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EGG LAYING BIRD WITH MALE PLUMAGE DEMONSTRATES THE PUZZLING
NATURE OF SEXUAL DIFFERENTIATION AND SELECTIVE MATE
PREFERNCES IN ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

by
John Aaron Howell

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of
the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2017

Approved by

Advisor: Dr. Lainy Day

Reader: Dr. Chris Leary

Reader: Dr. Debra Young

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ACKNOWLEDGEMENTS

Many people have contributed to the overall process of conducting my research and writing my thesis. Firstly, I would like to thank my family and friends for their constant support throughout this endeavor. Without each of you, I know that this would have been an even more daunting task than it already was. I would also like to thank the other members of the Day Lab for their numerous contributions to this project. Because this thesis is being written for publication in a scientific journal, I have included the work of former Day Lab students, in addition to my own, so the reader may place the work that I have done in a more complete context. The contributions of each student are outlined in the Preface (page 1). I would like to give a special thanks to Dr. Richard Buchholz, who allowed us to use his spectrometer, helped take initial measurements, and provided insights into how to process the color spectra. I would like to thank the Sally McDonnell Barksdale Honors College (SMBHC) for their unwavering support of my research throughout my undergraduate career. I sincerely appreciate the many opportunities that the SMBHC has given me to present this research at numerous conferences, including the Mississippi Academy of Sciences meeting and the Society for Neuroscience meeting. Many thanks go to my thesis readers, Dr. Christopher Leary and Dr. Debra Young. Your contributions did not go unnoticed. Lastly, I would like to thank my advisor, Dr. Lainy Day, for her incredible mentorship, support, and contributions both to this project and my personal development as a scientist.

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PREFACE

My thesis includes a series of experiments that place my work in a larger context and will expedite the process of preparing this body of work for journal publication. The methods and results for Experiments 1 and 2 are vastly edited versions from unpublished manuscripts of several of the contributors. The final format of the introduction and discussion is almost exclusively the creative output of the thesis author and his mentor. Material in the introduction and discussion, as well as all methods and results related to Experiments 3 and 4, are the work of the thesis author completed under the tutelage of his mentor.

Credits: Chelsea Vann Vaculcik and Dr. Amy Hribar discovered that we had an egg laying zebra finch in a male-plumaged aviary. Chelsea and Dr. Hribar initiated behavioral observations, and Dr. Hribar helped with the building of mate preference chambers and with the development of the mate preference tests in Experiment 1. Megan E. Jones was principal in caring for the gynandromorph lineage and conducting behavioral observations of the gynandromorphs, and recording nesting and singing behavior (data not reported here). Megan Jones, Rebekah E. Cummins, and Elizabeth Jenkins conducted the mate preference experiments with the assistance of Roberto J. Perez. Roberto extended the subject numbers in the sex orientation preference test. Malcolm Barker-Kamps, Hannah Witchen, and Roberto J. Perez ran the PCRs for blood and tissue samples. Dr. Lainy Day trained students in all techniques, supervised all aspects of the experiments after the initial discovery of the gynandromorphs, guided the analyses and interpretation of results, and edited drafts of the manuscript.

ABSTRACT

Zebra finches (*Taeniopygia guttata*) are oscine Passerine songbirds that are native to Australia. Zebra finches have sexually dimorphic plumage, song nuclei, and behavior. The role of chromosomes, hormones, and genes on sexual differentiation are not completely understood. A gynandromorph in our aviary had male plumage, a male partner, and produced viable offspring. Mate preference tests revealed lower preference for the gynandromorph and its progeny than controls, suggesting that they had some traits that made them unattractive to other birds. Gynandromorph lineage males had greater same-sex preferences than control males. Chromosomes in birds are different than those in humans. Males are homozygous ZZ, and females are heterozygous ZW. All sampled gynandromorph tissues, including those in sexually dimorphic plumage regions, had ZW female chromosomes. Of zebra finches, males sing and females do not. Because of this, the regions associated with song learning and production, collectively called a song system, are larger in males than in females. We thought to measure the size of the song nuclei in our gynandromorph and compare it to control males and control females. For song system comparison of the gynandromorph, volume, cell number, and cell size of sexually dimorphic song nuclei were measured. The volumes of the song nuclei for the gynandromorph were consistently between the song nuclei volumes of males and females. Additionally, because birds can see in the ultraviolet spectrum of light, color spectra were analyzed to determine if the gynandromorph or its progeny had plumage differences that were undetectable by the human eye. Video analysis of the gynandromorph and its mate is being completed to determine if the gynandromorph sang and to observe nesting behaviors.

INTRODUCTION

Sexual differentiation, the acquisition of “male” typical and “female” typical traits, occurs via a series of multifaceted and integrated processes that begin at fertilization, proceed until birth, and are basically fully developed when the organism reaches adult reproductive status. Sexual differentiation of the chromosomes, gonads, internal reproductive structures, brain regions and associated behaviors, and external sexually dimorphic features do not occur during the same critical periods (Agate et al., 2003; Berenbaum & Beltz, 2011; Gahr, 2007; Schlinger, 1998; Tomaszycski et al., 2009). Thus, while a pattern of these “male” and “female” features form concomitant with sex-typical behaviors in most vertebrate species; a mosaic of features, many of which exist along a continuum rather than as binomial traits, are found in some individuals of most, if not all, vertebrate species (Berenbaum & Beltz, 2011). While the basic processes underlying sexual differentiation in mammals are understood and many of these processes have been studied in birds, we are far from a complete understanding in mammals and have further to go in understanding the processes in birds (Agate et al., 2003). Novel influences on sexual differentiation from cellular to environmental perturbations are regularly discovered and help shape a more complete model of development (Graves, 2014; Grisham et al., 2007; Itoh, Kampf, & Arnold, 2008; Tomaszycski et al., 2009). Gynandromorphs, animals that have both male and female features, have contributed to our understanding of the processes of sexual differentiation and how combinations of features influence mating preference and reproductive behaviors (Agate et al., 2003; DaCosta, Spellman, & Klicka, 2007). Here we studied a male-plumaged, egg-laying zebra finch (*Taeniopygia guttata*) and her progeny to further

understand the role of male plumage and sexual imprinting on two male-plumaged parents in mate preference, whether the appropriate sex chromosome is a prerequisite for male and female plumage and tissue types, and whether masculinized plumage is associated with brain masculinization in this gynandromorphy.

Zebra finch plumage patterns are known to be sexually dimorphic; males have bright orange cheek patches, zebra striped feathers on their chests, and brown and white spotted feathers down their sides. Females lack these features but have the species typical light grey plumage with a cream belly, and a white patch with a black dividing line on the cheeks (see Figure 1). Males are usually described as having redder beaks and feet than females (Simons et al., 2012), but this trait appears to vary greatly within sexes and may be influenced by hormones. Female sex chromosomes are heterozygous (ZW), and male sex chromosomes are homozygous (ZZ), the opposite of the human pattern (Itoh et al., 2008). In zebra finches, plumage pattern is not strongly associated with developmental (organizational) or adult circulating levels of hormones (activational), but appears to be influenced by sex-specific gene expression (A. Arnold, 1997). In zebra finches, males typically sing and have distinctly larger song nuclei in the brain than females, which do not sing (Nottebohm & Arnold, 2010).

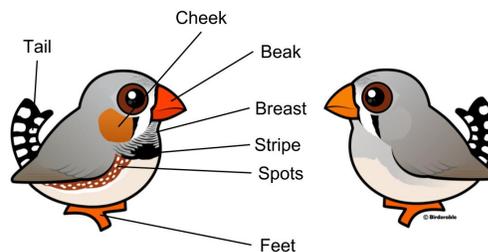


Figure 1: Plumage differentiation between male and female zebra finches (de Wolf, 2008)

Most studies of zebra sexual differentiation have focused on the song system, but many studies have also examined gonadal, plumage, and behavioral differentiation. While our understanding of sexual differentiation in zebra finches is far from complete, the most influential processes at several stages of sexual differentiation are clear. Sex chromosomes are known to control gonadal development in zebra finches (Schlinger, 1998); birds that are ZZ develop ovaries, and birds that are ZW develop testes. Markers for these sex chromosomes are found at different levels and appear to be dimorphic in the song nuclei of male and female zebra finches (Itoh et al., 2008). Gonadal, organizational, and activational hormones may also play a prominent role in the sexual differentiation of the zebra finch song circuit (Agate et al., 2003).

It is beyond the scope of this study to outline our current understanding of all the processes involved in zebra finch sexual differentiation, and the reader is encouraged to examine recent reviews on the subject (A. P. Arnold & Itoh, 2011; Schlinger, 1998; Wade & Arnold, 2004). Studies most pertinent to understanding the current study are those that involve gynandromorphic zebra finches, individuals that have both male and female typical characteristics. Zebra finches with bilateral gynandromorphy have female plumage and an ovary on one side of the body and male plumage and a testis on the other side of the body. Gonadal development is thought to result from a gene or genes on the Z or W chromosome (Schlinger, 1998), but may be guided by gene dosage effects (A. Arnold, 1997). Differentiation of the song nuclei, has long been thought to be primarily influenced by hormones because of their role in the formation of many other secondary sexual traits and because females induced with testosterone can sing (Schlinger, 1998).

However, sex atypical hormones given exogenously or endogenously via testicular induction do not fully masculinize females nor prevent masculinization in males. Furthermore, bilateral gynandromorphs, with both ovarian and testicular hormones circulating from early in development (Agate et al., 2003), demonstrate that not only is plumage unilaterally distinct, but the expression of Z and W linked genes is more prominently expressed in brain and other tissues on the male and female side respectively. Interestingly, the song nuclei of one of the studied bilaterally gynandromorphic zebra finches were somewhat feminized on the male side and somewhat masculinized on the female side (Agate et al., 2003). In addition, this bird sang a mostly typical male song. Thus, at least in bilateral gynandromorphs, gonads and plumage are strongly sex chromosome linked, while brain is influenced by sex chromosome genes and gonadal hormones (Agate et al., 2003). There are no studies, of which we are aware, that have published complete descriptions of the song systems of non-bilateral gynandromorphs with unilateral male plumage and female reproductive systems like the one in this study. However, two reports have been published in abstract form (Cui et al., 2015; Grisham et al., 2007). These two studies suggest the possibility for variation in degree of neural sexual differentiation when plumage is male typical but internal reproductive organs are female typical. Interestingly, it appears that only moderately masculinized song nuclei are necessary for song production (Agate et al., 2003), so birds with male plumage and female sexual organs may have the ability to produce male-typical song patterns.

Thus, we were interested in understanding the degree of sexual differentiation in our gynandromorphic bird. After decades of study, we have a fairly intricate, though far

from complete, knowledge of the song circuitry in zebra finches (Wade & Arnold, 2004). Of over a dozen known song nuclei, there are four that have been best studied and that are known to be highly sexually dimorphic (Wade & Arnold, 2004). The predominantly downstream song pathway involves a projection from nucleus HVC to the robust nucleus of the archistriatum (RA) and then to brain stem nuclei controlling singing. HVC also projects to Area X, which in turn projects to the lateral magnocellular nucleus of the neostriatum (LMAN), and this pathway is thought to be more related to hearing and learning song than the HVC/RA production pathway (Schlinger, 1998). Area X is not apparent in females, and HVC and RA are approximately five fold larger in males than in females (Schlinger, 1998). HVC and RA also have more and larger neurons in males than in females, and HVC, Area X, and LMAN have been found to contain more androgen receptors in males than in females (Wade & Arnold, 2004). LMAN volume is known to have similar volumes in both males and females, but the soma size is larger in males than in females (Tomaszycki et al., 2009). Differences in the size of these song nuclei have been found to correlate with singing behavior within and between sexes (Nottebohm & Arnold, 2010) and across species (Nottebohm & Arnold, 2010).

Parameters of song production are learned, generally from the father, but song quality in terms of mating success is also highly heritable (Adkins-Regan & Krakauer, 2000) and influenced by environmental factors affecting eggs and young (Adkins-Regan & Krakauer, 2000; Gong, Freking, Wingfield, Schlinger, & Arnold, 1999). Mate preference in zebra finches is also influenced by beak color, plumage, song rate, and male aggressiveness (Forstmeier & Birkhead, 2004). Thus, song nuclei development and the

songs produced should be an honest signal of ultimate and proximate mate quality, and appear to be an important factor in mate choice (Williams, 2004).

Zebra finches are socially monogamous and biparental (Banerjee & Adkins-Regan, 2014) though they typically live in colonies. In order to quantify mate preference in laboratory settings, various choice chamber procedures have been used to measure aspects of affiliate behavior, such as time spent near specific individuals. These measures of affiliate behavior correlate highly with mate preference in an open aviary (Rutstein, Brazill-Boast, & Griffith, 2007).

Despite the ease of measuring mate preference, we know of no study that has examined the mate preferences of gynandromorphic birds. This may be, in part, due to the unavoidable nature of the gynandromorph's uniqueness. If the gynandromorph is the only subject in its group, anecdotal evidence of this bird's mate preference or rejection by others may not hold up to the preferred standards of statistical evaluation. Fortunately, for us, our gynandromorph partnered with a seemingly typical male and produced 7 adult offspring. While we can not be certain that the young inherited qualities of the gynandromorph, we hypothesized that if progeny were in any way different from the average birds in our colony, mate preference tests would demonstrate these differences in the gynandromorphic lineage. By having the gynandromorph involved in mate preference tests as both a chooser and choice, we hoped to provide some quantification of the types of preferences the gynandromorph, her partner, and her progeny demonstrated.

In the current study we had a unique opportunity to verify the role of sex chromosomes and hormones in influencing sexual dimorphism of song nuclei, singing behavior, and plumage pattern. We also had the opportunity to determine the influence of

sexual imprinting, color pattern, and song production in influencing mate preference. Given the complex nature of sexual differentiation in “typical” zebra finches, we hesitated to make specific theoretical hypotheses related to our expected results. However, we did predict that the gynandromorph would have an absence of W linked genes in plumage tissues but not in reproductive tissues. Furthermore, given that the gynandromorph had several clutches with her mate, we predicted that she would exhibit female typical song nuclei and singing behavior. We did not have specific predictions about mate preference patterns for her progeny other than that the male progeny was likely to exhibit same sex partner preferences as shown in previous studies for males raised in the absence of females (Banerjee & Adkins-Regan, 2014). Once we found that the progeny were not preferred by other zebra finches in our colony, we predicted that color patterns would distinguish the progeny from other zebra finches in our aviary.

METHODS

Animals

For all experiments, we examined normal adult zebra finches and those of the gynandromorphic lineage bred in our aviary at the University of Mississippi (IACUC 16-024). The gynandromorph lineage consisted of the gynandromorph, its male partner, 6 female offspring, and 1 male offspring. All of the birds were given drinking and bathing water and fed a standard zebra finch diet *ad libitum*, except during habituation and testing periods, which lasted no longer than three hours. Food deprivation for longer periods, as much as 8 hours a day out of a 10 hour light cycle, have been shown to have no long term effects on behavior with weights, energetic output, and behavior recovering in a few days after 11 days of 8/10 hours a day food restriction. The aviary was on a 12-14 hour light schedule over the course of experiments, with lights on at 7 a.m. The aviaries used to house the breeders, the gynandromorph, and its partner were 182 x 182 x 121 cm. Juveniles were moved to same sex aviaries or cages (91 x 45 x 30 cm) upon sexing.

Experiment 1: Mate Preference

We performed two types of mate preference tests. To assess whether the gynandromorph lineage had qualities that were cryptic to humans but could be detected by the frequency that they would be chosen as mates, we measured the preference of control birds for other control birds versus gynandromorph lineage birds in a two choice

mate tests. To determine if being raised by two male plumage parents influenced the sexual orientation in the gynandromorph lineage, we compared the preferences of the gynandromorph lineage birds and control birds for same versus opposite sex plumaged birds.

Subjects

In the mate preference experiments, we tested 41 zebra finches including the gynandromorph lineage. The sexually naïve gynandromorph progeny were matched with other sexually naïve control females and males. The gynandromorph and partner were tested with other breeder adults. Subjects were age matched. No bird participated as a chooser more than once, and birds that were used as choices were not used more than once. Lineage birds were first tested as choices and then acted as choosers in the mate preference test. We thus matched choosers in the sexual-orientation task for experience with having been a choice bird in a mate preference test. However, all choice birds were similarly naïve to the mate preference task. Neither morphological nor behavioral differences were readily apparent between lineage birds and other birds in the aviary, though some hints of color differences in beaks were suspected. Lineage and 10 control birds matched for plumage, age, and reproductive state did not differ in body weight and no differences were found between any groups (\bar{X} ,SE,min:max; CF=15.8±0.59,14:19; PF=15.4±0.28,15:16; CM=15.2±0.47,14:18; PM=17). Controls for motivation and definitive choice behavior in mate preference tests (see procedures below) resulted in the exclusion of several subjects.

Materials

Six identical arenas (10.3 x 8 cm, see Figure 5) were split into two choice chambers, “A” and “B” (15.5 x 19 cm size) and a chooser chamber “C” (31 x 19 cm size). The choosers could view choices through a Plexiglass window. Choice birds were visually isolated with opaque dividers, but none of the birds were acoustically isolated. To make it difficult for the chooser bird to view both choices simultaneously, a “no choice zone” was created by occluding the middle 10 cm of the viewing window. A cardboard insert was also used to block the viewing window during habituation to the test chamber. To track the movements of the chooser bird, we used an ImagingSource (The Imaging Source, Germany) camera fed into Ethovision (Noldus Information Technology, Wageningen, The Netherlands) software with a multi-arena module to track the center point of chooser birds in the six arenas. Video footage of all birds was also captured for manual inspection of all bird behavior.

Procedure

Choosers were placed in C chambers, and choices were placed in A and B chambers (Figure 5) in one of six arenas. Testing was conducted starting with habituation between 7-9 a.m. or between 1-3 p.m. with controls and lineage birds balanced as much as possible (given even or odd numbers or greater numbers of control subjects) between morning and afternoon tests. Sexually inexperienced birds were all housed in same-sex group cages for several weeks before testing, while breeding adults remained in large nest active aviaries. To begin testing, birds were first captured and moved to single bird cages with dark cloth covers and were then placed into the appropriate chamber under infrared lighting conditions. Standard fluorescent lighting was then turned on from outside the

room and birds habituated to the arenas with the viewing window occluded for two hours prior to the preference trial. At the end of the habituation period, experimenters turned off the lights from outside the room, quietly entered the room turning on an infrared light to guide removal of the viewing barriers and begin Ethovision tracking. Lighting was returned to normal and the birds' behaviors were video recorded for 30 minutes. Birds' position in the A or B chamber was counterbalanced and re-tested 24 hours after the initial trial.

We calculated the proportion of the total time the birds were positioned in preference area A and preference area B based on the center point tracking ($A/A+B$ and $B/A+B$). Since birds were swapped between chamber A and chamber B, preference for the A side in initial and counterbalanced trials would indicate a preference to a side of the chamber, not a particular bird. Birds that showed a side preference (greater than 60% on the same side) were removed from the experiment. In addition, trials during which a bird did not spend time in both chamber A and B before remaining in one chamber or a bird spent the majority of the time in the no choice zone were not included in the experiment. If the chooser appeared to view both choices during the trial but remained motionless for most of the trial, regardless of which chamber it was in, the trial was considered a "no-choice" trial, and the chooser bird was retested with a new naïve pair of choices for sex-preference tests. We balanced the number of retested choosers across controls and gynandromorph lineage as much as possible. For mate preference tests, unmotivated or biased choosers were replaced with naïve choosers so that the lineage birds and controls being chosen between had the same exposure to the testing apparatus.

Test of Sex Preference

To determine whether the gynandromorph lineage and controls differed in their degree of same versus opposite sex preference, birds in each group were given a choice between randomly selected birds with matching or opposite plumage to their own in choice chambers (see Figure 2).

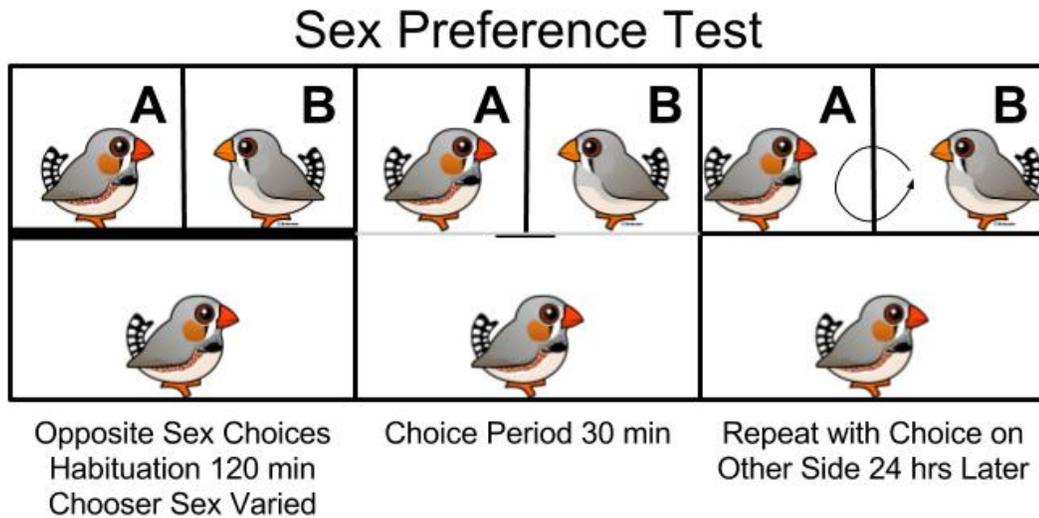


Figure 2: Illustration of sex preference tests conducted with gynandromorph lineage

Preference for Gynandromorphs vs. Controls

To determine whether control birds preferred control birds to gynandromorph lineage individuals, a gynandromorph lineage bird and a control bird that were both the plumage of the opposite sex of the chooser were placed in the choice chambers (see Figure 3).

Mate Preference Test

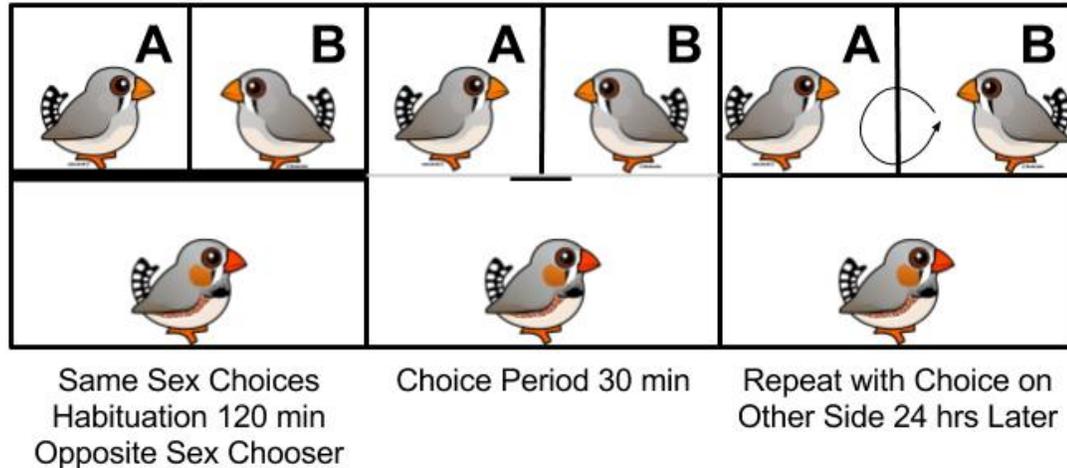


Figure 3: Illustration of mate preference tests conducted with gynandromorph lineage

Data Management and Statistics

After eliminating birds that had side preferences or were unmotivated to perform in the mate preference tests, we had 26 birds that chose between birds of the same plumage or different plumage to their own (GYN lineage $n=9$, 3 male plumage, 6 female plumage, 1 GYN, 1 Male Mate; Controls = 17, 10 male plumage, 7 female plumage; 3 birds were dropped for side preferences and 2 GYN lineage and 2 controls were tested twice with new choice birds on the retest). There were 9 control birds that selected between one of the 9 gynandromorph lineage birds and an age, reproductive status, and plumage matched control (2 choosers had side preferences and were replaced with new choosers, choices were maintained so that they were matched for exposure to testing arena). Proportion data was angular transformed to better fit assumptions of normality. Independent t-tests were used to compare same-plumage orientation between gynandromorph lineage and controls (no significant differences in variance were detected between groups, all Levene's test $p > 0.21$), and paired t-test were used to compare the

degree of same versus opposite sex preference within groups and to compare control birds preferences for gynandromorph lineage birds versus control birds.

Results

Control birds in our aviary spent more time interacting with other controls than with gynandromorph lineage birds ($t(8) = 2.39$, $p < 0.044$, see Figure 4) suggesting that birds in our aviary could identify some cryptic quality of the gynandromorph lineage that differed from controls. Gynandromorphs also differed from controls in having a lower degree of opposite plumage orientation in choice tests than control birds ($t(24) = 2.94$, $p = 0.007$) see Figure 4). Control birds had a strong preference for opposite plumage birds ($t(16) = 2.85$, $p = 0.01$), whereas birds of the gynandromorphic lineage did not show a preference for birds of the same or opposite plumage to themselves ($t(8) = 1.65$, $p = 0.12$). To more accurately assess orientation patterns, we analyzed male and female plumage birds separately and found that female plumaged birds of either group did not have plumage orientation preferences (GYN, ($t(5) = .0524$, $p = 0.623$), control, ($t(6) = 0.901$, $p = 0.402$) and did have a difference in the strength of their orientation to same sex-linked plumage ($t(11) = 0.96$, $p = 0.358$) in that they did not prefer a certain sex ($t(11) = 3.73$, $p < 0.03$). Thus, differences between groups is driven mainly by the males, with control males having a strong opposite plumage preference ($t(9) = 2.63$, $p = 0.027$), and the gynandromorph lineage male plumage birds strongly preferring the same sex plumaged birds ($t(2) = 8.07$, $p = 0.015$), resulting in greater strength of opposite plumage preference in control males compared to male plumaged gynandromorphs ($t(11) = 3.73$, $p = 0.003$). While there are only three male plumaged birds in the gynandromorph lineage, it should be noted that the similarities in female plumaged controls and the lineage was

quite similar, while those in the male lineage were very different from the strong opposite plumage orientation of control males. Arguably, we might only consider the gynandromorph's partner and her male progeny as acting against their gonadal orientation when choosing same plumage affiliation in the mate preference tests. But, the association with same plumage affiliation is not being driven by the gynandromorph, the male progeny chose same plumage 93% of the choice duration, and the male partner chose same plumage 96% of the choice duration. Obviously, the male partner also chose a same plumage partner in an open aviary test. Given the known association of same plumage preference in motherless males (Banerjee & Adkins-Regan, 2014) that is thought to be based on sexual imprinting of the young on the only available parental plumage reference, one might posit that our male progeny is fairly typical of other males under similar conditions. Therefore, it would seem that the greatest abnormality in regard to partner preference is actually driven by the same-sex plumage orientation of the gynandromorph partner.

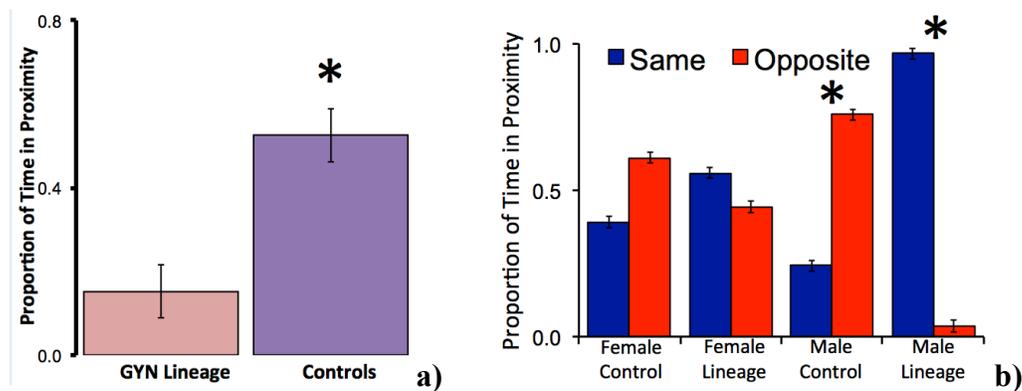


Figure 4: Results from the mate preference tests (a) show that control birds prefer other control birds to the gynandromorph lineage. Sex preference tests (b) shows that neither female group has a significant sex preference. Male controls prefer opposite sex birds; male lineage birds prefer same sex birds. Asterisks (*) indicate significance.

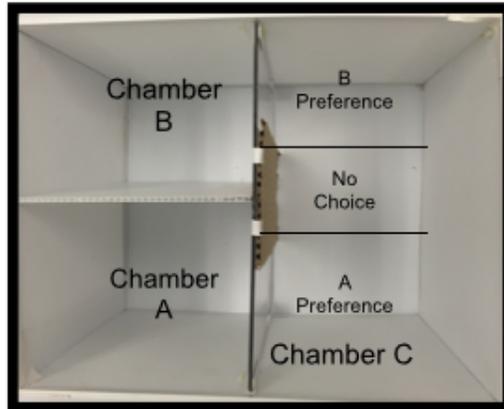


Figure 5: Mate Preference Apparatus

Experiment 2: Tests for Sex Chromosome-Specific Gene Markers

In the typical male and female zebra finch, all somatic cells contain two Z or a Z and W sex chromosome, respectively. Genes that appear on only one chromosome, or that differ in length between the chromosomes, can be used as markers to confirm the presence of Z and W chromosomes in various tissue types. We used Z and W gene markers to determine if the gynandromorph or its progeny are chimeric, having the presence of ZW chromosomes in some tissues and ZZ in other tissues, as do bilateral gynandromorphs (Graves, 2014).

Animals

Blood samples were taken from the gynandromorph, its male partner, three female plumaged progeny that are still living, one female plumaged progeny that later died, the one male plumaged progeny that is currently living, a control male, and a control female. Tail feathers were plucked from the gynandromorph, its partner, and the one male plumaged progeny. Tail feathers for controls and female progeny were plucked from different individuals to minimize disturbances to any one individual and diversify our sample. Samples of feather quills were removed from the check area and breast area

where males have reddish-orange checks and black and white zebra-striped plumage and females have more grey plumage. The smallest samples of feather quills adequate for DNA extraction were collected from a previously un-sampled female progeny, and the male and female progeny were returned to their cages. An ovary from a nestling progeny (approximately 12-15 days old) was collected after it was sacrificed by isoflurane overdose. The bird had been found on the floor of the aviary after caretakers had returned it to the nest box two other times. Feather quill samples for cheek and zebra-striped feathers areas in a control female, a control male, the gynandromorph, and her partner were taken upon sacrifice, as was the underlying skin from these areas. DNA samples of feather quills and underlying skin from the spot area (see Figure 1) as well as a gonad, and samples from the beak, liver, and heart of the gynandromorph, its partner, and a control male and female have all been extracted but are not yet represented in PCRs. Each PCR run is independent, but the same individual's extracted DNA is used for multiple genes and may be run several times for the same gene if the sample failed to amplify on the first run.

Materials

DNA from blood, skin, tissues and feathers was extracted using Chelex (Chelex 100 resin, Sigma) methods as previously described in (Jensen et al., 2003; Walsh et al., 1991; Day, McBroom, Schlinger, 2006). All other sample types and some blood, skin tissue, and feathers were extracted using the Qiagen blood and tissue kit (Valencia, CA) with user supplied protocol modifications for feathers posted on the Qiagen website (<https://www.qiagen.com/us/resources>). Primers for CHD1 employed the P2/P8 primers (Fridolfson & Ellegren, 1999; Griffiths, Double, Orr, & Dawson, 1998). The CHD1 gene

has different sequences on the Z and W chromosome but has conserved regions. The P2 and P8 primers bind to regions that are conserved on the Z and W versions of the CHD1-Z and CHD1-W gene but also amplify intron regions that differ in length for this portion of CHD1 such that products for ZZ individuals result in a single band while ZW individuals have both the shorter Z and the longer W band (forward primer, 5'-YTKCCAAGTTGAGAACTG-3; and reverse primer 5'-TCTGCATCACTAAAKCCTTT-3'). TGW is a W specific gene sequence and primers TGW1: 5'-GCAGCATGGATGTTTGGAGC-3' and TGW2: 5'-TGGCGCAGGTAGAAAATAGTC-3' will amplify a 293bp W specific sequence. This marker is run with myoglobin primers (Myoint.c (forward): 5' AGCCCTGGAGGATCCATTGG 3', Myoint2.nc (reverse): 5' CAGTGAGGTCTAGTATGCAAGG 3' that amplify sequences for both males and females of the same size as a positive control for male DNA amplification (Runciman, Zann, & Murray, 1999). ZBM is a W-specific sequence for which different lengths of the sequence can be amplified using ZBM1 and ZBM2 primers (Itoh et al., 2008). Primers used were ZBMF (5'-GCTCATTTAGTTGTTTCCTGT-3'), ZBM1R (5'-ATCTCCACCAACTCTTCTA-3'), ZBM2R (5'-TAAAATCACAGAGGTGACAT-3'), and internal control primers that amplify both Z and W forms of spindlin INT-F (5'-ATA GAA ACA ATG TGG GAC-3') and INT-R (5'-CTC TGT CTG GAA GGA CTT-3') (Itoh et al., 2001).

Procedures

For the extraction of blood of subjects, on average, 50-120 µL of blood was extracted from the jugular vein and stored at -20 °C or placed in lysis buffer and stored at

4°C. For the unfertilized egg, vitelline membrane tissue was extracted for PCR analysis (K. E. Arnold, Orr, & Griffiths, 2003). Chelex extraction methods were performed as in (Jensen et al., 2003; Walsh et al., 1991; Day, McBroom, Schlinger, 2006), and Qiagen extraction methods followed manufacturers suggested protocols. PCR methods followed published methods for each sequence (CHD, Day, McBroom, & Schlinger, 2006; ZBM, Itoh et al., 2001; TGW, Runciman et al., 1999). All products were run on a 2% agarose, sodium borate gel at 200V until there was clear separation of bands. Products that did not produce bands were first rerun on gels and then re-amplified with PCR. Results shown here represent all products that amplified in no more than two attempts for each gene shown.

Results and Discussion

Our results show that the gynandromorph, her female progeny, and female controls expressed markers for the W chromosome in all tissues tested, and the male mate, male progeny, and control males expressed a lack of W marker, or the Z length sequence for all tissues sampled. The PCR results are shown in Figures 6-9. The unfertilized egg contained the ZBM gene suggesting that the W chromosome is inherited only from the gynandromorph. While some samples for the GYN or the female plumage bird do not show a clear W, this is never the case when the control gene is also clearly amplified.

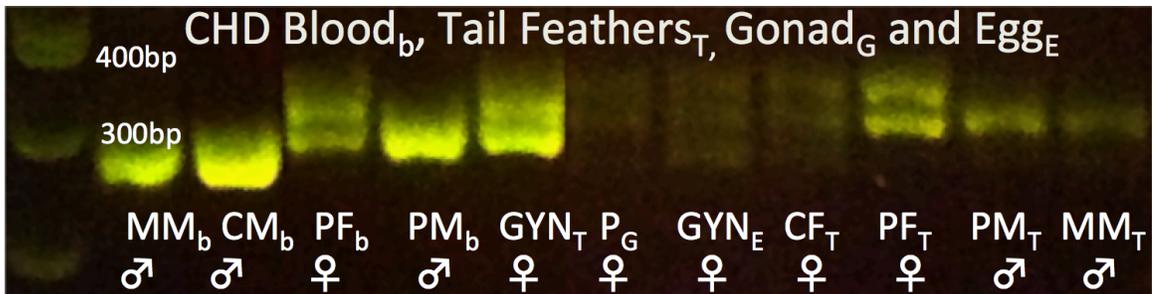


Figure 6: Female length of CHD gene is present in progeny female (PF) blood and gynandromorph (GYN) tail feathers, progeny female gonads, a gynandromorph egg, and control female (CF) tail feathers, yet is not present in control males (CM). Partner (MM) blood, control male blood, progeny male (PM) blood, progeny male tail feathers, and control male tail feathers do not show the female length of the CHD gene. A ladder is included in the first lane.

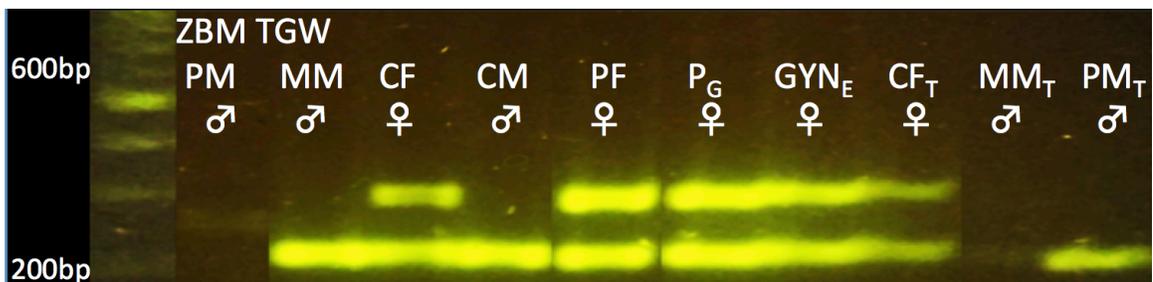


Figure 7: ZBM gene is present in control female (CF) tissue, progeny female (PF) tissue, progeny female gonads, gynandromorph (GYN) egg, and control female tail feathers. ZBM gene not present in progeny male (PM) tissue, male mate (MM) tissue, control male (CM) tissue, male mate tail feathers, or progeny male tail feathers. A ladder is included in the first lane.

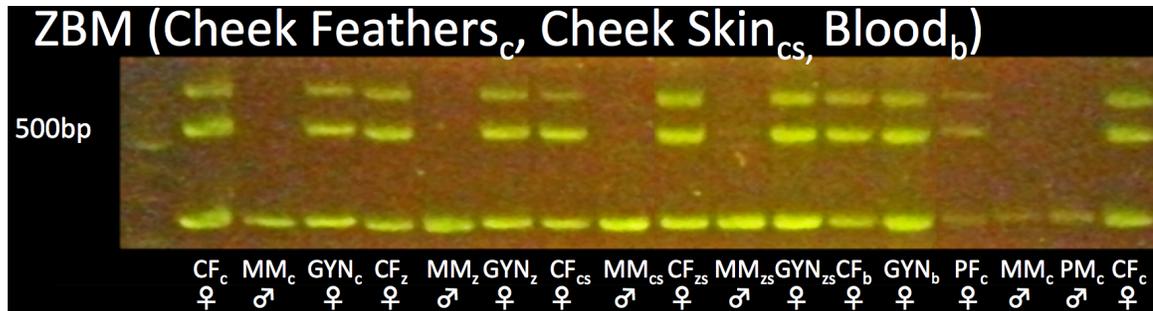


Figure 8: ZBM gene is present in cheek feathers, zebra stripe feathers, cheek skin, and blood of control females (CF) and the gynandromorph (GYN). ZBM is not present in the male mate (MM) or progeny male (PM). The control band at 200bp is amplification of spindlin. A ladder is included in the first lane.

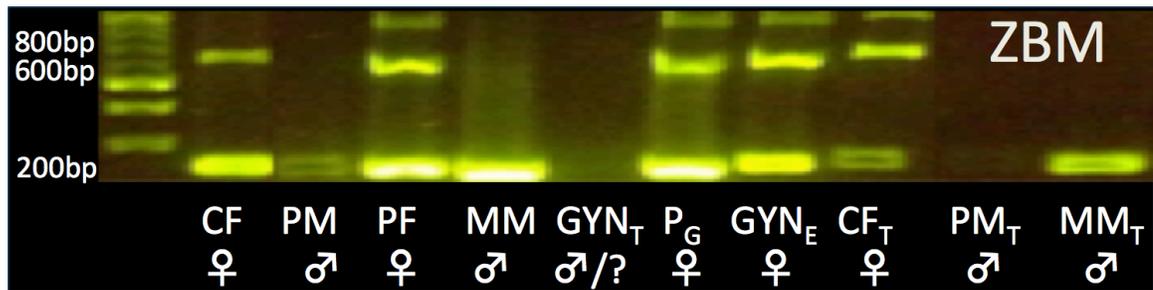


Figure 9: ZBM gene is present in control females (CF), progeny females (PF), progeny female gonads, and the gynandromorph (GYN) egg. ZBM is not present in progeny males (PM) or the male mate (MM). The small control band at 200bp in each lane represents the amplification of spindlin. A ladder is included in the first lane.

Experiment 3: Color Spectrometry

Mate choice experiments suggested that some cryptic trait of the gynandromorphs lineage reduced preference for these birds compared to matched controls. While a number of behavioral or morphological differences could influence mate choice, we noticed that at least one of the female progeny had a rather pale beak, but could not tell

from visual inspection alone whether this trait differed from the normal range in our aviary. In addition, like many avian species (Peters et al., 2004), zebra finches have retinal cones that are sensitive to the ultraviolet (UV) portion of the spectrum of light (Bowmaker et al., 1997). Since humans cannot see UV reflectance, it is important to use a spectrometer to measure reflectance of wavelengths from 300-700 nm and to calculate color variables based on a retina that has four cones absorbing spectra in the species-specific range. Higher overall brightness and bright UV reflectance are often associated with greater mate preference and often with health or fitness measures in birds (Simons et al., 2012), and increased red, especially in the carotenoid inducing red range, predict mate preference in both male and female zebra finches (Peters et al., 2004). Thus, we predicted that the gynandromorph lineage would have lower values of red in the beak and feet, especially in the carotenoid range. We predicted higher brightness for beaks and feet of gynandromorph lineage overall, because less saturated colors will reflect more. However, we predicted that for other body parts, the gynandromorph lineage might show less brightness and lower UV reflectance as indicators of abnormal sexual differentiation of plumage.

Animals

We measured color patterns of the gynandromorph, its mate, their 7 progeny (1 male plumaged), 6 control males, and 6 control females. All birds were housed in aviaries as in Experiments 1 and 4. Due to experimenter error, the spectra for the breast of one progeny female were omitted. The narrow variation of this group for this measurement suggested this one measurement would not alter the overall results.

Obtaining Color Spectra

Color reflectance spectra were measured for seven sexually dimorphic plumage and body regions of zebra finches: beak, cheek, breast, feet, stripes, spots, and tail (see Figure 1). Progeny and control birds were carried in cages covered with black cloth from aviaries to be measured in an adjacent room. Care was taken to hold birds in such a way as to not spread finger grease on areas to be measured. The bodies of the gynandromorph and its mate were removed from -80 °C storage, and the body regions to be measured were dissected, cutting under the skin to collect plumage regions. This was done to avoid thawing the entire carcass while allowing dissected areas to thaw and dry to prevent interference of ice and water on the reflectance spectra. Measurements taken from museum preserved skins, arguably far more disturbed than frozen specimen, have proven to be indistinguishable from measurements taken from wild caught birds (Endler & They, 1996). To keep feather tracts flat and in as normal a configuration as the original, we taped the top of skin sections to white card stock, taking care that plumage in the section was thick enough to be opaque.

Color reflectance spectra were collected in the bird visual spectrum (300-700 nm) using a spectrometer (Ocean Optics, Dunedin, FL, USB2000+) with a custom probe affixed to an Ocean Optics synchronized xenon light source (model PX2), which emits light at a desired wavelength from 5 fiber optic channels and gathers the reflected light from one fiber optic channel (Maddocks, Church, & Cuthill, 2001). The probe allowed for a consistent angle of capture at 90° and 10.13mm from the samples, a sampling distance that resulted in 80% reflectance intensity for the white standard (Spectralon). SpectraSuite software (Ocean Optics, Dunedin, FL, 2.0.162) was used to capture the reflected light. The reflectance sensor was calibrated after every third measurement using

the white reflection standard and a black standard, a solid matte black surface that emits nearly 0% reflectance. Each region was sampled three times. The photon captures for each 1 nm captured by the SpectraSuite, were extracted and various color parameters were calculated using the CLRVars program in the CLR 1.05 JAVA suite (Montgomerie, 2008).

This program allowed us to view many different aspects of our color spectra. From the available calculated values, we selected those pertinent to our study. Brightness is the total amount of light reflected from the surface (Saks et al., 2003). Chroma is a measure of purity or saturation (Saks et al., 2003) of a color (Meadows, Morehouse, Rutowski, Douglas, & McGraw, 2011), which can be thought of as the amount of “grays” of a certain brightness added to a hue (color). So a particular hue that has a high peak of reflectance in the range of that hue’s wavelength (nm) and little reflectance of other wavelengths would be highly saturated or have a high chroma value. Thus, red chroma is the sum of the reflectance in the red portion of the spectra (Meadows et al., 2011) relative

to overall reflectance: $\frac{\sum_{\lambda_{605}}^{\lambda_{max}} Reflectance}{Total Reflectance}$ (Montgomerie 2008). UV

chroma is the sum of reflectance in the UV portion of the spectra relative to the overall

reflectance: $\frac{\sum_{\lambda_{min}}^{\lambda_{400}} Reflectance}{Total Reflectance}$ (Montgomerie 2008). Carotenoid chroma

is specific for reds for which the reflectance at 450 nm is less than the reflectance at 700

nm (Peters et al., 2004), indicating that the reflected light that is in the portion of red

wavelengths is related to the organisms level of carotenoids. Hue, the “color” of an

object, was measured across the zebra finch visual range and was calculated using the

approximate cone captures for birds (Bowmaker et al., 1997). While these differ slightly

for different song birds, these calculations give us a basic idea of what the color was for

the peak reflectance of the curve. The hue calculation is a correlate of the reflectance data, measured in degrees, around a circular spectrum or color wheel (Saks et al., 2003). These measurements were used to plot the reflectance spectra and analyze differences in plumage coloration parameters between groups.

Statistics

Measurements for each of the five sexually dimorphic plumage regions were averaged for individuals. These subject means were used to calculate the CLR spectra values of brightness, red chroma, UV chroma, overall chroma, carotenoid saturation, and hue as discussed above. SPSS (Version 22.0, IBM) was used to compare the control male (CM), control female (CF), and female progeny (FP) groups using one-way ANOVA. The gynandromorph (GYN), its male mate (MM), and its male progeny (MP) were qualitatively compared because these groups have an $n = 1$. We considered these to be planned comparisons, and we obtained all p values from the ANOVA output. Significance is indicated in Table 1 by superscripted letters. Shared letters indicate a significant difference between the groups that also have that letter. If no letters are present, the values did not differ significantly. If all values in a row have an asterisk, then they all differed significantly from one another.

Results and Discussion

Only beak, breast, and cheek calculated color spectra variables differed significantly between the groups tested (see Table 1). In the beak, there were differences in red chroma and hue between the control males and females, regardless of whether they were control females or female progeny. The progeny females and control females had

slight variations among one another for the measured variables, but they did not differ significantly for the beak measurements. This is expected because males are known to have redder beaks than females (Simons et al., 2012). In red chroma, control males were significantly redder, indicating higher values of reflectance in the red wavelengths, than control females, and progeny females did not differ from control females. If we look at overall hue, there is a similar result in that control males differ from progeny females, but control females and progeny females do not differ from one another. This suggests that both female groups differed from males as expected, and that beak coloration is not a characteristic that would have identified the progeny females in the mate preference tests. The gynandromorph was actually lower than both the female range and the male range for red chroma, but its value was closer to the range of female controls. This means that the gynandromorph had less reflectance than both male and female controls for the red wavelengths but was closer in value to the reflectance in red wavelengths for females. For hue, the gynandromorph value was higher than both the female and male ranges, indicating that it had a beak with slightly different coloration from both males and females.

For the breast, progeny females were noted to have more white feathers present than the other groups. Breast feathers are typically gray in both males and females. When we measured the breasts of the progeny females, however, we aimed to include as much gray as possible for an accurate comparison among the groups. The spectra data indicated that the progeny females had the brightest breasts, indicating higher overall reflectance, which was expected due to the amount of white feathers present. Control males stood out for UV chroma, with a higher reflectance in the UV range than both control and progeny

females, and hue. Each group had different overall chroma, suggesting that there are variations in the patterns of gray across the spectra. The gynandromorph is in both the male and female control range for breast brightness, but it is not in the range of the progeny females. In UV chroma, the gynandromorph is in the range of both control and progeny females, but in hue, the gynandromorph is in the range of the control males. This yields interesting results because the gynandromorph does not stand out from control females in the variables of brightness or UV chroma, but it is within the range of typical male hue. In mate preference tests, breast brightness could have been an indicator to chooser birds that progeny females were different.

The cheek coloration is thought to be a prominent secondary sexual characteristic and is suspected to be an area where the gynandromorph lineage could be identified as unusual even though we did not observe any differences without the aid of a spectrophotometer. The cheek region has the most differences among groups. This is expected because male zebra finches have bright orange cheeks, and females have gray cheeks. In each of the variables produced by the CLR program, the control males stood out, and female groups did not differ from one another. Control males had a lower brightness than females, which is expected because gray feathers reflect more light than colored feathers. The red chroma of the control males is higher than the females, due to the orange coloration of their cheeks compared to the gray coloration of the females' cheeks. The UV chroma is lower in control males than female groups, and the overall chroma for control males is nearly three times higher in males than females. Carotenoid chroma in male cheeks is less than female groups, and the overall hue of the male cheeks is different than the hue of female cheeks. The gynandromorph falls into the female

ranges for brightness and hue, but it falls into the male ranges for red chroma, UV chroma, overall chroma, and carotenoid chroma. This indicates that cheek coloration was likely not a factor that prevented the progeny females from being chosen in the mate preference trials. However, cheek coloration is definitely a distinguishable factor for the gynandromorph.

Spots are only found on male zebra finches, so a comparison between the spots of males and females could not be done. Since our gynandromorph did have spots, we could compare it to control males. The gynandromorph was within the control male range for spots for both brightness and hue, but it was slightly out of range for UV chroma and overall chroma. Thus, it can be determined that the gynandromorph closely resembled a control male in the region of spots as well.

None of the groups showed significant differences in the variables measured for feet. This was unexpected because we thought we would see similar results for the beak and the feet, as they are both thought to have color differences due to carotenoids.

Because the gynandromorph is a group of one, there is no test to determine if the values of the color spectrometry for the gynandromorph are significant. However, we can look at the ranges for the other groups (control males, control females, and progeny females) and see where the gynandromorph falls within these ranges to determine if the gynandromorph value would be significant (see Table 1). By this logic, the gynandromorph would differ significantly from other control groups in the chroma of the beak and the brightness of the cheeks. Beak coloration has been correlated with mate choice in zebra finches, with both males and females preferring mates with redder beaks (Simons et al., 2012). Also, while the gynandromorph had orange cheeks, the brightness

of its cheeks was closer to that of a control female than a control male (see Figure 12). It is possible that the mate looked at the cheeks for brightness and not coloration when he decided to mate with the gynandromorph.

Beak							
Variable		CM	PF	CF	MM	PM	GYN
Brightness	Mean	68.5151	69.2886	80.003	55.61	43.09	99.96
	Range	(45.3402 – 91.6899)	(44.2053 – 94.372)	(70.6619 – 89.3441)	MM-CM	ALL>PM	ALL<GYN
Red Chroma	Mean	0.5253 ^a	0.4944	0.4527 ^a	0.49	0.57	0.41
	Range	(0.4639 – 0.5867)	(0.4221 – 0.5667)	(0.4269 – 0.4785)	MM-CM	PM-CM	ALL>GYN
UV Chroma	Mean	0.1714	0.1716	0.1972	0.15	0.11	0.18
	Range	(0.1521 – 0.1907)	(0.1334 – 0.2098)	(0.1799 – 0.2145)	MM-PF	ALL>PM	GYN-ALL
Overall Chroma	Mean	24.0742	22.6455	24.7482	20.75	19.73	25.99
	Range	(20.1672 – 27.9812)	(19.0916 – 26.1993)	(22.6177 – 26.8787)	ALL>MM	PM-PF	GYN-ALL
Carotenoid	Mean	-0.8055	-0.802	-0.7568	-0.83	-0.9	-0.73
	Range	(-0.8662 – -0.7449)	(-0.8791 – -0.7249)	(-0.789 – -0.7246)	MM-CM, F	ALL>PM	GYN-PF,CF
Hue	Mean	0.0495 ^a	0.1345 ^a	0.0824	0.26	0.29	0.29
	Range	(0.0341 – 0.0649)	(0.0273 – 0.02417)	(0.0304 – 0.1343)	ALL<MM	ALL<PM	ALL<GYN
Breast							
Variable		CM	PF	CF	MM	PM	GYN
Brightness	Mean	69.0492 ^a	89.3884 ^{a,b}	51.7825 ^b	60.08	67.62	76.19
	Range	(58.5537 – 79.5447)	(89.3884 – 132.5047)	(51.7825 – 96.8005)	MM-CM, C F	PM-CM, C F	GYN-CM,CF
UV Chroma	Mean	0.215 ^{a,b}	0.1622 ^b	0.1793 ^a	0.17	0.21	0.18
	Range	(0.2075 – 0.2225)	(0.1341 – 0.1902)	(0.1602 – 0.1983)	MM-PF,CF	PM-CM	GYN-PF,CF
Overall Chroma	Mean	2.3559 [*]	8.421 [*]	4.3333 [*]	6.74	2.58	6.17
	Range	(2.0311 – 2.6807)	(5.8732 – 10.9688)	(2.9353 – 5.7314)	MM-PF	PM-CM	GYN-PF
Hue	Mean	0.5288 ^{a,b}	0.8283 ^a	0.7915 ^b	0.68	0.62	0.65
	Range	(0.3707 – 0.6869)	(0.7186 – 0.938)	(0.7068 – 0.8762)	MM-CM	PM-CM	GYN-CM
Cheek							
Variable		CM	PF	CF	MM	PM	GYN
Brightness	Mean	42.0244 ^{a,b}	63.2842 ^a	68.3129 ^b	61.68	54.81	62.58
	Range	(27.7569 – 56.292)	(52.7052 – 73.8632)	(49.56 – 87.0658)	MM-PF,CF	PM-ALL	GYN-PF,CF
Red Chroma	Mean	0.4626 ^{a,b}	0.3023 ^a	0.3059 ^b	0.48	0.55	0.38
	Range	(0.3702 – 0.5551)	(0.2882 – 0.3163)	(0.2914 – 0.3204)	MM-CM	PM-CM	GYN-CM
UV Chroma	Mean	0.1136 ^{a,b}	0.1861 ^a	0.1814 ^b	0.1	0.07	0.14
	Range	(0.0584 – 0.1689)	(0.1673 – 0.2048)	(0.1629 – 0.2)	MM-CM	PM-CM	GYN-CM
Overall Chroma	Mean	12.9864 ^{a,b}	4.2869 ^a	4.6772 ^b	18.85	21.77	10.55
	Range	(6.5435 – 19.4294)	(2.9958 – 5.5781)	(4.2426 – 5.1118)	MM-CM	ALL<PM	GYN-CM
Carotenoid	Mean	-0.7099 ^{a,b}	-0.3084 ^a	-0.3261 ^b	-0.78	-0.86	-0.56
	Range	(-0.9518 – -0.468)	(-0.3692 – -0.2475)	(-0.3829 – -0.2693)	MM-CM	PM-CM	GYN-CM

Hue		Mean	0.434 ^{a,b}	0.6262 ^a	0.6379 ^b	0.45	0.44	0.6
		Range	(0.4012 – 0.4667)	(0.4592 – 0.7932)	(0.4895 – 0.7863)	MM~CM	PM~CM	GYN~PF,CF
Foot								
Variable		CM	PF	CF	MM	PM	GYN	
Brightness	Mean	52.5975	59.0853	54.9526	60.74	58.73	59.63	
	Range	(45.6467 – 59.5482)	(55.7658 – 62.4048)	(48.1998 – 61.7054)	MM~PF,CF	PM~ALL	GYN~PF,CF	
Red Chroma	Mean	0.4012	0.3772	0.405	0.35	0.37	0.38	
	Range	(0.3701 – 0.4323)	(0.3551 – 0.3993)	(0.379 – 0.431)	ALL>MM	PM~CM,P F	GYN~ALL	
UV Chroma	Mean	0.2049	0.2168	0.2097	0.2	0.2	0.21	
	Range	(0.1896 – 0.2202)	(0.2057 – 0.228)	(0.1966 – 0.2229)	MM~ALL	PM~ALL	GYN~ALL	
Overall Chroma	Mean	11.9658	10.8598	12.0363	9.47	10.71	12.33	
	Range	(9.113 – 14.8187)	(8.626 – 13.0935)	(9.5039 – 14.5686)	MM~CM,P F	PM~ALL	GYN~ALL	
Carotenoid	Mean	-0.6744	-0.6211	-0.679	-0.52	-0.62	-0.63	
	Range	(-0.7381 – -0.6108)	(-0.6908 – -0.5515)	(-0.738 – -0.6201)	ALL>MM	PM~ALL	GYN~ALL	
Hue	Mean	0.1035	0.0468	0.0324	0.33	0.21	0.18	
	Range	(0.0474 – 0.1596)	(-0.0344 – 0.128)	(0.0411 – 0.1059)	ALL<MM	ALL<PM	ALL<GYN	
Spots								
Variable		CM	PF	CF	MM	PM	GYN	
Brightness	Mean	61.2099	N/A	N/A	52.45	72.34	65.3201	
	Range	(53.24 – 67.22)	N/A	N/A	CM>MM	CM<PM	~CM	
UV Chroma	Mean	0.1682	N/A	N/A	0.13	0.14	0.1241	
	Range	(0.14 – 0.20)	N/A	N/A	CM>MM	~CM	CM>GYN	
Overall Chroma	Mean	9.5968	N/A	N/A	12.04	15.02	14.9017	
	Range	(2.40 – 14.28)	N/A	N/A	~CM	CM<PM	CM<GYN	
Hue	Mean	0.4245	N/A	N/A	0.44	0.44	0.4737	
	Range	(0.19 – 0.65)	N/A	N/A	~CM	~CM	~CM	

Table 1: Means and ranges of multiple comparisons of brightness, red chroma, UV chroma, overall chroma, carotenoid chroma, and hue (as described above) for beak, breast, cheek, and foot. Shared superscripts indicate significant differences between groups, and an asterisk (*) indicates significant differences between all groups ($p < 0.05$). For groups or variables with no superscripts, there were no significant differences. Because the GYN, MM, and PM were individuals, no range is reported. Instead, the range row for these individuals includes information about how they relate to the ranges of the comparison groups. A greater than (<) or less than (>) symbol means that the individual's value was greater than or less than all comparison groups, respectively. A

congruence symbol (~) followed by a group name indicates that the individual's value was within a particular group range.

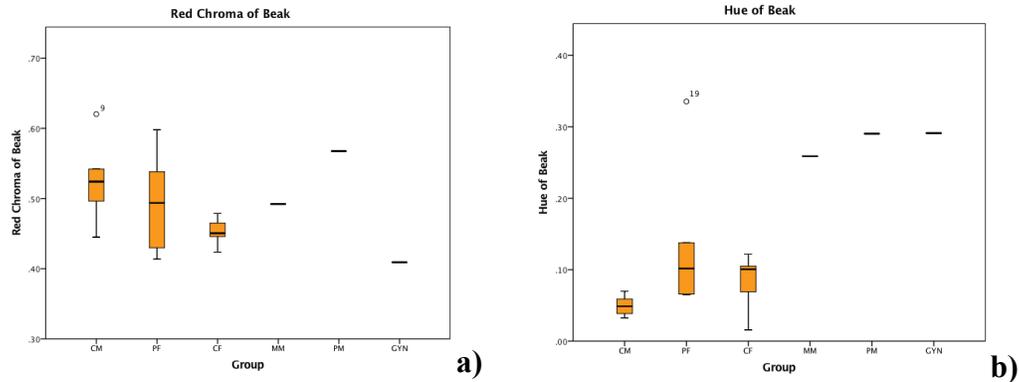


Figure 10: Beak – Box plots showing the ranges for CM, PF, and CF groups and where MM, PM, and GYN fall within these ranges for the red chroma (**a**) and hue (**b**) measurements of beak. Panel **a** shows that control males have a significantly higher amount of red chroma, relative to total reflectance, than females. This simply means that their beaks are redder. Panel **b** shows that control males and progeny females have different hues, or general coloration, of their beaks. This is expected because male zebra finches are known to have redder beaks than females (Simons et al., 2012).

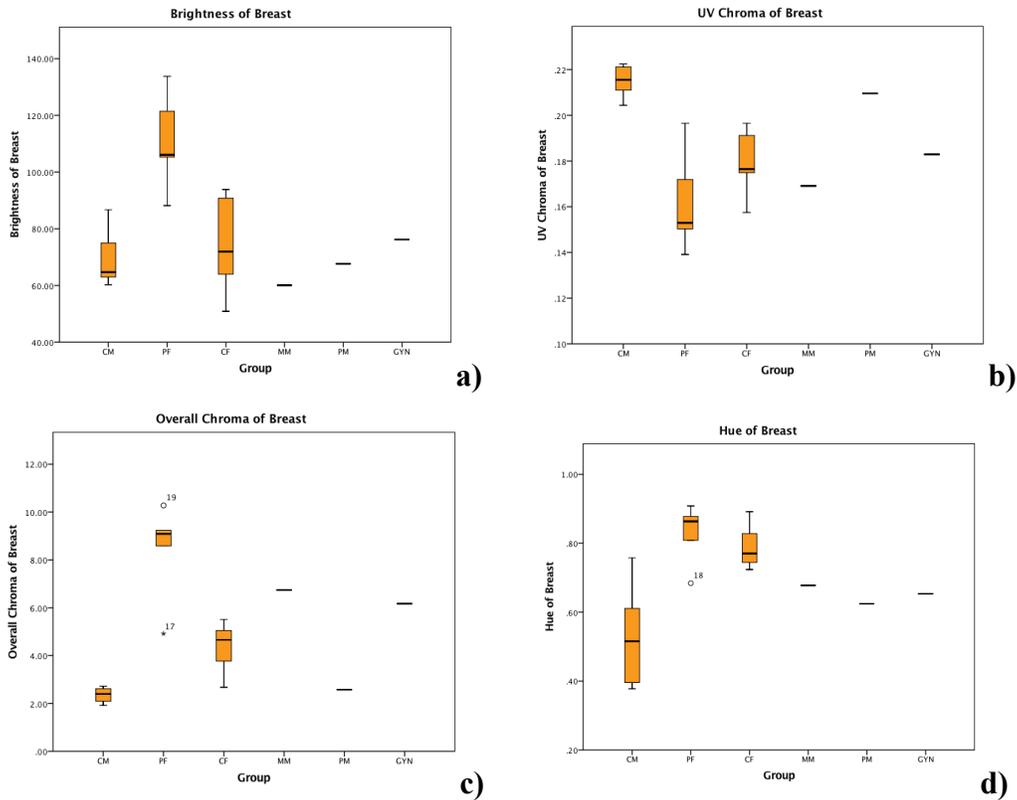


Figure 11: Breast – Box plots showing ranges for CM, PF, and CF groups and where MM, PM, and GYN fall within these ranges for brightness (a), UV chroma (b), overall chroma (c), and hue (d) measurements of the breast. Panel a shows that the progeny female are significantly brighter than other groups. We attribute this to the increased amount of white feathers present on their breasts, causing them to reflect more light than the typical gray feathers. Panel b shows that control males have higher reflectance in the UV regions of light than either female group. Panel c shows that the progeny female had a different overall chroma for their breast, again attributed to the large amount of white feathers present. Panel d shows that males had a different hue than both female groups.

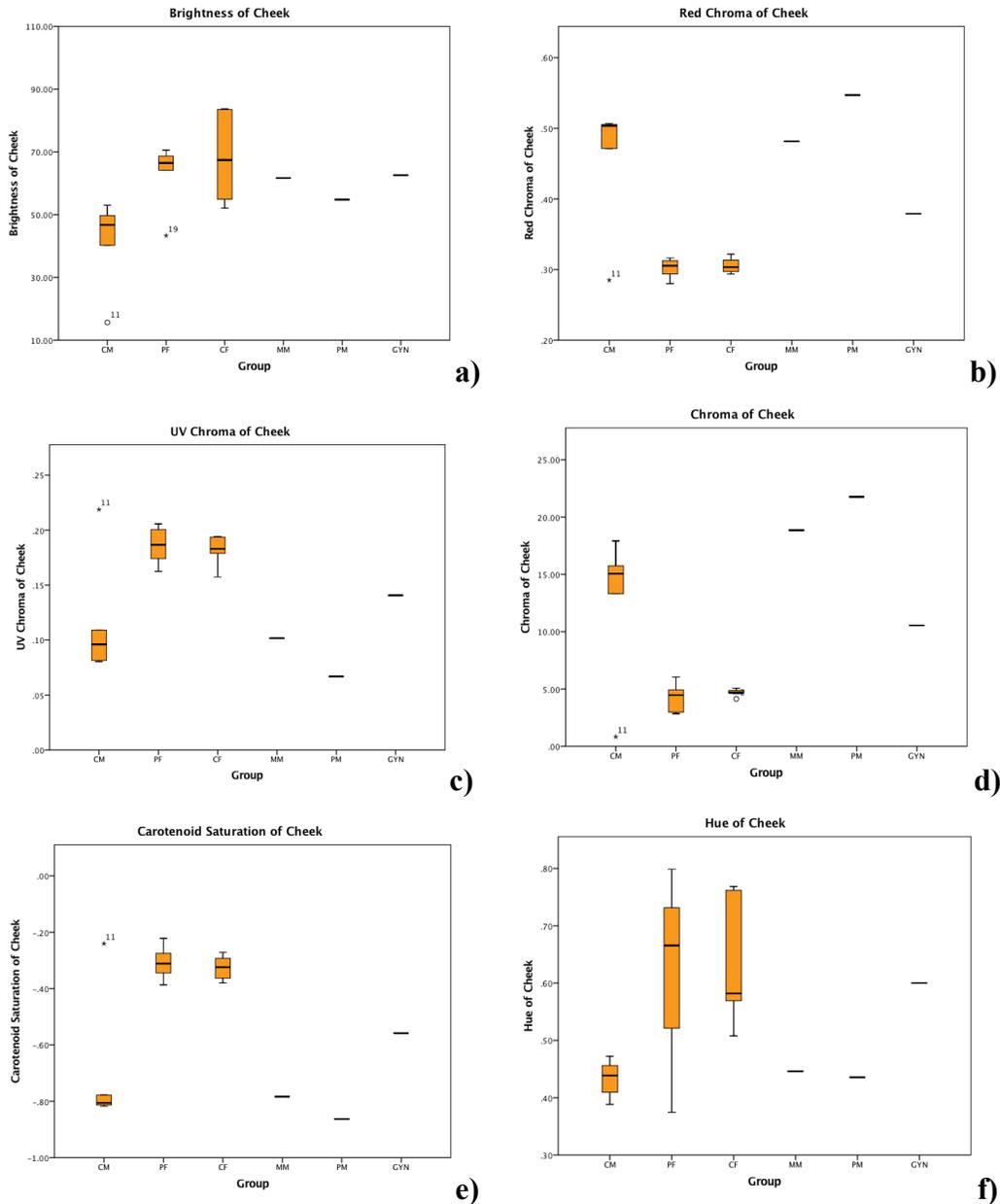


Figure 12: Cheek – Box plots showing ranges for CM, PF, and CF groups and where MM, PM, and GYN fall within these ranges for brightness (**a**), red chroma (**b**), UV chroma (**c**), overall chroma (**d**), carotenoid saturation (**e**), and hue (**f**) measurements of the cheek. Panel **a** shows that both female groups were significantly brighter than the control males. This is due to the gray female cheek plumage, which reflects more light than the orange cheeks that males have. Panel **b** shows that males have increased

amounts of red chroma present in their cheeks than females, which is expected. Panel **c** shows that males have significantly less UV chroma in their cheeks than females. Panel **d** shows the chroma differences between males and females. Males have higher overall chroma because their cheeks have orange coloration, while female cheeks are gray. Panel **e** shows differences in carotenoid levels in the cheeks of the male and female zebra finches. Males have a more negative value for carotenoid saturation, indicating that the cheek region coloration is likely to be carotenoid dependent. Panel **f** shows that males have a different hue for the cheek region than female groups, which is expected.

Experiment 4: Sexual Differentiation of the Gynandromorph Brain

Male zebra finches, which sing, have larger volumes of three song nuclei (LMAN, HVC, and RA) (Schlinger, 1998; Tomaszycski et al., 2009; Wade & Arnold, 2004) than females, which do not sing and also lack a noticeable fourth song nucleus, Area X. Masculinization of the song circuit is influenced during development by genetic and hormonal factors and during adulthood, mainly by circulating levels of testosterone. Given that our gynandromorph showed W genes in all tissues tested and clearly had sufficiently normal levels of female hormones for reproduction and lacked testes, we predicted that the song nuclei would be similar in size to other females. To determine the extent of the masculinization of our gynandromorph, we felt it necessary to obtain precise measures of the song nuclei in the gynandromorph's brain.

Animals

We sacrificed 4 female controls, and 4 male controls, the gynandromorph, and its male partner when all birds were within a few months of 3.3 years old. All birds had been

breeders for most of their adult life. Approximately 23 months prior to tissue collection, control males had been used in another study that involved a 30 minute relatively non-invasive surgery. A sham lesion was performed which involved sedation, and dissecting a skin flap, but no craniotomy or penetration of the underlying tissue (IACUC protocol 16-024).

Tissue Preparation

Birds were overdosed via isoflurane inhalation, and brains were dissected, sliced into two hemispheres, and fast frozen on a dry ice/isopentane slurry and then stored at -80 °C until cutting.

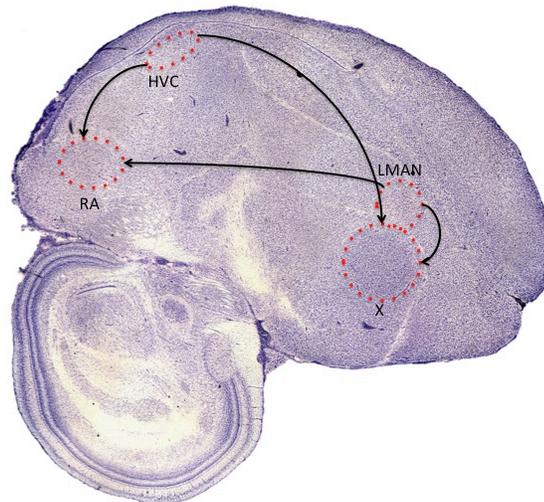


Figure 13: HVC, RA, LMAN, and Area X outlined in a male zebra finch in a sagittal section approximately 2.07 mm from the midplane. Arrows indicate projection patterns among the nuclei.

Slide Preparation

We used optimal cutting temperature compound (O.C.T.) to stabilize the tissue and affix it to a chuck for sagittally cutting at 30 μm in a cryostat. Only one hemisphere of each brain was cut, to preserve the remaining hemispheres for further studies involving

the zebra finch song system. Brain slices were placed on slides in three series. Two of the series were Nissl stained with cresyl violet, to allow nuclear and cytoplasmic visualization, the remaining series was stored in the -80 °C freezer for future experiments.

Measurements of Brain Region Volumes

The neuroanatomical boundaries of the four song nuclei to be measured are very well defined in print and online versions of zebra finch brain atlases (Nixdorf-Bergweiler & Bischof, 2007; Brain Architecture Project) that delineate their appearance throughout the brain making identification relatively easy (see Figure 13). We used stereological methods for all measurements using a Stereologer software to analyze images (Stereological Resource Center, St. Petersburg, FL) captured by an Imi Tech IMC-3145FT camera (Imi Tech, Seoul, South Korea) from a Zeiss light microscope (Zeiss AxioImager M1, Germany). Each slice containing the region from lateral to medial was identified to determine the number of total slices and develop efficient sampling strategies that kept the coefficient of error within samples and averaged across samples below 10% . Using a Cavalieri point counting, $volume = V_{ref} = T \cdot \Sigma A$, where V_{ref} is region volume estimate, T is the thickness of your section, and A is the sum of all the sampled surface areas obtained from point counts (Mouton, 2011). Region volume was estimated on 8-10 sections (90 μ m apart) using a grid size of 0.25% screen height² resulting in a CE of 0.0319. Region boundaries were identified and point counts performed at 2.5x objective and 100x images were used to determine section thickness. The camera and phototube magnify objects by 10x making the total magnifications 25x and 1000x for region point counting and depth measurements respectively. The thickness

of each slice is determined by focusing through the Z-axis and identifying the first and last Z value at which an object is in focus and applying an upper and lower guard zone of 0.50 μm to avoid the artifacts on the cut surfaces of slices and measure only the central portion. This Optical Dissector method is also used for counting cells and measuring cell size. An inclusion frame size of 25% screen height² was sampled for every 175 μm within each region throughout the 10 μm height of the Optical Dissector across all cells for which a clearly identifiable nucleolus comes in focus, that is within the inclusion frame, and does not touch the non-inclusion lines is counted. Our sampling strategy was to aim for an average of 100 cells counted and measured. For cell size, we used the independent uniform random (IUR) method with three dissector lines that were 3 μm apart. The sampling frames are the same as for cell counts except that only one dissector plane is randomly chosen, and only cells in focus in this plane are measured. The CE for cell size was 0.100 and for cell counts was 0.133.

Statistics

Measurements for volume, cell number, and cell size were averaged for both the control males and control females. Since the gynandromorph and its mate were $n = 1$, their values could not be averaged. We used SPSS (Version 22.0, IBM) to perform independent t-tests on the group averages for the three parameters of volume, cell number and cell size to obtain p-values and ranges for each group. We also performed Levene's test for heterogeneity of variances. For any comparisons where Levene's test for heterogeneity of variance was significant, we used the t that was corrected for unequal variances. Significance between the control male and control female groups is indicated

in Table 2 by asterisks (*). General linear models were used to adjust the volumes of the regions for the size of the telencephalon.

Results and Discussion

Our results show that the volume of the gynandromorph's song nuclei was within the range of control males for HVC, RA, and LMAN. The male mate, however, had a larger volume than the control males for each of these regions. For cell number, the gynandromorph was within the control male range for each of the three regions. The gynandromorph's mate was within control male range for RA and HVC, but the mate had more cells in LMAN than control males. HVC and RA are known to be approximately five times larger, and contain more and larger neurons, in males than in females (Schlinger, 1998). LMAN, however, has not been shown to differ in size between sexes, but is shown to have an increased number of larger cells in males than in females (Tomaszycki et al., 2009).

Our results show that RA volume, both relative and absolute, is larger in males than in females ($t(7) = 9.3037$ $p < 0.0001$). The volume of RA for our control males was found to be 4.56 times larger than control females, which is consistent with the literature (Schlinger, 1998). The volume of RA in the gynandromorph brain was found to be within the range of our control males, and the volume of RA for the gynandromorph's partner was found to be slightly larger than our control male range (see Figure 14). RA cell number was found to be marginally significant between control males and control females ($t(3.052) = 2.660$, $p = 0.075$), with males having more cells in the RA nucleus than females. The gynandromorph and its mate were both within range of control males for RA cell number (see Figure 15). Our two control groups had no significant difference

for RA cell size ($t(7) = -0.241$, $p = 0.816$), but males were expected to have larger cell size in the RA nucleus. The gynandromorph and its mate were both within male range for cell size (see Figure 16).

Our control males had a significantly larger absolute volume of HVC compared to control females ($t(6) = 3.122$, $p = 0.021$), but there was no significant difference when the volumes were relative to the telencephalon. The volume of HVC in our control males was only 2.13 times larger than control females, which is less than the published ratios that suggest that HVC is approximately five times larger in males than in females (Nottebohm & Arnold, 2010). Our gynandromorph was within the range of HVC volumes for control males, while its mate was slightly above range for control males (see Figure 14). While our control males had a larger number of cells present in HVC, and those cells were larger in size, the differences between control males and control females for cell number and cell size were not significant ($t(3.008) = 1.399$, $p = 0.256$; $t(6) = 0.749$, $p = 0.482$). Our gynandromorph and its mate were both within male range for cell number (see Figure 15), but both contained cells larger than control males (see Figure 16). Though previous studies have reported LMAN to have similar volumes between males and females (Tomaszycki et al., 2009), we found both the absolute and relative volume of LMAN in our control males to be larger than control females ($t(3.909) = 4.365$, $p = 0.013$). Control male volume was 2.51 times larger than control females, and our gynandromorph was within the range of control males, but the gynandromorph's partner had a volume of LMAN that was larger than the range of control males (see Figure 1215). Males also had a significantly larger number of cells than females ($t(7) = 2.754$, $p = 0.028$), but cell size did not show any significant differences ($t(7) = -0.316$, $p = 0.761$).

Our gynandromorph was within male range for cell number, but the male partner had more cells than the control male range (see Figure 15). For cell size, both the gynandromorph and the male partner had larger cells than control males and control females (see Figure 16).

The experimenter was blind to expectations in size differences between subjects when performing most volumetric measurements and only became aware of expectations for Area X volume after measuring Area X in both males and the gynandromorph. Later, the experimenter was confused by an inability to find Area X in females, at which time it was revealed that Area X is not detectable in normal females (Schlinger, 1998). Because volumes of males and the gynandromorph were measured first, and the gynandromorph has an $n=1$, it was not possible to be blind to the identity of females. However, our results are in the opposite direction to our expectation of a female like brain, suggesting little expectation bias during measurement of female brains and cell measurements.

We also found a putative Area X, which is only found in male zebra finches, in our gynandromorph. The volume of Area X for our gynandromorph is 2.20 times smaller than that of a control male, but the volume of Area X for the male mate was within control male range (see Figure 14).

In all three regions that we measured, the volume and cell numbers of each region in the gynandromorph brain was within range of control males. In HVC and LMAN, the cell size of the gynandromorph was larger than both control males and control females. After noticing the masculinization of the song nuclei in our gynandromorph, we began to observe nesting video footage to determine if the gynandromorph ever produced viable song. We discovered a segment of video that clearly shows our gynandromorph

producing song. As far as we know, this is the first time that a unilaterally plumaged gynandromorph produced viable song. This also adds an interesting facet to the mating patterns of the gynandromorph and its mate, because typical females do not produce song.

	CM	CF	GYN	MM
<i>RA</i>	0.0735	0.0161	0.0734	0.0959
<i>Vol mm³*</i>	(0.0606 – 0.0909)	(0.0104 – 0.0217)	<i>GYN~CM</i>	<i>MM>CM</i>
Cell Number	11691.692	2534.861	7903.396	12283.85
	(4801.003 – 19782.44)	(1689.05 – 3287.63)	<i>GYN~CM</i>	<i>MM~CM</i>
Cell Size	29.901	31.456	25.298	43.856
	(16.558 – 44.807)	(20.838 – 42.290)	<i>GYN~CM,CF</i>	<i>MM~CM</i>
<i>HVC</i>	0.0444	0.0208	0.0436	0.0759
<i>Vol mm³*</i>	(0.0251 – 0.0662)	(0.0149 – 0.0261)	<i>GYN~CM</i>	<i>MM>CM</i>
Cell Number	8180.810	2614.0742	3571.677	7592.167
	(1660.322 – 19198.641)	(2422.095 – 3037.584)	<i>GYN~CM</i>	<i>MM~CM</i>
Cell Size	29.160	26.689	86.458	63.286
	(20.902 – 32.124)	(21.745 – 29.84)	<i>GYN>ALL</i>	<i>MM>ALL</i>
<i>LMAN</i>	0.0382	0.0152	0.0431	0.0792
<i>Vol mm³*</i>	(0.0226 – 0.0548)	(0.00998 – 0.0155)	<i>GYN~CM</i>	<i>MM>CM</i>
<i>Cell Number*</i>	5336.567	1779.901	6100.118	9548.742
	(2065.393 – 9080.791)	(1252.673 – 2511.959)	<i>GYN~CM</i>	<i>MM>CM</i>
Cell Size	29.843	32.443	47.509	45.0706
	(28.837 – 41.574)	(17.548 – 48.898)	<i>GYN~CF</i>	<i>MM~CF</i>
<i>X</i>	0.4087		0.1857	0.4205
<i>Vol mm³</i>	(0.322 – 0.473)		<i>GYN<CM</i>	<i>MM~CM</i>
Cell Number	47681.29			
	(9293.553 – 105704.397)			
X Cell Size	38.316			
	(33.964 – 46.694)			
<i>Tele*</i>	18.336	13.893	19.827	15.571
<i>Vol mm³</i>	(14.984 – 21.066)	(11.341 – 16.800)	<i>GYN~CM</i>	<i>MM~CM,CF</i>

Table 2: Mean and range for song nuclei measurements of control males (CM), control females (CF), the gynandromorph (GYN), and its mate (MM). Asterisks (*) indicate significance between male and female controls. The volumes reported are the raw volumes (mm³), and the ranges reported are the maximum and minimums for each group.

Because the GYN and MM were individuals, no range is reported. Instead, the range row for these individuals includes information about how they relate to the ranges of the comparison groups. A greater than (<) or less than (>) symbol means that the individual's value was greater than or less than all comparison groups, respectively. A congruence symbol (~) followed by a group name indicates that the individual's value was within a particular group range.

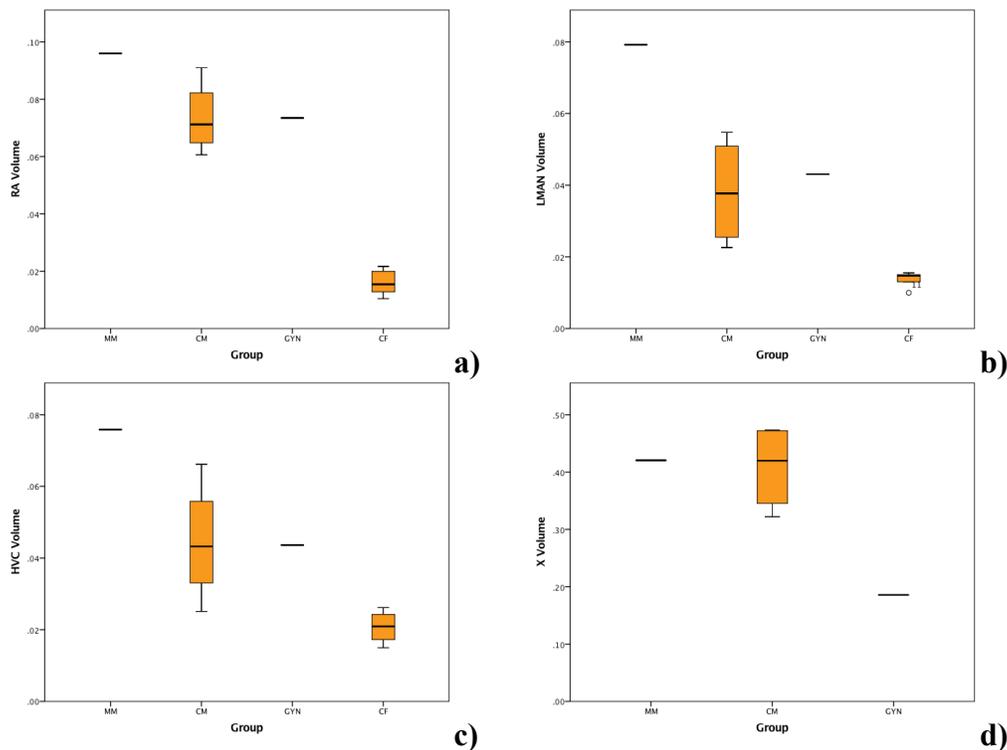


Figure 14: Volumes and ranges of the four song nuclei measured: RA (a), LMAN (b), HVC (c), and Area X (d). Control Females are not included in the graph of Area X volume because they do not have Area X. All units are in mm³. Panels a, b, and c show that the volume of RA, LMAN, and HVC for the gynandromorph is within range of male controls, while the volume of these regions for the male mate is larger than male controls. Panel d shows that the volume of the gynandromorph's putative Area X is below the

range of control males, and the volume of Area X for the male mate is within the range of male controls.

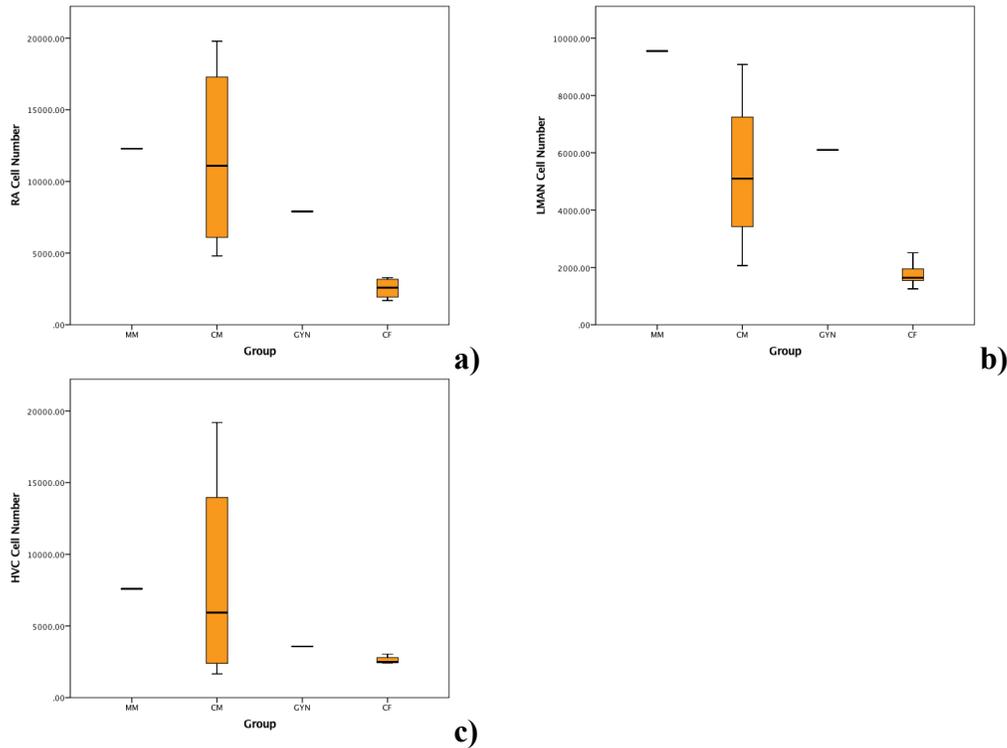


Figure 15: Cell number of three song nuclei measured: RA (a), LMAN (b), and HVC (c).

Panels a and c show that the gynandromorph and male mate are within control male range of cell number for RA and HVC. Panel b shows that the gynandromorph is within male control range for cell number of LMAN, while the male mate has more cells than control males.

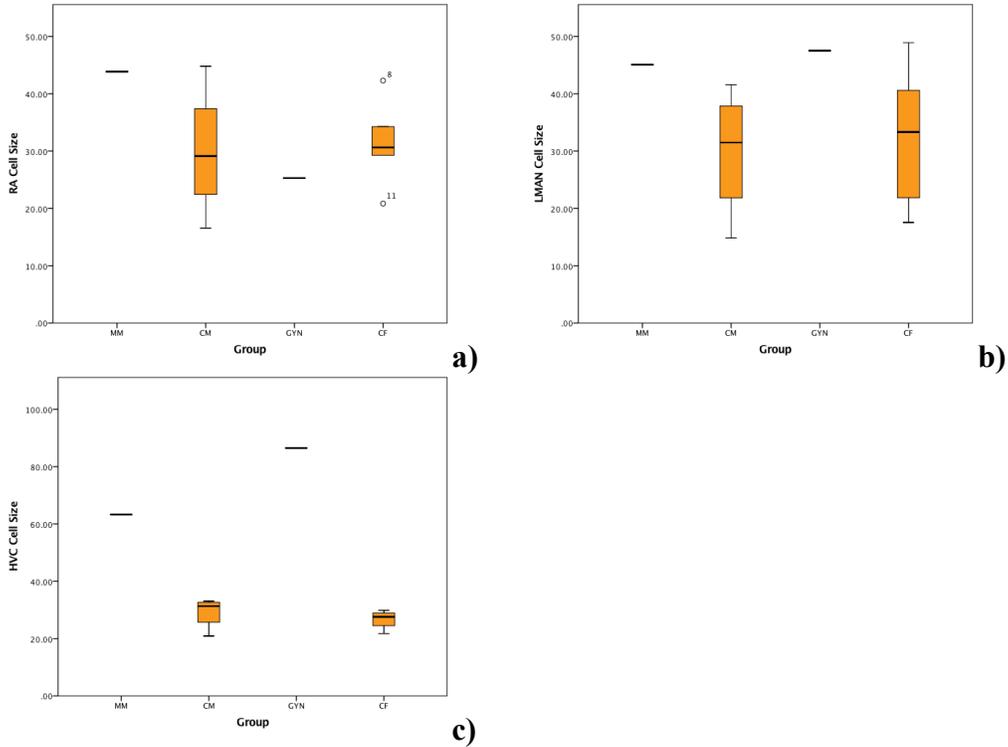


Figure 16: Cell size of three song nuclei measured: RA (a), LMAN (b), and HVC (c).

Panel **a** shows overlap between the male and female ranges for cell size and also shows that the gynandromorph and male mate are within range of the control males for RA.

Panel **b** shows that the gynandromorph and male mate are within range of control females for cell size of LMAN. Panel **c** shows that the gynandromorph and male mate have larger cells than both control males and control females in HVC.

GENERAL DISCUSSION AND CONCLUSION

Results of our experiments suggest that the gynandromorphic zebra finch and her lineage support previous studies on sexual orientation in mate preference test, suggest that color patterns of plumage might influence mate preference and that these color patterns might be heritable, and extend our understanding of sex chromosome expression and hormonal influences on sexual differentiation of plumage and song nuclei .

The gynandromorph lineage was less preferred in two-choice mate preference tests, and the gynandromorph mate and male progeny had strong same plumage orientation in the sex preference tests. Abnormal plumage patterns were found in the gynandromorph lineage, and the gynandromorph did not have sex chromosomes that differed from those expected from her reproductive sex even in the male-typical plumage samples and her progeny and mate did not have any sex chromosome unexpected for their plumage appearance. The gynandromorph has masculinized song nuclei and produced song. Due to the gynandromorph's reproductive output and normal appearance of her ovaries and reproductive tract, we assume that circulating reproductive hormones would be within the normal range of normal females.

Mating in zebra finches is known to be influenced by song (Gahr, 2007), ultraviolet plumage differences (Maddocks et al., 2001), and beak coloration (Simons et al., 2012). Less is known about how particular aspects of color such as red chroma, brightness, and hue influence mate preference. Our color spectra data shows that the gynandromorph plumage was distinct from both males and females. The gynandromorph was within male ranges for some regions (beak, cheek, and spots) and within female ranges for breast. Some of these plumage differences may be heritable, as seen in

measures where the gynandromorph and her partner were within the female progeny range but not the control male or female range. This occurred for UV chroma of the beak and overall chroma of the breast. Initially we assumed most differences in the breasts of the progeny females to be due to the increased amount of white feathers present.

However, we did not note any excess of white feathers in the gynandromorph or mate, so the overall chroma difference seen in the progeny females for breast could be a heritable factor instead of the distribution of white feathers.

We know that mate preference, and song nuclei size, can be heritable as well, but this has typically been studied in the context of song (Airey et al., 2000; Rutstein et al., 2007). Our results suggest the possibility that zebra finches may attend to specific aspects of plumage when choosing mates and that abnormalities unapparent to us can be detected by other zebra finches. However, we cannot determine in the current experiments if these plumage differences directly influenced mate preference. One other distinct possibility for the gynandromorph lineage being less preferred is that the sexual orientation biases of the gynandromorph lineage also influenced the preference of choosers when selecting between them and a control.

Having same sex plumage parents has been shown to increase same sex preference due to sexual imprinting (Adkins-Regan & Krakauer, 2000; Banerjee & Adkins-Regan, 2014). The gynandromorph progeny had two same sex plumage parents, and males were shown to have same sex orientation. Female zebra finches generally have less strong sex preference than males (Rutstein et al., 2007). Our female progeny sex preference was not significant, but they chose opposite sex controls more than same sex

controls. Our results suggest that sexual imprinting plays a role in the same sex preferences of our gynandromorph lineage.

This sexual imprinting may have also caused behavioral abnormalities that would influence preference tests. Previous studies have shown that sexually naïve birds, that have never had contact with a bird of the opposite sex, may require time to develop attractiveness towards the opposite sex due to lack of exposure to sex specific behavioral and plumage differences (Adkins-Regan & Krakauer, 2000). While our gynandromorph progeny were sexually naïve, they were housed in an aviary with each other. This lack of exposure to potential normal mates of the opposite sex could have influenced their choice in the mate and sex preference trials.

Interestingly, our gynandromorph's male mate has an extremely masculinized song system, but he chose a male plumaged mate that also produced song. Given the masculinization of our gynandromorph's brain, we immediately suspected that she would have unusual behaviors for a female. Zebra finches typically learn their song from their father (Williams, 2004), and our gynandromorph sang. This may have affected the song that the male progeny learned, and thus affected the male progeny's ability to sing directed song during the mate and sex preference experiments.

Our gynandromorph is different from the other two reported unilaterally plumaged gynandromorphs. One of the reported gynandromorphs has male plumage, ZZ male typical chromosomes, an ovary and no testis, produces song, and has a partially masculinized song system (Grisham et al., 2007). The other reported gynandromorph has male plumage, ZW female typical chromosomes, normal ovaries, does not sing, and has a feminine song system (Cui et al., 2015). Our gynandromorph has male plumage, ZW

female chromosomes, normal ovaries, produces song, and has a masculinized song system. We believe that the combination of these attributes in these three gynandromorphs could further our knowledge of the processes of sexual differentiation.

Zebra finch plumage differentiation is thought to be controlled by an imbalance of Z and W genes present in an individual, with more Z expression leading to masculinized plumage (A. P. Arnold & Itoh, 2011). In this case, our gynandromorph may have had more Z expression, which caused her to develop masculine plumage. Our gynandromorph's particular combination of features suggests that both singing behavior and plumage differentiation in zebra finches may be determined by gene expression on the Z chromosome. We do not think that circulating gonadal hormones determined the plumage or singing ability in our gynandromorph since she had female typical hormone levels, as evident by her reproductive abilities.

Analyzing our gynandromorph's song for male typical singing patterns would help us better understand the nesting behavior that she and her mate exhibited as well as the effect that would have had on the progeny. We also hope to obtain hormone levels from the gynandromorph's remaining tissue to verify that her hormone levels were within female range and did not affect her plumage or song system differentiation.

Our data suggest that genes that influence plumage may also masculinize the brain and create subtle differences in plumage coloration. These coloration differences, behavioral differences, and interest for the opposite sex could influence how often birds are chosen for mates and also suggest the possibility that variation in these variables in the normal population could influence mate selection.

Our gynandromorph provides a unique perspective into the sexual differentiation mechanisms of zebra finches. We had a bird with male plumage, *ZW* sex chromosomes, and masculinized song nuclei that produced song and 7 viable offspring. Through our studies, we have gotten a better grasp of what differences birds may notice when choosing a mate and how zebra finch behavior can be altered when both parents share plumage coloration. We hope that our research, combined with the efforts of other scientists studying the zebra finch sexual differentiation pathways, can lead to the full understanding of this complex system and the process of sexual differentiation overall.

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