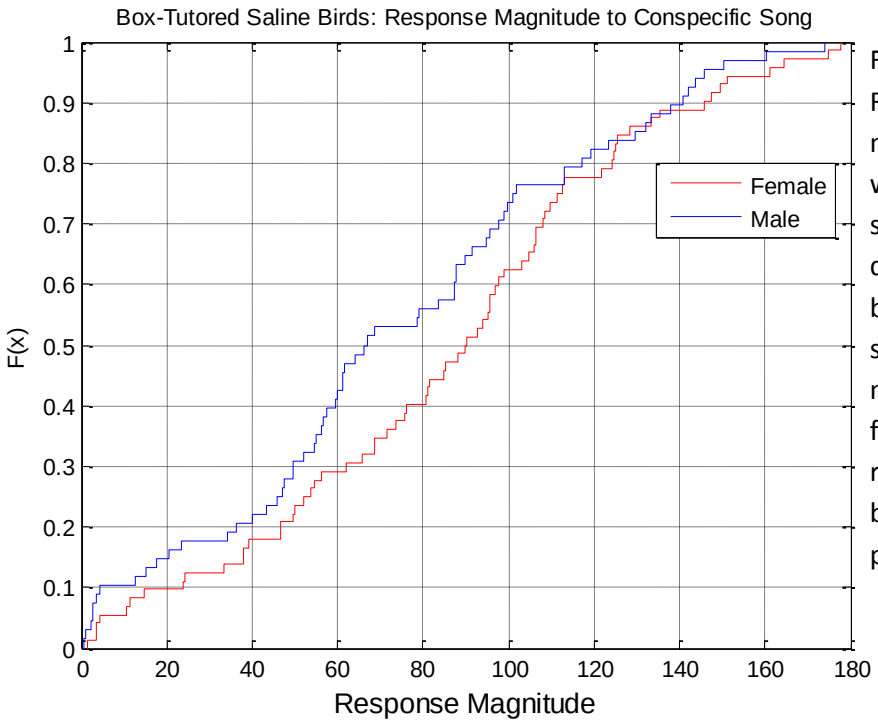

Figure 4.12: Response magnitudes were not significantly different between saline-treated males and females reared in a box-tutoring paradigm.

Adaptation Rates in Box-Tutored Birds:

Fadrozole did not induce significant changes in adaptation rate in box-tutored females (KS, p=.8057, **Figure 4.13**) or in males (KS, p=.1765, **Figure 4.14**). However, comparison of adaptation rates between saline-treated males and females showed a strong trend for females to have lower adaptation rates **(Figure 4.15),** but no significant difference (KS, p=.0662).

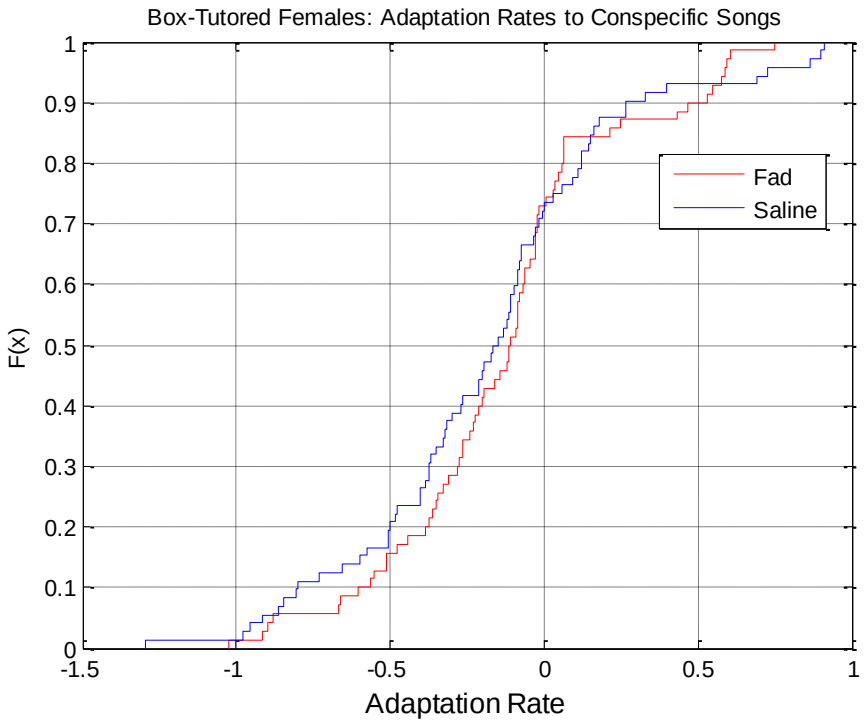

Figure 4.13: Adaptation rates were not significantly different in Fadozole- and saline-treated females reared in a box-tutoring paradigm.

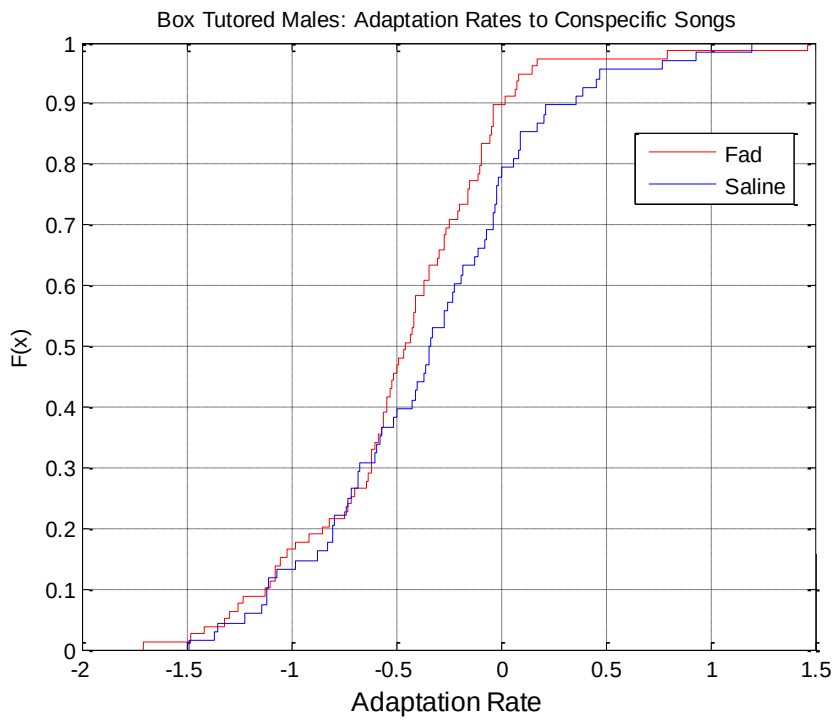

Figure 4.14: Adaptation rates were not significantly different in Fadozole- and saline-treated males reared in a box-tutoring paradigm.

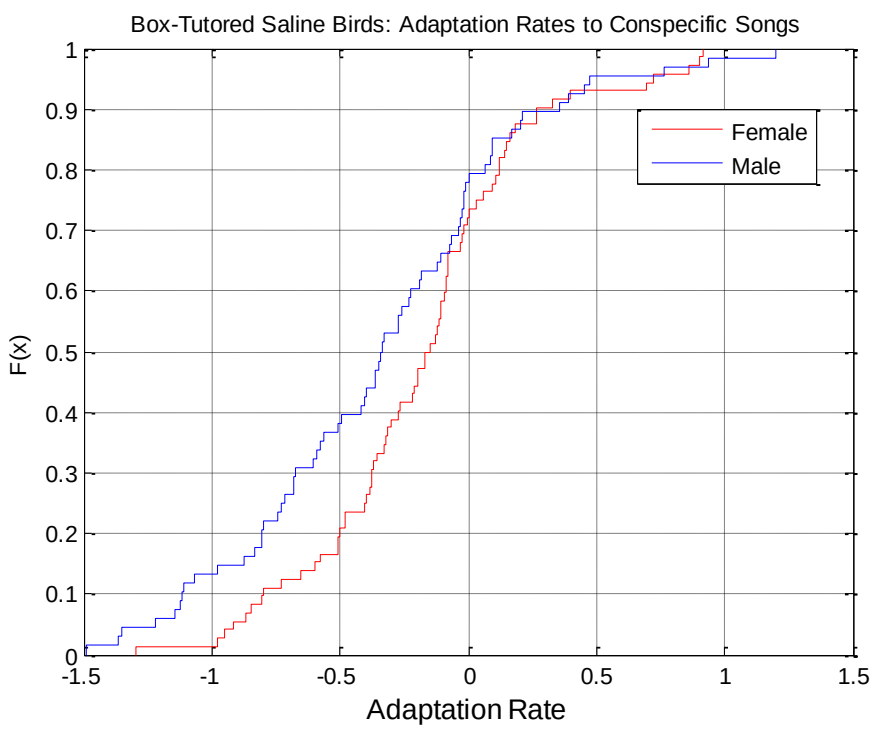

Figure 4.15: Adaptation rates were not significantly different between saline-treated males and females reared in a box-tutoring paradigm.

Memory for Recently Heard Songs in Box-Tutored Birds:

Fadrozole treatment did not significantly influence FIs for the training songs in box-tutored females (KS, p=.3394, **Figure 4.16**). In contrast, Fadrozole significantly *increased* FIs in box-tutored males (KS, p=.02, **Figure 4.17**).

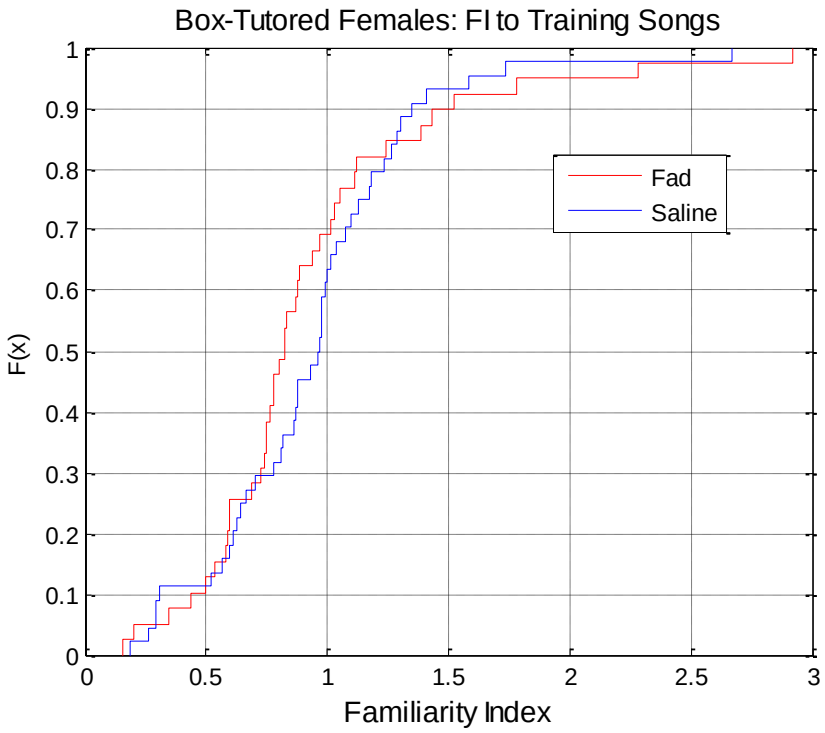

Figure 4.16: FIs for the training songs were not significantly different in saline-and Fadrozole-treated females reared in a box-tutoring paradigm.

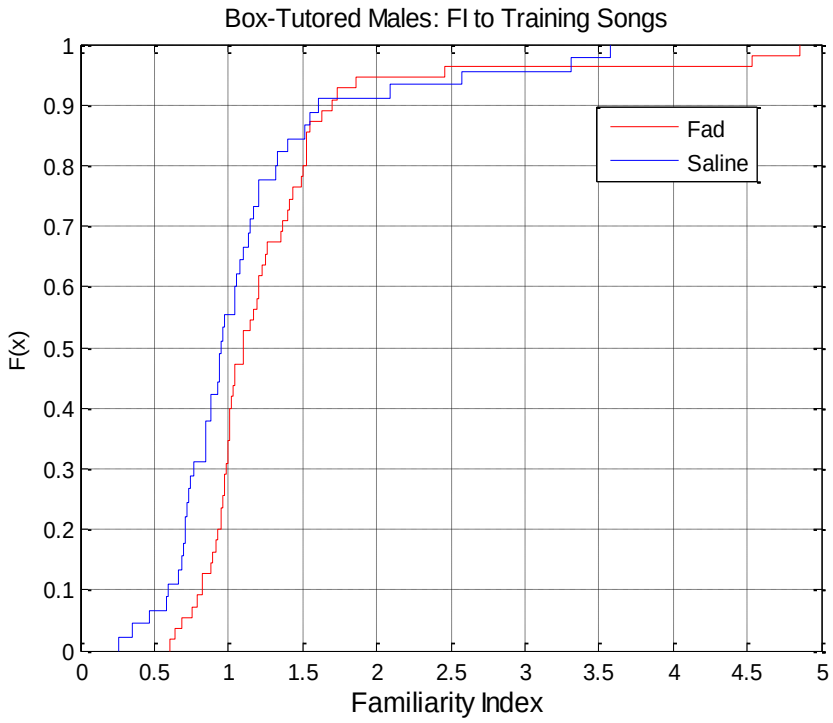

Figure 4.17: FIs for the training songs were significantly higher in Fadrozole- than in saline-treated males reared in a box-tutoring paradigm.

Conclusions:

The results of this experiment suggest that 1) Fadrozole decreases response magnitudes to conspecific songs in box-tutored females but not in box-tutored males 2) Fadrozole does not influence response magnitudes in aviary males or females. 3) Fadrozole does not alter adaptation rates to conspecific songs in aviary or box-tutored birds. 4) Fadrozole decreases familiarity to recently heard songs in aviary males, but not in aviary females 5) Unexpectedly, Fadrozole appears to *increase* FI for songs in box-tutored males, but not in box-tutored females.

The result that Fadrozole decreases FIs in aviary males is in agreement with other studies (Remage-Healey, et al., 2008; Pinaud et al., 2011) that have shown that Fadrozole

administration to male zebra finches disrupts the behavioral recognition of familiar songs. These studies have also shown that application of Fadrozole (or other aromatase inhibitors or estrogen receptor antagonists) into NCM prevents the increased firing rate of individual NCM neurons that normally occurs when the bird hears conspecific song (Tremere et al., 2006; Remage-Healey et al., 2008). The Fadrozole-treated aviary males that had significantly lower FIs also showed a weak but non-significant trend toward lower response magnitudes compared to saline-treated males (see Figure 4.17), an effect which has been reported in male NCM with direct injection of aromatase inhibitors or estrogen receptor blockers (Tremere et al., 2006).

The apparent Fadrozole-induced *increase* in FIs in box-tutored males is difficult to interpret, but leads to the hypothesis that NCM in box-tutored males responds differently to estradiol depletion as a result of their restricted developmental experience with song. Another possibility is that in aviary birds, estradiol could normally be modulating inhibition in NCM (Tremere et al., 2009) that could participate in maintaining adapted responses to familiar songs, and that Fadrozole injection disrupts this balance. Because the FI compares novel to familiar adaptation rates, I inspected the CDFs to check for differences in adaptation to the novel songs in box-tutored males (see Figure 4.14). There was a strong trend for Fadrozole-treated males to show higher adaptation rates to novel song than saline males (but this was not significant). If adaptation rates to familiar songs were unaffected by the treatment, the higher adaptation to novel would significantly increase FI, leading to an apparent increase in memory.

My experiment differs from previous studies in that my treatment was chronic (8 days) and was administered as subcutaneous injections, whereas previous studies administered local injections directly into NCM (Tremere et al., 2006; Pinaud et al., 2011; Remage-Healey et al., 2008). My daily treatment route could have influenced hormonal feedback systems whereas acute injections would have done so to a lesser degree.

In females, I found that FIs were not significantly different in saline- and Fadrozole-treated birds reared in the aviary or in the box-tutoring condition. However, it appears that only ~20 percent of female sites (box-tutored or aviary) have FIs greater than 1 (meaning no differences in adaptation rate to novel versus familiar songs). It may be that the daily injections were stressful for the females and suppressed estradiol production in itself. In answer to this question, I returned to the cumulative frequency distributions to compare the Fadrozole and saline animals with estradiol implanted females (that also received injections) from my previous study in box-tutored birds (**Figure 4.18**). Though the distributions are not significantly different there is a trend (KS, p=.07), and ~60 percent of the estradiol distribution is above 1, compared with ~30 percent in the saline-treated birds. I then examined the distributions of saline-treated versus untreated females (**Figure 4.19**), which were also not significantly different but showed the same strong trend (KS, p=.0595). Finally, I compared the distributions of Fadrozole birds to birds that were implanted with Estradiol and found that FIs in the estradiol group were significantly higher than those in the Fadrozole-treated group (KS, p=.0042; **Figure 4.20**). This could mean that significant differences in female memories are only seen when the “control” group has estradiol knocked down to begin with. So, the results of

these comparisons support the hypothesis that estradiol levels were lowered by the saline-injections administered to females, but are restored with estradiol. It appears that estradiol is suppressed by the injection and/or handling stress associated with administration of Fadrozole or saline in box-tutored females, and are in line with studies that have shown negative effects of stress on some types of learning and memory tasks in females (but not in males), such as eye-blink conditioning (Shors et al., 2000).

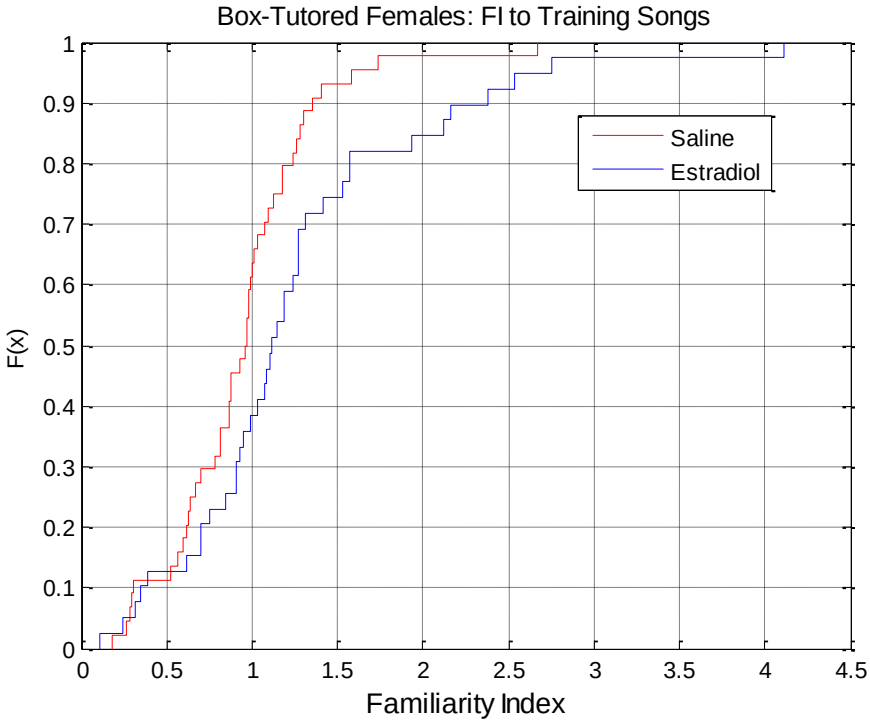

Figure 4.18: FIs for the training songs were not significantly different between saline- and estradiol-treated females

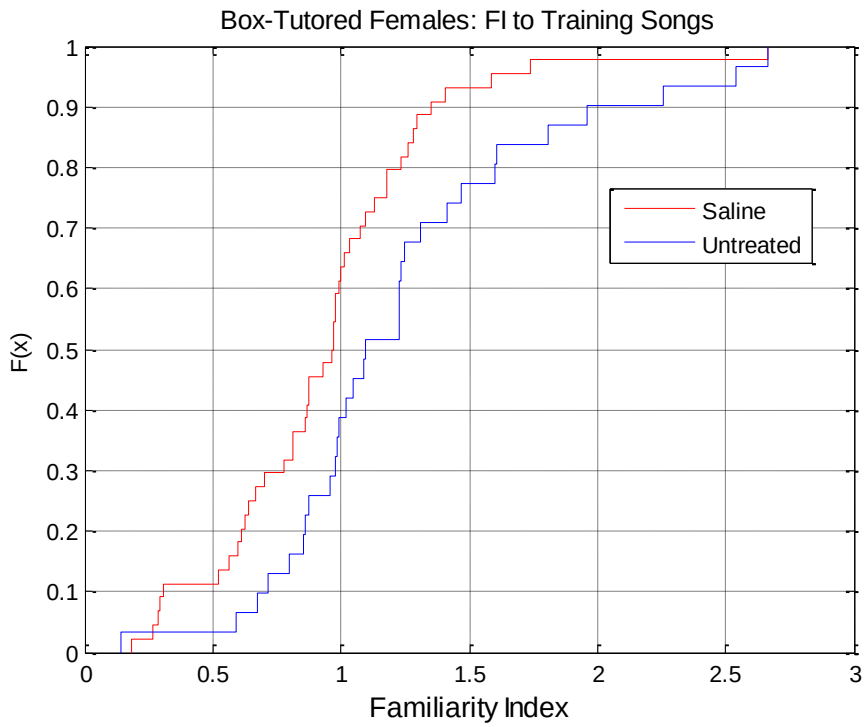

Figure 4.19: FIs for the training songs were not significantly different between saline- and untreated females

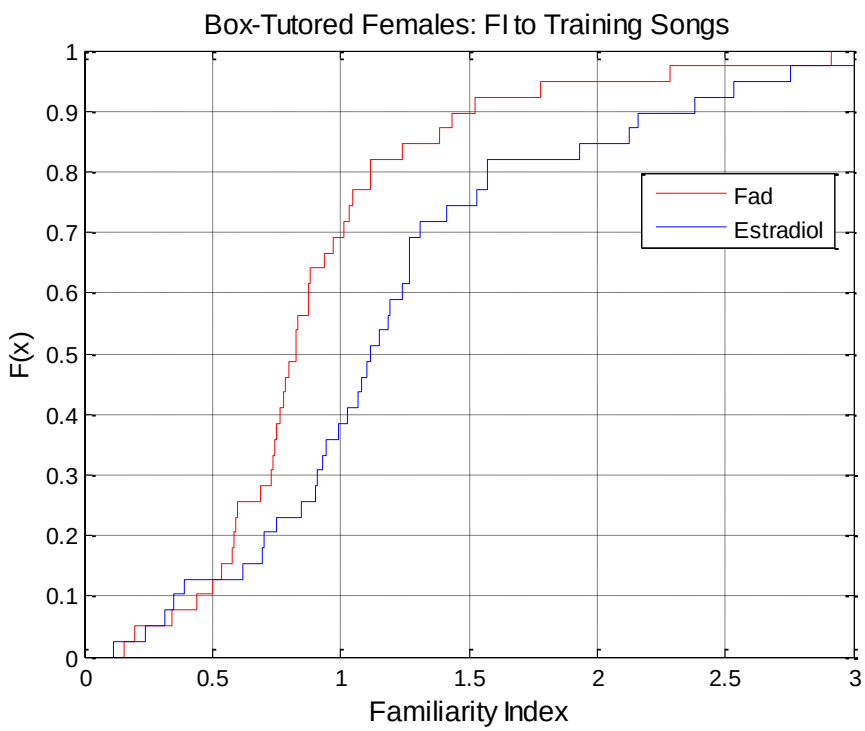

Figure 4.20: FIs differed significantly between Fadrozole- and estradiol-treated females

<u>Chapter 5:</u> Effects of Estradiol Depletion on Tutor-Song Memory in Box-tutored Males and Females

Rationale:

Previous work in the laboratory found clear evidence of tutor song memory in NCM of male birds (Phan et al, 2006), but the data in females were variable, hard to interpret, and not included in that publication. My previous work in females showed that estradiol depletion did not disrupt tutor song memory, but did disrupt the memory for more recently heard songs and decreased response bias towards conspecific song. In male songbirds, estradiol suppression in NCM is known to induce deficits in the behavioral and neural discrimination of the tutor-song from novel songs (Tremere and Pinaud, 2011), but the neuronal memory for tutor-song has not been tested in males that are deprived of estradiol. To test the hypothesis that estradiol depletion would degrade the neuronal memory for tutor-song, I employed the methods I previously developed in females to manipulate estradiol in adult males that had been artificially tutored during the sensitive period for song learning, and then recorded NCM responses to playbacks of novel songs and the Tutor song. For comparison, I re-analyzed the results of my previous study on females and provide them along with the male data.

Experimental Question 5a: Do untreated males and females express differences in memory for the Tutor song as measured by the Familiarity Index (FI)?

Experimental Question 5b: Does estradiol manipulation affect neural memory for the Tutor song in NCM of females?

Experimental Question 5c: Does estradiol manipulation affect neural memory for the Tutor song in NCM of females?

Results:

Experimental Question 5a: Do untreated males and females express differences in memory for the Tutor song as measured by the Familiarity Index (FI)?

The distributions of FIs for tutor song in untreated males and females were not significantly different (KS, p=. 9; **Figure 5.1**). This suggests that males and females do not differ in their memory for the Tutor song, as measured by FI.

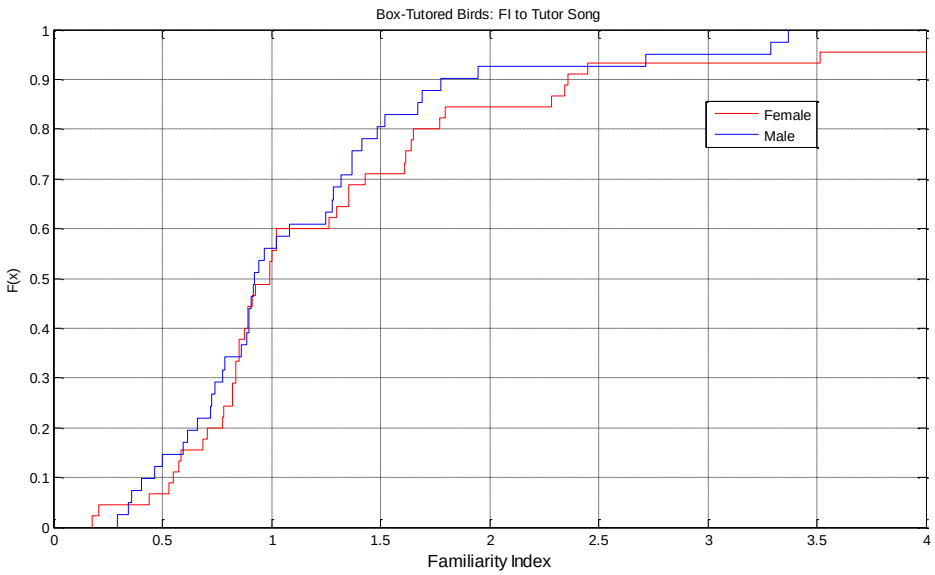

Figure 5.1: FIs for tutor song did not differ significantly between males and females

Experimental Question 5b: Does estradiol manipulation in females induce differences in Tutor Song Memory?

The distributions of Fadrozole and saline-treated females are not significantly different (KS test, p=.41, **Figure 5.2**). These results suggest that estradiol depletion does not appear to disrupt tutor-song memory in females, in concordance with the results of my original experiment.

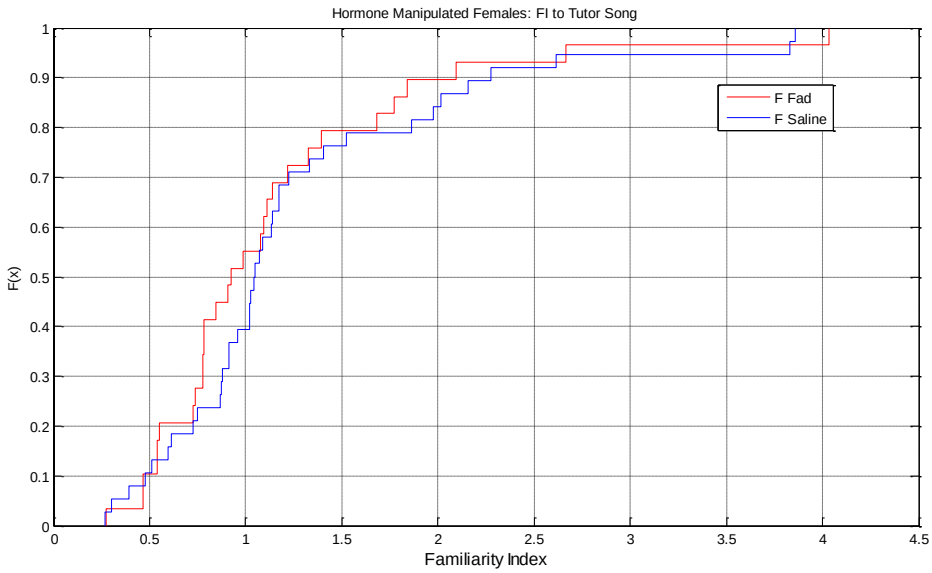

Figure 5.2: FIs for tutor song did not differ significantly between Fadrozole and saline-treated females

Experimental Question 5c: Does estradiol manipulation in males induce deficits in Tutor song memory?

FIs to tutor song were not significantly different for Fadrozole and saline-treated males (KS, p=.98; **Figure 5.3**). The overlap of the FIs in the cumulative frequency distributions suggests that Fadrozole did not significantly impact FI to tutor song. However, it is impossible to draw a clear conclusion that FAD had no effect in males because, surprisingly, there was no indication of a tutor-song memory (FI > 1.0) in the saline-treated males, as would have been expected from earlier results in box-tutored males (Phan et al, 2006).

Because of the discrepancy with earlier results, I compared the current group of saline males to untreated males (no injections) who had been tested according to the same experimental design. FIs in untreated birds did not differ significantly from FIs in saline-treated birds (KS, p=.42). However, the Fadrozole-treated males also did not differ significantly from the untreated males (KS, p=.41). In the original Phan et al. study (2006), the FIs were shown to correlate with the bird's Song Similarity Index (SS, a measure of how well the bird copied). I measured SS in my male cohort, and found that copying was comparable to what was seen in the Phan cohort in both Fadrozole (t-test, p=.18) and saline-treated birds (t-test, p=.48). However, when I regressed SS against FI (as in Phan et al., 2006), there was no significant regression in either saline (r^2=.12) or FAD (r^2=.004) or the combined group (r^2=.009) of males. However, this analysis differs from the prior analysis in that the SIs were calculated for birds, whereas I based this

analysis on sites. From these discrepancies, I cannot reach a firm conclusion one way or the other about any effects in my current datasets.

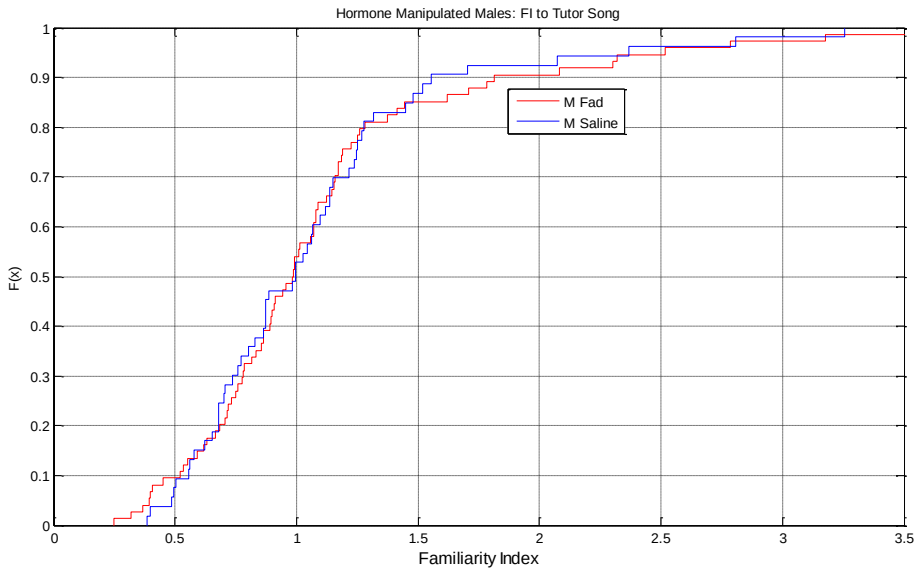

Figure 5.3: FIs for tutor song did not differ significantly between Fadrozole and saline-treated males.

Conclusions on Tutor Song Memory:

These analyses suggest that the neuronal memory for the Tutor song is similar in males and females, and that this memory is not altered significantly by estradiol depletion in males or females. However, the distributions of data do show interpretable effects.

First, the FI distributions for females are not significantly different from those of males. This result is surprising, given that my examination of female Tutor-song Memory in NCM was motivated by the fact that 1) previous NCM recordings in our lab had suggested that, in females, this memory might be weaker than in males (Phan,

unpublished observations) 2) the Tutor Song memory in females might be stored in CMM, not in NCM based on immediate early gene observations (Bolhuis, 2000). On the other hand, females do show behavioral recognition for their Tutor's song (Miller, 1979; Riebel, 2000) so this memory must exist somewhere. Based on this comparison with males, I cannot conclude that females differ from males in their memory for Tutor song, at least in NCM.

Second, FI distributions for Fadrozole treated females are not significantly different from those of Saline-treated females. These results were presented as part of my Masters thesis and were re-presented here to provide comparison with the male data. Nonetheless, from the current analysis, the safest conclusion is that the tutor-song memory does not differ with estradiol manipulation in females.

Third, FI distributions for Fadrozole treatment in males are also not significantly different from those of Saline males. This result was surprising, given that other studies have shown that blocking estradiol in male NCM disrupts behavioral recognition of the Tutor song, disrupts neural discrimination, as well as inducing lower overall firing rates in NCM.. However, because the saline treated males in my experiment did not show any memory, as measured by FI, it is impossible to conclude anything about the effect of FAD in these birds. The birds imitated the tutor song so they must have formed a memory of it, but for unknown reasons, the memory was not detected in my NCM data.

Chapter 6: Influences of Rearing, Sex, and Estradiol on Response Bias for Conspecific Song

Rationale:

A characteristic feature of NCM is that it shows selectivity for conspecific songs over heterospecific songs or other categories of sounds (Chew et al., 1996). In males and females, the ability to discriminate members of their own species by song may be an essential part of social and reproductive communication. Females in particular need to make this discrimination in order to select a mate on a gross categorical scale. Therefore, in females, it was hypothesized that species discrimination may be most important when females are in a breeding state, and circulating estradiol levels are high. Results of my previous study in females depleted of estradiol suggested that these females had reduced responses bias for conspecific versus heterospecific song, and these results were in agreement with the work conducted in other species that show that estradiol must be present for demonstrating IEG induction differences to playback of conspecific songs over tone stimuli (Maney et al., 2006). I used datasets from Chapters 3 and 4 that included responses to heterospecific as well as conspecific stimuli to determine whether 1) aviary males and females show a response bias for conspecific versus heterospecific songs 2) whether estradiol depletion influences this response bias in either sex.

Experimental Question 6a: Do males and females differ in their response bias, in box-tutored and aviary birds?

Experimental Question 6b: Does estradiol manipulation in male and female aviary reared songbirds decrease the response bias for conspecific songs?

Experimental Question 6c: Does estradiol manipulation in male and female box-tutored birds influence the response bias for conspecific songs?

Experimental Question 6d: Do adaptation rates to heterospecific songs change as a result of rearing environment or hormone manipulation?

Methods:

All birds included in this dataset heard playbacks of novel zebra finch and canary songs presented in randomized order while recording NCM. In order to compare absolute response magnitudes between these two stimulus classes, I created a ConHet and a Response Bias Index (RBI; see Methods). These measures were then compared between saline and Fadrozole treated birds to determine whether the treatment influenced song discrimination. I also computed a ConHet Adaptation Index (CI; see Methods). Using this measure, values at zero represent no difference in adaptation rate to conspecific versus heterospecific songs. Values greater than zero indicate that adaptation rates are greater to heterospecific song, and values less than zero represent adaptation rates are greater to conspecific song.

Results:

Experimental Question 6a: Do untreated males and females differ in their response bias, and does this differ in aviary and box-tutored birds?

In aviary reared birds, there was no significant sex difference in RBIs (**Figure 6.1**; KS, p=.47) or ConHet ratios (KS, p=.47). In box-tutored birds, males did show significantly higher RBIs (**Figure 6.2**) and ConHet ratios than females (KS test, p=.006 for both measures).

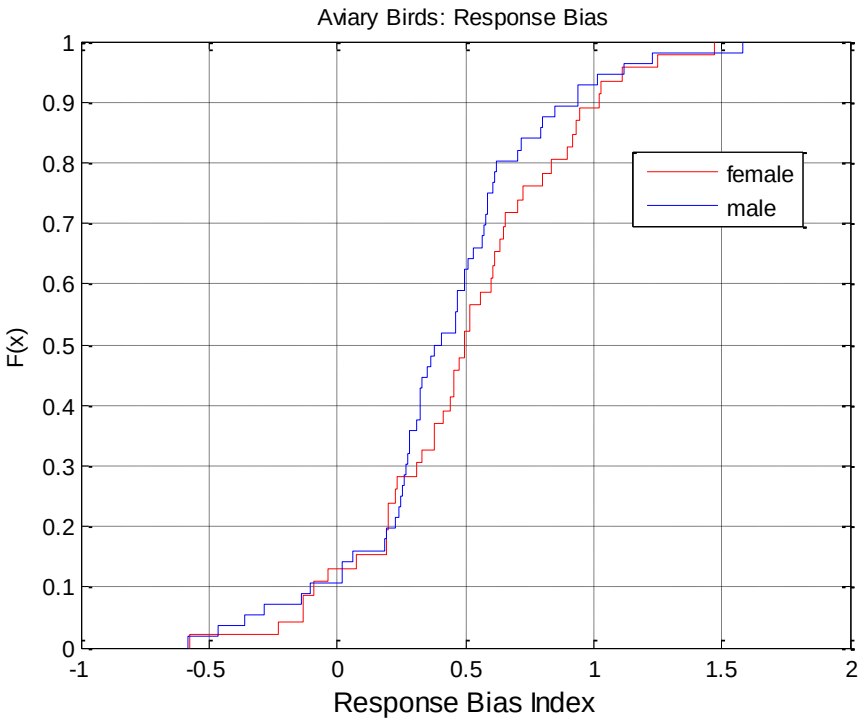

Figure 6.1 RBIs do not differ significantly between aviary males and females

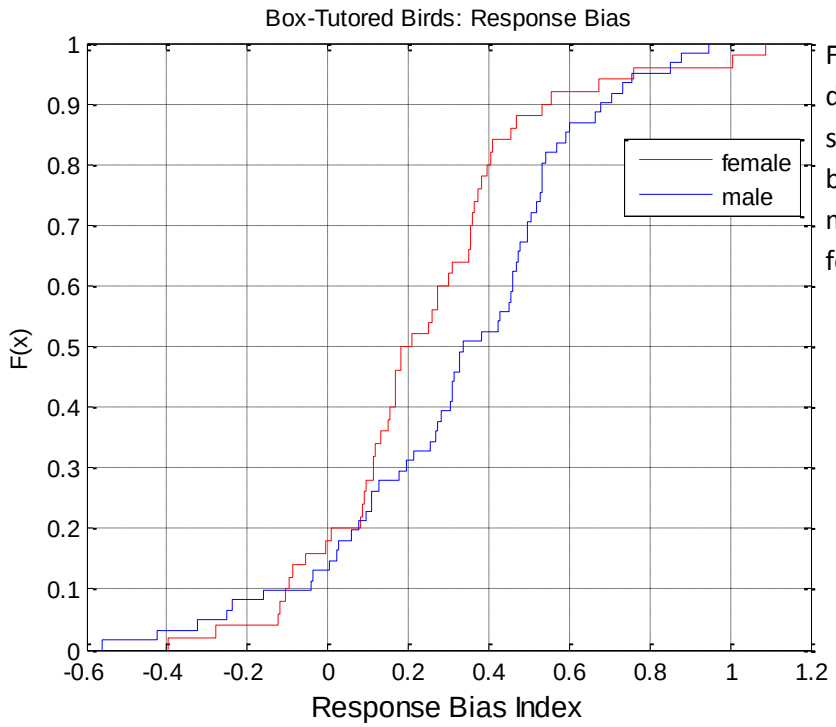

Figure 6.2 RBIs differ significantly in box tutored males and females

Experimental Question 6b: Does estradiol manipulation in male and female **aviary** reared songbirds decrease the response bias for conspecific songs?

In aviary reared males, Fadrozole and Saline treated males had similar distributions of RBIs and Conhet ratios, and no differences were significant (KS, p=.93 for both measures; **Figure 6.3**). In aviary females, the distribution of RBIs and ConHet ratios among Fadrozole treated birds was significantly lower than that of saline treated birds (KS test, p=.03 for both measures; **Figure 6.4**).

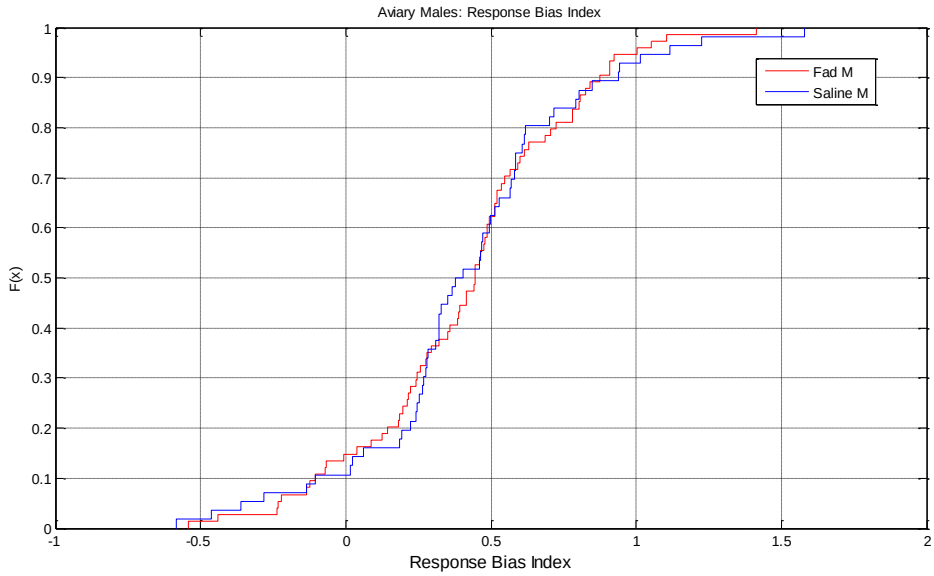

Figure 6.3 RBIs did not differ significantly between Fadrozole- and saline-treated males reared in the aviary

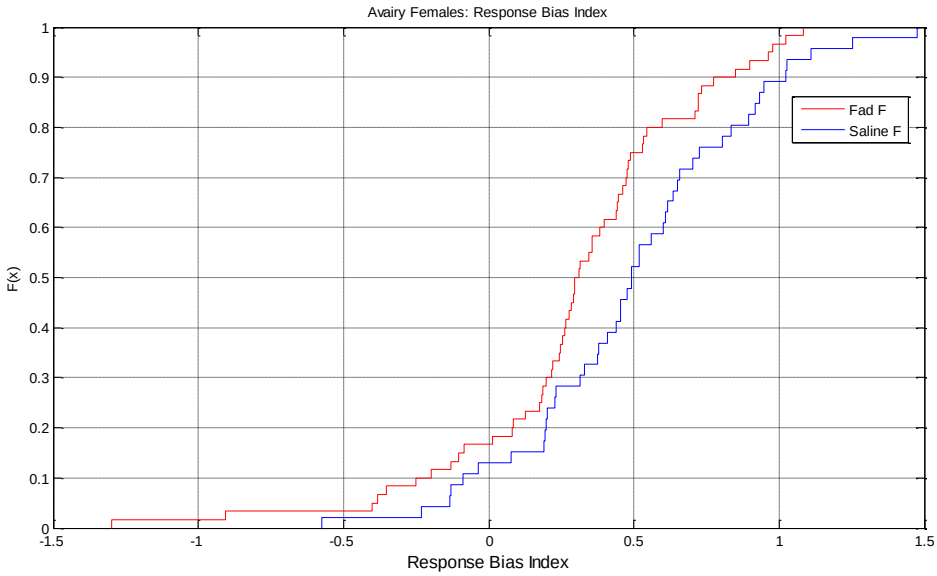

Figure 6.4 RBIs differ significantly between Fadrozole- and saline-treated females reared in the aviary

Experimental Question 6c: Does estradiol manipulation in male and female box-tutored birds influence the response bias for conspecific songs?

Although the distributions of RBIs and Conhet ratios in the Saline treatment group of males appear to be different than in Fadrozole treated males (**Figure 6.5**), KS tests were not significant (p=.32 for both measures). There was a trend for FAD-treated males to have higher RBIs. In box-tutored females, the distributions of RBIs or of ConHet ratios were not significantly different (KS test= .16 for both measures; **Figure 6.6**).

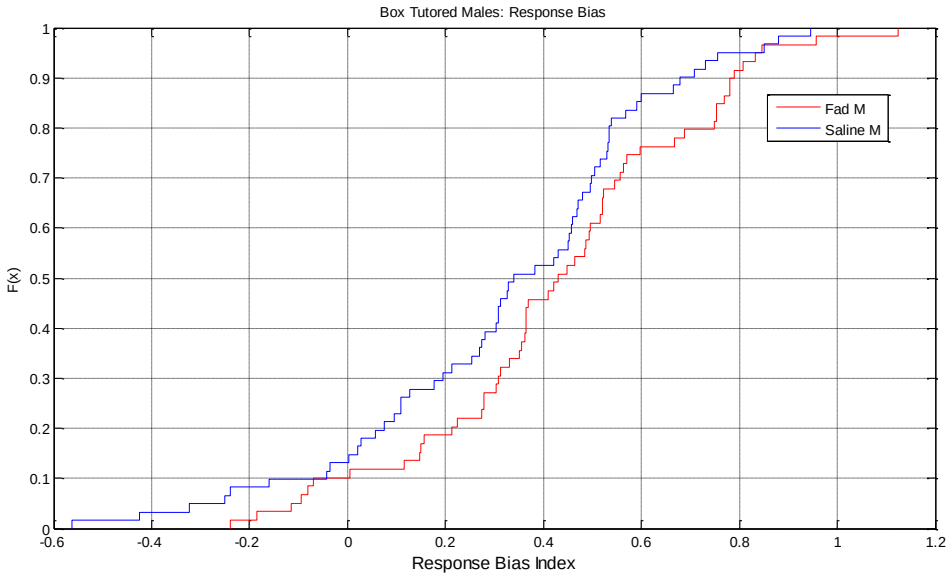

Figure 6.5 RBIs were not significantly different between Fadrozole and saline treated males that were box-reared.

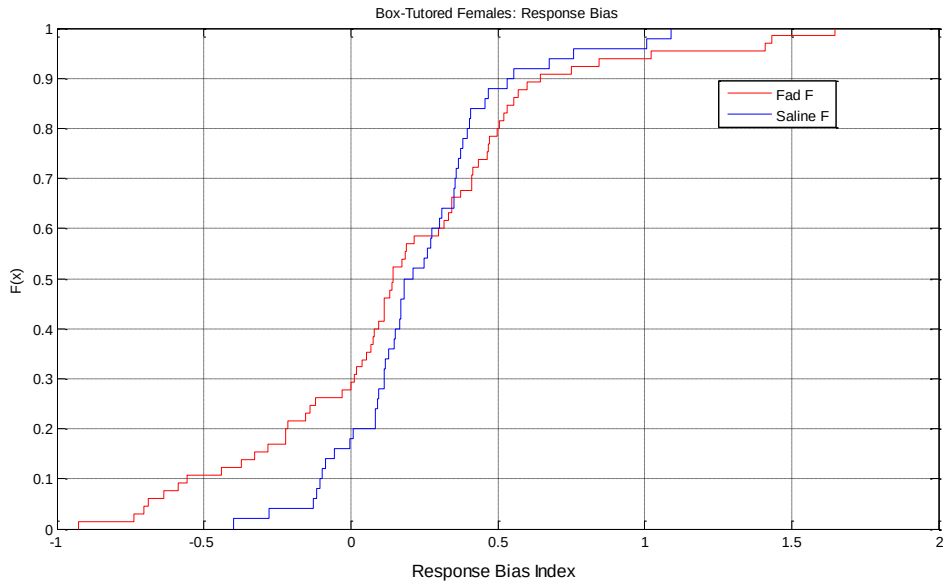

Figure 6.6 RBIs did not differ significantly between Fadrozole and saline-treated females that were box-reared

In order to test the relationships among the factors of housing, sex, and the treatment, I used ANOVA to test for interactions among these variables using RBI as the dependent measure. The ANOVA showed a main effect of sex (F (1,463) =6.74, p=.01), in that males had higher RBIs (.40, SD=.02) than females (.30, SD=.03). There was also a main effect of rearing (F (1,463) =10.43, p=.001); aviary birds had higher RBIs than box-tutored birds (.41, SD=.03 in 236 sites versus .29, SD=.03 in 235 sites, respectively).In addition, there was an interaction between rearing and sex, in that box-tutored males had higher RBIs (.37, SD=.03 in 120 sites) than Box-tutored females (.21, SD=.04 in 115 sites), while that aviary birds did not show this sex difference (.41, SD=.04 in 106 sites and .40, SD=.04 in 130 sites for males and females, respectively). This interaction can be inferred from the distribution plots presented in Figures 1a and 1b.

Further, the ANOVA showed an interaction between sex and treatment (F (1,463) =6.43, p=.01), in that male RBIs were unaffected by the Fadrozole treatment (FAD: .42, SD=.03 in 133 sites versus Saline: .37, SD=.04 in 117 sites), but female RBIs were lower in

Fadrozole (.24, SD=.03 in 125 sites) than in saline-injected females (.37, SD=.04 in 96 sites).

Experimental Question 6d: Do adaptation rates to conspecific versus heterospecific songs change as a result of rearing environment or hormone manipulation?

No significant differences were found in my comparisons of adaptation differences to conspecific and heterospecific songs, using the CI as a measure. Males and females had similar distributions in both the aviary (KS, p=.95) and in box-tutored birds (KS, p=.2). Within aviary birds, Fadrozole- and saline-treated females had similar distributions in CI (KS, p=.21), as did Fadrozole and saline-treated males (KS, p=.41).

Discussion:

First, the results of this experiment showed that, although both sexes discriminate conspecific from heterospecific songs, males show a significantly greater response bias in NCM than females. This is true in aviary and box reared birds. This effect may be explained by the fact that both box and aviary males hear themselves sing - producing the vocalizations of their own species - which may produce a stronger reference for conspecific song than in females, who do not produce these modulated vocalizations. For females, the bias for conspecific song is lower in box-tutored birds (who experience only the Tutor-song during development) than in aviary-reared birds (who hear many different songs), suggesting that the strength of this response bias is modulated by experience with conspecific song.

Second, estradiol depletion significantly reduced response bias in aviary females, but not in males. This suggests that in females, this discrimination is estrogen-dependent, and so fits the hypothesis that females may need to make this discrimination when they are in a breeding state and looking for a mate within their own species. The fact that Fadrozole did not induce lower bias in box-tutored females most likely results from their limited experience with conspecific song; if the discrimination is already weak, Fadrozole may not be able to decrease it much more.

Fadrozole does not seem to affect RBIs or Conhet ratios in males. This suggests that this type of discrimination is estrogen *independent* in males. This result is in line with the fact that males do not rely on songs to select a mate (as females do). Nonetheless, species discrimination may be important in male-male interactions, but may operate independently of estradiol levels. The fact that adaptation rate differences were not altered by estradiol depletion suggests that the phenomenon of adaptation – a part of the process of forming recognition memories for individual songs - is estrogen-independent in both males and females.

Chapter 7: General Discussion of Results

The results of experiments described in the preceding chapters show several interesting phenomena that begin to elucidate the effect of sex, developmental experience, estradiol, and the interactions among these variables on processing of communication signals within the auditory system. Even more exciting, I found that estradiol can apparently modulate the neuronal memory for these communication signals, as seen in aviary males and in females reared in an artificial tutoring paradigm. When placed into a larger context, what may seem like small quantitative changes in how auditory area NCM responds to songs, - and is modulated by sex, experience with song, and estrogen - actually sheds light on how the auditory brain works within a functional framework to enable behavioral differences in response to the social environment. The following sections examine the results and attempt to explain them in terms of their behavioral relevance.

Sex differences in the processing of songs

Based on my own experience recording NCM in both sexes, I had hypothesized that sex differences exist in the way that males and females process song, which would be revealed by comparing adaptation rates or response magnitudes. I tested this hypothesis by comparing untreated males and females for NCM responses to novel songs. In an initial analysis that collapsed housing conditions, I found that females exhibited shallower adaptation slopes to novel song than males do, but there was no sex difference in response magnitude or in the memory for recently heard songs. Because the adaptation rate reflects the decrease in response magnitude over repeated presentations

(across trials 6-25) of a song, this means that in males, the responses drop quickly and for females, the responses drop more slowly. In other words, a female may have more sustained responses in NCM when she hears a song compared to a male. This sex difference in the adaptation rate could reflect the different functional role that song plays for males and females in this species. For females, song serves as an "honest" signal of a potential mate's fitness, and so the sustained response to a song may be evidence that a female has sustained attention to a song even as she hears it over and over again. This sustained response potentially contributes to memory acquisition for the songs of potential suitors. Since females will hear these songs sequentially within many different social interactions and across various timepoints, storing a memory for the songs she hears would enable her to compare many males' songs against one another and return to the male of her choice.

For male zebra finches, songs do not function in the same way. Males do NOT choose a mate based on song but instead sing their own songs to females to court them. In zebra finches, males seldom counter-sing to other males, as occurs in territorial species, so the responses in males may decrease quickly to repeated songs, perhaps reflecting decreased attention to the song of another male. Speculating from this line of reasoning, I would predict that I would <u>not</u> find a sex difference in NCM adaptation rates within species in which song functions in territorial defense, such as the song sparrow. For a male of a territorial species, nearby song serves as a threatening signal that another male is attempting to invade his territory, and he would need to respond by singing back in order to defend it (Vehrencamp, 2001; Molles and Vehrencamp, 2001). Were I to test NCM adaptation in such a species, I speculate that I would find similar adaptation rates

for males and females, since songs heard in adulthood serve as salient signals for both (mate choice and territorial defense). It would also be interesting to test whether juvenile males have shallower adaptation rates than adult males. I would predict that they would exhibit more sustained responses to song, since during this time they are listening to and attempting to reproduce the songs they hear.

An analysis of ZENK expression might follow a similar pattern. The IEG *zenk* is induced in NCM when a bird hears a novel song, but induction of *zenk* is low in response to a familiar song. If a novel song induces high electrophysiological responses and high ZENK expression, then we might expect that a sustained electrophysiological response in NCM might be paralleled by a similarly high level of ZENK. This line of thinking – that ZENK levels are proportional to total electrophysiological activity over repeated songs - would lead to the prediction that males express less ZENK than females. However, no sex differences were reported in original studies measuring ZENK induction in response to conspecific song playbacks (Mello et al., 1992) or in a more recent study by Tremere et al. (2009). Thus, additional factors may influence ZENK expression, factors that may not be identical in males and females.

I also attempted to test sex differences for tutor song memory in NCM. The data provide novel evidence of a tutor song memory in female NCM similar to that reported in males (Phan et al., 2006) and are at variance with a study that failed to find increased ZENK expression for tutor-song in female NCM (Terpstra et al., 2006) . However, I was unable to complete the intended sex comparison because I could not detect a clear tutor-song memory in my control group of males, for unknown reasons.

Estradiol influences on the processing of songs

Recent studies have shown that estradiol is produced in response to song playback in both males (Remage-Healey et al. 2009) and females (Remage-Healey, 2012) NCM. In addition, estradiol is necessary for NCM to respond normally to conspecific song in male and female NCM (i.e., increase firing rate, express IEGs associated with memory formation: Remage-Healey et al., 2008, Tremere at al., 2009). Therefore, preventing estradiol from acting on NCM neurons could influence behaviors in males (juveniles learning to sing or adults engaging in song-matching) and females (choosing a mate based on song comparison with the memory of tutor song). I interpreted the results of the experiments in which the training stimuli were administered and then re-presented with novel songs as "memory" effects, and concluded that suppressing estradiol production disrupted the "memory" for recently heard songs (as seen in reduced FIs to the training songs). However, the observed effects on "memory", thus defined, might instead be interpreted as merely deficits in "discrimination." I consider these two possible interpretations of the data below.

The term "neuronal memory" has been applied to the long-term song-specific adaptation phenomenon observed in NCM recordings in which lower adaptation rates are observed for songs the bird has previously heard compared with novel songs. This effect is calculated as the familiarity index (FI); when the familiar songs have adaptation rates that are the same as novel songs (FI is ~1.0), they are not "remembered". In the present experiments, I compare FIs between groups of birds with different hormone manipulations, and interpret low FI (~1.0) as a lack of "memory" in this sense. However,

the hormone manipulation could have other effects which are observed as a low FI. In particular, other experiments have shown that blocking estradiol locally within NCM disrupts the neural discrimination among individual songs (Tremere and Pinaud, 2011), suggesting that any given song (whether novel or familiar) may not be encoded as different from another. Lowered levels of estradiol in my experiment might have reduced discrimination. Because birds in my experimental paradigm received daily treatments of Fadrozole, neural discrimination could have been disrupted both at the time of training (first time the songs were heard) and at testing (second playback session of training songs mixed in with novels). This leaves open the possibility that 1) memories for individual songs were not formed at the time of training because the individual songs could not be discriminated from one another (if all songs sound the same, then what is there to remember?) and 2) even if memories of the training songs had formed during training but Fadrozole was disrupting song discrimination during testing, then a familiar song would not be discriminated from a novel one.

There are three kinds of reasons to believe that my treatment did not affect discrimination severely enough to prevent individual songs from being discriminated. First, tutor-song memory was detected with FI >1.0 in at least some of my FAD groups. This could only have been observed if the tutor song was discriminated from the novels. Second, the novel songs played back at the time of testing showed independent stimulus-specific adaptation. Again, this could only have occurred if the songs were discriminated at the neural level. Third, adaptation rates to novel songs were not different in Fadrozole versus saline treated sites. However, the adaptation rates to familiar song *were* presumably affected by the Fadrozole treatment, in that the FI is the ratio of adaptation

rates to novel versus familiar; if adaptation to novel is not different between treatments, the change in adaptation to familiar must be the deciding variable that leads to FIs closer to 1.

The fact that discrimination was present in my Fadrozole-treated birds and was reduced in the earlier study (Tremere and Pinaud, 2010) could be for several reasons: 1) my effective doses may have been lower; 2) my birds had received drug for a week prior to training and testing, not acutely, and may have adapted in some way; 3) discrimination was reduced, but not abolished, in the earlier studies.

These considerations suggest that we can interpret the results as a "memory" deficit rather than merely a deficit in discrimination among songs presented during the training and/or playback sessions. Thus, my results are novel, suggesting that estradiol is necessary for new songs to be consolidated into auditory memories. However, in my paradigm, it is impossible to definitely attribute the lack of "memory" to a deficit in acquisition versus a deficit in recall, because drug treatment was in effect at <u>both</u> time points. However, the fact that tutor-song memory could be detected in some of my treated groups suggests that recall was not affected. Of course, tutor-song memory is formed during the juvenile period, very long-lasting and may be especially robust. Nonetheless, these arguments tend to favor the interpretation that reducing estradiol synthesis affects some aspect of memory acquisition and consolidation.

This interpretation is consistent with what is known about estradiol action in the songbird brain. Tremere et al (2009) showed that local action of Fadrozole prevented the expression of several IEGs when the bird heard song playbacks. These included *zenk, c-*

fos, and *Arc*, which have been associated with memory formation (Swett, 2003; Clayton, 2000). If my Fadrozole treatments were having the same effect within NCM (even though it was administered peripherally), then blocking aromatase could also have prevented the memory formation during training by interfering with the events either upstream or downstream of the IEG induction, and thereby prevent changes within the neuron that are associated with the formation of an auditory "memory" as represented by the maintenance of adaptation in NCM. Chew et al (1995) showed that the adapted response to a familiar stimulus requires gene transcription. They presented stimuli in the playback "training" period and injected RNA- or protein-synthesis inhibitors (Actinomycin D or Cyclohexamide) directly into NCM at different time points following song presentation. When RNA or protein synthesis was prevented at particular time points following playback (.5-3.5hours, 5.5 hours, and 6.5-7 hours), NCM neurons showed a "forgetting" response, meaning that adaptation rates for previously heard songs were more like those of novel songs (the adaptation rates became steeper as the songs were forgotten). Furthermore, the experimenters tested the degree to which their treatments blocked *zenk* induction and showed that either treatment had prevented its expression in response to song playbacks. Since the protein synthesis inhibitors injected at particular time points had induced "forgetting" of NCM neurons and had blocked ZENK expression, these results suggest that *zenk* may be required to maintain the neuronal memory for recently heard songs. If *zenk* is required for memory formation (this experiment has not been conducted), and blocking estradiol action prevents *zenk* induction, then estradiol blockade may also prevent the neuronal memory from forming to recently heard songs. In my experiment, I used Fadrozole to decrease the level of

estradiol within NCM. As was observed in the Chew et al (1995) experiments, there was no effect of drug treatment on immediate adaptation; adaptation rates to novel songs were not different in Fadrozole versus Saline treated sites. Though ZENK expression and the maintenance of the neuronal memory for songs has not been shown to be directly related, Chew et al (1995) showed that blocking protein synthesis at particular time points during song playback induces neural "forgetting" suggesting that *zenk* could be involved in the process of memory formation.

Locus of action of Fadrozole: Perception versus Motivation

Sensory processing: Because I administered Fadrozole peripherally, it could have disrupted sensory processing as a result of lowering estrogen availability not only in NCM, but also at earlier points in the auditory processing stream. Although NCM expresses an abundance of estrogen receptors and aromatase (Saldanha et al., 2000) estrogen receptors and aromatase are also co-localized within the inner ear of this species (Noirot et al., 2009). Therefore, it is possible that my treatments were the result of estrogen depletion at the earliest stages of sound processing. If my treatments decreased the detection of sounds at such an early stage, then subtle changes in frequency or duration from one note to another would be more difficult for the bird to hear. In this scenario, more central processing stages might also be affected by decreased estradiol. Overall, the poor representation at an early stage could lead to poor representation of the song in NCM, which could lead to poor discrimination of a particular song from others, and result in "memory deficits" for the training songs. However, this is essentially

another version of the interpretation of Fadrozole effects as effects on discrimination, which was discussed above. Discrimination deficits may contribute, but my data show sufficient evidence of song discrimination that this is unlikely to be the main mechanism.

From an ethological perspective, estradiol could influence the sensory processing of signals that are relevant to reproduction at a time when reproduction is more likely to occur. In the case of songbirds, estradiol could act at individual auditory structures concurrently to influence the processing of song stimuli (which reflect mate fitness). At peripheral auditory structures, estrogens could lower the threshold of detection of particular frequencies of sounds for example. This has been shown to occur in midshipman fish: females are better able to hear the sounds of males when the female has eggs to release, and at this time reproduction is more likely (Sisneros, 2009). Furthermore, these changes are associated with behavioral changes in that females will approach the hum of males attempting to mate with them only when they have eggs to release, but not when they are infertile and chances of reproduction are low. Similar, albeit small effects of estrogen fluctuations have also been observed in humans. Aside from its role in modulating memory and cognitive abilities, estradiol may also modulate hearing thresholds in women. Changes in the auditory brainstem responses (ABRs) and Event Related Potentials (ERPs) have been shown to vary across the female menstrual cycle, and are correlated with changing estradiol levels (Walpurger et al., 2004). Such studies suggest that low estradiol is associated with higher hearing thresholds, and that hearing is poorer during menses when estradiol is at its lowest (Davis and Arhoon, 1982; McFadden, 1998). In addition, post-menopausal women undergoing hormone replacement therapy (HRT) exhibit slightly better hearing than post-menopausal women

who do not undergo HRT. Women with Turner's Syndrome, who are deficient in estradiol, experience early hearing loss relative to age-matched controls (reviewed in Hultzcrantz et al, 2006).

Changes in other sensory modalities can also affect perception of cues associated with mate-choice during more fertile periods. Women's ratings of facial attractiveness are higher for men with higher testosterone when women are nearing ovulation (Roney et al., 2011). Furthermore estradiol levels in female raters positively correlated with the males' testosterone levels they were rating (Roney and Simmons, 2008). Females prefer men with more symmetrical and masculine faces more during their periovulatory phase (Gangestad et al., 2007). These preferences are not only apparent in visual but also olfactory judgments, in that women who are in the periovulatory period prefer the smell of symmetrical men (Gangestad and Thornhill, 1998). Males also appear to be attuned to the reproductive status of women. In one study, men were asked to smell the t-shirts of women that were ovulating versus non-ovulating women or control t-shirts (Miller and Maner, 2010). Only the men who had smelled t-shirts of ovulating women had increased testosterone, suggesting that males and females are both attuned to the reproductive signals of the opposite sex, although female perception may be more variable as a result of their menstrual cycle and the modulatory effects of estradiol. More recent investigations have shown that female fertility is positively correlated with the degree to which a male mimics the female in face-to-face interactions (Miller and Moner, 2011).

If estradiol influences sensory processing, it could exert its actions at lower and higher levels of the auditory system by sharpening discrimination of the amplified

sound—essentially "turning up" the gain at many areas that express estrogen receptors could enable not only better detection of the sounds an animal encounters, but better discrimination of what is being heard. Fadrozole treatment in both males and females could have reduced detection and discrimination by preventing estradiol action both at the periphery and in the central auditory system.

Motivation: On a related note, it is also possible that the peripheral administration of Fadrozole lowered availabity of estradiol supplied to (or produced in, through the action of aromatase) other brain areas outside NCM. This could have altered the "motivational" state of the birds to respond to songs, and probably affected responses within hypothalamic areas that modulate responses to particular stimuli that are relevant to an animal's needs at a given time. When an animal is food-deprived and hungry, an animal may respond more to stimuli associated with food. When an animal is in a reproductively active state, the animal may respond more to stimuli that are associated with reproduction and the opportunity to mate. Behavioral responses to auditory stimuli are modulated by breeding state and estrogen in several species. Female frogs move toward a speaker emitting a male mating call only when they are in breeding condition or are treated with estradiol (Chakroborty and Burmeister, 2009). Female songbirds display more copulation solicitation displays (CSDs) for males singing a song they prefer (Brenowitz, 1991; Zann, 1996). Female canaries that are photostimulated by increasing the number of daylight hours (to increase estradiol) perform CSDs to playback of male songs, whereas females concurrently treated with an estrogen-synthesis inhibitor do not exhibit CSDs during the course of the treatment, and show delays in displaying and egg-laying after treatment has ended (Leboucher et al., 1998). Estradiol treatment administered

peripherally induces receptive behavior in female birds in species that ordinarily do not exhibit CSDs to song stimulation in captivity (e.g. zebra finches; Vyas et al., 2008). Furthermore, in some species the degree of CSDs observed in response to male song is positively correlated to the dose of estradiol (Searcy and Capp, 1997). Presumably, the peripheral estradiol treatments (or the natural levels associated with females in breeding condition) affected the animals' motivation to mate, and to attend more to signals associated with reproduction (e.g., mating calls or songs) than to signals that are less related to reproduction (e.g., tones, silence). It is important to note that these changes in motivational state are probably not exclusively due to changes in sensory processing or behavior, but to a combination of both.

Attempts have been made to associate the concept of "motivation" with a set of brain structures that can be studied in response to hormonal manipulations. A model was described originally in mammals (Newmann, 1999; reviewed in Maney et al., 2008) and more recently in songbirds (Maney et al., 2008) as the "Social Behavior Network". The model identifies a group of brain structures including hypothalamic nuclei and areas of the limbic system that are modulated by both hormones and social context: medial amygdale (MeA), bed nucleus of the strius terminalis (BNST), lateral septum (LS), ventral tegmental area (VTA) and Central Gray (GCt) in the midbrain, ventromedial hypothalamus (VMH), anterior hypothalamus (AH) and the medial preoptic area (mPOA). All structures are reciprocally connected to each other and are sensitive to gonadal steroids. Newman hypothesized that all social responses, including courtship and aggression could be controlled by this network, and that the pattern of activity as a

unit is what determines the behavioral responses to social stimuli, rather than activity within a single brain area (in Maney et al., 2008, p. 174).

Maney et al (2008) describe a homologous network within songbirds, and tested the effects of estradiol manipulation on ZENK expression within each "node" of the network in response to male song or tones. They also recorded the number of CSDs displayed in response to the playbacks. They found that ZENK was higher to song than to tones in nearly all areas of the network in estradiol-treated females, whereas blank-implanted females on the same short-day cycle had similar levels of ZENK to tones and song. These results complement the results of this group's previous study showing that estradiol-treatment similarly increases selectivity for song versus tones in auditory areas NCM, CMM, and the homolog of inferior colliculus MlD (Maney et al., 2006). Also similar to their previous study, the authors point out the selectivity that resulted from estradiol was not due to an increase in the response to song, but due to suppression in response to tones. This physiological selectivity fits nicely with the view that hormones shift an animal's perception towards stimuli associated with the animal's motivational state/goal and away from stimuli that are less relevant to the animal's motivational state to influence behavioral responses to the same stimulus. Beach originally stated that "social signals, which can take the form of courtship displays, aggressive threats, or hungry offspring, act as triggers for behavioral responses, and hormones raise or lower the threshold for performing those responses" (in Maney and Pinaud, 2011, p. 287). The findings of Maney et al., 2006 and 2008) support a role for estradiol in modulating motivation (estradiol increased selectivity of ZENK levels across the Social Behavior Network), auditory processing (estradiol increased selectivity of ZENK in three auditory

areas), and behavior (estradiol induced CSDs in all estradiol treated birds in response to songs only in both studies). Though I did not test behavioral responses but only auditory responses to songs in my birds, lowering estradiol would have lowered the responses to songs within the Social Behavior Network (as described) while at the same time affecting processing of the song stimuli in NCM (and possibly CMM and other areas). These effects, along with the possibility that Fadrozole decreased hearing sensitivity at the cochlea, could contribute to my own result that Fadrozole-treated females had lower responses to songs than Saline-treated controls.

Further evidence from studies in quail has shown that preventing estradiol synthesis could modulate motivational state without necessarily inducing changes in auditory processing. One area within the social behavior network, the hypothalamic preoptic area (POA) has been extensively studied for its role in regulating aggression and sexual responsivity in male quail, a non-songbird species. Cells within this area express aromatase and estrogen receptors. Schlinger and Callard (1990) showed that, for testosterone to be effective in inducing aggressive behaviors in response to presentation of a male conspecific, it must be metabolized to estradiol within the POA. Male aggressive behaviors toward another male were only induced in males that had received injections of testosterone (T) or estradiol (E2), but not with dihydrotestosterone (DHT; which is non-aromatizable) or testosterone + the aromatase inhibitor 4-hydroxyandrostenedione (OHA). Other studies on male sexual responses have shown that T-injections increase sexual behavioral responses (e.g., mounting attempts) during interactions with a female, but again, that the T within this area must be converted to estradiol to affect behavior (Cornil et al., 2006). Injections of the aromatase inhibitors

prevented T-injections from increasing sexual responses in males during interactions with a female. These behaviors are also decreased when aromatase inhibitors are implanted directly into the POA (Balthazart et al., 1996). Similar experiments conducted in male zebra finches have yielded similar results (Harding et al., 1983; Balthazart et al, 1999). Castration of male finches reduced courtship behaviors, including directed singing, beak-wiping, and copulation attempts but these behaviors were restored when castrated animals received supplementation with either T or E2 (reviewed in Balthazart et al, 1999). These results suggest that Fadrozole, in addition to reducing estradiol in the NCM, likely reduced estrogen within the POA and could have influenced sexual motivation or even aggressive behaviors in my male zebra finches. If zebra finches were known to use song in aggressive encounters to defend their territory, I would expect that decreasing estradiol might decrease responses in the POA and possibly auditory areas as a result of decreased motivation. However, zebra finches are not known to engage in song as a means of aggression, so the role of estradiol affecting motivation and subsequent response to song in auditory areas is difficult to predict.

The interconnection among an animal's internal/hormonal state and the responses to external social signals becomes more complicated to study because the signals themselves can play back onto the hormonal state of the animal. Gonadal hormones have been shown to increase in response to sexually relevant stimuli in multiple species. Playback of male vocalizations can increase circulating estradiol in female frogs (Lynch and Wilczynki, 2006; Wilczynki and Lynch, 2011; reviewed in Arch and Narins, 2009), ring doves (Lehrman and Friedman, 1969; Cheng, 1986), as well as zebra finches. Tchernichovski et al. (1998) showed that playback of male zebra finch songs induced

increased levels of circulating estradiol in females after five days, and the females subsequently began to lay eggs. Another interesting demonstration of how the social environment can affect an animal's hormonal milieu has been documented in a non-songbird species, the ring-dove. In this species, males court females by approaching them with bowing and cooing displays and then settles into a nest site and begins to coo to her. The female responds by settling into his nest site and the pair engage in a duetting of nest coos for a day or two, and copulation occurs multiple times during this duetting routine. Thereafter, the female engages in solo cooing, while the male perches nearby, and begins to lay eggs shortly after the onset of this solo cooing behavior (reviewed in Cheng, 2008). Initial experiments suggested that ovarian maturation was induced as a result of females hearing the male vocalizations (Lehrman and Friedman, 1969). However, a set of follow-up experiments showed that the ovarian maturation and egg-laying was not induced by solely exposure to male cooing, but by production of her <u>own</u> vocalizations. In other words, hearing herself coo back to male vocalizations "self-stimulated" her hypothalamic-pituitary-gonadal (HPG) axis, leading to the increased ovarian hormones (including estradiol), maturation of follicles, and subsequent egg-laying (reviewed in Cheng, 2008).

Similar increases in circulating androgens in males occur in male hamsters when placed into contact with a conspecific female (Harding, 1981). In some species of fish in which males emit calls to attract females and defend their territory (reviewed in Bass, 2008), playbacks of conspecific male calls increase testosterone in male toadfish within minutes (Remage-Healey and Bass, 2005, 2006). In male song sparrows, a territorial songbird species in which song functions in territorial defense, testosterone also increases

following exposure to conspecific song (Wingfield, 1985). Thus, hormones can alter motivational state of the animal and alter behavioral responses to stimuli, but social signals themselves can induce changes in hormonal state and, in turn, alter the motivational state during a social interaction. As others have suggested (Pfaff et al., 2008) these are all examples of the idea that hormones act to establish coherency between the behavioral response to a sexually relevant stimulus and the reproductive capability of the body within a given time period.

NCM within a functional network of song-processing

I recorded NCM in my studies because my primary goal was to test the effect of estradiol depletion on the neural memory for conspecific songs, with respect to tutor-song and more recently heard songs. NCM has been shown to exhibit memories for both tutor-song in males (Phan et al, 2006; Terpstra et al., 2004) recently heard songs in both sexes (Chew et al., 1995, 1996), to be sensitive to estradiol (Schlinger, 1997; Saldanha et al., 2000). Previous studies have shown that NCM expresses estrogen receptors and aromatase (Schlinger, 1997; Saldanha et al., 2000) and that estradiol increases in NCM when the bird hears conspecific songs (Remage-Healey et al., 2008; 2011). In addition, when estradiol action is prevented in NCM (by blocking receptors or preventing estradiol synthesis), responses that are typically observed when a bird hears conspecific song playback are eliminated: *zenk* induction is blocked, and the increased firing rate to conspecific song is prevented. Importantly, behavioral discrimination is also disrupted for Tutor versus novel song when estradiol action is blocked locally within NCM (Pinaud

and Tremere, 2011). These results provide a compelling evidence that estradiol action within NCM is required for NCM to process song normally and to enable the behavioral preference or discrimination among conspecific songs (Tremere et al., 2009; Pinaud and Tremere, 2011; Remage-Healey et al., 2008). These observations elucidate how just one area of the auditory system can enable (or disrupt) a behavioral response to a stimulus. However, the contribution of NCM and its dependence on estrogen provides a richer understanding of how the auditory system may function as a set of interconnected "units" working together to allow an auditory stimulus to be identified and to influence behavioral responses to a song stimulus. NCM is one of several auditory processing areas that show selectivity for conspecific songs. Subregions of Area L project not only to NCM, but to CMM. NCM and CMM are reciprocally connected (Gentner et al., 2004; Theunnissen et al., 2004). In addition, the lateral most extent of CM (CLM) sends projections to HVC via the shelf region and indirectly through area Nif (Ball and Gentner, 1998; Gentner et al., 2004).

NCM and CMM are similar in that both areas respond more strongly to conspecific relative to heterospecific song, in IEG and electrophysiological response magnitudes (Chew et al, 1995; Mello et al., 1992; Gentner et al., 2004; Theunnissen et al., 2004). However, their responses among conspecific songs differ according to the category of stimulus presented, and the bird's previous experience with that stimulus. NCM responds more strongly to novel conspecific songs (measured as ZENK and electrophysiology), and both the ZENK and electrophysiological responses adapt with repeated presentations (Mello et al. 2002; Chew et al, 1995). NCM responds uniquely to individual songs of other birds (i.e., exhibits stimulus specific adaptation and these

adapted responses are maintained for many hours), and appears to be important for the process of learning the song of one's tutor (Phan et al., 2006; Terpstra et al., 2004; London and Clayton, 2008). In general, NCM shows changes in responses to a song as it becomes familiar; over repeated presentations, the responses adapt and are maintained, suggesting that NCM participates in forming a representation of the songs a bird hears (a form of non-associative learning: Gentner et al., 2004). For a bird to respond differentially to the songs of other individuals requires the auditory system be able to associate the song with the individual or circumstance. Unlike NCM, which responds more strongly to novel songs, CMM responds more strongly to familiar songs that have been paired with a behaviorally relevant stimulus (e.g., food) relative to novel songs that are unpaired, and so is thought to contribute to the associative learning that occurs between a song and a behaviorally relevant consequence (Gentner et al., 2004). For example, starlings trained on a go no-go task in which correct choices result in food reward exhibit higher ZENK to the go versus the no-go stimulus.

Together, NCM and CMM might enable the learning of a song's identity and its relevance. Gentner et al (2004) suggest that "novel information may be admitted into both NCM and CMM at all times, but only held or consolidated in CMM if coupled with an associative signal (p. 1009)". For a male songbird, this connection may be important in deciding whether or not he is hearing a threatening song and engages in aggressive behavior (including singing) to defend his territory. For females, the NCM-CMM connection might underlie the behavioral preference for songs that resemble her tutor (Reibel, 2000), the song of her mate (Milller, 1979b), or even songs that sound like her own species (Lauay et al, 2003). Gentner et al. (2001) showed in female starlings that

CMM exhibits higher ZENK in response to long-bout songs, which the females prefer, than to short-bout songs. Under normal conditions, female zebra finches exhibit CSDs toward conspecific song relative to heterospecific song. When CMM is lesioned, this selectivity is lost, and the females display to both song types (MacDougall-Shackleton et al., 1998). One interpretation is that, in the intact female, CMM enables the female to recognize the conspecific song as more salient than the heterospecific song, and displays more to the male song of her own species as a result. However, in other species of songbirds, the HVC, an area of the song control system, also appears to play a role in the perception or behavioral response to song. In female canaries, lesions to the HVC induce the same behavioral preference deficit as CMM lesions induce in zebra finches (Brenowitz, 1991). The species difference might be due to the fact that female canaries can sing and that HVC sends input to the song control system, whereas female zebra finches do not sing and their HVC does not send inputs to the song control system (Macdougall-Shackleton et al., 1998). However, the authors point out that HVC might still be used in female zebra finches to make more subtle discriminations among conspecific songs. For females displaying to particular songs, CMM or HVC (or possibly both) could contribute to the behavioral display rates of certain songs depending on the species.

For males, a song's behavioral relevance differs from females because they do not rely on song for mate-selection. Instead, a song may indicate an aggressive which they can respond aggressively to or not by singing. If HVC is involved in the song perception and/or recognition in males and in females that sing, then lesions to the HVC in males should also induce behavioral changes that reflect a loss of selectivity for songs of

different behavioral relevance. Gentner et al. (2000) examined the role of HVC in perceptual and associational learning in a series of operant experiments. Birds were trained on an operant task that required them to first discriminate among songs and then to classify them as go-or no-go songs through many trials of experience with the songs and reward or punishment that resulted from their behavioral responses to playback. All birds learned to discriminate and categorize the go from the no-go stimuli and respond accordingly (i.e., peck for food at the go-, withhold from pecking for the no-go). After the initial training and testing trials, the go and no-go stimuli were reversed, and birds were required to learn the new associations of song stimuli. Following these training and testing sessions, HVC was lesioned and animals were tested for recognition of the previously formed associations and for acquisition of new associations (i.e., the go- and no-go stimuli were reversed). Although HVC-lesioned birds responded correctly to the previously learned categorization of go and no-go stimuli, they showed deficits in the acquisition of new associations when the stimuli were reversed, and the degree of impairment correlated with the size of the lesion in HVC. This result suggests that HVC plays an important role in forming new associations for behaviorally relevant stimuli.

As a collection of functional units then, auditory areas NCM, CMM and HVC appear to underlie the behavioral discrimination and recognition for songs that <u>are becoming</u> or <u>have become</u> behaviorally relevant as a result of auditory experience with those songs. The processing of new associations could be performed by CMM, HVC, or both, depending on the species and sex of the bird (e.g., the role of HVC in female zebra finch processing of songs is not known). In the Gentner et al (2000) experiments, no sex differences were reported for starlings.

NCM within this functional network is in a prime position to influence the formation of new associations for songs, and furthermore to ensure new representations for songs at a time when probability of reproductive success is high. Activity in NCM has been shown to be estrogen-dependent (Tremere et al., 2009) and additional estradiol contributes to better discrimination of songs from one another (Pinaud and Tremere, 2011). For females in a reproductively active state (modulated by photoperiod or humidity and food availability), high circulating estradiol produced by the ovaries could ensure that songs of potential mates are discriminated from one another, and that IEG expression associated with memory formation of those songs in NCM is induced (Tremere et al., 2009 showed that estradiol is necessary within NCM for ZENK responses, associated with memory formation, to be observed). The enhanced responses in NCM could ensure that the behavioral relevance attached to a particular stimulus is strongly/specifically attached to that stimulus. A second way that estradiol-dependent NCM might influence the associations formed within CMM and HVC is by sending these areas information about the quality or relevance of stimuli that are important for reproduction (e.g., Maney et al., 2006 showed that ZENK responses to tones are suppressed in NCM when estradiol is high).

In my experiments, I found that Fadrozole treatment reduced the response bias for conspecific songs in females. This might represent one way that NCM could modulate the information that arrives at CMM or HVC and new associations are formed. Importantly, CMM does not express aromatase or estrogen receptors, and estradiol levels are not elevated in this area when a female hears male song (Remage-Healey et al., 2012) suggesting that NCM processing of novel songs could be modulated directly by the

reproductive state of the female, and that the “filtered” song signals could be passed on to associative areas CMM or HVC. The shelf-region of HVC also expresses aromatase (Saldanha et al., 2000) and estrogen receptors (Gahr and Metzdorf, 1997), so HVC could also participate in the hormonal modulation of auditory perception in females for which HVC is important in discrimination. If this were the case, then the perception of signals more relevant to reproduction (e.g., quality conspecific songs versus unlearned songs versus heterospecific songs versus tones) would be sharpened and less-relevant signals filtered out at a time when reproduction is likely to occur.

For males, who do not use song in choosing a mate but rather in territorial defense, at least in many species, estrogen-dependent NCM could bolster success in acquiring or defending a territory at a time when circulating testosterone is high and reproduction with a female is more likely. Within a potential aggressive encounter, the male bird hearing another male’s song would increase estradiol in NCM by converting the plentiful testosterone precursor into estradiol (Remage-Healey et al., 2008). Estradiol could then act on NCM neurons to improve discrimination of the songs he is hearing, form memories for them, and possibly form better associations between the songs and the invader male who is singing them. For males, it may be that HVC, rather than CMM, plays an important role in forming the association between the song and the encounter.

Putting it all together

These experiments examined how communication signals are processed in the auditory system under the different conditions of sex, hormones, and developmental experience. Estradiol may be required in both males and females for the neuronal memory for songs to be formed and/or discriminated from novel songs. In females, estradiol may facilitate mate selection by altering auditory detection/discrimination independently or in the context of an overall motivational state. Given that estradiol is produced in both male and female NCM in response to song playbacks (Remage-Healey et al, 2009; Remage-Healey et al., 2012), estradiol production in NCM in response to song could enable neuronal memory for songs. Furthermore, in females, estradiol produced by the ovaries could "ensure" that memories for songs form in NCM and subserve the song-dependent selection of mates at times when she is fertile. During these times, estradiol could also guarantee that, in addition to forming memories of potential suitors, she could make sure she was selecting a mate from her own species. Other studies have shown that social variables modulate estradiol's rapid production and can modulate behavioral responses. Whereas I administered a peripheral aromatase inhibitor that most likely lowered estradiol availability to hypothalamic as well as peripheral and central auditory processing structures, investigations that manipulate or measure local estradiol suggest that estrogens exert their actions on the brain more like neuro-modulators, or even neurotransmitters, than as "endocrine" hormones (Saldanha et al., 2011 refer to this as "synaptocrine" signaling). These findings of the interaction of social context and hormonal modulation of communication signals leads to the exciting possibility that estradiol may be modulating behaviors on a moment to moment basis by

acting within perceptual areas that process communication signals. This function of estradiol is distinct from its well-known role in facilitating reproduction in females.

I found that in both males and females, estradiol depletion had negative effects on memory and/or discrimination of recently heard songs. If these effects are also produced in human males and females, then discrimination of communication signals will be affected and could contribute to deficits in verbal memory. The role of estradiol in human learning and memory has also gained interest, especially because of its potential importance in an aging population concerned about the onset of dementia. One study conducted in healthy older men showed a positive correlation between estradiol levels and scores on a verbal memory task, in which subjects were required to name a set of 16 objects presented pictorially to them, and then recall them after the objects were removed (Zimmerman et al., 2011). Two other studies tested the effect of testosterone depletion in elderly men. In the first, Cherrier et al (2003) tested verbal and spatial memory in males prior to and during treatment with testosterone or DHT (which is a non-aromatizable androgen). In men undergoing T treatment, both T and estradiol concentrations were higher in circulating plasma during treatment than during baseline, and this group showed improved verbal memory during the treatment, which was measured as recall of a spoken list of words and information from stories read to them. Men receiving DHT supplementation did not show improved scores of verbal memory, suggesting that the action of either T or E2 was contributing to the improvement in memory. In a follow-up study (Cherrier et al., 2005), the same group of researchers administered T alone or T plus the aromatase inhibitor Anastrozole and found that verbal memory increased in the men treated with T alone but not in the group co-treated with the aromatase inhibitor,

suggesting that testosterone's conversion to estradiol was facilitating verbal memory. Studies in women who underwent ovariectomy showed a decline in verbal memory in addition to other types of cognitive tasks, compared to women who had undergone the same surgery but received estradiol or testosterone supplementation (Sherwin, 1998; Philips and Sherwin, 1992; Hogervorst et al., 1999). The cognitive effects of blocking aromatase have also begun to be studied in women undergoing treatment for estrogen-dependent cancers, such as breast cancer (Lejbeck et al., 2010). So far, studies on the effects of preventing estradiol action have revealed that cancer patients receiving tamoxifen (to block receptors) have smaller hippocampal volumes and decreased glucose metabolism in the frontal lobes (Eberling et al, 2004). Jenkins et al (2004) showed that postmenopausal women taking aromatase inhibitors performed more poorly than age-matched controls on tests of verbal memory recall.

The role of estrogen in other forms of learning and memory has been extensively studied in the hippocampus within rodent models of spatial and recognition learning. Estradiol levels substantially modify the electrophysiological and morphological characteristics of hippocampal neurons. For example, estradiol greatly influences spine density of neurons in area CA1 of the hippocampus, and studies have been conducted to test how these changes may relate to processes of learning and memory. Gould et al (1990) found a 30% decrease in spine density in ovariectomized versus intact female rats, and that implanting the females with estradiol following ovariectomy prevented this decrease. Such differences in spine density were also observed during the natural fluctuation of estrogens across the female estrus cycle. As females transition from diestrus to proestrus, estrogen levels begin to increase, peak during proestrus, and then

decrease again in estrus. Woolley et al (1990) sacrificed females at different stages and showed that the changes in estradiol associated with each stage are paralleled by changes in spine density. Spine densities were moderately high in diestrus, were highest in proestrus, and then decreased by 30% between proestrus and estrus, when estrogen levels are lowest. The number of spines per dendrite was later found to correlate with the number of synapses (Woolley and McEwen, 1992). Estradiol also influences the electrophysiological responses of neurons within the same region. In one study (Wong and Moss, 2001), hippocampal slices were bathed in an aCSF solution and recorded for responses to afferent stimulation before and after estradiol application. Estradiol application resulted in increased response amplitude in the excitatory post-synaptic potential (EPSP) in response to afferent stimulation. In the same set of experiments, hippocampal slices were recorded using the same method, but slices were obtained from ovariectomized females that had received injections of saline or estradiol for two days prior to sacrifice. The slices obtained from estradiol-treated females exhibited more spikes in response to stimulation compared with slices from saline-treated females.

Estrogenic effects at the physiological level could facilitate learning and memory formation of stimuli or events associated with a specific outcome, and therefore contribute to better performance on behavioral tasks that involve these memory processes. In the Morris Water Maze, for example, animals learn to swim to a hidden platform, form a memory of its location, and then rely on the memory to find the platform more quickly over subsequent trials. Estradiol administration appears to modulate performance on this task. In one study, ovariectomized female mice injected with estradiol after the task was acquired found the platform significantly faster than saline-

treated controls (Gresack and Frick, 2006). The same animals were tested in an Object Recognition task, in which they first were allowed to explore two objects, then a day or two later, were presented with one familiar and a novel object and exploratory behaviors are compared to each object (novel objects will induce greater exploratory behaviors than familiar ones, so the "familiar" object should induce lower levels of exploratory behavior). Parallel to the results of the Water Maze task, estradiol injections immediately following the acquisition period led to enhanced memory (measured as significantly lower exploration of the familiar object than the novel one, relative to saline-injected females). Together, these results suggest that estradiol injections facilitated memory for the objects within the animals' environment and, as a result females treated with estrogen found the platform faster. Similar results of estradiol's memory enhancement effects in ovarectomized females have been observed in other tests of memory using the water maze (e.g., Rissannen et al., 1999; Sandstrom and Williams, 2004), the T-maze (Fader et al., 1998), radial arm maze (e.g., Luine et al., 1998).

One confound in the mammalian studies is whether the effects of estrogen treatment on memory are the result acquisition, recall, or both and are difficult to resolve since the treatments are administered chronically. The Gresack and Frick (2006) experiments were carefully designed to avoid these confounding variables associated with a prolonged treatment regimen. They specifically tested the effect of estradiol on acquisition of the memory for objects (Object Recognition Test) and the platform location (Water maze test). Injections of estradiol were administered immediately <u>after</u> the learning trials had occurred, at least a day before the retest trials began, thereby eliminating the confound I encountered in my own experiments. Because estradiol was

injected during a time when the animal is not actively learning the task or recalling a memory, estradiol is interpreted to facilitate memory acquisition, and acting during the consolidation process. Other studies have shown that estradiol is also effective when injected directly into the hippocampus immediately following the learning trials and (Packard and Teather, 1997; Fernandez et al., 2008). This effect has also been demonstrated in male rats (Packard et al., 1996). This approach would be useful and could be easily applied to my own experiments; injection of estradiol, saline, or Fadrozole in male AND females would aid in clarifying whether the estradiol depletion effects were in fact due to the acquisition of a memory for the auditory stimuli during the first playback session. Further analysis on the effects of estradiol within mammalian models that include estrogen effects on males as well as females will be especially informative of the degree to which estrogens influence various types of learning and memory.

As more about the role of estradiol action within the brain is discovered, side effects of drugs that lower estradiol on cognition in humans will become essential to consider. This may prompt the development of drugs that are effective but do not reach or affect the brain to prevent deficits in auditory processing. Estradiol may be modulating not only auditory perception, but other sensory areas in the brain. It may be that estradiol (perhaps in addition to other neuro-hormones) is necessary for adequate processing of other types of communication signals. These results are potentially applicable to and should continue to be examined in women (and possibly men) receiving estrogen receptor antagonists or aromatase inhibitors as part of the treatment for estrogen-dependent cancers. Several studies have already begun to examine these effects, although the

effects on memory may be confounded by the potential effects of chemotherapy the brain and cognition via other mechanisms, e.g. neurogenesis. Estradiol depletion induced similar deficits in neural memory or discrimination in males and females in my study, and these findings could be extended to men receiving similar estradiol inhibiting drugs and possibly drugs that suppress estradiol precursors, such as testosterone, since this also could lead to very low concentrations of brain estradiol.

Future directions

The studies presented here investigated the importance of estradiol in adult memory and auditory processing, but an examination of the importance of estradiol in the juvenile brain is well warranted, since estradiol deprivation may influence the development of these brain areas as well as when birds are learning to discriminate (e.g., Gobes et al. 2010) among communication signals. The types of studies I have done in adult birds should also be conducted in juvenile songbirds during song-tutoring, to determine whether local estradiol within auditory processing areas is necessary for learning to discriminate (and, in males, later produce) communication signals. Considering the effects of reducing estradiol availability on auditory areas in adults, treatments that potentially limit the availability of estradiol and its precursors (e.g. drugs used to lower cholesterol) in children should be carefully considered. Future studies of the kind I have conducted in a songbird model will be valuable in determining variables that induce deficits in learning and processing of communication signals.

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