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The Contribution Of Genes And Hormones To The Sexual Differention Of The Zebra Finch Song System

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THE CONTRIBUTION OF GENES AND HORMONES TO THE SEXUAL DIFFERENTION OF THE ZEBRA FINCH SONG SYSTEM

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DEDICATION

This work is dedicated to my parents Priscilla Cure and Christopher Zito, for the wellspring of support and encouragement I've been lucky enough to experience my entire life; to the rest of my family for their advice and faith; and to my husband Andrew for providing a patient ear when I needed to talk things through, humor when I got frustrated, cheers when I needed to be reminded of my little successes, and a gentle tug here and there to pull me back from the brink of procrastination. I love you.

"*There is no cure for laziness but a large family helps.*"

~Herbert Prochnov

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CHAPTER 1

INTRODUCTION

There has long been an interest in understanding the mechanisms driving sexual differentiation in humans. Ethical considerations prevent the scientific community from conducting research with enough experimental control to generate cause and effect relationships. As such studying the underpinnings of sexual differentiation in an animal model can broaden our understanding of general mechanisms governing sexual differentiation. One animal model uniquely suitable for this task is the zebra finch. Zebra finches exhibit striking sex differences in behavior that are linked to differences in brain morphology. One of the most striking differences is song production. Male finches sing and females do not and their brains clearly reflect this sex difference. A large portion of the zebra finch brain is devoted to learning and producing song. Many of these areas are larger in males than in females (Arnold, 1997b). Zebra finches and other songbirds have a long period of song learning that is so far unparalleled in any other species except humans, allowing us to study the mechanisms of sexual differentiation from a developmental perspective. Zebra finches also live in complex social groups. One practical benefit is that the zebra finch genome has recently been completely mapped and annotated. These benefits, coupled with a burgeoning understanding of zebra finch endocrinology, allow us to study sexual differentiation from a behavioral, hormonal and genetic standpoint.

1.1 The Zebra Finch Song System

Male zebra finches produce a stereotypical song that is thought to be solely important for attracting a female, since zebra finches are not territorial (Searcy & Yasukawa, 1996). The use of temporary and reversible vocal distortion techniques such as the transection of the tracheosyringeal nerve (which is the nerve that connects the song production pathway to the

syrinx, the vocal apparatus, the transection ofwhich results in low quality song of similar length and complexity) and inter-clavicular air sac puncturing (which results in a temporary absence of audible song output from the loss of pressure in the air sac surrounding the syrinx), has revealed that when given the choice between a control male and a male singing distorted song, the female is significantly more likely to choose a male singing unaltered, high quality, song (Tomaszycki & Adkins-Regan, 2005). Females also prefer tutored song (song learned from an adult male, preferably the father) over song by males reared without adult males (Lauay, Gerlach, Adkins-Regan, & Devoogd, 2004). Thus, song is learned, is highly important for adult courtship and pairing behavior, and involves a large portion of the brain.

1.2 Sex Differences in Brain Morphology

Research on song production and learning in zebra finches has focused primarily on areas in the telencephalon (Arnold, 1996). Area X and the lateral part of the magnocellular neostriatum (LMAN) are known to be crucial for song acquisition, as lesioning these areas during development inhibits song learning in males (Doupe & Solis, 1997). The HVC (used as proper name) and the robust nucleus of the archistriatum (RA) have been identified as areas important for song production, specifically the motor aspects of song (Wade, 2001). HVC has projections to RA which in turn innervates the tracheosyringeal nerve via the hypoglossal nucleus (nXIIts), providing a direct link between the brain and the syrinx (Wild, 2004).

Concurrent with their role in singing behavior, there are significant sex differences in the size and/or structure of these regions. The most marked difference is in Area X, which never develops in female zebra finches. HVC and RA are larger in volume in male zebra finches with greater cell densities and larger soma sizes (Nottebohm & Arnold, 1976). The projection from HVC to RA is denser in males and XIIts is correspondingly larger in volume (M. E. Gurney,

1981). Unlike other song nuclei, there are no sex differences in LMAN volume in adulthood. However, there are differences which are apparent at the cellular level. The somas in female finches show a reduction between days 35 and 60 post-hatching, resulting in a sexual differentiation of cell size (Nixdorf-Bergweiler, 2001). These differences in the song system and in singing behavior have been thought to be regulated by hormone exposure early in development.

1.3 Organizational effects of hormones on sexual differentiation

In all mammals females posses the chromosome pair XX, while males possess XY. The SRY gene on the Y chromosome causes testes to form in male fetuses, which, in turn, begin to produce testosterone which sets of a cascade of events assumed to generate sex differences in peripheral morphology, neural morphology and behavior. The understanding that steroid hormones have a profound effect on the sexual differentiation of brain and physiology is decades old. In 1959, Phoenix and colleagues, proposed the "organizational" and "activational" mechanisms of hormone activation. They proposed that "organizational" effects of sex hormones differentially organize neural pathways in permanent ways during critical periods early in development. "Activational" effects occur much later and emergent behaviors or physical features are dependent on earlier organization (e.g. male facial hair or female menarche in humans). Cause and effect relationships for sexual differentiation in humans are difficult to determine due to ethical reasons, but correlational studies involving individuals with sex chromosome disorders shed some insight into hormone and behavior relationships in humans. The best example is complete androgen insensitivity syndrome (CAIS). Due to multiple mutations in the androgen receptor gene, it is not possible for the testosterone secreted by the testes to act at their receptors and thus effect development (Hughes & Deeb, 2006). A genetic

male develops female neural morphology and external genitalia. They are frequently assumed to be female at birth until following breast development, menarche does not occur (Griffen, 1992). This genetic disorder not only highlights the importance of steroid hormones in normal development, but also the importance a properly functioning X chromosome (Lubahn et al., 1988).

Other mammals have provided excellent experimental models of sex differences. Female rats have been shown to take significantly longer in spatial navigation tasks due to smaller hippocampal volumes in comparison to males, and treatment with neonatal testosterone in females eliminated this difference (Roof & Havens, 1992). There is also evidence of sex-typical mating behavior in rats (Seward, 1945), and this behavior can be masculinized by neonatal hormonal manipulations.

Supporting the organizational hypothesis, there are concurrent brain regions that are also affected by steroid hormones in rats. These include: the sexually dimorphic nucleus of the preoptic area of the hypothalamus (Gorski, 1978) and the hippocampus, which has been implicated in spatial navigation (Jacobs, Gaulin, Sherry, & Hoffman, 1990). Non-human primates have also been studied extensively. In rhesus macaques, androgen secretion begins at D40 prenatally and continues until 3 months postnatally (Mann et al., 1984). Contrary to the rodent literature, neonatal manipulation of androgens in rhesus monkeys affect mother-infant relationships in small ways (Wallen, Maestripieri, & Mann, 1995). Castration (Goy, 1978) or neonatal suppression of gonadatropic hormone did not have any effects on stereotypically sexually dimorphic behaviors in male rhesus monkeys, suggesting that the masculinization process occurs during the prenatal period, and does not continue postnatally.

Support for the organization of morphology and behavior during the prenatal phase comes first from the substantial modification of genitalia in prenatally-treated females (Wallen, 1996). Furthermore, we know that sexual differentiation is strongly dependent on the timing of administration, which, for genital morphology, occurs during the second trimester (Wallen, 1996).

However, prenatal androgens may not be the entire story in rhesus macaques. Changes in sexually dimorphic behavior can also be seen in the rhesus monkey in the absence of androgen manipulation. Restricted rearing contexts such as peer groups (reared only with same-aged individuals, without adults, including mothers) induced changes in stereotyped male behavior like rough and tumble play, such that there was an increased frequency of rough and tumble play in the peer-group reared males in comparison to normally (socially) reared animals (Wallen, 1996). Changes in sexually dimorphic behavior without concurrent changes sex hormones suggest mechanisms of sexual differentiation that exist independently of circulating hormones, such as changes in social environments. Administration of prenatal androgens to females masculinized behavior regardless of rearing conditions, but particular behaviors were dependent on hormones administered at specific periods during gestation (the second or third trimester) and could occur in the absence of genital masculinization, which happens during the second trimester (Goy, Bercovitch, & McBrair, 1988). Furthermore, masculinization was not complete, suggesting that higher doses of androgens are needed to masculinize behavior relative to genital morphology, or that other mechanisms are important.

1.4 The organizational hypothesis: does testosterone always masculinize morphology and behavior?

One assumes that behavior is masculinized by androgens, but the "aromatization hypothesis" (Feder, 1981; MacLusky & Naftolin, 1981) suggests otherwise. Estradiol is formed by testosterone via the aromatase enzyme, which then masculinizes the individual. Studies of rodents have shown that estrogens play a large role in the normal development of males. Thus, the theory suggests that masculinization is paradoxically occurring in response to the presence of the "female" hormone estradiol. Evidence in female rats and mice exposed to high amounts of exogenous estradiol lends support to this theory (Christensen & Gorski, 1978). In the normal course of development alpha-feto protein binds to estradiol in females and prevents it from crossing the cell membrane (MacLusky & Naftolin, 1981). However, exposure to exogenous estradiol overwhelms this process permitting some estradiol to cross, thus causing masculinization of sexual behavior in the female animal (E. Adkins-Regan & Ascenzi, 1990; Bakker et al., 2006).

Due to the popularity of the aromatization hypothesis in rodents, many studies in zebra finches have focused strongly on estrogen-driven development to explain the masculinization of the song system. Indeed, a similar phenomenon has been observed, to some extent, in female zebra finches. Early post hatch exposure to estradiol (E2) has been shown to masculinize singing behavior of the female finch (Arnold, 1997a). When females are implanted with both testosterone (T) and E2 on the day of hatching, volumes of HVC and RA increased as did soma size in RA, HVC and LMAN by day 60. E2 was found to be more effective in masculinization than T, consistent with the aromatization hypothesis. However, these regions were still consistently smaller in females than males, even when coupled with T treatments in adulthood(Adkins-Regan, Mansukhani, Seiwert, & Thompson, 1994).

Other research on female zebra finches does not support the aromatization hypothesis. Treatment with tamoxifen (an anti-estrogen) also has masculinizing effects: increasing cell size in LMAN and HVC in both males and females (Mathews & Arnold, 1990). Furthermore, these same treatments in the first 25 days induced development of area X in females (Mathews & Arnold, 1991). Also puzzling is the fact that *inhibiting* aromatase activity also causes masculinization. Pre-hatch treatment with fadrozole (an aromatase inhibiter) caused the formation of an ovitestis on the left side of the female where the ovary normally develops, and a testis on the right side where there is normally no gonadal tissue (Wade & Arnold, 1994). In adulthood the testis was functional and produced sperm. Brain morphology was also similar to males (Gong, Freking, Wingfield, Schlinger, & Arnold, 1999). The results in the female zebra finch, though paradoxical, strongly suggest the role of sex hormones steroids in the masculinization of the song system.

Although data from female zebra finches partially supports the aromatization hypothesis, data from male finches undermines the theory that sex steroids regulate sex differences in zebra finch brain and behavior. Castration in male finches eliminates copulatory behavior and reduces courtship behavior, but does not eliminate song (Harding, Sheridan, & Walters, 1983). Additionally, blocking E2 in multiple ways and at multiple ages fails to prevent masculinization in the male finch (Grisham & Arnold, 1995). Inhibiting aromatase activity with vorozole (a competitive inhibitor of the aromatase enzyme) decreased, but did not eliminate, song in males, nor did it alter brain morphology (Balthazart, Absil, Fiasse, & Ball, 1995). If E2 were necessary for masculinizing the song system then it makes sense that treatment with anti-estrogens should have had a demasculinizing effect. Pre-hatch treatments in males have likewise yielded conflicting results. Pre-hatch treatment with fadrozole (and aromatase inhibiter) despite the

dramatic results in females, showed no effect on males (Wade & Arnold, 1996). In fact, pre-hatch treatment with fadrozole paradoxically hyper-masculinized singing in adult males (Wade & Arnold, 1996).

Thus, various treatments which alter the exposure of estradiol in females have yielded partial masculinization; however there is no treatment has been found that will reliably demasculinize the male finch. If sex steroids were the whole story behind sexual differentiation of the song system, this would not be the case. Similarly we would expect there to be sexually dimorphic expression of estrogen receptors (ER) during relevant developmental stages, however, ER is similar in male and female finches as late as P20. These results strongly suggest another mechanism underlying the development of masculine behavior and morphology in zebra finches.

1.5 Sex Steroids in the Zebra finch song system

Given the focus of past literature on the aromatization hypothesis, it is interesting to note that, compared to other songbirds, the zebra finch has relatively few aromatase receptors (ARO) in song nuclei (Metzdorf, Gahr, & Fusani, 1999). However, the highest level of AROs exist in HVC (Saldanha et al., 2000). LMAN seems to be other only other area which expresses ARO and only at moderate levels compared to HVC (Metzdorf, et al., 1999).

In the adult zebra finch, the greatest levels of androgen receptor (AR) expression occurs in the HVC and the magnocellular neostriatum (MAN) (K. W. Nordeen, Nordeen, & Arnold, 1986). Expression of AR is sexually dimorphic in HVC as early as P12-P20 (Bottjer, Glaessner, & Arnold, 1985). Starting at P15 until P30 the size of HVC increases by almost 3 times in male finches (K. W. Nordeen, et al., 1986). Despite assumptions of the masculinizing effects of estradiol, estrogen receptors (ER) do not appear until P15 when HVC is already sexually

dimorphic (Gahr & Metzdorf, 1999) and thus are not responsible for the early development of sexually dimorphic morphology.

What role, then, does E2 play in masculinizing the song system if it isn't responsible for initiating the process? One suggestion is that E2 may be acting during song template formation to promote survival and addition of neurons in HVC and MAN (E. J. Nordeen & Nordeen, 1989). It has also been suggested that E2 may be acting on HVC by promoting or suppressing genes that effect the process of sexual differentiation (Burek, Nordeen, & Nordeen, 1995). This is further supported studies examining the effect of later T administration to females previously treated with E2. Along with increases in HVC, RA and LMAN there is also the appearance of Area X with a greater likelihood of song attempts than in females treated only with E2 (Gurney, 1982).

1.6 Genetic contributions to sexual differentiation of brain and behavior

Based on previous evidence described above it would seem steroid hormones are not solely responsible for masculinization of the zebra finch. A rare opportunity to explore this hypothesis was presented with the discovery with a completely gynadromorphic zebra finch. On one side of its body the finch was phenotypically male and on the other side female (Agate et al., 2003). Sexual differentiation between the male and female hemispheres was identical to that observed between normal male and female finches. *In situ* hybridization confirmed that expression of the Z chromosome was higher on the left side (the male side—males are ZZ, females are ZW) than the female side, and W chromosome expression was restricted to the right side (Agate, et al., 2003). With both halves of the brain exposed to the same level of circulating hormones, a solely hormonal theory of sexual differentiation would expect similar feminization or masculinization across both hemispheres.

In order to understand the relevance of recent findings implicating genetics in the sexual differentiation of the song system, a working knowledge of function of gene dosage compensation in the zebra finch is necessary. In mammals, females express the homozygous chromosome pair XX, while males express a heterozygous chromosome pair XY. Despite having two dosages of the X chromosome, males and female show the same levels of X activation. During early development one of the X chromosomes in the somatic cells of female mammals becomes inactive (Lyon, 1989). Through X-inactivation the phenomenon, first discovered in *Drosophila malanogaster* (Bridges, 1922a, 1922b) referred to as "dosage compensation" occurs (Muller, 1932). In zebra finches, the homozygous chromosome pair belongs to the male (ZZ) with the females having the heterozygous pair (ZW). Unlike mammals, dosage compensation does not seem to be as effective in birds as it is in mammals (Ellegren, 2002; Itoh et al., 2007). Across bird species Z genes are expressed at consistently higher levels in males compared to females.

The discovery of the gynadromorphic finch led to a microarray analysis to identify genes which are differentially expressed in the telencephalons of male versus females zebra finches (Wade, Tang, Peabody, & Tempelman, 2005). Several genes have found to be sexually dimorphic and specific to song nuclei. Increased expression of ribosomal proteins L17 and L37 has been found in the song system of juvenile male zebra finches compared to both adult zebra finches and juvenile females (Tang & Wade, 2006). Expression of L7/SPA an estrogen receptor co activator has shown increased expression in juvenile males compared to females using western blot analysis (Duncan & Carruth, 2007). Increased expression of a secretary carrier membrane protein (SCAMP1) has been shown in HVC and area X of juvenile male zebra finch relative to same aged females (Tang, Peabody, Tomaszycki, & Wade, 2007). The zebra finch

genome has been completely sequenced and annotated by the Songbird Neurogenomic Initiative (Replogle et al., 2007). Studies have since identified genes that are both sex-linked and expressed more in the male song system versus the female song system at post-hatch day 25, which gives us the opportunity to begin to examine the time course of this expression (Tomaszycki et al., 2009). Day 25 is particularly relevant to development of the song system. Song-template formation is well underway in both males and females, and sensorimotor integration has begun for males (fig 1). All six genes were shown to map onto portions of the zebra finch Z-chromosome.

There are three genes that are of particular interest, due to their known identities and effects on brain and behavior. Genbank: CK313884 (17-β-hydroxysteroid dehydrogenase type IV) converts estradiol into its inactive component estrone. Genbank: CK310795 (Methycrontonyl-CoA carboxylase beta chain) may facilitate song learning due to its link with NMDA receptors, and therefore, long-term potentiation (Aamodt, Nordeen, & Nordeen, 1996). Humans deficient in this gene exhibit motor deficits, learning disabilities, attention-deficit disorder and reduction in white matter (Baumgartner et al., 2004). Genbank: DV946640 (sorting nexin 2) was shown to be differentially expressed in Area X. Deletion of sorting nexin 1 and 2 has lethal consequences in developing mice (Griffin, Trejo, & Magnuson, 2005). Sorting Nexin 2 (SNX2) may be related to maintaining neural circuitry essential for learning in human males (Small et al., 2005), as well as promoting the survival and incorporation of new cells in area X and HVC (Tomaszycki et al., 2009). This research suggests that genes on the Z chromosome may play a role in the masculinization of the song system.

In light of data showing conflicting results with estradiol treatment, a possibility exists that exposure to exogenous estradiol may, in part, compensate for the reduced gene expression in

females. The present study focused primarily on the expression of CK313884 in HVC in male and female zebra finches. CK313884 codes for 17 beta hydroxysteroid dehydrogenase type IV (17BHSD4) which converts into its inactive metabolites. 17-β estradiol has previously been implicated in differentiation of the song system in zebra finches (M. E. Gurney & Konishi, 1980). It is reasonable to assume that 17 beta hydroxysteroid dehydrogenase type 4 would be expressed in the same location as ER responding to 17 β estradiol as its role is to break down estradiol into its inactive component estrone. Examining distribution of estrogen receptors (ER) in the song system, it was found that ERs were mainly localized to HVC starting at post hatch day 15, but in low levels across development (Gahr, 1996). To examine the relationship of hormones and genes it seems prudent to start with a familiar paradigm. We know that estradiol when administered to the developing female finch masculinizes song system morphology. This study asks two primary questions: First, how does estrogen treatment affect expression CK313884 mRNA expression? Secondly, is this gene located in the same cells as androgen receptors? This co-localization might play a part in the masculinization process, since estrogen receptors are low in the song system (Gahr & Metzdorf, 1999).

Chapter 2

Methods

2.1 Animals and Tissues

All tissues for all experiments were collected from animals living in large colonies cages containing multiple males and females, as well as their offspring. Birds were implanted with either a 1mm pellet containing 50µg of 17β-estradiol or a blank pellet on the third day post hatching. At post hatch day 25, the brains were collected by rapid decapitation, frozen in cold methyl-butane and stored at -80°C. Sex was determined by examining the gonads post-mortem under a dissecting microscope. The presence of the pellet and sex of the animal was confirmed in all subjects. Subjects who did not have visible pellets were not included in the study.

2.2 Histology

Brains were sectioned coronally (20µm) and mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). Six series of sections representing the whole brain were collected and store at -80°C with dessicant. The final sample included 6 animals in each group (6 females and 6 males treated with estradiol; 6 females and 6 males implanted with a blank pellet). Thus, a total of 24 animals were included in the study.

2.3 Probe Preparation

Colonies used to generate probes were obtained from glycerol stocks, and plasma DNA was isolated and confirmed through sequencing. To obtain enough clones for *in situ* hybridization, a Qiagen Maxi Prep kit (Valencia, CA) was used, and the templates were then linearized using the restriction enzymes Xhol (T3) and NotI (T7). In all cases, T3 was the anti-sense strand and T7 was the sense strand.

2.4 Double-label Fluorescence In Situ Hybridization

In situ hybridization was adapted from (Pinaud et al., 2004). Briefly, slides were brought to room temperature, fixed in 3% paraformaldehyde and rinsed in phosphate buffered saline (PBS). Slides were incubated for 10 minutes in 0.1M triethanolamine hydrochloride with 0.25% acetic anhydrate then rinsed three times in 0.2M sodium phosphate, sodium chloride and EDTA (SSPE), dehydrated in ethanols and air dried for 10 minutes. Slides were hybridized overnight at 55°C 200µl of hybridization buffer, which included 12µl of probe.

Posthybridization was accomplished as follows: parafilm coverslips were removed by rinsing in 2X SSPE, than washed in 2X SSPE at room temperature for 30 minutes on shaker. This was followed by a wash in 2X SSPE/50% formamide for 1 hour and 65°C, then washed two times in 0.1X SSPE for 30 min at 65°C. Anti-DIG-FITC signal detection was accomplished by incubating slides in 0.3% hydrogen peroxide in TNT buffer for 10 minutes followed by rinsing slides in TNT buffer for 5minutes on shaker 3 times. Slides were then washed in TNB buffer (TNT buffer with 2mg/BSA) for 30 minutes, then incubated in TNB buffer containing Anti-DIG-POD antibody (1:100; 10 µg/ml, Roche Diagnostics, Indianapolis, IN) for 2 hours, followed by further washes. This was followed by an incubation for 30 minutes in a 1:100 tyramide-conjugated fluorophore in manufacturer's buffer (Alexa 594, Molecular Probes, Carlsbad, CA). Slides were then incubated in 0.3% Hydrogen peroxide in TNT buffer for 10 minutes, then washed for 5 minutes in TNT buffer. For Biotin detection, slides were incubated for one hour in TNT buffer containing Anti-Biotin antibody (1:500; 10µg/ml). After a final series of washes, the slides were coverslipped with Slow Fade (Molecular Probes, Carlsbad, CA), dried in a light proof box overnight, and the edges were sealed with clear nail polish the following day.

2.5 Analysis

Images were analyzed using a Nikon (Eclipse 80*i*) microscope with Nikon Elements (AR 3.0) software. Each brain area of interest was first located in adjacent sections stained with thionin using brightfield microscopy (*fig* 2). Observers were blind to sex and treatment condition. Cells were counted in an area that was 2560 x 1960μm^2. Cells were counted in 3 slices and both hemispheres per animal for each area. For each section, three separate images were analyzed, FITC illuminating cells expressing CK313884, TRITC illuminating cells expressing AR and a merged image showing the co-localization of CK313884 and AR in each area. The average number of cells per area was analyzed using PASW (version 18.0, Chicago,Il). We first ran an ANOVA to confirm sex differences in untreated animals. We then ran a Multivariate Analysis of Variance (MANOVA) was run to examine the effects of sex and estradiol treatment. To determine the degree of co-localization we examined the proportion of merged cell to cells expressing AR. A Mann-Whitney U-test and Kruskal-Wallis test was run on the calculated percentages to examine any sex or treatment differences in the degree of co-localization.

Chapter 3

Results

A One-way Analysis of Variance (ANOVA) confirmed previously established sexually dimorphic expression of CK313884 and AR in area HVC. Untreated male zebra finches had significantly more cells than untreated females as expected as was indicated by both cells expressing CK313884 ($F(1, 10)=17.54$, $p<.01$) and AR ($F(1,10)=8.94$, $p<.05$), (fig,3c). Expression of AR and CK313884 did not differ in LMAN ($F(1,10)=.867$, $p=.374$); ($F(1,10)=.001$, $p=.973$) or Area X ($F(1,10)=.023$, $p=.882$); ($F(1,10)=.029$, $p=.867$).

We next examined the effects of estradiol treatments on sex differences in gene expression. A main effect of treatment was observed in Area X for cells expressing AR ($F (1, 10) =11.348$, $p<.01$) (*fig* 4) and the co-localization of AR and CK313884 ($F(1,10)=16.293$, $p<.01$). No other main effects were significant.

A significant sex by treatment interaction was found for AR expression in HVC ($F(1,10)=17.758$, $p<.001$), such that treatment with estradiol increased expression of AR in females to levels similar to control males but decreased expression in males to levels similar to control females (*fig* 3a). Furthermore, there was a significant sex X treatment interaction for CK313884 expression (*fig* 3b), such that treatment with estradiol increased the expression of CK313884 in females but decreased expression in treated males ($F(1,10)=7.213$, $p<.05$); co-localization ($F(1,10)= 11.319$, $p<.01$) . No significant interactions were found in either LMAN or Area X

To examine whether or not sex affects the degree of co-localization in control animals, a Mann-Whitney U was run comparing the percent of co-localization for each area. Only Area X showed a significant difference with control males having a higher percent of cells expressing

both AR and CK313884 (95.5%) compared to control females (81.2%), ($p<.05$). In both HVC and Area X a Kruskal-Wallis test showed treatment with estradiol trended towards an effect in female animals. Co-localization in HVC showed a decreased from 88% in control animals to 81.2% in females treated with estradiol ($p=.072$). In Area X co-localization increased from 81.2% in control females to 92.1% in females treated with estradiol ($p=.072$) (*fig* 6).

A one-way ANOVA was run to make multiple comparisons there was a significant main effect for co-localization in HVC ($F(3)=3.109$, $p<.05$) and Area X ($F(3)=4.121$, $p<.05$). Post-hoc analyses LSD revealed a treated females (M=.812, SD= .091) showed significantly less co-localization than untreated males in HVC (M=.914, SD= .079) indicating treatment with estradiol did not successfully increase co-localization in females zebra finches (*fig* 6). In Area X untreated males (M=.955, SD=.05) showed significantly higher co-localization than untreated females (M=.822, SD=.089) (*fig* 6). This difference disappeared with treatment, as estradiol treatment significantly increased co-localization in females (M=.921, SD .091) (*fig* 6).

Chapter 4

Discussion

This study successfully replicated the work done by Tomaszycki et al. (2009) confirming sexually dimorphic expression of the Z-linked gene CK313884 in the HVC of male and female zebra finches. Increased expression of CK313884 during development in HVC of males compared to females is consistent with the hypothesis that this gene is involved in the masculinization process. This supports the idea that CK313884 is involved in masculinization of song nuclei morphology and may be related to the early phases of song learning (template formation). This was successfully accomplished by employing a double label fluorescence *in situ* hybridization protocol (FISH) (*fig 5*). The use of FISH provides many benefits over older radio-labeling techniques. The most notable of which are safety, more consistent probe specificity and shorter exposure times (Levsky & Singer, 2003).

HVC

We hypothesized that estradiol would increase CK313884 mRNA expression in females. Gene CK313884 codes for 17 beta-hydroxysteriod dehydrogenase type 4 (17BHSD4). 17BHSD4 converts estradiol into a less active component estrone, for which estrogen receptors have a lower affinity (de Launoit & Adamski, 1999). The presence of 17HSD4 in the zebra finch telencephalon helps confirm the presence of estradiol, perhaps from regions near HVC. Though treatment with estradiol significantly increased both the expression of CK313884 and AR in females, treatment with estradiol significantly decreased the same expression in male zebra finches. Estradiol treatment had a much stronger impact on AR expression than expression of CK313884 (*fig* 4). That 17BHSD4 mRNA expression is elevated at day 25 and exists in greater quantity in the male finch suggests a potential cytotoxic effect of estradiol on HVC during

development. Since 17HSD4 converts E2 into estrone, it suggests that the presence of this gene may be slowing down the effects of estradiol to protect HVC from excitotoxicity.

LMAN

LMAN showed no sex differences CK313884 either before or after estradiol treatment. This confirms previous findings that, though, there is global labeling of CK313884 in the zebra finch telencephalon, sex differences in gene expression can be localized to specific areas (Tomaszycki, et. al, 2009) (*fig* 3). Surprisingly, LMAN did not show sex differences in AR mRNA expression. Sex differences in AR expression have previously been reported in MAN (K. W. Nordeen, et al., 1986) (*fig* 3). The lack of sex differences in LMAN may suggest that sex differences in the numbers of androgen receptors in MAN are isolated to the medial magnocellular neostriatum (mMan).

Area X

Sex differences were not found in control animals in Area X which confirms previously reported findings (Tomaszycki, et al., 2009). These differences are likely due to the absence of Area X in female finches and not absence of AR per se. Treatment with estradiol had no effect of expression of CK313884 in either males or females. However, a dramatic down regulation of AR mRNA expression was seen in both treated males and females compared to control animals. The increase in co-localization for treated females in this area is likely an artifact of this down regulation. This again lends support to the finding in HVC which suggests that estradiol may have a cytotoxic effect on AR (*fig* 5).

Summary

That the phenotypic expression of CK313884 can be changed by developmental exposure to estradiol suggests an epigenetic mechanism underlying the sexual differentiation of the song

system. However, there may be a limit to which exposure to exogenous estradiol post-hatching can further masculinize a male, as treatment may have overrun the ability of 17HSD4 to protect the song nuclei from the cytotoxic effects of estradiol.

We also predicted that this gene would be located in the same cells as androgen receptors. This co-localization might play a part in the masculinization process, since estrogen receptors are low in the song system (Gahr & Metzdorf, 1999).

The use of the double labeling FISH protocol allowed us to show the co-localization of CK313884 and AR in HVC. Co-localization was slightly higher in male controls than in female controls however, this difference was not significant. Though treatment with estradiol significantly increased the expression of CK313884 and AR in females, the degree of co-localization was dramatically reduced. These results show that aromatization may have some role, since the AR expression suggests the presence of androgens and the 17HSD4 expression suggests the presence of estradiol, due to its role in converting estradiol into estrone. Perhaps these androgens are being converted into estrogens via the aromatase enzyme, which is present in HVC at this time. These results also suggest a possible explanation for why estradiol only partially masculinizes the female zebra finch. Though estradiol increases the size and number of cells in HVC mimicking male morphology, it is not affecting co-localization in the same way. This suggests that not only are larger quantities of cells expressing AR and CK313884 necessary for masculinization, but this expression needs to occur with a high degree of co-localization for complete masculinization. Together, these results shed light on the relationship between hormones, genes and the sexual differentiation of song nuclei.

In reviewing studies examining the effects of estradiol on sexual differentiation in the zebra finch (see above), it becomes clear that estradiol does play a role in masculinization of the

song system based on the effects shown in developing females. The confusing aspect of these findings is the inability to feminize the male finch by reversing the techniques used in females. Unfortunately, though this study provides new avenues for answering this question, the exact mechanisms by which estradiol is masculinizing the song system are still unknown. There is the possibility that E2 may be setting the stage for sensitivity to AR later in development by increasing the number of AR cells in HVC and MAN (Noordeen, Noordeen and Arnold, 1986). There is also the possibility that masculinization of the song system is the default developmental trajectory activated by genes on the Z chromosome. Males are ZZ and females are ZW, it's possible that expression of genes on chromosome W in female finches may act to inhibit masculine development of the song system (Arnold, 1996), or activate the development of the feminine song system. The female zebra finch may have separate genes that code for their song system (Bailey & Wade, 2003), and it may be such genes that should be the focus of a demasculinization or feminization study.

There are many difficulties inherent with doing this type of research. As is typical when examining the role of genes in development it is problematic to focus on one and determine its unique role in the system. There are five other genes that are part of song system development, sexually dimorphic and z-linked CK310795 (Methycrotonyl-CoA), CK303566, DV956689, CK3038959, CK306803 (Sorting Nexin 2). Future work researching how these six genes work together may provide us with a more complete picture. Also, we are not yet in a position to examine what would happen to the system if we turned certain genes "on" or "off" in development, making it more difficult to test cause and effect relationships.

Future work should study expression of CK313884 at other developmental time points. From the microarray data, CK313884 remains sexually dimorphic from day 1 through adulthood.

The current study examined the male and female birds at P25. By this time finches are well into the song learning phase of their development (Bottjer, 2002). The HVC has been sexually dimorphic since P15 and cell number and size is continuing to increase in male (K. W. Nordeen, et al., 1986). At this time HVC is forming connections with area X marking the beginning of the sensorimotor integration period for male finches (K. W. Nordeen & Nordeen, 1997). The next interesting time point marks the closing of song template formation at P40 (Bottjer, 2002). Finally at P60 neural development has just completed and the animal has reached adulthood (Clayton, 1997). Following up with CK313884 at these different time points would help to tease apart the functions of this gene. For example is CK313884 expressed in the same quantities at P40 and P60 as it is at P25? If it is involved in sexual differentiation we would expect the expression CK313884 to decrease after the closing of the song template P40.

Understanding how multiple genes work with development, hormones and each other will lend a greater understanding the sexual differentiation of the zebra finch song system.

APPENDIX A-FIGURES

Fig 1
The development of the song system in the zebra finch through adulthood (P60). Brains for this study were collected at P25 (from Tomaszycki et al., 2009).

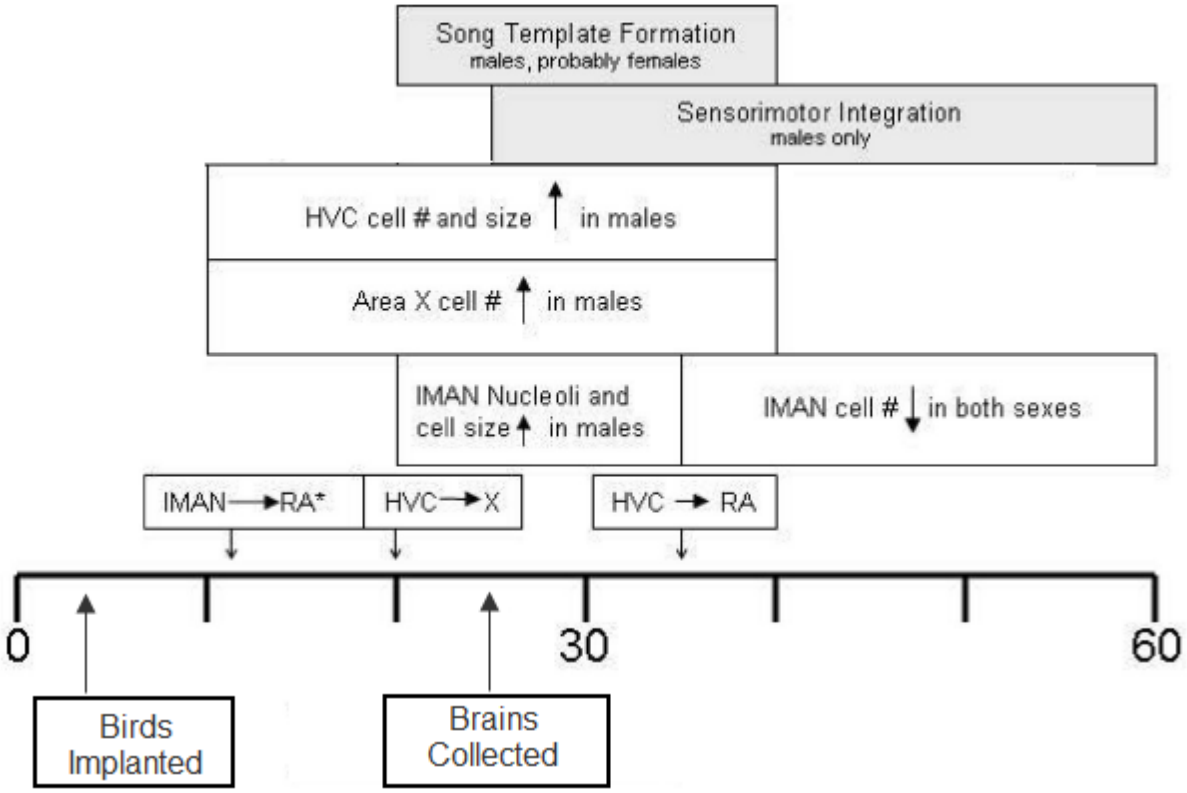


Fig 2

a) Schematic showing locations of target brain areas in zebra finch. Brightfield images from thionin stained sections depicting areas b) HVC, c) Area X and d) LMAN at 40x .

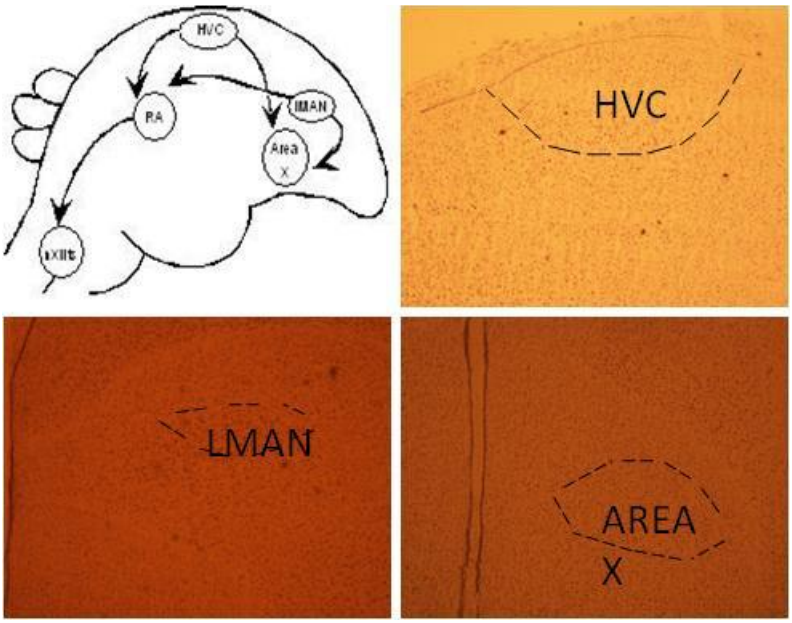


Fig 3

Sex by treatment interaction in HVC for CK313884 and androgen receptor mRNA expression in developing male and female zebra finches. A) A significant sex X treatment interaction for mRNA expression of AR in HVC. B) A significant sex X treatment interaction for cells expressing CK313884 mRNA in HVC. C) A bar graph representing the average number of cells expressed for both AR and CK313884 in each treatment condition. compared to control females. FC=female control; MC= male control; F+E= estradiol treated females; M+C= estradiol treated males.

*p<.05

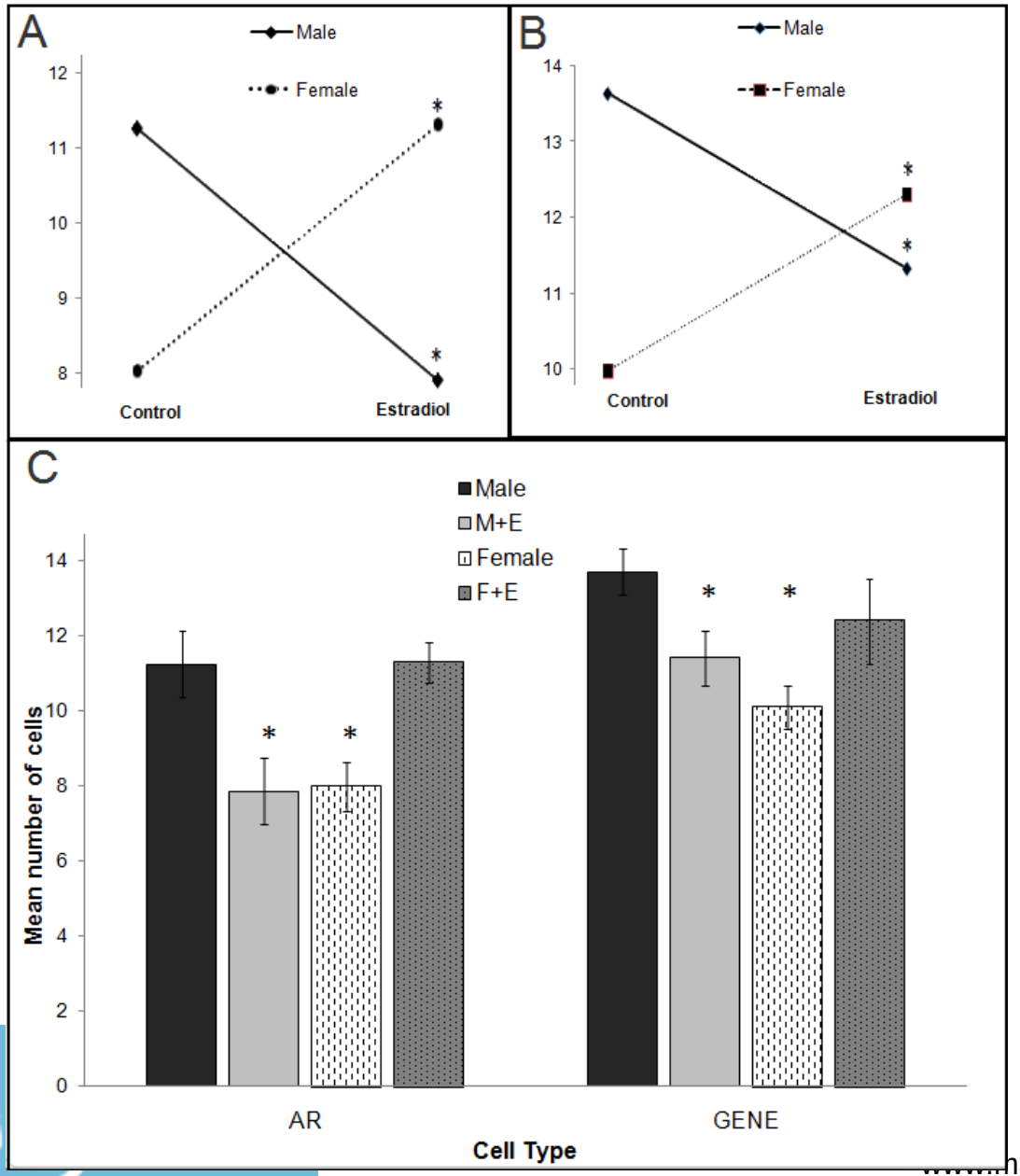


Fig 4

Effect of Treatment on mRNA expression of CK313884 in Area X Area X. A) A significant treatment interaction for mRNA expression of AR in Area X. B) A bar graph representing the average number of cells expressed for both AR and CK313884 in each treatment condition. compared to control females. FC=female control; MC= male control; F+E= estradiol treated females; M+C= estradiol treated males.

*p<.05

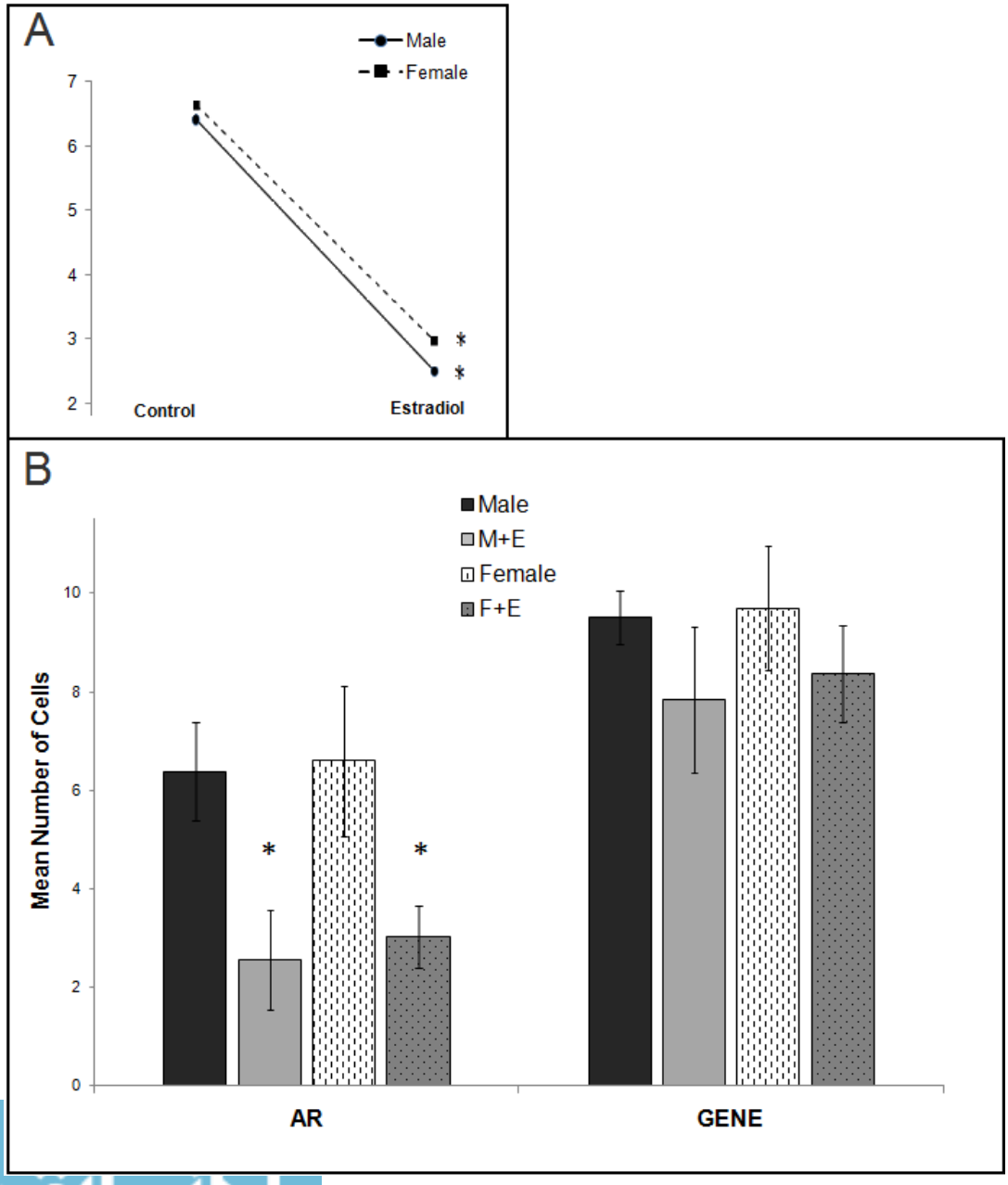
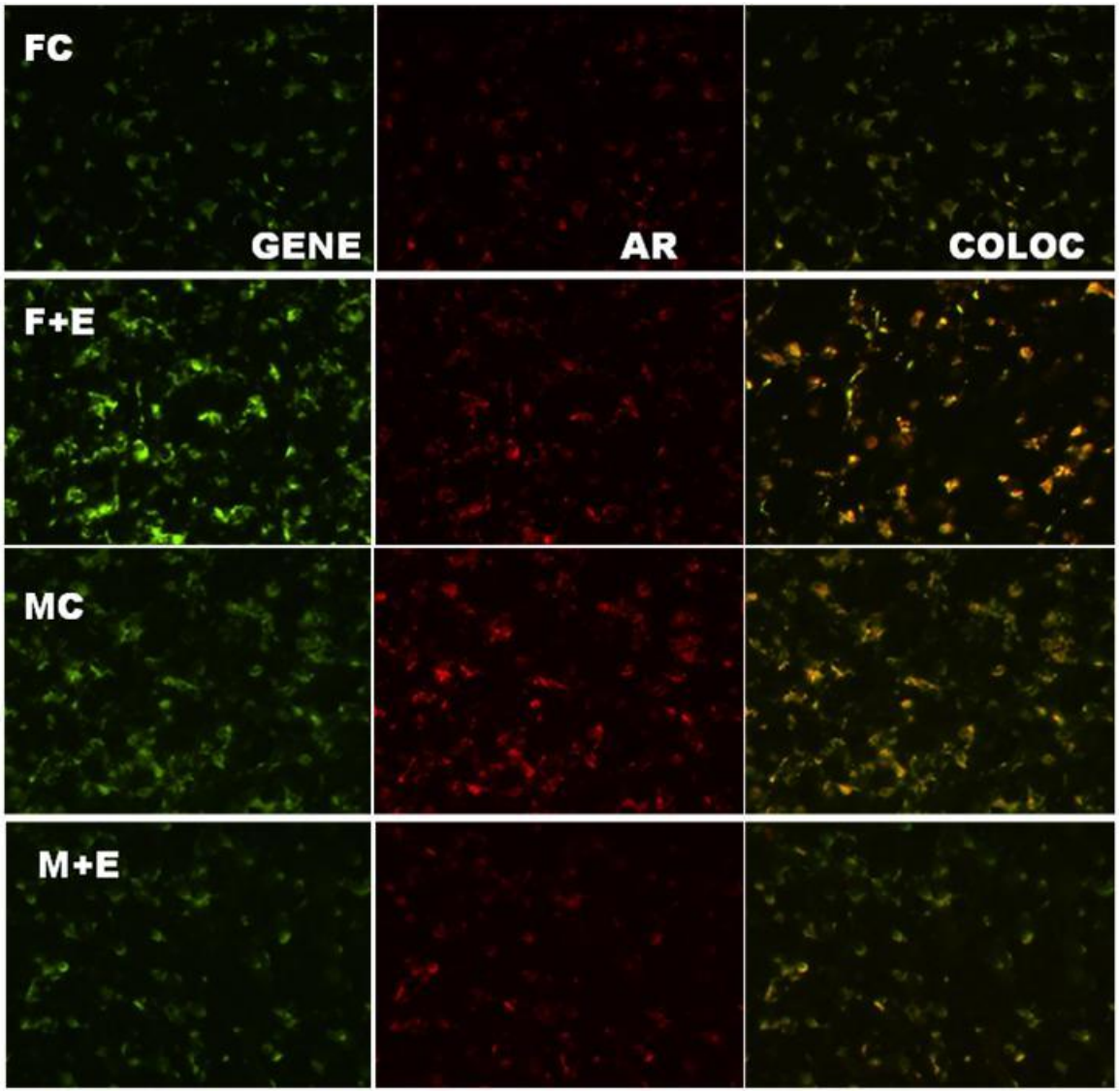
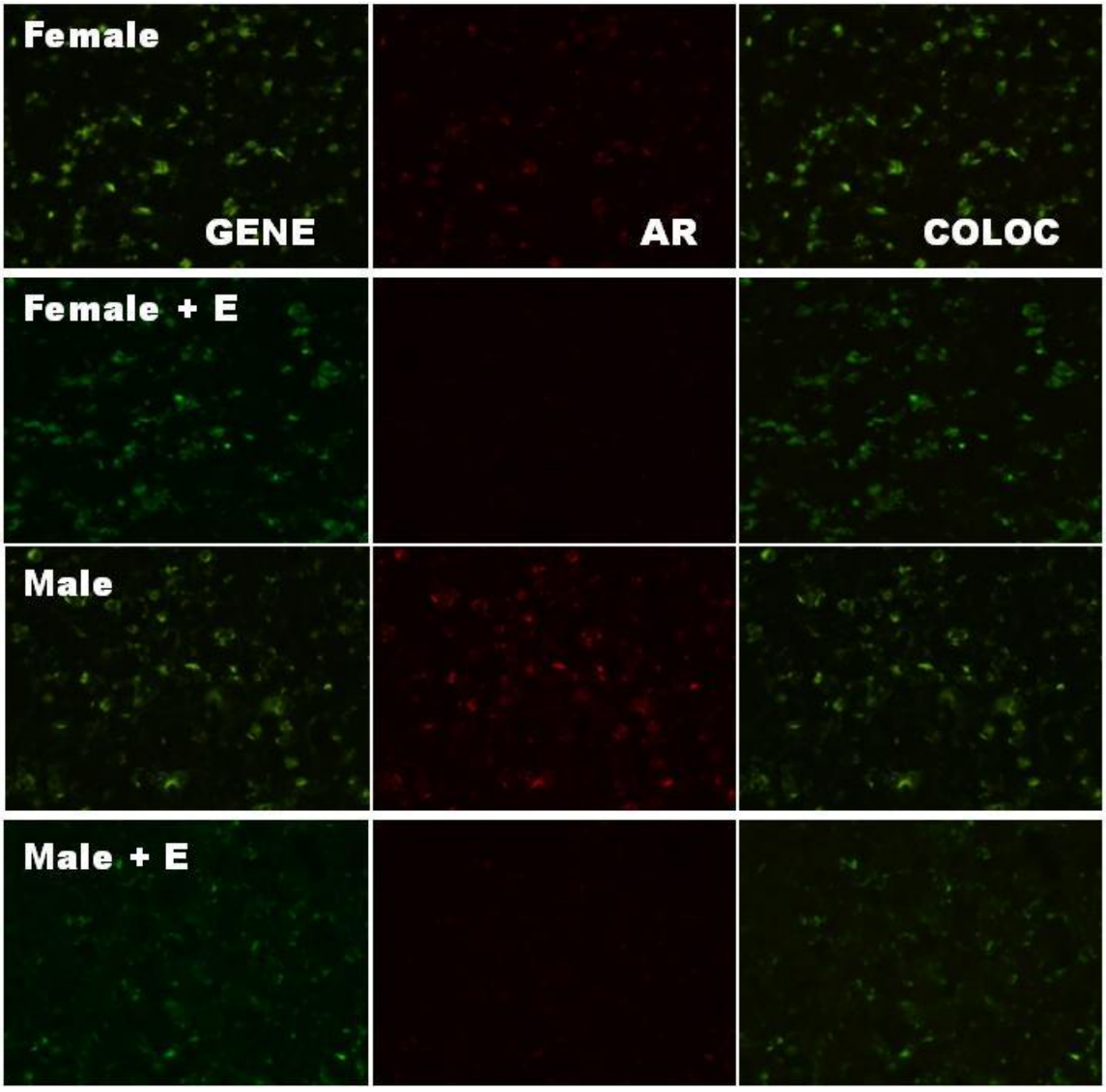


Fig 5 *In situ* hybridization using DIG-labeled probes showing cells expressing CK313884 in male and female zebra finches, compared to females treated with estradiol, early in development. AR: *In situ* hybridization using Biotin-labeled probes for androgen receptors. COLOC: co-localization of CK313884 and androgen receptors. A) HVC, B) Area X, C) LMAN

A. HVC



B. Area X



C. LMAN

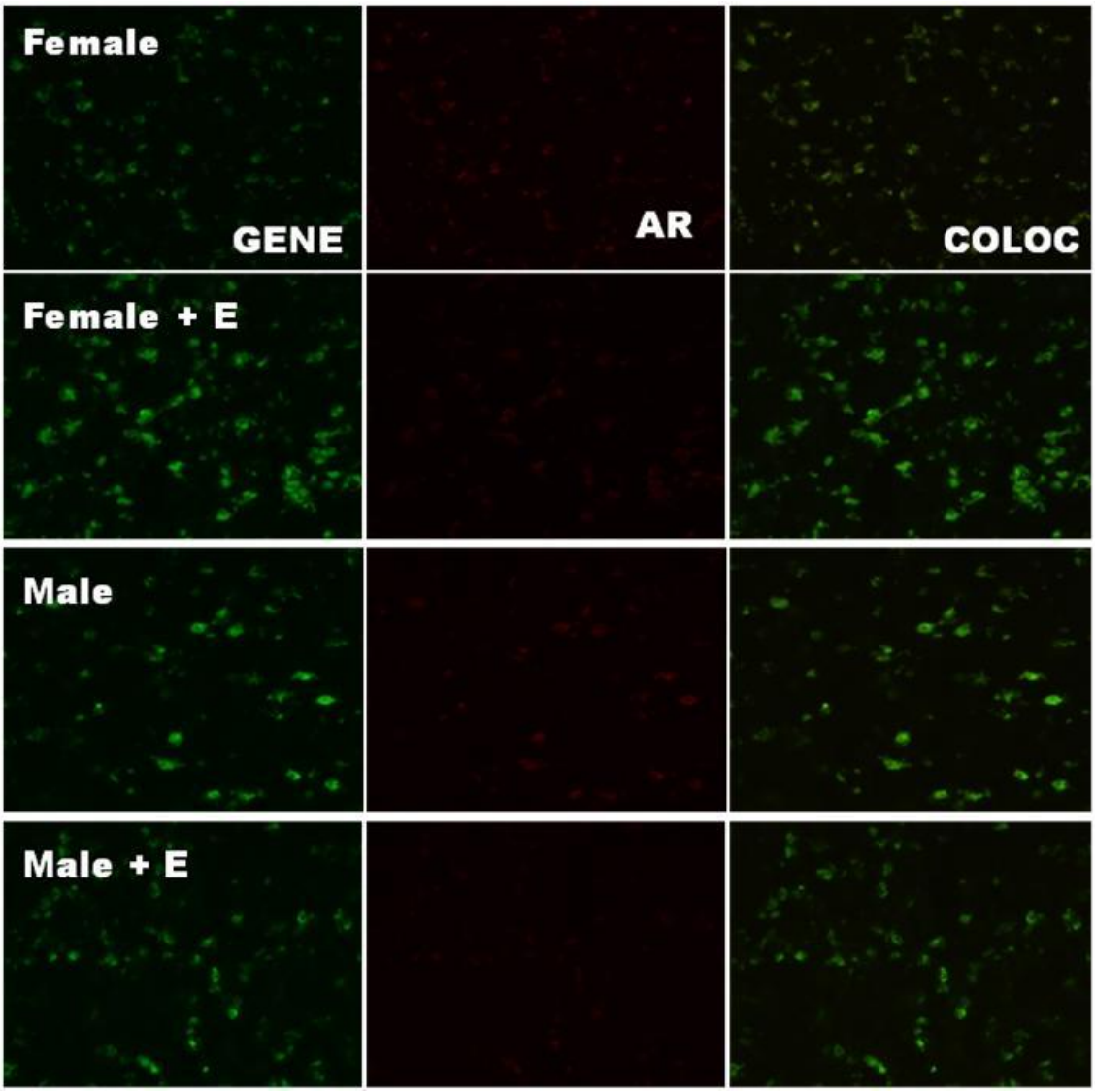
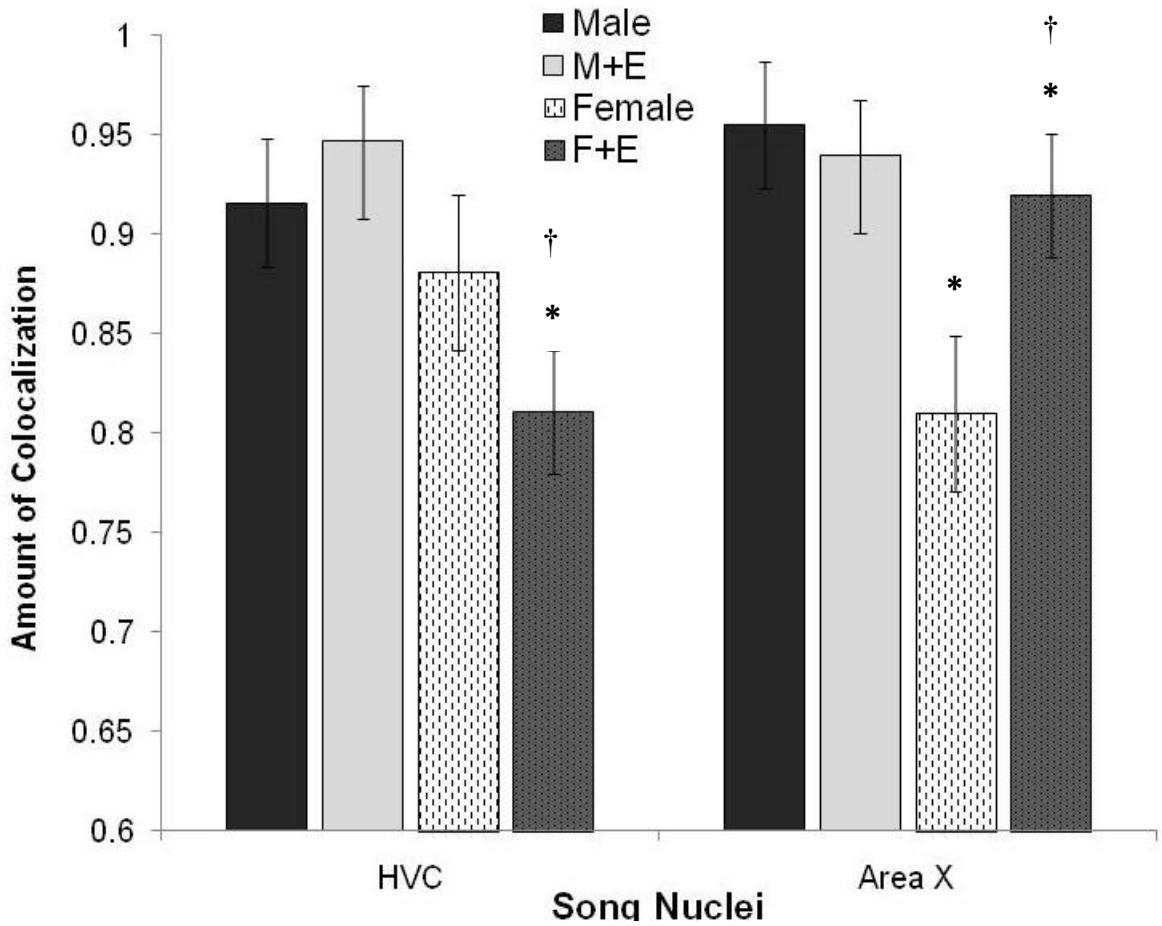


Fig 6: **Co-localization of CK313884 and AR mRNA in HVC and Area X**

In HVC there were no sex differences between control animals in degree of co-localization. A Kruskal-Wallis indicated a decrease in co-localization for female zebra finches after treatment with estradiol. A post-hoc LSD confirmed co-localization for treated females to be significantly less than untreated males. Mann-Whitney U test indicates control males show significantly higher degrees of co-localization in Area X compared to control females. Also in Area X a Kruskal-Wallis and post-hoc LSD indicate a significant increase in co-localization in females after estradiol treatment.

*$p<.05$; † $p<.08$



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ABSTRACT

THE CONTRIBUTION OF GENES AND HORMONES TO THE SEXUAL DIFFERENTIATION OF THE ZEBRA FINCH SONG SYSTEM

by

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Recent studies in the zebra finch suggest the sexual differentiation of the song system and singing behavior may not be solely driven by steroid hormones organizing the brain, and may involve direct genetic effects. In fact, genes and hormones might act together to produce sexual differentiation of the brain. To address this idea, animals were implanted with estradiol or a blank pellet on the third day post-hatching. At day 25, the brains were collected and a double label fluorescence *in situ* hybridization protocol using biotin and digoxigenin-tagged mRNA probes was used to simultaneously label androgen receptor and 17β-Hydroxysteroid Dehydrogenase type IV mRNAs.

Estradiol increased the number of cells expressing of 17HSB4 in the HVC of the female zebra finch, but did not affect co-localization of 17BHSD4 and AR. In male zebra finches, estradiol decreased the number of cells expressing AR and 17BHSD4 in HVC and the number of cells expressing AR in Area X. This pattern suggests a limit to which estradiol will contribute to masculinization and exposure to greater amounts results in cytotoxicity. These results lend further evidence to support the hypothesis that genes and hormones act in concert to sexually differentiate the song system in the zebra finch.

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April 2007 – September 2008 – Treasurer, GEOC
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April 2010 – September 2011 – President, Graduate Student Neuroscience Society
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2004 – Certificate of Teaching Development
2008 – Departmental Teaching Award (Lecture)
2009 – Departmental Research Award
2009 – Graduate School Research Recognition