

THE ROLE OF NEURAL MECHANISMS AND EARLY SOCIAL
ENVIRONMENT IN AFFILIATIVE RELATIONSHIPS IN
ZEBRA FINCHES (TAENIOPYGIA GUTTATA)

A Dissertation

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by

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The neural mechanisms underlying affiliative behaviors such as pair bonding are best studied in a mammalian rodent, the prairie vole (*Microtus ochrogaster*). Although a large number of avian species are socially monogamous, the neural mechanisms underlying monogamy remain largely unexplored. The experiments presented in the first part of this thesis explore the role of the neurotransmitter dopamine in pair bonding in the socially monogamous avian species, the zebra finch (*Taeniopygia guttata*). Dopamine is a key player in the mediation of reward in vertebrate species. We hypothesized that pair bonding is a rewarding behavior and therefore would involve this neuromodulator. The dopaminergic pathway consists of dopaminergic neurons that project from the ventral tegmental area (VTA) that project into the medial striatum. We measured levels of dopamine in the medial striatum and activity of dopaminergic neurons in the ventral tegmental area (VTA) in unpaired and paired zebra finches. Additionally, we observed the effects of dopaminergic antagonists on pairing behaviors. The activity of VTA dopaminergic neurons and dopamine levels in the medial striatum were higher in paired versus unpaired birds but blocking dopaminergic neurotransmission via two receptor subtypes did not prevent the formation of pairs in this species. The second major theme in this dissertation has been to explore the role of early social environment and its role on the development of the stress response as well the development of mate choice and pairing behaviors in adulthood. Zebra finches were raised solely by their fathers (female-deprived) in the

absence of their mothers. When these offspring reached adulthood, they were subjected to stressors such as isolation and restraint. We found that female-deprived adults were hyperresponsive to isolation as compared to control birds that were raised with both parents. Additionally, corticosterone receptor levels were altered in female-deprived offspring. Finally, a significantly higher percentage of female-deprived offspring formed pairs with other female-deprived males. These results suggest that early life perturbation of the social environment affects mate choice as well as stress physiology of offspring in adulthood.

BIOGRAPHICAL SKETCH

Sunayana ‘Nina’ Banerjee was born on December 17th, 1979 in Mumbai, India to Swati and Biswarup Banerjee. Her physicist father and biology teacher mother played a key role in molding her interests in research and biology. She schooled in the J.B Petit High school for girls, Mumbai, where she was taught by liberal and open-minded teachers, who urged their students to be strong and independent young women. In addition to pursuing academics, she took an avid interest in an Indian classical dance form, which she proceeded to learn for 14 years. After high school, she went on to pursue a Bachelor of Science degree from St. Xavier’s college, Mumbai, where she majored in Life Sciences and Biochemistry. After graduating from St. Xavier’s college in 2001, she joined the Master’s program in Biology at the Tata Institute of Fundamental Research (TIFR), Mumbai. Here, she worked with Vidita Vaidya Ph.D. and after three years, defended her Master’s thesis titled: Neurobiology of depression: role of signaling pathways and postnatal development. During her master’s, strongly encouraged by Vidita, she developed a keen interest in the neural mechanisms of animal behavior. Confident that this was the area that she wanted to do her Ph.D. work in, she joined the Department of Psychology at Cornell University in August, 2004. After a year of lab rotations she joined the laboratory of Prof. Elizabeth Adkins-Regan. During her 5.5 years at Cornell she had the privilege of being advised and mentored by Elizabeth as well as the opportunity to perform collaborative research projects in the labs of Professors Andy Bass and Tim DeVogd. The next step for Nina will be a postdoctoral position at the laboratory of Prof. Peg McCarthy at the University of Maryland, Baltimore.

To Ma and Baba,
for believing in me and never letting me lose
faith in myself.

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CHAPTER 1

INTRODUCTION

The experiments described in this thesis have attempted to elucidate the role of neural pathways and social environment underlying affiliative behaviors in an oscine passerine bird- the zebra finch (*Taeniopygia guttata*). Although a vast body of work exists on the song system of this species, the neurobiology behind social behaviors and relationships remains unexplored. The studies presented describe the role of dopamine in pair formation in zebra finches and the role of mothers in the development of mate choice. Additionally, the effect of a social stressor (isolation) on the hypothalamic-pituitary-adrenal (HPA) axis of this highly social species and whether conspecifics can buffer this stress response, was studied. The attempt has been to combine molecular and behavioral approaches to shed light on the mechanisms involved in affiliation in this species. The experiments presented in the first part of this thesis (chapters 2 and 3) include manipulations performed during adulthood to tease out the neural pathways involved in mate choice and pair formation. The second part of this thesis (chapter 4) includes experiments that have addressed the effect of a social challenge-isolation in this highly social species. Additionally, this segment addresses the role of social buffering by conspecifics in when individuals were exposed to isolation. The next segment (chapter 5) addresses the importance of biparental care and the impact of the loss of a parent on the hypothalamic-pituitary –adrenal (HPA) axis and stress responsiveness to isolation in adulthood. Finally, the last segment (chapter 6) presents the role of biparental care on the development of mate choice and neuronal activation in response to potential mates.

Neural mechanisms of pair bonding

Although a large number of avian species are socially monogamous, the neural mechanisms underlying this monogamy are unknown. In contrast, there is a large body

of work elucidating the neural pathways involved in pair bonding in a socially monogamous rodent- the prairie vole (*Microtus ochrogaster*). Studies performed in this species, suggest that neuromodulators oxytocin, vasopressin and dopamine play a key role in the formation and maintenance of pair bonds in this species (Young and Wang, 2004; Young et al., 2005; Lim and Young, 2006). Although there is one study that suggests that mesotocin (avian homologue of oxytocin) and vasotocin (avian homologue of vasopressin) does not play a key role in pair bond formation in zebra finches (Goodson JL, 2004), the role of dopamine in this species remains largely unexplored. Dopamine is an interesting molecule as apart from playing a role in pair bond formation in voles, it has been widely studied for its role in the mediation of reward in response to drugs of abuse, food and sexual behaviors (Ikemoto and Panksepp, 1999; Wise, 2004; Carlezon and Thomas, 2009). Zebra finches (*Taeniopygia guttata*) are a highly social, monogamous and biparental species that form life-long pair bonds (Zann, 1996a). It is possible that the behaviors observed in zebra finches such as clumping and allopreening and spending time with partners in a nest are hedonistic behaviors and involve a component of reward. We therefore decided to explore the role of this molecule in pair bonding in this species. To do so, we first addressed if dopamine levels were enhanced in the mesolimbic dopaminergic pathway. This pathway consists of dopaminergic neurons in the midbrain ventral tegmental area (VTA) that project to limbic areas such as the hypothalamus, hippocampus and nucleus accumbens. Dopamine synthesized by the dopaminergic neurons in the ventral tegmental area is then transported into the various limbic areas. Studies suggest that the release of dopamine in the nucleus accumbens is responsible for the processing of rewarding stimuli resulting in the hedonia that results from drugs of abuse, sex and food. Therefore we hypothesized that paired birds would have higher levels of dopamine in the nucleus accumbens if pair formation included a component

of reward. As the exact stereotaxic co-ordinates of the nucleus accumbens are not known in the zebra finch, we measured levels of dopamine and its metabolite DOPAC in paired and unpaired birds, in the medial striatum, the area that contains the nucleus accumbens. To throw light on the neural activity of dopaminergic neurons that synthesize dopamine in ventral tegmental area, we used a double-labeling technique to label dopamine-synthesizing neurons that also expressed a marker for neuronal activity, the immediate early gene product Fos. The results of both sets of experiments suggested that the mesolimbic dopaminergic pathway was more active in paired versus unpaired birds. Having addressed if dopamine levels and activity of dopaminergic neurons was higher in birds and unpaired birds, we sought to address if interfering with dopaminergic neurotransmission would interfere with pairing in this species. We administered dopamine receptor antagonists both systemically and directly into the medial striatum and observed that although courtship behaviors specifically the frequency of directed singing to females by males, was lower in birds injected with antagonists versus control birds injected with saline, there was no difference in the number of birds that formed pair bonds in the control and experimental groups. Although dopamine neurotransmission might play a role in pair formation, the blockade of the receptors for dopamine for a short period of time during adulthood was not sufficient to prevent pair formation.

Corticosterone responses to challenges

Zebra finches are highly social species and live in large flocks in the wild. We hypothesized that separating a zebra finch from its flock and placing it in isolation would result in HPA axis activation as due to its highly social nature, isolation would be perceived as a challenge. We sought to examine the effects of social isolation in zebra finches by separating and isolating them for 10 or 30 minutes. In addition we also handled and released a group of birds so that were sure that the effects we

observed were due to the manipulation of isolation and not merely human handling. To compare the effects of isolation on corticosterone levels in zebra finches, we also restrained a group of birds for 10 and 30 minutes. Our results suggest that indeed isolation is a stressful event for this social species although the birds seem to adapt to this manipulation soon after 10 minutes and levels at 30 minutes are no longer different from hormone levels in birds that were undisturbed. Given this effect, we sought to address if there would be a buffering effect of the pair bonded partner or another familiar bird on the stress response of a finch that was isolated for 10 minutes. Given that these birds had life-long pair bonds, we hypothesized that the partner would alleviate the effects of isolation from the flock. We were proved wrong, as when a zebra finch was isolated for 10 minutes, the presence of another birds whether partner or familiar bird did not make a difference. Regardless of whether a bird was in isolation alone or with another bird, the corticosterone levels were higher than in birds that were undisturbed and housed with their flocks.

Female or maternal deprivation and its effects on the HPA axis

A large number of studies in mammals have shown that disruption of maternal care through periods of separation of offspring from their mothers, alters the stress responsiveness of the HPA axis in these offspring. The effects of these perturbations are long-term and last into adulthood. When exposed to stressors in adulthood, the corticosterone responses of maternally deprived offspring are higher than control offspring. In addition maternally deprived offspring performed poorly in tests of cognition and memory (Plotsky and Meaney, 1993; Francis and Meaney, 1999; Bremner and Vermetten, 2001; Lippmann et al., 2007). As these experiments were performed in species that are largely uniparental where mothers provide all the parental care, we sought to address the effect of maternal deprivation in zebra finches as they are a biparental species where both male and female parents contribute to

raising their young. It is possible that in such a species, the parent left behind, in our case the male parent, would compensate for the absence of the mother. In our experiment, mothers were removed from breeding aviaries when the offspring were 2-12 days old and the fathers raised their offspring to independence. We refer to these offspring as female-deprived as all adult females were removed from the breeding aviaries and as unrelated females may also socially interact with offspring we cannot be certain that the effects we observe are solely due to the absence of the offspring's mothers versus all adult females in their social environment. Once offspring reached adulthood we exposed them to isolation and restraint and measured their corticosterone responses. In comparison to the control group, the female-deprived birds had elevated levels of corticosterone on exposure to 30 minutes of isolation in comparison to female-deprived offspring that were not subjected to isolation. Control offspring exposed to 30 minutes of isolation had corticosterone levels similar to control birds that were not exposed to isolation. Additionally, mRNA levels of corticosteroid (glucocorticoid and mineralocorticoid) receptors were different between control and female-deprived offspring. This study suggests that maternal deprivation in a biparental species does have long-term consequences on stress-responsiveness potentially mediated through the expression of glucocorticoid receptors.

Female or maternal deprivation and its effects on mate choice and neuronal activation

An important component of pair bonding is choosing an appropriate mate. This is especially important in species in which partnerships last a lifetime and where the offspring depend on biparental care for survival. Zebra finches are one such species. It is not clear as how the decision to choose a specific individual as a partner over others is made. Another question that arises is how do birds choose a mate of the opposite sex versus one of the same sex? We hypothesized that mate choice is influenced by

early learning during development by observation of adult birds and partnerships in the social environment of young offspring. To disrupt partnerships in the early social environment of birds, we removed all adult females including mothers from breeding aviaries when offspring were 2-12 days old in a manipulation. We observed that when male female-deprived offspring reached adulthood, and were given a choice between male and female birds, they chose to pair with male birds. This observation was specific to males as female-deprived offspring that were female, chose to pair with other males. It is possible that female-deprived males sexually imprinted on the adult males during development and thereby chose to pair with males in adulthood. As a majority of female-deprived males chose to pair with other males, it was possible that when exposed to both male and female stimuli, the neuronal activation in areas involved in sensory processing such as pallial regions differed from control males that chose to pair with females. We performed an experiment to test this prediction and indeed we did observe differential expression of immediate early gene Fos (a marker of neural activity), in pallial regions between the two treatment groups.

In summary, a multipronged approach consisting of adult and developmental manipulations was used to study the neural mechanisms of pair formation, mate choice as well as HPA axis responsiveness to stressors in the zebra finch.

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CHAPTER 2

DOPAMINE LEVELS AND FOS EXPRESSION IN PAIRED AND UNPAIRED ZEBRA FINCHES

Abstract

Little is known about the neural pathways underlying monogamy in avian species. A large body of work has shown that the neuromodulator dopamine plays a critical role in pair bond formation in a socially monogamous rodent species, the prairie vole. The mesolimbic dopaminergic pathway that arises from the ventral tegmental area and projects to the nucleus accumbens is important for pairing in this species. Interference of dopaminergic neurotransmission in the nucleus accumbens using dopamine receptor agonists and antagonists prevents the formation and maintenance of pair bonds. We sought to explore the role of the mesolimbic dopaminergic pathway in pair bond formation in zebra finches, a socially monogamous avian species that forms life-long pair bonds. We observed that dopamine and DOPAC levels were higher in the medial striatum of paired birds than unpaired birds. Additionally, numbers of dopaminergic neurons that expressed the immediate early gene Fos were higher in the ventral tegmental area of paired birds versus unpaired birds. These data suggest that the mesolimbic dopaminergic pathway plays a role in pair bond formation in zebra finches, pointing to a possibility of conserved neural mechanisms of monogamy in birds and mammals.

Introduction

Zebra finches, like the majority of avian species, are socially monogamous and biparental. In the wild, they form long-term pair bonds that usually last for life (Zann, 1996a). Little is known about the neural mechanisms underlying the formation and maintenance of these long-term pair bonds. We hypothesized that the neurochemical

dopamine has a key role in this form of affiliation in zebra finches, based on the vast body of literature that implicates dopamine and the mesolimbic dopaminergic pathway in the processing of reward linked to stimuli such as food, sex and addictive drugs. The mesolimbic dopaminergic pathway comprises dopaminergic neurons that arise from the ventral tegmental area (VTA) situated in the midbrain and project to limbic regions such as the hypothalamus, bed nucleus of stria terminalis, septum and nucleus accumbens. Studies have shown that extracellular levels of dopamine are increased in the nucleus accumbens during the experience of reward (Blackburn et al., 1992; Schultz, 1998; Ikemoto and Panksepp, 1999; Adinoff, 2004; Alcaro et al., 2007). Recent studies suggest that although dopamine does not create the sensation of pleasure or hedonia, stimulation of the midbrain dopaminergic pathway results in individuals desiring or expecting a pleasurable stimulus (Adinoff, 2004). It is possible that individuals in long-term pair bonds experience activation of the mesolimbic dopaminergic pathway while associating with their mates and this activation contributes to the maintenance of pair bonds in monogamous species.

Most of our knowledge regarding the neurochemicals involved in pair bond formation comes from studies performed in a monogamous rodent species – the prairie vole, *Microtus ochrogaster* (Young et al., 2001; Wang and Aragona, 2004; Curtis et al., 2006). Studies have shown that neurohormones dopamine, oxytocin and arginine vasopressin play a key role in partner preference formation and maintenance in this species. With respect to dopamine, studies have shown that dopamine receptors in the nucleus accumbens facilitate or inhibit the formation and maintenance of pair bonds. The activation of the dopamine D1-like receptor prevented partner preference formation whereas activation of the D2-like receptor facilitated this process in male and female prairie voles (Aragona et al., 2003; Liu and Wang, 2003; Aragona et al., 2006). The ventral tegmental area has also been implicated in the formation of pair

bonds in the voles. Injection of either a GABA or a glutamate receptor antagonist into the ventral tegmental induced partner preference in male prairie voles in the absence of mating (which is usually critical for the formation of a partner preference) in the short time interval of 6 hours. The authors proposed that pair formation involved a reduction of excitatory input to the ventral tegmental that in turn reduces the activity of GABAergic cells within this region, ultimately leading to increased dopamine release in the nucleus accumbens (Curtis and Wang, 2005).

In our study we explored the role of dopamine in pair formation in male and female zebra finches by measuring the levels of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the medial striatum, hyperpallium apicale, preoptic area and ventral tegmental area. When dopamine is released into a synapse it either binds to its receptors on pre and post-synaptic neurons or it is taken up into presynaptic neurons by dopamine transporters (DATs). It is then oxidized by mitochondrial monoamine oxidase to form DOPAC (Giros and Caron, 1993). Additionally, we counted numbers of dopaminergic neurons (using tyrosine hydroxylase as a marker for dopaminergic neurons) that express the immediate early gene protein Fos (the expression of which indicates neuronal activity) in the ventral tegmental area, substantia nigra, rostral and caudal A11 and preoptic area. Our hypothesis was that if dopamine (specifically in the mesolimbic pathway) is involved in pairing in this species, paired birds of both sexes would have higher levels of dopamine in the nucleus accumbens (situated in the medial striatum) in addition to greater numbers of Fos expressing dopaminergic neurons in the ventral tegmental area, in comparison to unpaired birds.

There is no clear consensus on the exact location of the nucleus accumbens in zebra finches and therefore we measured dopamine and DOPAC in the ventro-medial striatum. The striatal region receives dopaminergic input from the ventral tegmental

area and the substantia nigra and displays fine-grained dopaminergic processes (Bottjer, 1993; Durstewitz et al., 1999). In addition, we measured dopamine in the hyperpallium apicale that is situated in the same coronal sections as the ventro-medial striatum and therefore serves as a control region. We also measured dopamine levels and Fos expression in the preoptic area. The preoptic area is a key area involved in male sexual behavior as described in vertebrate species such as rats and Japanese quail. Studies in rats have shown that dopamine levels are increased in this region in conjunction with the expression of sexual behaviors. Administration of dopamine agonists into the medial preoptic area of rats facilitated male sexual behavior whereas dopamine receptor antagonists suppressed copulation in addition to sexual motivation (Dominguez and Hull, 2005; Hull and Dominguez, 2007). Although it is not clear if copulation is necessary for pair formation in zebra finches, it is possible that male zebra finches that engaged in sexual behaviors during pair formation would have increased dopaminergic activity as reflected by dopamine levels and Fos expression in this region. Fos expression was also measured in the hypothalamic and central grey A11 midbrain nuclei. Dopaminergic neurons in the A11 region project to song nuclei and showed increased expression of Fos in male zebra finches exposed to sociosexual situations (Bharati and Goodson, 2006; Goodson et al., 2009b). Finally, we measured dopamine levels and Fos expression in the ventral tegmental area and in the adjoining substantia nigra as a control region. Dopamine is synthesized in both these areas but the ventral tegmental area is primarily associated with limbic function whereas dopamine in the substantia nigra is associated with non-specific motor function and therefore is an appropriate control region.

Experimental procedures

Animals and housing

All male and female zebra finches used in this experiment were sexually naïve and housed in single-sex aviaries (0.94 X 0.76 X 0.94 m) prior to the onset of the experiment. Seed and water were provided *ad libitum*. All housing, testing and sacrifice procedures were in accordance with Federal and State regulations and were approved by the Cornell University IACUC.

Aviary design for courtship and pairing behaviors, behavioral observations and sacrifice

Courtship behaviors:

There were four groups: two control groups (one of each sex) and two experimental groups (one of each sex). The ‘control male group’ consisted of 4 sexually naïve male birds with 4 sexually male experienced birds (subjects) whereas the ‘experimental male group’ consisted of 4 sexually naïve male birds (subjects) with 4 sexually experienced female birds. The ‘control female group’, consisted of 4 sexually naïve females (subjects) with 4 sexually experienced females and the ‘experimental female group’ consisted of 4 sexually naïve females (subjects) with 4 sexually experienced males. Individuals in each group were allowed to interact for 30 minutes after which the subjects from the control groups and the subjects from the experimental groups that engaged in courtship behaviors were sacrificed. This experiment was repeated so that there were 6-8 subjects in each group.

Pairing behaviors:

There were four groups: two control groups (one of each sex) and two experimental groups (one of each sex). The ‘control male group’ consisted of 4 sexually naïve male birds (subjects) with 4 sexually male experienced birds whereas the ‘experimental male group’ consisted of 4 sexually naïve male birds (subjects) with 4 sexually experienced female birds. The ‘control female group’, consisted of 4 sexually naïve females with 4 sexually experienced females (subjects) and the ‘experimental female group’ consisted of 4 sexually naïve females (subjects) with 4 sexually experienced males. Individuals in each group were allowed to interact for 2-4 days after which the subjects from the control groups and the subjects from the experimental groups that formed pairs were sacrificed. This design, with experienced stimulus birds, was to ensure that maximum number of naïve male or female birds were paired in the experimental group as experienced birds do tend to pair over a shorter duration of time. . This experiment was repeated to so that there were 6-8 subjects in each group.

Behavioral observations

Behaviors in control and experimental birds were observed for 15 minutes during 30 minutes of interactions or during days 2-4 (after the onset of the experiment) prior to sacrifice.

The following behaviours were recorded during each 15 minute observation period by an observer using custom-designed software (Goldstein & Brodsky 2006).

Directed song bouts (recorded as number of bouts of song in 15 minutes): a salient male courtship behaviour in which the male sings to a target bird while perched close to the target. Males produce several repetitions of the song before stopping, marking the end of the song bout.

Undirected song bouts (recorded as number of bouts of song in 15 minutes): singing that is not obviously directed at another bird.

Aggression (recorded as number of bouts in 15 minutes): consisted of one bird attacking another.

In nest box (recorded as duration in seconds): birds in the process of pairing spend substantial time in the chosen nest box together. Time spent in a nest box alone, with male or with female was recorded separately.

Pair status: at the end of 2-4 days, birds that frequently spent time in a nest box together were categorized as paired.

Tissue collection for HPLC analysis

Subjects from the 30 minute interaction experiment and the 2-4 interaction experiment were decapitated, brains rapidly dissected, frozen on dry ice, and stored at -80°C until the time of sectioning. Using a cryostat (Microm HM 500 OM), 200- μm coronal sections were thaw mounted onto Superfrost Plus slides (Erie Scientific, USA). Sections were then rapidly frozen using a cooling block set at -20°C (Physitemp Instruments Inc., USA), and the brain regions of interest (ventral tegmental area, preoptic area, hyperpallium and ventro-medial striatum) were dissected using a 300- μm diameter micropunch. Tissue samples from each animal were assayed independently of each other and not pooled. The punched tissue was stored in ice-cold 70 μl homogenization solution [a mixture of 60 μl homogenization buffer: 0.1 M Perchloric acid (Sigma-Aldrich) containing 347 μM sodium bisulphate (Sigma-Aldrich) and 134 μM EDTA disodium salt (Fluka, USA), and 10 μl of 100 nM Epinine-internal standard; Sigma-Aldrich]. Tissue samples in homogenization solution were frozen at -80°C overnight and thawed after 24 h. The thawed samples were centrifuged at 14,000 rpm at 4°C for 20 min, after which the supernatant was collected

and used for HPLC analysis. Protein content in the resulting pellet was determined by resuspending and agitating the pellet in 45 μ l ice-cold 0.3N NaOH for 24 h at 4°C and carrying out a modified Bradford assay thereafter (Pierce Biotechnology Inc., USA).

HPLC analysis

Levels of dopamine and its metabolite DOPAC were determined by HPLC-EC using modifications of Bai et al. (1999) by Dr. Heng-Hsiang Lo in the CRED Analytical Instrumentation Facility Core (UT-Austin). Briefly, 50 μ l of sample was injected into an HPLC system that comprised a Shimadzu SCL-10A system controller, LC-10AD pump, an SIL-10A auto-sampler (Shimadzu, Columbia, MD), and coupled with a four-channel CoulArray electrochemical detector (ESA, Chelmsford, MA). The isocratic mobile phase contained 4 mM citric acid, 8 mM ammonium acetate, 120 μ M 1-octanesulfonic acid sodium salt, 60 μ M EDTA disodium in water and 5% MeOH, pH 3.5. The flow rate of the mobile phase remained at 1 ml/min. Separation was achieved by a 4.6 mm \times 80 mm reverse-phase HR-80, 3- μ m particle size column (ESA, Chelmsford, MA). The potential of channels 1 through 4 of CoulArray was set at - 50, 0, 300, and 400 mV, respectively. Peak area (nC) DA and DOPAC at the corresponding retention time on the chromatogram resulted from 300 mV, and was used to quantify the amount based on the standard curve of each neurotransmitter. Recovery of internal standard was consistently high across all experimental runs (95%–100%). Levels of dopamine and DOPAC were expressed as pg/ μ g of protein in the microdissected tissue extract.

Tissue collection for immunohistochemistry

Subjects from the 2-4 day interaction group were deeply anesthetized with Nembutal. Following that they were perfused transcardially with 0.9% saline followed

by 4% paraformaldehyde solution. The brains were removed and post-fixed overnight in 4% paraformaldehyde. They were then placed in a 30% sucrose solution for 2-3 days following which they were embedded in gelatin and stored in a 30% sucrose and 10% formaldehyde solution till they were sectioned. Brains were sectioned at 40um thickness using a freezing microtome and stored in 4°C until immunohistochemistry.

Tyrosine hydroxylase and Fos immunohistochemistry

Double labeling for Tyrosine hydroxylase and Fos was performed in the following manner; adapted from (Bharati and Goodson, 2006). Tissue was subjected to two 10-minute rinses in PBS, followed by 1 hour in blocking serum (PBS, 5.0% Normal goat serum, 0.3% Triton-X). Tissue was then incubated for 40–48 h at 4 °C in mouse anti-TH (Immunostar Incorporated, Hudson, WI, USA) and rabbit anti-Fos (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the former diluted 1:10000 and the latter 1:1000 in PBS 2.5% Normal goat serum, 0.3% Triton-X. This was followed by two 30-min rinses in PBS, 2 h in donkey anti-rabbit secondary conjugated to Alexa Fluor 594, donkey anti-mouse conjugated to Alexa Fluor 488 in PBS 2.5% Normal goat serum and 0.3% Triton-X. Alexa Fluors were purchased from Invitrogen, Eugene, OR, USA. Sections were then rinsed extensively in PBS and transferred to PB before mounting. Tissue was mounted on superfrost slides (VWR, USA) and coverslipped with SlowFade Light containing DAPI nuclear stain (Molecular Probes).

Confocal imaging and cell counting

All confocal imaging was performed at the Microscopy and Imaging Facility, a part of the Lifesciences Core Facility Center at Cornell University, using a Leica SP2 scanning laser confocal microscope. Dopaminergic neurons were visualized using a 20X oil immersion lens and were manually counted on a computer attached to the

microscope. A Z-stack was created for each dopaminergic area on a section and the dopaminergic neurons were counted in each Z-stack image. Fos-positive neurons were counted manually in each dopaminergic area of a section while scanning along the Z-axis.

Statistics

Behavioral data were analyzed using Kruskal-Wallis tests followed by Dunn's multiple comparison tests. HPLC data as well as Fos data were analyzed using mixed linear model analysis after correcting for repeated sampling of different brain regions from the same individual. Percentages of TH-positive neurons expressing Fos were analyzed. Data were natural log transformed to satisfy assumptions for equality of variances and normality. Bonferroni corrections were performed to correct for multiple comparisons. Significance level was set at 0.05 for all tests.

Results

Courtship behaviors in male and female zebra finches

During 30 minutes of interactions, male subjects in the 'experimental male group' performed more directed song bouts and beak wipes to females than subjects in the 'control male group' (Table 2.1, $p < 0.05$). Subjects in the 'experimental female group' received greater number of beak wipes and bouts of directed song than females in the 'control female group' (Table 2.1, $p < 0.05$).

Pairing behaviors in male and female zebra finches

After 2-4 days of interaction, subjects in the 'experimental male group' spent more time in nests with females, spent more time in nest boxes alone and displayed a higher

frequency of aggressive bouts in comparison to subjects in the ‘control male group’ (Table 2.2, $p < 0.05$). After 2-4 days of interaction, subjects in the ‘experimental female group’ spent more time in their nests with their male partners in comparison to subjects in the ‘control male group’ (Table 2.2, $p < 0.05$). All male and female birds that were used for the experiment formed pairs between 2-4 days of being placed in the experimental aviary. Same-sex pairs were not observed in the control aviaries.

Dopamine (DA), Dihydroxyphenyl acetic acid (DOPAC) and DOPAC/DA ratio in control and experiment male and female zebra finches in response to courtship and pair bond formation

After 30 minutes of interaction dopamine, DOPAC, and DOPAC/DA levels were not significantly different between the ‘control male group’ and ‘experimental male group’ in any brain region (Table 2.3, $F = 38.21$, $p > 0.05$). The same was true for female birds (Table 2.3, $F = 53.56$, $p < 0.05$).

After 2 -4 days of interaction (Figure 2.1), dopamine levels were significantly higher in male subjects of the ‘experimental male group’ in comparison to the ‘control male group’ in the medial striatum ($p < 0.001$) but not in the preoptic area ($p = 0.318$), hyperpallium apicale ($p = 0.773$) or ventral tegmental area ($p = 0.865$). Overall, there was a significant interaction between treatment and region ($F_{3,27} = 70.859$, $p = 0.029$), a significant main effect of treatment ($F_{1,9} = 6.58$, $p < 0.03$) and region ($F_{3,27} = 70.85$, $p < 0.001$). DOPAC levels in the medial striatum were significantly higher in male subjects of the ‘experimental male group’ in comparison to the ‘control male group’ ($p < 0.016$), and there were no significant differences in the hyperpallium apicale ($p = 0.924$), preoptic area ($p = 0.316$) and ventral tegmental area ($p = 0.932$). There were no significant differences in DOPAC/Dopamine ratios in any region ($p > 0.05$).

After 2-4 days of interactions between female zebra finches (Figure 2.2), dopamine levels were significantly higher in the medial striatum ($p < 0.001$) of female subjects of the ‘experimental female group’ in comparison to the ‘control female group’. There were no significant group differences in the hyperpallium apicale ($p = 0.995$), preoptic area ($p = 0.842$) or ventral tegmental area ($p = 0.892$). Overall there was a significant interaction of treatment and region ($F_{3,24} = 10.117$, $p < 0.001$), a significant main effect of treatment ($F_{1,24} = 8.69$, $p = 0.007$) and region ($F_{3,24} = 48.51$, $p < 0.001$). DOPAC levels were also significantly higher in the medial striatum of female subjects of the ‘experimental female group’ in comparison to the ‘control female group’ ($p < 0.001$). There were no significant differences in DOPAC levels in the hyperpallium apicale ($p = 0.913$), preoptic area ($p = 0.77$) or ventral tegmental area ($p = 0.891$). There were no significant differences in DOPAC/DA ratios in any region ($p > 0.05$).

Tyrosine hydroxylase (TH) +Fos expression in male zebra finches after pair formation

Overall, there was no significant interaction between treatment and region ($F_{4,38.96} = 0.832$, $p = 0.513$). There was a significant main effect of treatment ($F_{1,12.34} = 14.30$, $p = 0.002$) and region ($F_{4,38.96} = 35.21$, $p < 0.001$). Percentages of TH positive neurons expressing Fos (Figure 2.3) were higher in the medial striatum ($p = 0.002$), the caudal A11 nucleus ($p = 0.031$) and the rostral A11 nucleus ($p = 0.048$) of subjects in the ‘experimental male group’ in comparison to subjects of the ‘control male group’. Percentages did not differ between subjects of both groups in either the preoptic area ($p = 0.343$) or substantia nigra ($p = 0.265$). Cell numbers are shown in Table 2.4.

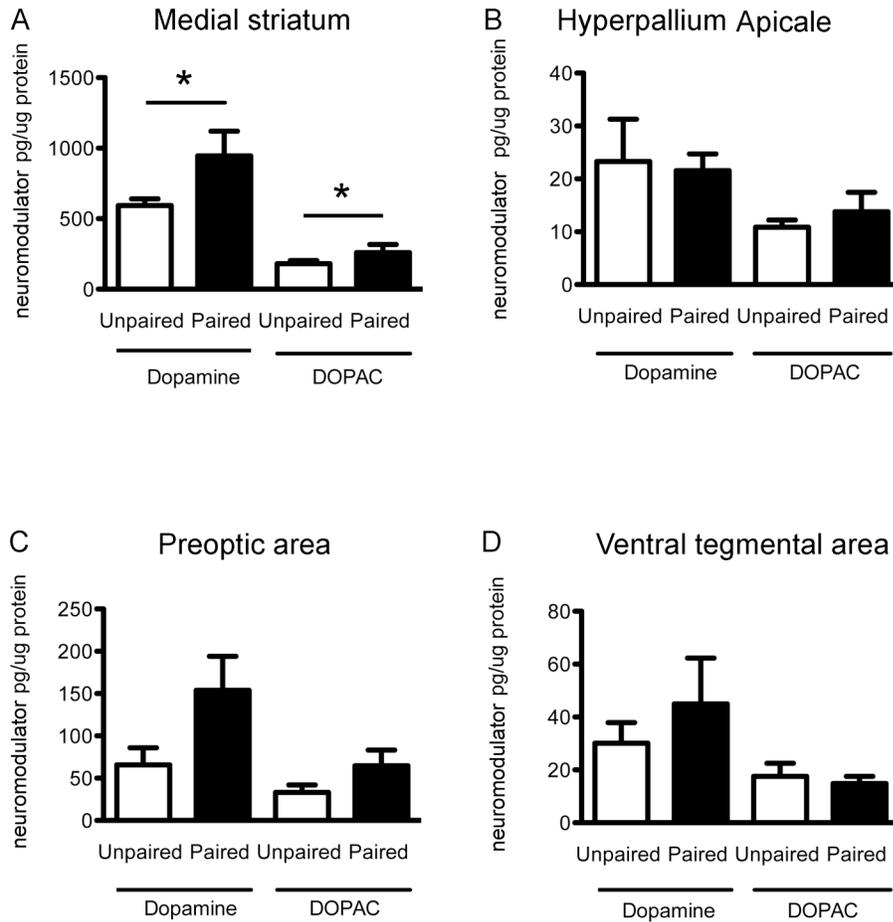


Figure 2.1. Levels of intracellular dopamine and DOPAC (mean \pm SEM) measured by HPLC in the (A) medial striatum (B) hyperpallium apicale (C) preoptic area (D) ventral tegmental area of unpaired and paired male zebra finches after 2-4 days of interaction. N=6-8/group, * p <0.05.

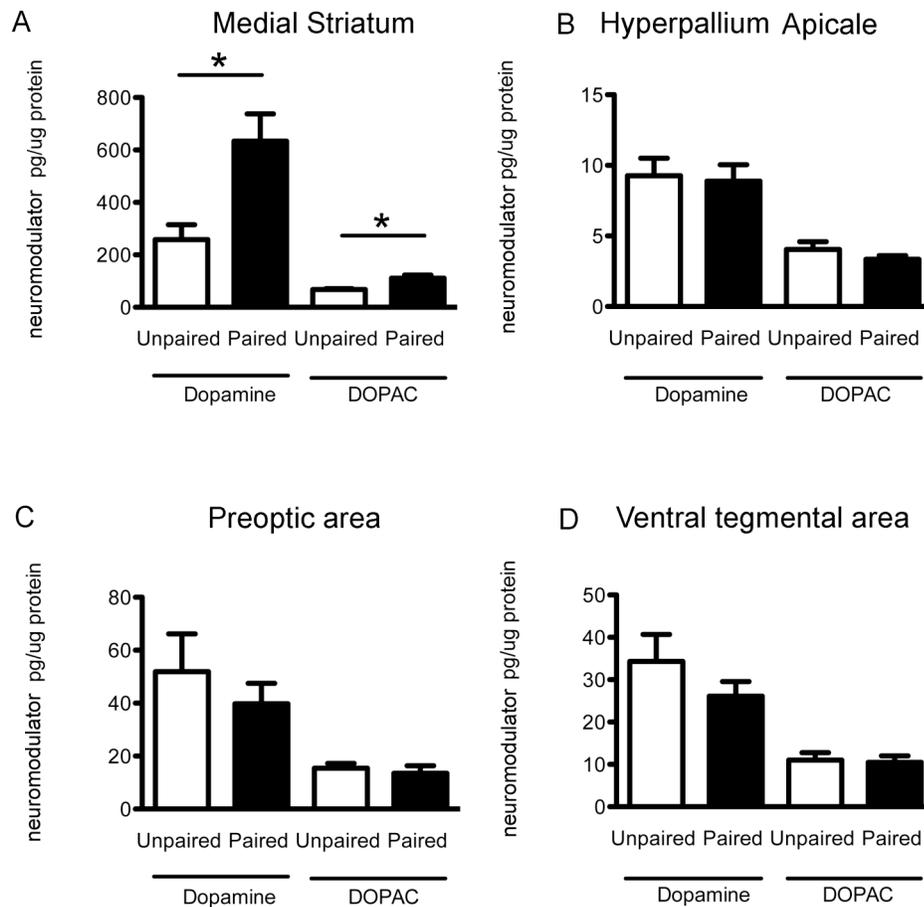


Figure 2.2. Levels of intracellular dopamine and DOPAC (mean \pm SEM) measured by HPLC in the (A) medial striatum (B) hyperpallium apicale (C) preoptic area (D) ventral tegmental area of unpaired and paired female zebra finches after 2-4 days of interaction. N=4-8/group, *p<0.05.

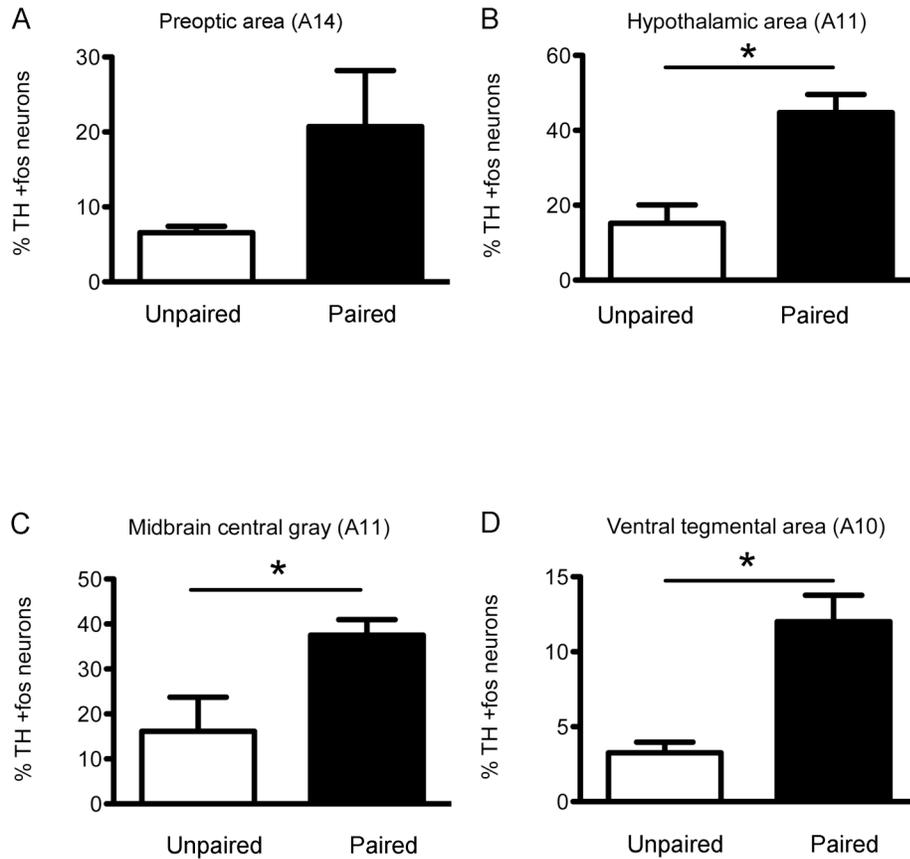


Figure 2.3. Percentage of tyrosine hydroxylase immunoreactive neurons that express Fos protein (mean \pm SEM), in unpaired and paired male zebra finches in (A) A14 population in the preoptic area of the hypothalamus, (B) A11 population in the rostral hypothalamus, (C) A11 population in the central gray and (D) A10 population in the ventral tegmental area. N=6-8/group, * $p < 0.05$.

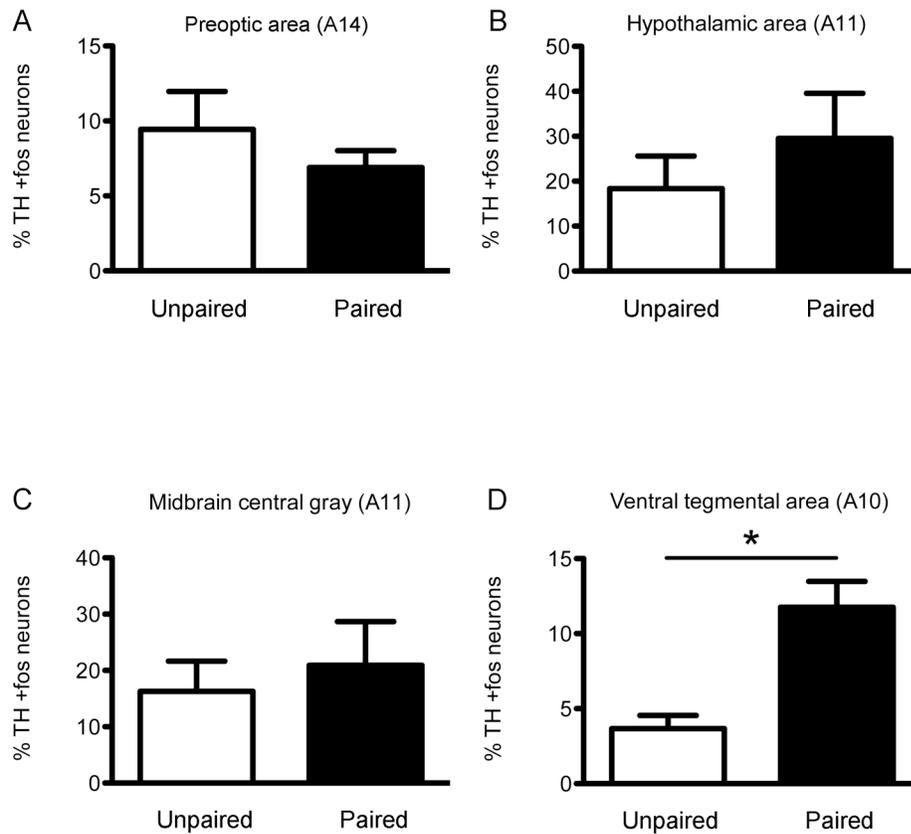


Figure 2.4. Percentage of tyrosine hydroxylase immunoreactive neurons that express Fos protein (mean \pm SEM), in unpaired and paired female zebra finches in (A) A14 population in the preoptic area of the hypothalamus, (B) A11 population in the rostral hypothalamus, (C) A11 population in the central gray and (D) A10 population in the ventral tegmental area. N=6-8/group, * p <0.05.

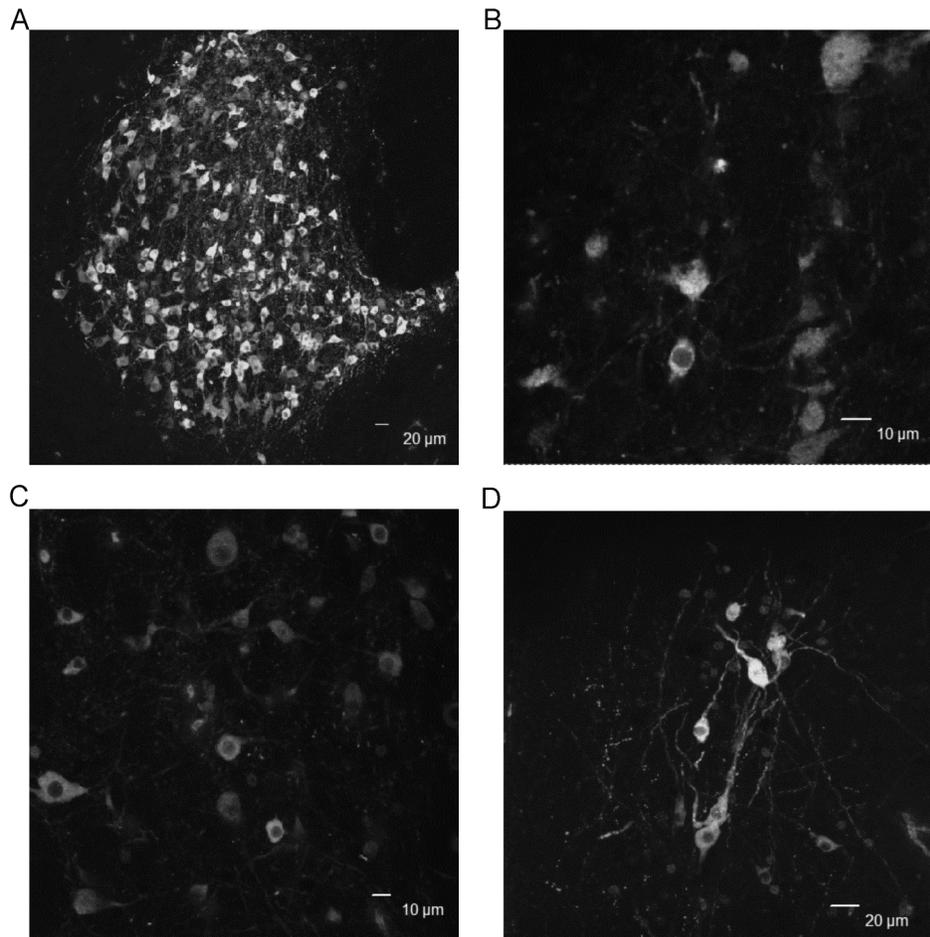


Figure 2.5. Immunocytochemical labeling of Fos-IR nuclei (red) and Tyrosine hydroxylase-IR neurons (green) in (A,B) the ventral tegmental area of an unpaired bird, (C) ventral tegmental area of a paired bird and (D) rostral A11 region of a paired bird.

Table 2.1. Behaviors observed in male zebra finches (mean \pm SEM) during 30' of interaction with males or females. Data were analyzed using Kruskal-Wallis tests followed by Dunn's multiple comparison tests.

| Behavior | Duration (seconds) or frequency | | P<0.05 |
|-----------------|---------------------------------|---------------------|--------|
| | With males | With females | |
| Directed song | 2.571 \pm 1.66 | 15.57 \pm 1.962 | Yes |
| Beak wipe | 0 \pm 0 | 5 \pm 1.414 | Yes |
| Undirected song | 0.1429 \pm 0.1429 | 1.143 \pm 0.4041 | No |
| Aggression | 0.4286 \pm 0.2974 | 0.2857 \pm 0.1844 | No |

Behaviors observed towards or by female zebra finches (mean \pm SEM) during 30' of interaction with females or males.

| Behavior | Duration (seconds) or frequency | | P<0.05 |
|-------------------------|---------------------------------|--------------------|--------|
| | With females | With males | |
| Directed song (by male) | 0 \pm 0 | 31.43 \pm 6.904 | Yes |
| Beak wipe (by male) | 0 \pm 0 | 3.286 \pm 0.9689 | Yes |
| Aggression (by female) | 0 \pm 0 | 3.571 \pm 1.556 | No |

Table 2.2. Behaviors observed in male and female zebra finches (mean \pm SEM) during 3-4 days of interaction with males or females. Data were analyzed using Kruskal-Wallis tests followed by Dunn's multiple comparison tests.

| Behavior | Duration (seconds) or frequency | | <i>P</i> |
|--------------------------------|---------------------------------|-------------------|----------|
| | Unpaired males | Paired males | |
| Directed song | 0.0 \pm 0.0 | 0.5 \pm 0.3416 | No |
| Undirected song | 13 \pm 4.86 | 3.667 \pm 2.076 | No |
| Aggression | 0.0 \pm 0.0 | 5.667 \pm 1.944 | Yes |
| Time spent in nest with female | 0.0 \pm 0.0 | 244.2 \pm 62.53 | Yes |
| Time spent in nest alone | 8.571 \pm 8.571 | 120.7 \pm 87.62 | Yes |

| Behavior | Duration (seconds) or frequency | | <i>P</i> <0.05 |
|------------------------------|---------------------------------|-------------------|----------------|
| | Unpaired females | Paired females | |
| Aggression | 0.8571 \pm 0.4041 | 1.000 \pm 0.574 | No |
| Time spent in nest with male | 0.0 \pm 0.0 | 191.7 \pm 117 | Yes |
| Time spent in nest alone | 0.0 \pm 0.0 | 88.77 \pm 75.70 | No |

Table 2.3. Levels of Dopamine and DOPAC (mean \pm SEM) in male zebra finches after 30' minutes of interactions with male or female zebra finches.

| Brain area | With male | With female |
|--------------------------|--------------------------------------|--------------------------------------|
| Medial striatum | 413.9 \pm 57.41, 113.6 \pm 11.97 | 325.8 \pm 34.27, 100.9 \pm 15.15 |
| Hyperpallium apicale | 19.75 \pm 6.451, 7.525 \pm 1.651 | 27.17 \pm 10.17, 12.11 \pm 1.965 |
| Hypothamic preoptic area | 83.73 \pm 11.63, 43.58 \pm 9.015 | 48.27 \pm 19.68, 28 \pm 8.316 |
| Ventral tegmental area | 13.7 \pm 4.158, 8.561 \pm 1.202 | 14.81 \pm 3.725, 11.15 \pm 1.666 |

Levels of Dopamine and DOPAC (mean \pm SEM) in female zebra finches after 30' minutes of interactions with female or male zebra finches.

| Brain area | With female | With male |
|--------------------------|---------------------------------------|---------------------------------------|
| Medial striatum | 821.1 \pm 120.6, 128.6 \pm 12.56 | 826.7 \pm 59.37, 165 \pm 29.38 |
| Hyperpallium apicale | 20.47 \pm 3.316, 6.204 \pm 0.3439 | 15.84 \pm 2.931, 5.877 \pm 0.8593 |
| Hypothamic preoptic area | 83.28 \pm 26.38, 26.03 \pm 7.474 | 78.33 \pm 25.29, 30.26 \pm 8.022 |
| Ventral tegmental area | 42.88 \pm 8.06, 16.40 \pm 2.204 | 27.62 \pm 5.327, 11.29 \pm 1.478 |

Table 2.4. Numbers of TH-IR and TH-IR + Fos-IR positive cells (mean \pm SEM) in unpaired and paired zebra finches.

| Brain area | Unpaired males | Paired males |
|----------------------------|---------------------------------------|---------------------------------------|
| Hypothalamic preoptic area | 90.33 \pm 15.74, 9.66 \pm 4.14 | 139 \pm 11.74, 36.5 \pm 16.40 |
| Hypothalamic A11 region | 47.33 \pm 19.80, 7.16 \pm 2.34 | 31.33 \pm 6.93, 13.83 \pm 3.124 |
| Central gray A11 region | 156 \pm 68.50, 14.75 \pm 4.66 | 94.5 \pm 29.40, 35.5 \pm 11.80 |
| Ventral tegmental area | 636.28 \pm 117.67, 28.85 \pm 6.97 | 616.57 \pm 78.60, 74.85 \pm 16.44 |
| Substantia nigra | 417.57 \pm 97.30, 1.85 \pm 0.55 | 450.42 \pm 76.56, 5.42 \pm 1.52 |
| Brain area | Unpaired females | Paired females |
| Hypothalamic preoptic area | 30.25 \pm 9.66, 2 \pm 0.707 | 50.25 \pm 15.07, 2.75 \pm 1.37 |
| Hypothalamic A11 region | 41.2 \pm 10.39, 5.6 \pm 2.18 | 40.8 \pm 10.61, 12 \pm 3.20 |
| Central gray A11 region | 52.5 \pm 12.15, 10.83 \pm 5.35 | 68.6 \pm 27.40, 23.8 \pm 17.86 |
| Ventral tegmental area | 490.8 \pm 85.51, 13.8 \pm 3.89 | 439.6 \pm 63.66, 53.33 \pm 19.34 |
| Substantia nigra | 361.5 \pm 84.32, 1.83 \pm 0.74 | 336.8 \pm 57.61, 2 \pm 0.54 |

Tyrosine hydroxylase (TH) +Fos expression in female zebra finches after pair formation

Overall, there was no significant interaction of treatment and region ($F_{4,34.86} = 1.67$, $p=0.179$). There was a significant main effect of treatment ($F_{1,11.97} = 10.08$, $p=0.008$) and region ($F_{4,34.86} = 38.85$, $p<0.001$). Percentages of TH positive neurons expressing Fos (Figure 2.4) were higher in subjects belonging to the ‘experimental female group’ in comparison to the ‘control female group’ in the medial striatum ($p=0.005$) but not in the preoptic area ($p=0.609$), the rostral A11 ($p=0.18$), caudal A11 ($p=0.15$) or substantia nigra ($p=0.231$). Cell numbers are shown in Table 2.4.

Discussion

Summary of findings

The present experiments have shown that paired male and female zebra finches had higher levels of dopamine and DOPAC in the medial striatum as compared to unpaired birds after 2-4 days of interaction. We did not observe any differences in dopamine or DOPAC in the preoptic area, hyperpallium apicale or the substantia nigra. We also did not observe in differences in dopamine or DOPAC levels between control and experiment groups, in either males or females, in any brain region, when they interacted for a period of 30 minutes. The percentage of dopaminergic neurons in the ventral tegmental area (VTA) that expressed Fos, were higher in paired versus unpaired birds of both sexes. Additionally, the percentage of dopaminergic neurons expressing Fos in the rostral and caudal A11 nucleus was higher in paired male birds in comparison to unpaired male birds. There were no differences in percentages of dopaminergic neurons expressing Fos in the preoptic area or the substantia nigra of paired and unpaired birds.

The mesolimbic dopamine pathway

Our hypothesis was that the mesolimbic dopaminergic pathway was involved in pair formation in male and female zebra finches. A vast body of literature has described the role of the mesolimbic dopaminergic system in the mediation of euphoria or hedonia in response to rewarding stimuli such sex, food, drugs of abuse and more recently pair bonding. Most of these studies have been performed in mammals, namely rodents and non-human primates. The mesolimbic dopaminergic pathway arises from the ventral tegmental area in the midbrain and leads into the nucleus accumbens situated in the mammalian forebrain as well as other limbic structures such as the hypothalamus and hippocampus. Dopamine is synthesized in the VTA and directly transported into the nucleus accumbens (NA) via the VTA-NA pathway. Concomitant with the intake of drugs of abuse such as cocaine or food such as sugar solution, dopamine is released into the nucleus accumbens. Here dopamine binds to the D1-like and D2-like receptors that triggers off downstream signaling cascades responsible for reward processing. The intake of rewards such as sex, food and drugs of abuse leads to hedonia. This in turn leads to the initiation of learning processes that enable the consolidation and coupling of the hedonia associated with certain cues, actions that allow consumption of the reward as well as assigning a value and motivational status to the reward to enable the organism to decide what amount of resources should be expended to achieve the reward. Incentive motivation is the priming of a rewarding encounter with a neutral stimulus that is associated with reward. Dopamine is important for the development of incentive-motivational value for previously neutral stimuli and blocking the dopamine system leads to a decoupling of reward-predicting cues and behaviors associated with consumption of reward. The dopamine hypothesis of reward states that dopamine is important both for reinforcement that arises from earning a reward and incentive motivation that precedes

the earning of a reward (Kalivas and Nakamura, 1999; Wise, 2004; Arias-Carrion and Poppel, 2007; Carlezon and Thomas, 2009).

It is likely that the mesolimbic dopaminergic pathway is conserved between mammals and birds. Studies in chickens and pigeons have shown that dopaminergic neurons from the midbrain ventral tegmental area and substantia nigra project into the striatum. The nucleus accumbens has been identified in the striatum of chickens and has been shown to have core and shell divisions as in mammals. Additionally, immunohistochemistry using an antibody against tyrosine hydroxylase (an enzyme essential for dopamine synthesis) has shown the presence of dopaminergic nuclei in zebra finches regions such as the ventral tegmental area. Although the exact stereotaxic co-ordinates and structure of the nucleus accumbens in the zebra finch have not been elucidated till date, it is generally thought to be situated in the ventro-medial striatum (Bottjer, 1993; Wynne and Gunturkun, 1995; Durstewitz et al., 1999; Jarvis et al., 2005; Balint and Csillag, 2007).

Role of the nucleus accumbens

The nucleus accumbens receives dopaminergic input from the ventral tegmental area as well as excitatory glutamatergic input from areas such the prefrontal cortex, amygdala and hippocampus. These inputs are integrated here linking motivation with action (Ikemoto and Panksepp, 1999; Wise, 2002). In socially monogamous species such as prairie voles (*Microtus ochrogaster*) dopamine plays a role in pair bond formation and maintenance. Mating with a female resulted in a 33% increase in dopamine turnover in male prairie voles that was followed by the development of a partner preference for the female (Aragona et al., 2003). In addition blocking dopamine receptors specifically in the nucleus accumbens prevented the development of a preference for a specific individual when control animals formed partner preferences. Additionally, stimulation of dopamine receptors by the administration of

agonists into the nucleus accumbens facilitated partner preference formation even in the absence of mating. These studies indicate dopaminergic neurotransmission in the nucleus accumbens of voles is critical for partner preference formation in this species. In our studies, we observed that dopamine and DOPAC levels were higher in the medial striatum of paired versus unpaired birds of both sexes. This suggests that the behaviors that are typical to paired birds such as spending time with their partners in a nest involve a component of reward and mesolimbic dopaminergic pathway activation.

In addition to playing a role in pair bonding, nucleus accumbens dopamine is also involved in sexual behaviors (Pfaus, 2009). In rats, the release of dopamine at the level of the nucleus positively regulated the anticipatory/motivational phase of copulatory behavior (Giuliano and Allard, 2001). As paired birds do engage in copulation, it is possible that this behavior contributes to enhanced levels of dopamine in the nucleus accumbens of paired birds.

Role of the ventral tegmental area

As we observed higher levels of dopamine and DOPAC in the nucleus accumbens we proceeded to find out if dopamine synthesizing neurons in the ventral tegmental area were more active in paired birds in comparison to unpaired birds. As predicted, we observed a higher percentage of dopaminergic neurons that expressed Fos in the ventral tegmental area of paired male and female birds in comparison to unpaired birds. This observation further strengthens our hypothesis that the mesolimbic dopaminergic pathway is more active in paired birds versus unpaired birds thereby playing an important role in the pairing process. It is known that these birds form long-term pair bonds and the activation of the reward pathway is one mechanism by which these bonds could be formed and maintained. There is a large amount of evidence to suggest that the ventral tegmental area and mesolimbic dopaminergic areas play an important role in socio-sexual behaviors. Romantic love in humans, pair

bonding in voles and sexual behavior in rodents and zebra finches, have been associated with enhanced activation of the ventral tegmental area (Curtis and Wang, 2005; Fisher et al., 2005; Fisher et al., 2006; Goodson et al., 2009b). Studies in zebra finches have demonstrated that neuronal activity indicated by Fos expression in the caudal ventral tegmental area is associated with courtship singing, sexual behavior and aggression (Bharati and Goodson, 2006; Goodson et al., 2009b). Additionally, sexual behavior in rats activates dopaminergic neurons in the ventral tegmental (Balfour et al., 2004). In prairie voles, enhancing dopamine synthesis and neurotransmission of dopaminergic neurons using GABA and glutamatergic agonists and antagonists, resulted in a facilitation of partner preference formation (Curtis and Wang, 2005). Our results are therefore consistent with the hypothesis that ventral tegmental area plays an important role in socio-sexual behavior across species.

Rostral and caudal A11 dopaminergic nuclei

We also measured Fos expression in dopaminergic neurons in the rostral A11 neurons and caudal A11 neurons of the central gray. We observed that that numbers of Fos expressing dopaminergic neurons were greater in paired versus unpaired male birds. Studies suggest that these neurons play role in courtship singing in zebra finches (Bharati and Goodson, 2006; Bharati IS, 2006; Goodson et al., 2009b). A study in quail also demonstrated increased activation of dopaminergic A11 neurons in response to sexual activity (Charlier et al., 2005). Therefore it is likely that sexual behavior and courtship behaviors in paired male zebra finches contributed to the enhanced Fos expression in the A11 region.

The preoptic area

In mammals such as rodents and humans as well as avian species such as quail, dopaminergic signaling in the preoptic area has been shown to be critical for male sexual behavior. In rats, dopamine was released in this area as measured by

microdialysis in response to exposure to females prior to copulation. In addition, dopamine agonists when injected into this area facilitated and antagonists inhibited sexual behavior (Ball and Balthazart, 2004; Coolen and Hull, 2004; Dominguez and Hull, 2005; Hull and Dominguez, 2007). Sexual behavior in male zebra finches (namely males that courted and mounted females) had enhanced neuronal activity of dopaminergic neurons in the preoptic area as compared to control subjects that did not court or mount females (Bharati and Goodson, 2006). In Japanese Quail (*Coturnix japonica*) copulation results in enhanced neural activity as shown by immediate early gene expression in the medial preoptic area (Charlier et al., 2005). Lesioning this area in the same species quail resulted in a decrease in copulatory behavior (Balthazart and Surlemont, 1990; Ball and Balthazart, 2004). In starlings, there was a positive correlation between numbers of song bouts performed in a breeding context and Fos expression in the medial preoptic area (Heimovics and Riters, 2005, 2006). Additionally, lesions to the medial preoptic area decreased the number of bouts of sexually motivated song and nest-associated behaviors in this species (Alger and Riters, 2006). Therefore it is clear that the preoptic area is important for sexual behaviors as well as sexually motivated song in avian species. Although we did not observe any significant elevations in dopamine levels or a higher percentage of Fos expressing dopamine neurons in the preoptic area in birds that engaged in courtship and pairing behaviors, it is quite likely that the upward direction of change in dopamine levels in the preoptic area of paired birds is related to sexual behavior and directed singing in paired male birds versus unpaired birds.

Conclusions

Our study suggests that the mesolimbic pathway is involved in pair formation in male and female zebra finches. We observed that the percentage of dopaminergic neurons in the ventral tegmental area that expressed Fos was higher in paired versus

unpaired birds. The increased neuronal activation of dopaminergic neurons in the ventral tegmental area might have contributed to the enhanced levels of intracellular dopamine in the medial striatum as dopaminergic neurons in the ventral tegmental area are known to project into the medial striatum via the medial forebrain bundle. Additionally, a recent study has demonstrated that glutamatergic receptor mediated firing of ventral tegmental area neurons plays a major role in dopaminergic neurotransmission in the nucleus accumbens (Sombers et al., 2009). We also observed that the percentage of A11 dopaminergic nuclei expressing Fos was higher in paired male birds in comparison to unpaired male birds. The increased activity in these nuclei likely contributes to sexual behavior and courtship singing independent of the pair status of a bird.

In our experiments, paired males not only demonstrated longer durations of pairing behaviors towards females but also displayed greater bouts of aggression towards other birds. Aggression towards unfamiliar conspecifics and affiliation towards partners is also observed in paired bonded male prairie voles. A study in which immunohistochemistry for tyrosine hydroxylase, vasopressin and Fos proteins was performed, suggests that dopamine and vasopressin in limbic regions play a role in aggression associated with pair bonding in this species (Gobrogge et al., 2007). A study in zebra finches has shown that neural activation in dopamine neurons of the ventral tegmental area is increased in response to fighting with conspecifics (Bharati and Goodson, 2006). Therefore it is possible that elevated dopamine levels in the medial striatum and increased Fos expression in dopaminergic neurons is also associated with increased aggression in paired male zebra finches.

It has been suggested that sex differences in the activation of the mesolimbic dopamine circuit in mammals may be due to sex differences in the neuroendocrine control of the same. As female mammals have an estrus cycle or and periods of

gestation, the regulation of the reward circuitry differs from male rats in that its activity is influenced by ovarian, placental and lactational hormones. Therefore it is likely that sexual behavior is not rewarding for a female rat if it is not in estrus (Becker, 2009). Female zebra finches on the other hand do not have an estrus cycle and therefore the regulation and activation of the mesolimbic pathway in relation to sexual behavior is likely similar to that of male zebra finches. Additionally, as both male and female birds contribute to raising their young, (unlike in most mammals) it is likely that reward circuit is activated to the same extent by pairing and nesting in both sexes. As expected, we observed that both male and zebra finches that were paired had higher levels of dopamine and DOPAC in the medial striatum as well as a greater percentage of Fos expressing dopaminergic neurons in the ventral tegmental area in comparison to the unpaired birds.

Overall, our experiments suggest that the mesolimbic dopaminergic pathway is involved in pairing in both male and female zebra finches. Given the role of this pathway in reward processing in a number of mammalian species, it is possible that pairing and associating with a specific individual might be rewarding for zebra finches of both sexes. On the other hand, the enhanced dopaminergic activity as indicated by Fos expression in nuclei that have been implicated in courtship singing and aggression in this species, suggests that regardless of pair status, dopaminergic signaling in these nuclei contributes to these behaviors. Finally, it is not known if dopamine is necessary for pairing in this species and blockade of dopaminergic signaling or lesioning dopaminergic neurons might shed light on this question. The experiments presented in chapter 3 suggest that the blockade of D1 and D2 dopamine receptors does not affect pair formation in male and female zebra finches. Thus, further study is needed to throw light on how signaling pathways downstream of dopamine influence reward mediation and pair formation in this species.

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CHAPTER 3

DOPAMINE RECEPTOR ANTAGONISTS DECREASE COURTSHIP SINGING BUT DO NOT AFFECT PAIRING BEHAVIORS IN THE SOCIALLY MONOGAMOUS ZEBRA FINCH

Abstract

Previous studies have shown that dopamine receptor antagonists decrease the frequency of male-specific courtship behaviors such as directed song in socially monogamous zebra finches. Zebra finches form permanent pair bonds expressed through behaviors such as clumping, allopreening and spending time in their nests with their partners. These behaviors are shown by both sexes and as pairs form courtship behaviors decline. Work from this laboratory has shown that paired zebra finches have higher levels of dopamine in the medial striatum as compared to unpaired birds. Therefore we sought to address the role of dopaminergic receptors (D1-like and D2-like receptors) in pairing and courtship behaviors in this species. Dopamine receptor antagonists haloperidol and SCH-23390 were administered systemically to male and female birds once every day over a 5-day period. We observed that males administered either drug sang fewer bouts of directed song but did not differ in their pairing status in comparison to saline administered control birds. To specifically target the dopamine D1-like receptor in the medial striatum and hence minimize any peripheral effects of this drug on behavior, we administered SCH-23390 into the medial striatum via cannulae twice every day, for 5 days. Once again we observed that males that received SCH-23390 sang fewer bouts of directed song but both sexes formed similar numbers of pairs as did control birds that were injected with saline. Our experiments suggest that the D1-like receptor in the medial striatum may not play an important role in pair bond formation in zebra finch but might contribute to

motivation to perform sexually motivated behaviors such as directed song towards females.

Introduction

Dopamine is a monoaminergic neurotransmitter that plays an important role in the reward processing in vertebrate species. The hedonia experienced as a result of food, sex or drug intake is mediated via dopaminergic neurotransmission in the nucleus accumbens (Pfaus and Phillips, 1991; Ikemoto and Panksepp, 1999; Arias-Carrion and Poppel, 2007; Wise, 2008; Carlezon and Thomas, 2009). Dopamine has been implicated in sociosexual behaviors in a large number of vertebrate species including pair bonding in a socially monogamous rodent species- the prairie vole (*Microtus ochrogaster*). Studies using specific agonists and antagonists of dopamine receptors have found that dopaminergic transmission within the rostral shell of the nucleus accumbens but not the core or the caudal shell, facilitates pair bond formation in prairie voles. In addition, within the rostral shell, D1-like dopaminergic receptor activation prevented pair bond formation and D2-like receptor activation promoted this process (Curtis and Wang, 2001; Young et al., 2001; Aragona and Wang, 2004; Wang et al., 2004; Young and Wang, 2004; Aragona et al., 2006).

Although the majority of avian species are monogamous, the neural mechanisms that enable monogamy are unknown. However, dopamine has been implicated in bird song and sexual behaviors, both of which contribute to the formation of a pair bond. When Japanese quail (*Coturnix japonica*) were injected with the dopamine D1-like and D2-like receptor agonist apomorphine, copulatory behaviors were inhibited (Absil et al., 1994). Another study using agonists and antagonists specific for receptor subtypes showed that a D1-receptor agonist stimulated whereas D1-receptor antagonist inhibited consummatory sexual behavior in Japanese quail. In contrast a

D2-receptor agonist inhibited and a D2-receptor antagonist facilitated this behavior in the same species (Balthazart et al., 1997).

In addition to copulatory behaviors, dopaminergic drugs have been found to modulate sexually motivated singing. In starlings (*Sturnus vulgaris*), the administration of a drug that prevents dopamine re-uptake, and as a result increases levels of dopamine in synapses, increased singing behavior in this species. In addition, the systemic administration of a D1-like receptor antagonist decreased singing behaviors (Schroeder and Riters, 2006). In oscine passerine birds such as zebra finches, directed song performed by males towards females is an important courtship behavior that usually precedes copulation along with courtship dancing (Zann, 1996a). The administration of a combined D1-like and D2-like receptor antagonist resulted in male zebra finches singing fewer bouts of directed song towards females. In addition, frequencies of other courtship displays such dancing, beak wiping and female following were also reduced by the administration of this drug (Rauceo et al., 2008).

Although the studies just reviewed have explored the role of dopaminergic signaling via the D1-like and D2-like receptors in sexually motivated behaviors such as directed song and copulation in passerine and non-passerine birds, information on the role of these receptors in the formation of pair bonds is scarce. In the present study we hypothesized that pair bonding in zebra finches involves dopaminergic reward pathways and mechanisms. Therefore we sought to understand the role of dopaminergic signaling via D1-like and D2-like receptors in this process. Zebra finches are socially monogamous birds that form life-long pair bonds. We hypothesized that blocking D1-like and D2-like receptors would decrease courtship behaviors such as directed song (as shown previously in this species), but also inhibit pair formation. We used a non-specific dopamine receptor antagonist haloperidol in our study as a study in female prairie voles using haloperidol decreased partner

preference formation (Wang et al., 1999). We also used a D1-like receptor antagonist as it has been shown to affect sexually motivated song in starlings (Schroeder and Riters, 2006). We performed two separate experiments performing systemic treatments using each drug. In addition we performed a third experiment administering the D1-like receptor antagonist SCH-23390 directly into the medial striatum via cannulae. As there is no consensus about the exact co-ordinates of the nucleus accumbens in zebra finches, we targeted the medial striatum, which is known to contain this area. In vertebrates, the nucleus accumbens receives dopaminergic input from the ventral tegmental area important for processing rewarding stimuli. As in prairie voles, pair bonding in zebra finches likely includes a component of reward and therefore inhibiting neurotransmission vital to reward processing, might interfere with pair bonding itself.

Materials and Methods

Animals and housing

Adult male and female zebra finches (120-200 days of age) that had no prior sexual or pairing experience were used in these experiments. Prior to being introduced into pair test aviaries, birds were housed in same-sex aviaries (0.93 X 1.03 X 0.8 m each). The rooms were maintained at 21 degrees Celsius on a 14:10 light cycle. Bird- seed mix and water were provided ad libitum. All housing and experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee (IACUC).

Pharmacological manipulations and behavioral observations

Systemic haloperidol and SCH-23390 treatments

Behavioral tests were carried out in testing aviaries (0.94 X 0.76 X 0.94m) containing nest boxes and nest material. The effects of systemic administration of haloperidol and SCH-23390 on courtship and pairing behaviors were studied in both male and female zebra finches as birds of both sexes engage in pairing behaviors. As these behaviors are not sexually dimorphic, we hypothesized that the drugs would affect pairing behaviors in males and females. These drugs were injected into the peritoneum of subjects once every day for 5 days. Two dosages of each drug, high and low, were tested. For haloperidol, the high dose was 0.5 mg/kg and the low dose was 0.05mg/kg. For SCH-23390, the high dose was 0.1mg/kg and the low dose used was 0.01 mg/kg. These dosages were based those used in previous studies in Japanese Quail, *Caturnix japonica*, (Balthazart et al., 1997).

Medial striatal SCH-23390 treatment (surgery and infusions)

The effects of medial striatal SCH-23390 were studied in male zebra finches based on our observations on pairing behaviors with systemic treatments of this drug. Subjects were anesthetized using Nembutal solution (2 ml 50 mg/ml Nembutal, 0.8 ml 100% ethanol, 3.2 ml propylene glycol, 4 ml water). Each bird was stereotaxically fitted with bilateral 28-gauge cannulae directed towards the medial striatum. Cannulae were positioned 0.5mm lateral of the midline, 6mm rostral of the cerebellum and 0.5mm deep at a 30° angle and were anchored to the skull with dental acrylic. Cannulae were kept closed by using dummy wires.

After a two-week recovery period, birds were injected using a Hamilton syringe with either 50ng or 200ng of SCH-23390 in 0.5ul saline or 0.5ul saline twice every

day for 5 days. The dosages were based on previous studies in voles (Aragona et al., 2006).

Drug regime and behavioral tests

All treatments were performed for 5 days as in our experiments, most birds are usually paired within 5 days and therefore any disruption of pairing due to drug administration would be observed within this period. To test the effect of the drugs (haloperidol or SCH-23390) administered systemically on courtship and pairing behaviors in male zebra finches, 4 saline-treated males and 4 unmanipulated sexually experienced females were placed in a testing aviary. In another aviary, 4 male birds injected with the low dose of drug interacted with 4 unmanipulated sexually experienced females, and a third aviary contained 4 male birds injected with high dose of drug and 4 unmanipulated sexually experienced females. Male birds were injected with either saline, low dose or high dose of drug once every day over 5 days for systemic treatments. A second cycle of treatment were carried out with new birds resulting in n=8 per treatment group. Different pools of birds were used for the haloperidol and SCH-23390 treatments.

To test the effect of a drug on courtship and pairing behaviors in female zebra finches, 4 saline treated females and 4 unmanipulated sexually experienced males were placed in a testing aviary. In another aviary, 4 female birds injected with the low dose of drug interacted with 4 unmanipulated sexually experienced males and a third aviary contained 4 female birds injected with high dose of drug and 4 unmanipulated sexually experienced males. Each female subject was injected with either saline, low dose or high dose of drug once every day over 5 days. A second cycle of treatment were carried out with new birds resulting in n=8 per treatment group. Different pools of birds were used for the haloperidol and SCH-23390 treatments.

For male birds administered SCH-23390 into the medial striatum, 3 saline injected males interacted with 3 females, 3 male birds injected with the low dose of drug interacted with 3 females, and a third aviary contained 3 birds injected with high dose of drug and 3 females. Birds were injected twice per day. Three cycles of treatment were carried out resulting in n=9 per treatment group.

The courtship and pairing behaviors of subjects (saline and drug injected birds) were observed for 15 minutes on day 1 and day 5 of treatment. Observations were carried out within 30 minutes of drug administration. The following behaviors were recorded during each observation period by an observer blind to the subject's group using custom-designed software (Goldstein & Brodsky 2006).

Directed song bouts (recorded as number of bouts of song in 15 minutes): a salient male courtship behaviour in which the male sings to a target bird while perched close to the target. Males produce several repetitions of the song before stopping, marking the end of the song bout.

Undirected song bouts (recorded as number of bouts of song in 15 minutes): singing that is not obviously directed at another bird.

Aggression (recorded as number of bouts in 15 minutes): consisted of one bird attacking another.

Clumping and Allopreening (recorded as duration in seconds): perching in direct physical contact with another bird often accompanied by the birds preening one another. These are indicators of pair formation.

In nest box (recorded as duration in seconds): birds in the process of pairing spend substantial time in the chosen nest box together.

Pair status: at the end of 5 days, birds that frequently spent time in a nest box together were categorized as paired.

Statistical Analysis

Pair status at the end of 5 days was analyzed using Fisher's exact probability tests. Courtship and pairing behaviors were analyzed using Kruskal-Wallis tests followed by Dunn's posthoc tests. Behavioral data from 8 (systemic treatments) or 9 (medial striatum treatment) birds from each treatment were averaged to provide means for day 1 and day 5 of observation. Data were analyzed using Graphpad Prism for Macintosh (version 5.0). Significance level was set at 0.05 for all tests.

Results

Effects of haloperidol in male zebra finches

The high dose of haloperidol resulted in a decrease of the number of bouts of directed song on day 1 of the experiment (fig. 1A, $H=16.03$, $n=24$, $p<0.05$). Other behaviors such as undirected song, aggression and time spent in nest with partner were not significantly altered by either a low or high dose (fig. 1B-D, $p>0.05$). Clumping and allopreening behaviors were observed infrequently and did not differ significantly among groups (data not shown). Song, aggression and pairing behaviors did not differ significantly between groups during day 5 of the testing paradigm (fig. 1E-H).

Effects of SCH-23390 in male zebra finches

The administration of SCH-23390 had an effect similar to that of haloperidol on the number of bouts of directed song sung by male zebra finches. Birds injected with a high dose of the drug produced significantly fewer song bouts than the saline-treated group on day 1 of testing (fig. 2A, $n=24$, $H=16.03$, $p<0.05$). Behaviors such as the production of undirected song, aggression and time spent in nest with partner were not significantly altered by either a low or high dose of SCH-23390 during day 1 (fig. 2B-

D, $p > 0.05$). Clumping and allopreening behaviors were observed infrequently and did not differ significantly among groups (data not shown). During day 5 of the testing paradigm, birds that were treated with either a low dose and a high dose of drug sang fewer bouts of directed song (fig. 2E, $H = 11.12$, $p < 0.05$). Behaviors such as undirected song, aggression and pairing behaviors did not differ significantly between groups on day 5 (fig. 2F-H).

Effects of haloperidol in female zebra finches

Pairing behaviors such as clumping and allopreening did not differ significantly between groups (data not shown). During day 5 of the behavioral tests, time spent in a nest with partner was significantly less in females that were treated with a low dose of haloperidol (Table 3.1, $n = 24$, $H = 6.723$, $p < 0.05$). There were no other significant differences in the behaviors observed.

Effects of SCH-23390 in female zebra finches

There were no significant effects of treatment with a low dose or a high dose of SCH-23390 in females either on day 1 or day 5 of the behavioral testing paradigm (Table 3.1, $p > 0.05$).

Pair status

There were no significant differences in the pair status of male birds that were administered systemic haloperidol (fig. 3.4 A, $\chi^2 = 2.4$, $p = 0.3012$), systemic SCH-23390 (fig. 3.4B, $\chi^2 = 4.875$, $p = 0.0874$) or medial striatal SCH-23390 (fig. 3.4 C, $\chi^2 = 0.3068$, $p = 0.8578$). There were also no significant differences in the pair status of females injected with systemic haloperidol (fig. 3.5A, $\chi^2 = 1.33$, $p = 0.5134$) or systemic SCH-23390 (fig. 3.5B, $\chi^2 = 2.02$, $p = 0.3640$).

Figure 3.1. Courtship, aggression and pairing behaviors (mean \pm SEM) observed in male zebra finches administered systemic haloperidol, on day 1 (A-D) and day 5 (E-H). N=8 per group.

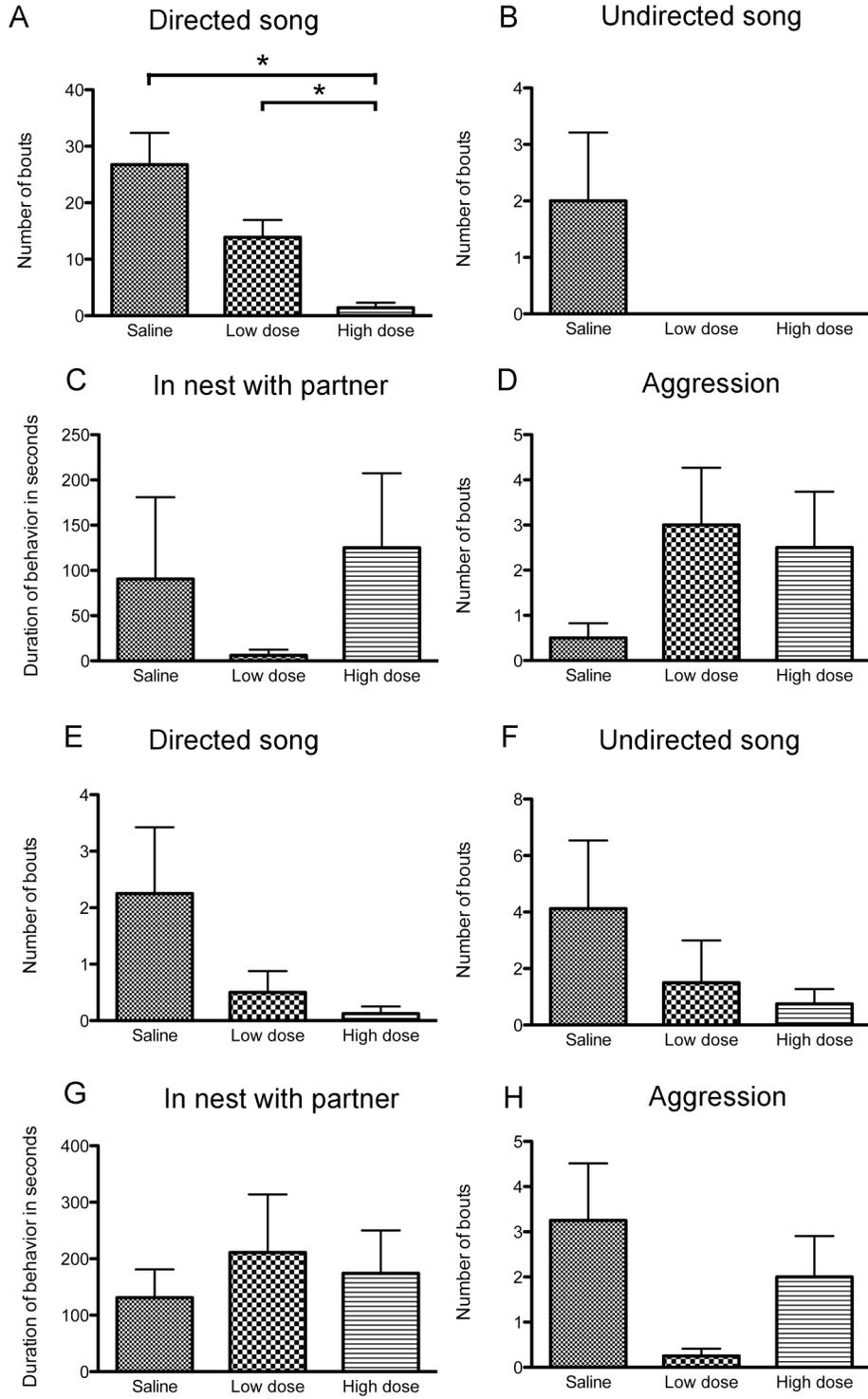


Figure 3.2. Courtship, aggression and pairing behaviors (mean \pm SEM) observed in male zebra finches administered systemic SCH-23390, on day 1 (A-D) and day 5 (E-H). N=8 per group.

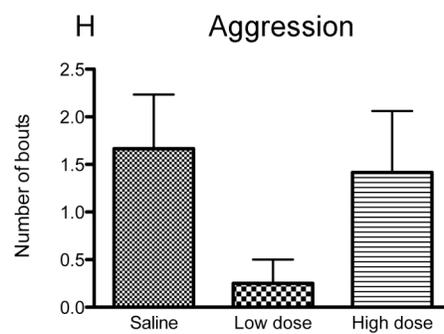
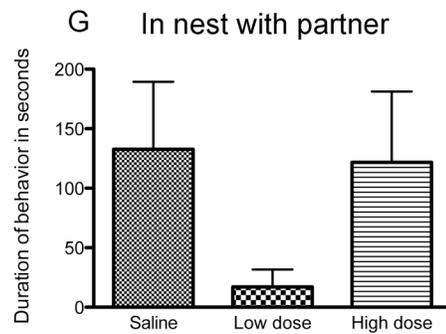
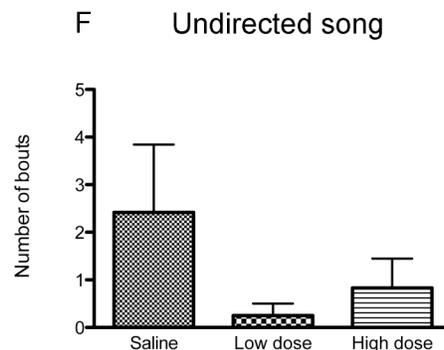
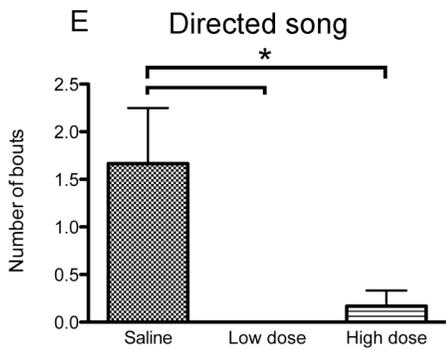
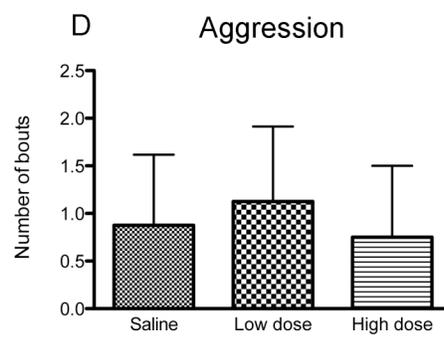
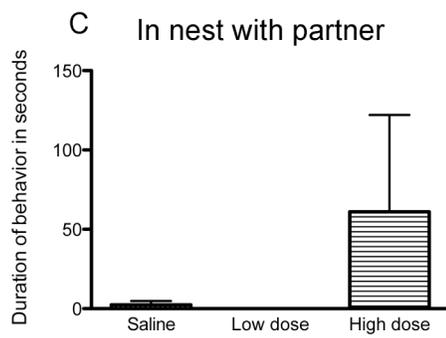
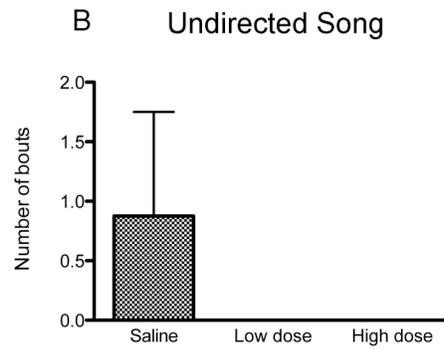
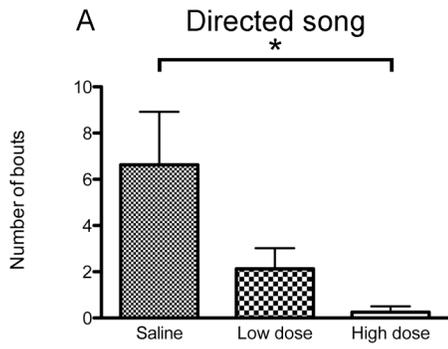
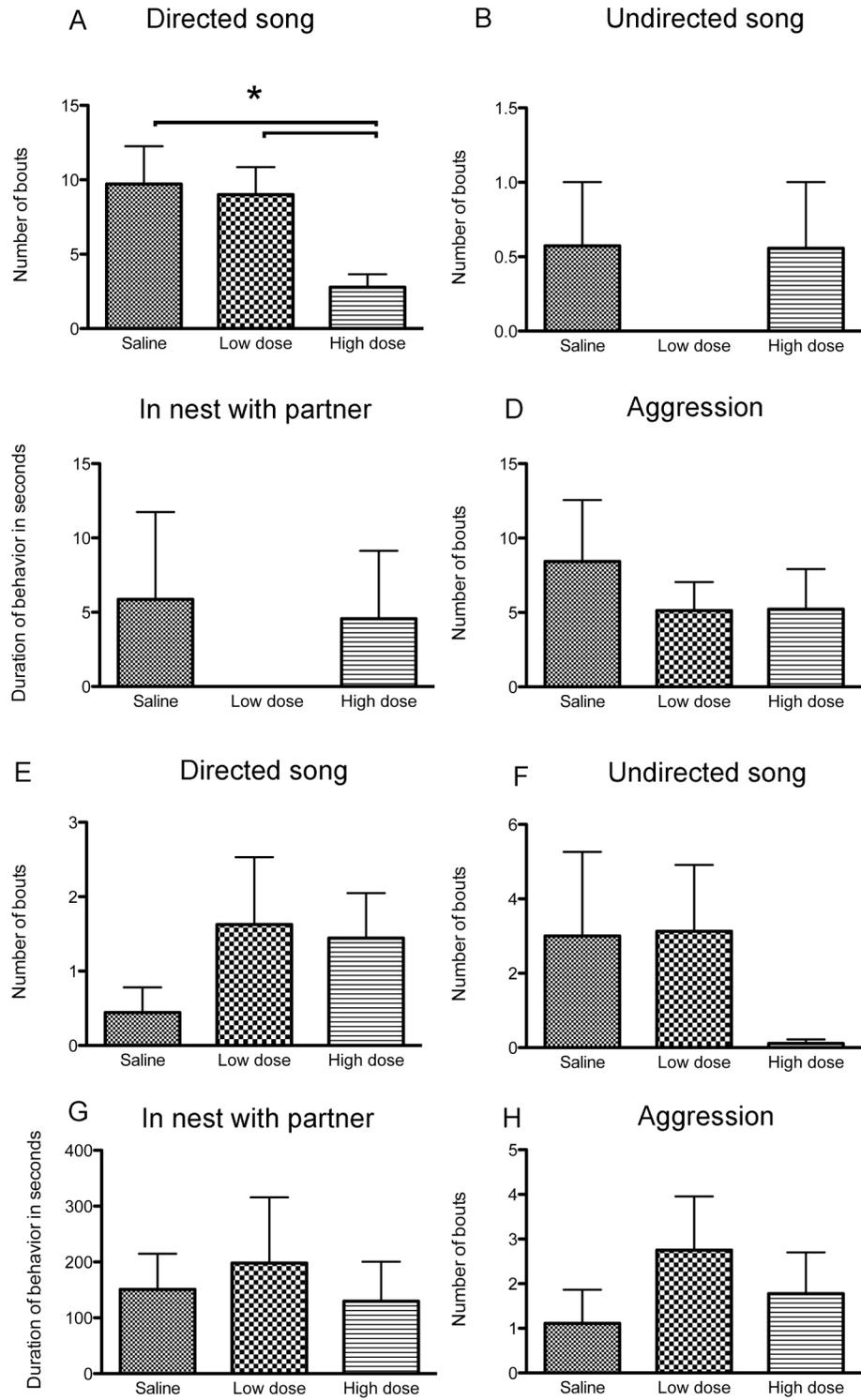


Figure 3.3. Courtship, aggression and pairing behaviors (mean \pm SEM) observed in male zebra finches administered SCH-23390 in the medial striatum, on day 1 (A-D) and day 5 (E-H). N=9 per group.



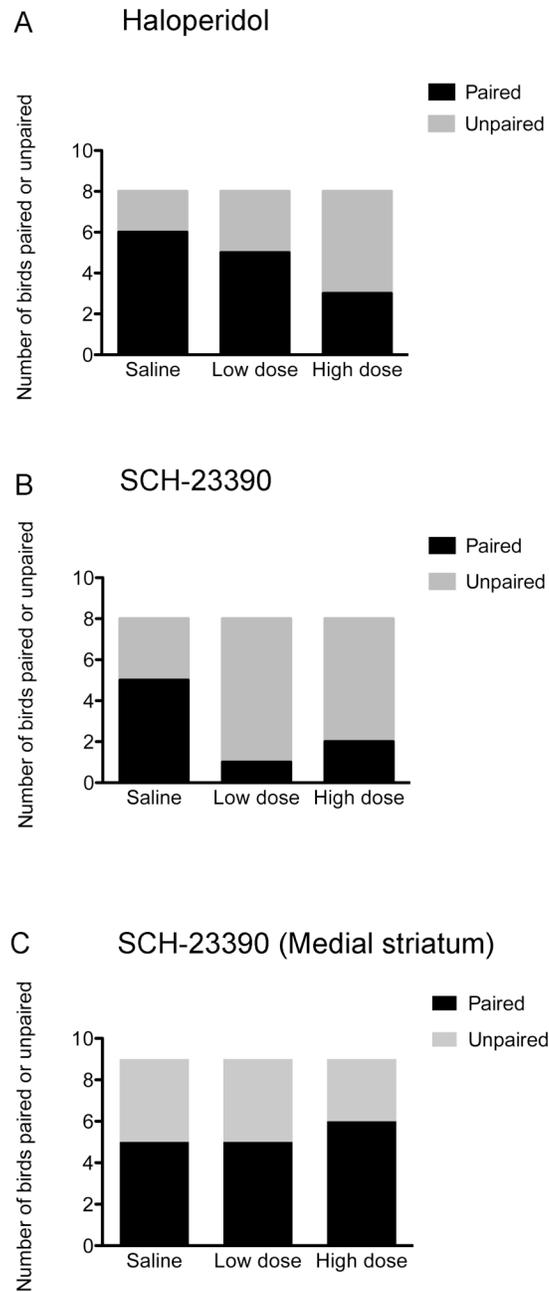


Figure 3.4. Numbers of male zebra finches paired and unpaired on day 5 after (A) systemic haloperidol, (B) systemic SCH-23390 and (C) medial striatal SCH-23390.

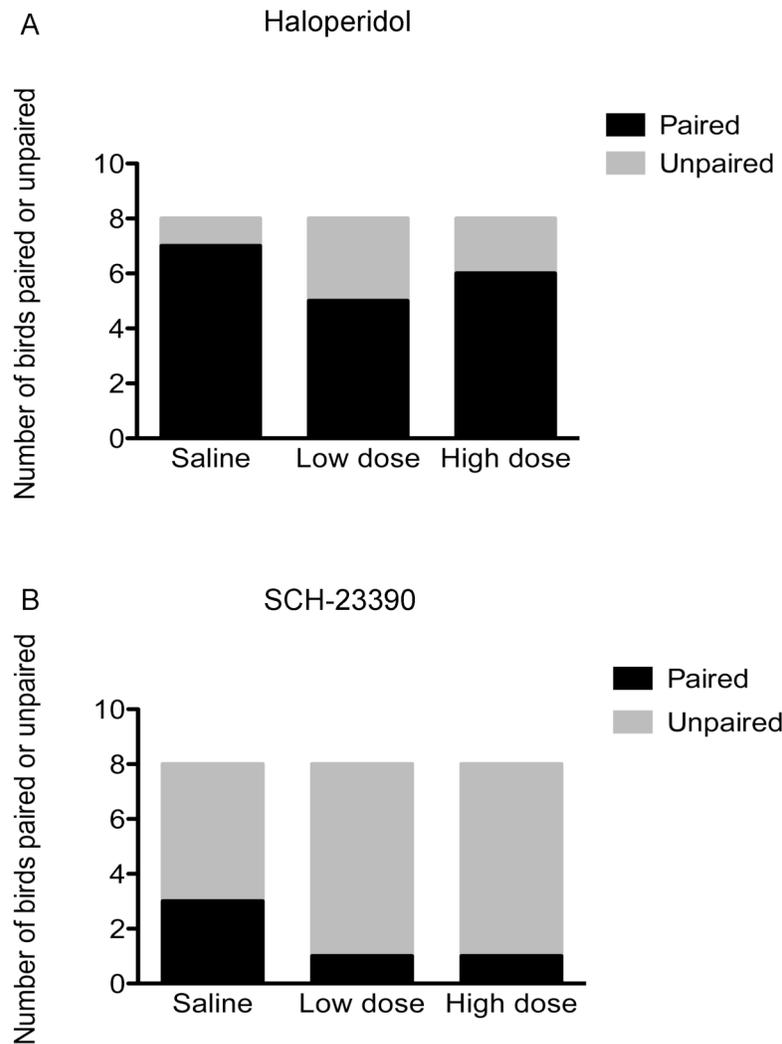


Figure 3.5. Numbers of female zebra finches unpaired and paired on day 5 after (A) systemic haloperidol and (B) systemic SCH-23390.

Table 3.1. Effect of Haloperidol administered systemically on courtship and pairing behaviors of female zebra finches.

| Behavior | Frequency/Duration (seconds) | | | <i>P</i> |
|------------------------------|------------------------------|--------------------|-------------|------------------|
| | Saline | Low dose | High dose | |
| Aggression (day 1) | 0.125±0.125 | 0.725±0.725 | 2.25±0.62 | p>0.05 |
| Aggression (day 5) | 1.5±0.80 | 1.125±0.58 | 2.37±0.90 | p>0.05 |
| In nest with partner (day 1) | 46.50±28.45 | 0 | 0 | p>0.05 |
| In nest with partner (day 5) | 473±101.9 | 42.49±18.77 | 275.7±121.7 | p<0.05 |

Effect of SCH-23390 administered systemically on courtship and pairing behaviors of female zebra finches.

| Behavior | Frequency/Duration (seconds) | | | <i>P</i> |
|------------------------------|------------------------------|-------------|-------------|----------|
| | Saline | Low dose | High dose | |
| Aggression (day 1) | 0.0 | 1.00±0.86 | 0.42±0.29 | p>0.05 |
| Aggression (day 5) | 0.0 | 1.25±0.81 | 1.50±0.71 | p>0.05 |
| In nest with partner (day 1) | 228.6±110.1 | 17.82±17.82 | 66.59±34.79 | p>0.05 |
| In nest with partner (day 5) | 349.5±182.5 | 28.54±25.28 | 2.493±2.493 | p>0.05 |

Discussion

In the current study we have shown that sexually motivated behaviors such as directed song in zebra finches, are decreased by dopamine antagonists haloperidol and SCH-23390. Both systemic and medial striatal administration of dopaminergic antagonists did not affect pairing status in zebra finches. In addition, female birds treated with a low dose of haloperidol spent less time in the nest with their partners than saline treated controls on day 5 of the behavioral test.

Our hypothesis was that dopaminergic antagonists would interfere with pairing behaviors as dopaminergic neurotransmission in the mesolimbic pathway was important for pair formation as dopaminergic neurotransmission via the D1 and D2 receptors in the nucleus accumbens, is thought to be critical for pairing in a socially monogamous rodent- the prairie vole. Furthermore, unpublished work from the lab (chapter 2) has shown that dopamine levels and neuronal activity of midbrain dopaminergic neurons are higher in paired versus unpaired zebra finches.

One of the functions of dopaminergic neurotransmission in mammals is the mediation of reward. The mesolimbic dopaminergic pathway is the key pathway involved in this process. It consists of dopaminergic projections that arise from the ventral tegmental area in the mid brain and synapse with neurons in the nucleus accumbens in addition to other limbic areas such as the hippocampus and hypothalamus. Administering a drug of abuse, gambling, sexual behavior as well as food is associated with increase in dopamine levels at synapses in the nucleus accumbens. Initially, dopamine was considered to be the molecule that caused the euphoria related with rewarding stimuli. Recent studies suggest that dopamine does not provide the reward itself but it is critical for signaling the incentive salience of events, driving motivated behavior, predicting reward or non-reward as well as

facilitating the consolidation of memories for salient events (Schultz, 1998; Berridge and Kringelbach, 2008; Goodman, 2008).

In avian species, as in mammals, the primary dopaminergic input into the telencephalon has been shown to arise from the ventral tegmental area, the substantia nigra (Durstewitz et al., 1999). The avian striatum (a part of the telencephalon) formally known as the *Lobus paraolfactorius* (LPO) is now thought to be homologous to the mammalian striatum and has a large number of dopaminergic fibers. The nucleus accumbens is thought to be situated medially in this area of the brain. In mammals, the nucleus accumbens has three distinct divisions; the rostral nucleus accumbens, the core and the shell. The shell is connected to limbic regions such as the ventral tegmental area and the bed nucleus of stria terminalis. In contrast, the core has connections with nuclei of the basal ganglia that are primarily involved with motor function. A recent study in chickens has been able to identify core and shell regions in the avian nucleus accumbens using immunocytochemical markers (Wynne and Gunturkun, 1995; Durstewitz et al., 1999; Balint and Csillag, 2007). Similar divisions of the nucleus accumbens in zebra finches have not been elucidated but it is likely that they exist. In addition to projections into striatal areas, the ventral tegmental area also sends dopaminergic projections into song system nuclei that are involved in song production. The caudal pathway, involved in the motor production of song consists of a series of discrete and interconnected brain nuclei. The HVC or high vocal center projects to the robust nucleus of arcopallium (RA), both situated in the telencephalon. The RA sends projections to the dorsomedial portion (DM) of the intercollicular complex. Both RA and DM project to the tracheosyringeal part of the nucleus of the XIIth cranial nerve (nXII_{ts}) that innervates the vocal production organ the syrinx and to nucleus retroambiguus (RA_m) and the rostral ventral respiratory group of neurons (rVRG) that regulates respiratory activity during song production

(Appeltants et al., 2000; Suthers and Margoliash, 2002; Mooney, 2009). Studies in canaries have found that dopaminergic projections from the ventral tegmental area (VTA) and the mesencephalic central grey (A11) project into the RA. Similarly, the HVC also receives input from these two dopaminergic areas (Appeltants et al., 2000; Appeltants et al., 2002). A study in zebra finches showed tyrosine hydroxylase immunoreactivity in song nuclei such as the HVC, the RA and Area X. Labeled neuronal processes were observed in these regions. In addition the striatum referred to as the LPO was also displayed densely labeled processes. As expected the ventral tegmental area appeared as darkly labeled cells and fibers (Bottjer, 1993).

A recent study has detailed the expression of dopamine receptors in the zebra finch and chicken brain. There are two main families of dopamine receptors, the D1-like and the D2-like receptors. The D1 receptors activate adenylate cyclase and downstream cell signaling whereas the D2 receptors inhibit adenylate cyclase and further signaling via this signaling cascade. The D1 family of receptors includes the D1A or D1, the D1B or D5, D1C and D1D receptors. The D1D receptor is thought to be restricted to avian species. The D2 family consists of the D2, D3 and D4 receptors. Kubikova and colleagues have shown that the D1A, D1B and D2 receptors are highly expressed in the striatum, the D1D receptors are expressed in the pallium, and D3 in the mesopallium while D4 receptors are primarily expressed in the cerebellum. Song nuclei as well had expression of these receptors. Both D1A, D1B and D2 receptors were expressed in Area X at high levels. The pallial song nuclei HVC and RA had higher expression of D1B, D2 and D3 receptors and lower expression of D1D in comparison to the surrounding pallium. The LMAN showed low expression for all receptors excepted for a few isolated cells expressing the D2 receptor. In sum, the three nuclei that had highest expression of dopamine receptors were the HVC, RA and Area X (Kubikova et al., 2010). As a result of the distribution patterns of dopamine

receptors, it is likely systemic treatment with dopaminergic drugs would likely affect a large number of areas within the zebra finch brain.

A large body of work performed in prairie voles implicates dopaminergic neurotransmission via D1 and D2 receptors in the nucleus accumbens in the formation of pair bonds in these socially monogamous rodents. Peripheral injection of a nonspecific DA receptor agonist induced partner preference formation in the absence of mating, while injection of a nonspecific DA receptor antagonist haloperidol blocked mating-induced partner preferences. Studies in both male and female prairie voles indicate that DA regulates pair bonding in both a receptor- and site-specific manner. The activation of D2 receptors, but not D1 receptors, in the nucleus accumbens facilitated partner preference formation in female and male prairie voles, whereas blockade of D2 receptors in the nucleus accumbens inhibited the formation of partner preferences. These data indicate that dopamine in the nucleus accumbens has opposing effects such that D2 receptor activation facilitates and D1 receptor activation inhibits partner preference formation. Further, dopamine acts in a sub-region specific manner within the nucleus accumbens, as activation of D2 receptors in the nucleus accumbens shell, but not the core, induces partner preference formation (Gingrich et al., 2000; Curtis and Wang, 2001; Curtis et al., 2003; Aragona and Wang, 2004; Aragona et al., 2006; Aragona and Wang, 2007; Young et al., 2008).

It should be noted that our experimental design was distinct from that used to test the effects of dopaminergic drugs in prairie voles. Prairie voles form partner preferences within 1-6 hours of cohabitation with mating or 24 hours of cohabitation in the absence of mating. Therefore if the blockade of a receptor results in the absence of a partner preference following 24 h of mating, it can be inferred that the receptor is necessary for pair bond formation. Alternatively, if pharmacological activation of a neurochemical receptor during a 1–6 h social cohabitation induces partner preferences,

it can be inferred that activation of this receptor is sufficient to induce pair bonding (Young et al., 2001; Wang and Aragona, 2004; Young et al., 2008). Therefore these experiments involved a single injection of a specific drug and results could be inferred within a far shorter period of time than 5 days as in our experiments. A longer time period was essential in our experiments as pair bonds in zebra finches housed in large aviary groups are observed within 5 days of cohabitation and forced pairing of two individuals is usually unsuccessful within a short period of time.

Our experiments suggest that a non-specific dopamine receptor antagonist haloperidol and a D1 specific receptor antagonist SCH-23390 do not block pair bond formation although they do decrease courtship behavior as measured by frequency of directed song bouts. It is interesting that on day 5 the duration of time spent with a partner was decreased in females that were injected with a high dose of haloperidol (Table 3.1). This might indicate that although females spend some time in their nest with their partners, their motivation to do so was less than the saline injected controls. Such an effect points to the interference in the motivation to perform normally rewarding pairing behaviors by a dopaminergic receptor antagonist. It would be interesting to know the effect of a D2 receptor antagonist in zebra finches, to see if this drug could block the development of partner preference based on its effects on pairing in prairie voles. Based on our results, it would be erroneous to conclude that D1 and D2 receptors do not play any role in pair bond formation in the zebra finches as to our knowledge the half-life of the drugs used in our experiments is not known in zebra finches. Therefore it is hard to be certain that the receptors were effectively blocked throughout the period of the experiment.

Several studies indicate that dopaminergic neurotransmission in the mesolimbic pathway is important for the expression of courtship behaviors such as directed song in zebra finches. In zebra finches, activity in the ventral tegmental area was modulated

when males sang to females. Single unit extracellular recordings from neurons in this area demonstrated that neuronal activity was much higher when males sang to females (directed song) versus undirected song in the absence of females (Yanagihara and Hessler, 2006). In addition, whole cell recordings from ventral tegmental area neurons demonstrated a potentiation of glutamatergic synaptic currents in neurons that synapsed with dopamine projection neurons in the ventral tegmental area after male zebra finches sang to females compared to after they sang alone (Huang and Hessler, 2008). In another study, numbers of dopaminergic neurons that expressed Fos were higher in the caudal ventral tegmental area and A11 in male zebra finches that performed bouts of directed song to females when compared to birds that did not sing or birds that were exposed to a non-social stimulus (Goodson et al., 2009b). In starlings, measures of tyrosine hydroxylase expression (optical density) in the ventral tegmental area along with the medial preoptic area (POM) and Area X, were positively correlated with song produced within a breeding context and not in situations that were outside of a breeding context (Heimovics and Riters, 2008). In the same species, the peripheral administration of SCH-23390, decreased sexually motivated song whereas a dopamine re-uptake inhibitor increased song production (Schroeder and Riters, 2006). Similarly, in our experiments, when SCH-23390 or haloperidol was administered systemically, a decrease in frequency of directed song bouts was observed. SCH-23390 in the medial striatum also decreased the frequency of directed song. Such an effect might have been observed due to interference in reward processing via receptors in the nucleus accumbens. This hypothesis is supported by studies in rodents that have shown that both D1 and D2 receptor antagonists reduce responding to rewarding stimuli in tasks such as operant responding to food, water, brain stimulation or drug self administration (Beninger and Miller, 1998; Sutton and Beninger, 1999; Dalley and Everitt, 2009).

It is possible that peripheral treatment of dopaminergic antagonists, decrease directed song due to direct effects in song nuclei themselves. At present it is unclear as to how dopamine in song nuclei modulates the production of directed song. A recent study has shown that dopamine levels are increased due to decreased breakdown of the neurotransmitter in synapses in the Area X of male zebra finches while they sang directed song but not undirected song (Sasaki et al., 2006). It is also known that D1 receptors are excitatory and D2 receptors are inhibitory in action in the Area X as in the mammalian striatum (Ding and Perkel, 2002). Although it is known that Area X is part of the anterior forebrain pathway not involved in song production, it is possible that it does modulate motivation to sing in zebra finches. The RA and HVC on the other hand are a part of the caudal song production pathway and therefore modulation of excitation and inhibition of these areas by dopaminergic drugs also remains a possibility. Given that in our experiments, a D1 receptor antagonist targeted specifically to the medial striatum caused a decrease in directed song, it is likely that peripherally administered antagonists also exerted their behavioral effects via receptors in the medial striatum as part of the mesolimbic dopaminergic pathway.

In summary, both haloperidol (non-specific dopamine receptor antagonist) and SCH-23390 (D1 receptor antagonist) decreased the frequency of courtship and pairing behaviors but did not interfere with the formation of pair bonds in male and female zebra finches. Perhaps species like zebra finches that have life long pair bonds have sufficient redundancy in their signaling cascades that the blockade of one or two receptors does not have any effect on pairing, given the importance of this behavior to the fitness of individuals in this species. It is also possible that pairing behaviors are developmentally organized in this species and therefore early life manipulations would be required to alter such behaviors.

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CHAPTER 4
EFFECT OF ISOLATION AND SOCIAL BUFFERING ON
CORTICOSTERONE LEVELS IN A SOCIAL AVIAN SPECIES,
THE ZEBRA FINCH

Abstract

Zebra finches are a highly social avian species. They are also socially monogamous and form life-long pair bonds. In the present study, we sought to address the effect of isolation on the stress response of individuals of this species. We observed that 10 but not 30 minutes of social isolation resulted in elevated levels of corticosterone in isolated unpaired and paired male zebra finches in comparison to baseline (undisturbed birds) levels of corticosterone. We hypothesized that as this was a socially monogamous avian species, the presence of the mate would act as a social buffer against the stressful effects of isolation. Our prediction was incorrect as the presence of another bird of the same or opposite sex did not have a buffering effect on the adrenocortical response to social isolation in paired male zebra finches. To compare the effects of isolation to a well-established stressor restraint, we subjected groups of birds to 10 or 30 minutes of restraint and measured corticosterone levels. Our results indicate that both 10 and 30 minutes of restraint lead to significantly higher levels of corticosterone as compared to baseline (undisturbed birds). To address the effect of handling a bird in the adrenocortical response, we measured corticosterone levels 10 or 30 minutes after handling involving capture and release without being followed up by restraint or isolation. Our results suggest that handling alone might contribute to the elevation of corticosterone 10 minutes after restraint but not 30 minutes after either manipulation.

Introduction

Zebra finches are a social avian species and live in large flocks in the wild (Zann, 1996a). We sought to study the effect of social isolation on stress responses in this species given their highly social nature. The stress response in birds and other vertebrates is mediated by the hypothalamic-pituitary-adrenal (HPA) axis. Both physiological and psychological adversities elicit the secretion of corticotrophin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, which then acts on pituitary cells, resulting in the release of adrenocorticotrophic hormone (ACTH), which is carried via the blood stream to the adrenal cortex resulting in the secretion of corticosterone from the adrenal cortex. Glucocorticoids (cortisol and corticosterone) are vital for making fuel available during high energy demands as well as enhancing cognitive abilities during duress. Over long periods of time, they can have damaging effects on cognition and neuron morphology as well as physiology. The HPA axis is turned off or inhibited by glucocorticoids themselves when they bind to their receptors in the hypothalamus and hippocampus and other limbic areas (McEwen et al., 1995; Brown et al., 1999; Sapolsky, 1999).

Studies in mammals have shown that isolation can enhance the adrenocortical response to a stressor such as novelty or an elevated plus-maze test. Rats that had been exposed to a 13 week period of social isolation had higher levels of corticosterone after exposure to a startle test in comparison to group-raised rats (Weiss et al., 2004). In another lab housed rodent species the prairie voles, female voles that were exposed to 24 hours isolation or a two week isolation period prior to being subjected to an elevated plus maze test, had elevated levels of corticosterone compared to voles that had not been isolated prior to the test (Stowe et al., 2005). Female prairie voles that had been housed in isolation for 60 days, had higher levels of corticosterone in

response to a resident-intruder paradigm as compared to voles that were pair housed without any previous history of isolation (Grippio et al., 2007). In wild birds such as the European starling (*Sturnus vulgaris*), 30 minutes of visual isolation resulted in elevated corticosterone levels compared to controls (Apfelbeck and Raess, 2008). In the present study we determined the effect of social isolation on corticosterone levels in zebra finches. As zebra finches are a highly gregarious species (Zann, 1996a), we predicted that social isolation would be a stressful event and result in elevated corticosterone levels in these birds. We expected to see higher levels of corticosterone in isolated birds compared to undisturbed or handled birds.

There is a large body of work on the buffering effects of conspecifics on stress. This phenomenon called ‘social buffering’ has been observed in rats (Davitz and Mason, 1955), guinea pigs (Graves and Hennessy, 2000; Hennessy et al., 2006; Hennessy et al., 2009), squirrel monkeys (Coe et al., 1978; Levine et al., 1997) as well as humans (Cohen and Wills, 1985; Kikusui et al., 2006). In these studies, the authors have found that the presence of a conspecific (usually the partner or a parent) when an individual is exposed to a stressor, alleviates stress and decreases glucocorticoid levels when compared to individuals who are isolated from any social contact when exposed to the stressor. In rats too, pair housing of males and females during exposure to chronic stressors such as foot-shock stress reduces stress-induced changes in behavior and physiology in both sexes when compared to rats who were isolated during exposure to a stressor (Westenbroek et al., 2005). Therefore, we sought to examine the role of a conspecific in buffering the stress response of a zebra finch, when subjected to 10 or 30 minutes of isolation. We used both paired and unpaired males as subjects. It is known that when paired zebra finches are separated from their partners, their corticosterone levels are elevated and return to baseline when reunited with their partners (Remage-Healey et al., 2003). Hence we wanted to understand if paired birds

that were removed from their home aviaries and isolated along with their partners would show an elevation in corticosterone levels. We tried to tease apart the importance of the relationship of the conspecific to the subject by using either the pair partner, a familiar opposite-sex bird, or a familiar same-sex bird as conspecific social buffers.

It is well established that capture and restraint causes a robust elevation of corticosterone in a number of avian and mammalian species. Thirty minutes of capture and restraint of wild free-living mammals such brown lemmings, golden-mantled ground squirrels and yellow-pine chipmunks results in elevated levels of corticosterone as compared to baseline levels of the hormone (Romero et al., 2008). This paradigm is commonly used in lab rats and results in elevated levels of corticosterone (Glavin et al., 1994; Gray et al., 2010). Capture, handling and restraint also results in elevated corticosterone levels, as compared to baseline, in a large number of wild and captive avian species. In zebra finches, when adult birds were placed in cloth bags for 15 minutes and 30 minutes, levels of corticosterone were higher than baseline at both time points (Wada et al., 2008). A similar observation was made in Gambel's white-crowned sparrows when subjected to 30 minutes of restraint (Romero and Wingfield, 1999). A capture and restraint paradigm in uniparental and biparental arctic shore birds, resulted in significantly elevated corticosterone levels compared to baseline (O'Reilly and Wingfield, 2001). Other free-living avian species such as snow buntings and Lapland longspurs, when subjected to 60 minutes of capture and restraint, showed significant elevations of corticosterone compared to baseline, and there were no differences in the stress response between the breeding season and just prior to migration (Wingfield et al., 1994). In feral pigeons as well, 30 minutes of restraint resulted in elevated corticosterone levels regardless of whether the birds were molting or non-molting (Romero and Wingfield, 2001). In the present

study we measured corticosterone levels in response to 10 and 30 minutes of restraint in male zebra finches to compare levels of corticosterone in response to two different paradigms- restraint and isolation. In addition, we also measured corticosterone responses to handling (capture and release) without following this manipulation with restraint or isolation to tease apart the contribution of handling alone on adrenocortical responses when birds were subjected to either paradigm of restraint and isolation.

Materials and Methods

Birds

Male zebra finches that were raised in the laboratory and had prior breeding experience were used in these experiments. Only male birds were used as subjects in all experiments. In experiments where we measured corticosterone levels in response to restraint and isolation in unpaired male birds, birds were housed in unisex aviaries. In experiments where we measured corticosterone responses to isolation in paired male birds, birds were housed in mixed sex breeding aviaries with nest boxes. Each breeding aviary held between 16-20 birds, males and females included. Pairs were confirmed through observation of pairing behaviors. Birds were used only once and there was no repeated sampling in any experiment; “baseline” levels were thus levels of separate groups of undisturbed birds. All blood sampling was performed between 9am-12pm. Birds were housed on a 14:10 hour light /dark cycle. Humidity ranged from 30-70% and room temperatures were maintained at 22°C. The dimensions of each housing and breeding aviary were 0.94 X 0.76 X 0.94 m. All animal procedures conformed to Federal and State regulations and were approved by the Cornell University IACUC.

Experiments 1 and 2: Corticosterone levels in handled, restrained and isolated males

Blood samples to measure baseline (undisturbed) levels of corticosterone were obtained by capturing birds and sampling them in less than three minutes from previously undisturbed aviaries. For the handling group, male birds were captured from their housing aviaries and released immediately into the same aviary. 10 or 30 minutes after this manipulation blood samples were acquired from the handled birds. Blood samples were acquired in a similar manner from male birds that were subjected to 10 or 30 minutes of restraint and 10 or 30 minutes of isolation. Each male was restrained by placing it in a brown paper bag for 10 or 30 minutes. Birds were isolated from their peers by placing them in single cages (45 X 25 X 23 cm) in a room that had no other birds in it for either 10 minutes or 30 minutes. There were 8-9 subjects in each group.

Experiment 3: Effect of a conspecific on corticosterone levels in response to social isolation

Blood samples to measure baseline levels of corticosterone were obtained by capturing birds and sampling them in less than three minutes from previously undisturbed aviaries. To obtain blood samples in response to isolation, birds were isolated from their peers by placing them in single cages (45 X 25 X 23 cm) with food and water, in a room that had no other birds in it, for either 10 minutes or 30 minutes. Unpaired male birds were subjected to isolation alone and not with any other conspecific. Paired male birds were placed in the cage alone or with their female partner or with a different female or with a male bird. Both non-partner female and male birds were housed in the same breeding aviary as the subject; therefore all conspecifics were familiar to the subjects. There were 4-10 subjects per group.

Blood collection

Blood samples of approximately 100ul was obtained by piercing the alar vein of the left wing using a 26-gauge sterile needle (used once for each bird) and gathering the welling blood using heparinized capillary tubes. This procedure was carried out within three minutes of the lights being turned off in aviary rooms for capture. Samples were centrifuged at 12000 rpm and plasma was stored at -80°C.

Enzyme-immunoassay to measure corticosterone

We used corticosterone EIA kits (Cayman Chemical, cat.no.500651). The assay has a detection limit of 30 pg/ml and a sensitivity of 150 pg/ml. We ran samples in duplicate at a dilution of 1:10 in buffer and took the average of both of readings to obtain the final value of each sample. The intra- and interassay CVs were 8.13% and 9.6% respectively.

Statistics

Data were analyzed using univariate ANOVA followed by Bonferroni corrections for multiple comparisons and Tukey HSD posthoc tests. Data were natural log (ln) transformed to meet assumptions for normality and equality of variances. Significance levels were set at $p < 0.05$ for all tests.

Results

Experiment 1: Corticosterone levels in handled and restrained male zebra finches (Figure 4.1A)

Overall, there was a significant main effect of group ($F_{4,40} = 12.41, p < 0.001$). Corticosterone levels in birds that were handled and sampled after 10 minutes (“Handled 10”) were significantly higher than in the baseline (undisturbed) group

($p=0.029$). Hormone levels in birds that were exposed to 10 minutes of restraint (“Restraint 10”) were also higher than in the baseline group ($p=0.008$). Hormone levels in birds exposed to 10 minutes of restraint were not significantly different from birds that were handled and sampled after 10 minutes ($p=0.986$). Corticosterone levels in birds that were handled and sampled after 30 minutes (“Handled 30”) were not significantly different from those in the baseline ($p=0.517$) or sampled and handled after 10 minutes groups ($p=0.573$). Hormone levels in birds that were subjected to 30 minutes of restraint (“Restraint 30”) were significantly higher than those in the baseline ($p<0.001$) and as well as “Handled 30” ($p<0.001$) and “Handled 10” groups ($p=0.007$). Finally, corticosterone levels of the group subjected to 10 minutes of restraint were significantly lower from those in the group exposed to 30 minutes of restraint ($p=0.029$).

Experiment 2: Corticosterone levels in isolated male zebra finches (Figure 4.1B).

Overall, there was a significant main effect of group ($F_{4,37}=6.09$, $p=0.001$). Corticosterone levels in the baseline group were not significantly different from those in the group that was handled and sampled 10 minutes later (“Handled 10”) ($p=0.974$). Corticosterone levels of birds that experienced 10 minutes of isolation (“Isolation 10”) were significantly higher than in the baseline ($p=0.002$) and “Handled 10” groups ($p=0.015$). These levels were also significantly higher from those in the group that was sampled 30 minutes after being handled (“Handled 30”) ($p=0.001$), but not from those in the group exposed to 30 minutes of isolation (“Isolation 30”) ($p=0.165$). Hormone levels of birds exposed to 30 minutes of isolation were not significantly different from those of the baseline ($p=1.0$), “Handled 10” ($p=1.0$) or “Handled 30” ($p=0.850$) groups.

Experiment 3: Effect of a conspecific on corticosterone levels in response to social isolation (Figure 4.2).

Overall, there was a significant main effect of treatment group ($F_{11,73} = 6.509$, $p < 0.001$). Corticosterone levels of undisturbed unpaired males (“Baseline single”) were not significantly different from levels in undisturbed paired males (“Baseline paired”) ($p = 0.992$). Unpaired birds exposed to 10 minutes of isolation (“10’ single”) had significantly higher levels than the “Baseline single” group ($p < 0.001$) but birds exposed to 30 minutes of isolation (“30’ single”) did not ($p = 0.332$). Each of the four groups of paired males that were exposed to 10 minutes of isolation had higher levels than the “Baseline paired group,” including paired males in isolation alone (“10’ paired alone,” $p < 0.001$), paired males in isolation with their female partners (“10’ paired + female p,” $p = 0.001$), paired males in isolation with a non-partner female (“10’ paired + female np,” $p = 0.037$), and paired males in isolation with another male (“10’ paired + male,” $p = 0.022$). In contrast, none of the groups of paired birds that were in isolation for 30 minutes had levels that differed from those of the “Baseline paired” group (“30’ paired alone,” $p = 0.079$; “30’ paired + female p,” $p = 0.253$; “30’ paired + female np,” $p = 0.939$; “30’ paired + male,” $p = 0.446$). When compared with unpaired undisturbed birds, paired birds that were exposed to 30 minutes of isolation had higher corticosterone levels if they were alone ($p = 0.002$), with their female partner ($p = 0.010$), or with a familiar male ($p = 0.028$), but not if they were with a non-partner female ($p = 0.362$). There were no significant differences between any of the groups exposed to 10 minutes of isolation or between any of the groups exposed to 30 minutes of isolation.

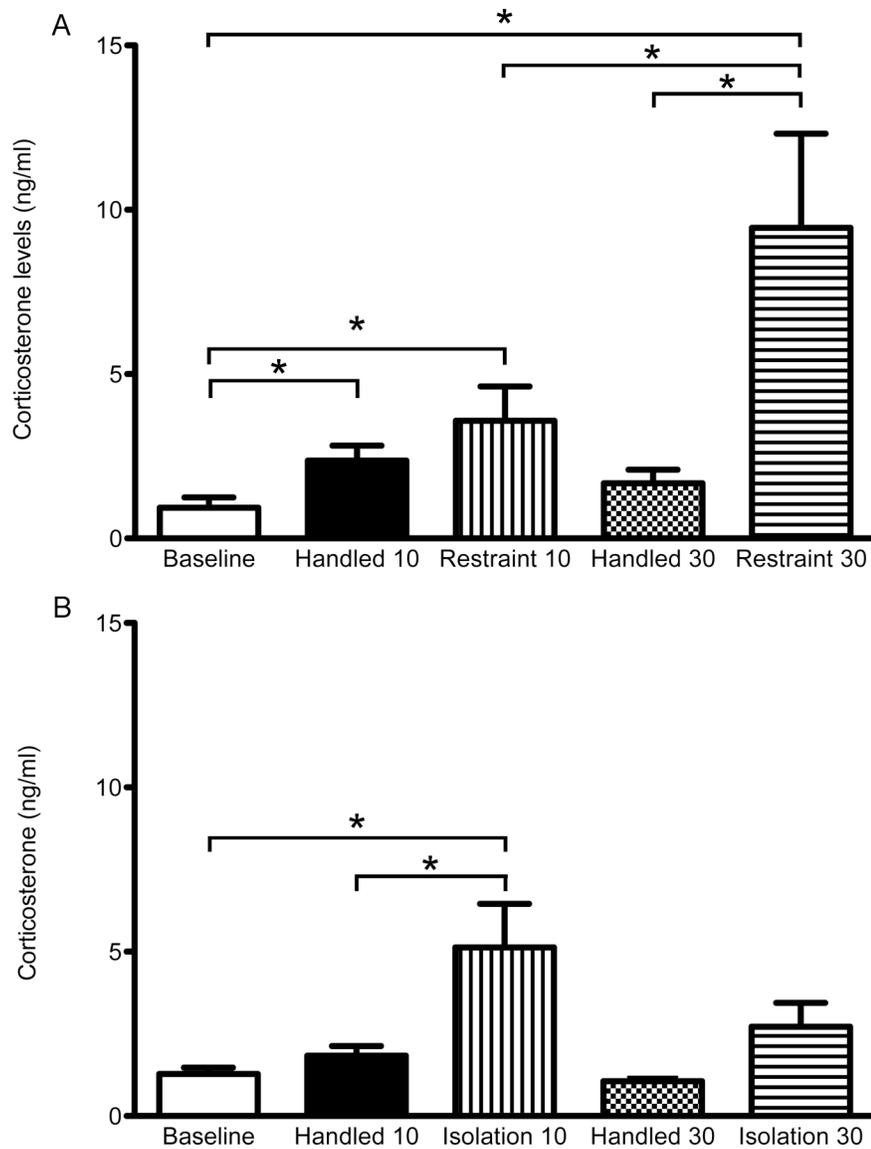
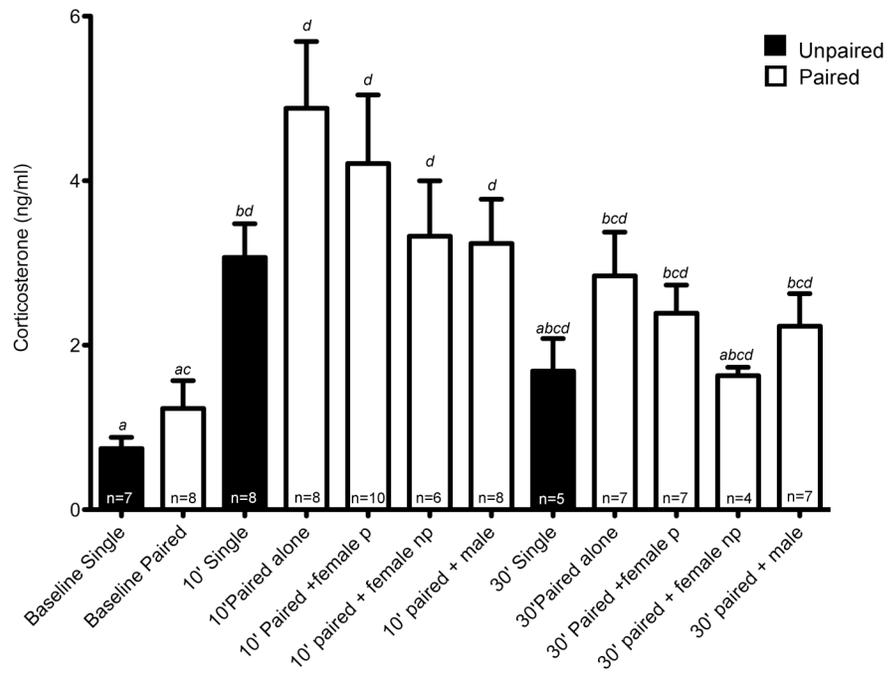


Figure 4.1. Corticosterone levels (mean \pm SEM) in groups of males measured at baseline, 10 minutes after handling, after 10 minutes of restraint, 30 minutes after handling or after 30 minutes of restraint (A), and levels measured at baseline, 10 minutes after handling, after 10 minutes of isolation, 30 minutes after handling and after 30 minutes of isolation (B) in male zebra finches. N =8-9/group, * p <0.05.

Figure 4.2. Corticosterone levels (mean \pm SEM) in groups of single or paired male zebra finches measured at baseline or in response to 10 or 30 minutes of isolation either alone, with the female partner (p), with a female non-partner (np) or another male. Both female non-partner and male conspecifics were obtained from the same aviary as the subject. Different letters above error bars indicate significant differences between bars, $p < 0.05$.



Discussion

Summary of Results

Through a series of three experiments we determined the effect of restraint and isolation on plasma corticosterone levels in zebra finches. In addition, we sought to address the role of conspecifics in the social buffering of stress responses in this highly gregarious and socially monogamous species. Our results from Experiment 1 are similar to the results from previous studies in mammalian and avian species. Both 10 minutes and 30 minutes of restraint results in higher levels of circulating corticosterone as compared to unstressed controls or baseline levels as well as the ‘handled 30’ group. When birds were handled (capture and immediate release into their home aviary) and sampled 10 and 30 minutes later, corticosterone levels were significantly higher than baseline in the group that was sampled 10 minutes after handling but not 30 minutes after handling, suggesting that the elevation of corticosterone that we observed after 10 minutes of restraint might be due to handling in addition to restraint.

In our second experiment, when a different set of birds were subjected to handling and isolation, we observed that corticosterone levels of the group that was exposed to handling and sampled 10 or 30 minutes later were not significantly different from baseline. Birds that were exposed to 10 minutes of isolation had significantly higher levels of corticosterone when compared to the baseline, ‘handled 10’ and ‘handled 30’ groups. In contrast, on exposure to 30 minutes of isolation, corticosterone levels of zebra finches were no different from baseline or handled groups. These data suggest that 10 minutes of isolation is seen as a stressful event therefore confirming our hypothesis that the removal of an individual from its home cage and placing it in a novel surrounding in isolation is a stressful event. From our experiment, it is hard to tease apart the effect of novelty of being placed in a small cage in a different room and

the effect of being alone and far away from one's conspecifics. It is likely that both these factors play a role in the higher levels of corticosterone in the 'isolation 10' group. Within 30 minutes of isolation, birds seem to habituate to the absence of conspecifics and their new surroundings, as corticosterone levels in the 'isolation 30' group were no different from baseline. To our knowledge this is the first study to study the effect of short periods of isolation on corticosterone in both unpaired and paired zebra finches. It should be noted that corticosterone levels in response to isolation were not as high and did not last as long as 30 minutes as the corticosterone response to restraint suggesting that zebra finches adapt rapidly to isolation. Restraint, on the other hand, is potentially viewed as a more threatening event.

In the third experiment, we addressed the role of social buffering by conspecifics when an individual is exposed to an adverse situation. To do so, we first exposed unpaired birds to 10 and 30 minutes of isolation as we did in the previously described experiment. We replicated our results and found that when exposed to 10 minutes of isolation, corticosterone levels of unpaired zebra finches are significantly higher than baseline but on exposure to 30 minutes of isolation, corticosterone levels are no different from baseline. When paired birds were subjected to the same treatment, the results obtained were similar to the unpaired birds in that 10 minutes but not 30 minutes of isolation resulted in higher levels of corticosterone compared to baseline. As paired birds had built nests with their partners and had laid eggs in their home aviaries, we wanted to know if being separated from their eggs and partners would elevate their corticosterone levels further but we did not observe any such effect. We also exposed paired male birds to 10 and 30 minutes of isolation or removal from their home aviary along with their partners to see if the presence of their female partners would prevent any stress response at the 10 minute time point but this was not the case. Corticosterone levels of paired male birds that were placed in the isolation

chamber along with their female partners were significantly elevated from baseline. Similar results were obtained when a paired male was isolated a familiar female (not partner) housed in the same aviary as the subject and when a paired male was isolated along with a familiar male also from the same aviary as the paired male. At the 30 minute time point, corticosterone levels of paired male zebra finches that were isolated alone or with another bird were no longer significantly different from baseline. These results suggest that the presence of conspecifics does not act as a buffer against stressors in paired male zebra finches. We did not perform the same experiments to test the effect of social buffers in unpaired zebra finches. It is possible that social buffering against stressors by conspecifics in paired males that have nest boxes might not be an effective way of reducing a stress response as the absence of their nest box and eggs might override any comforting stimuli provided by conspecifics.

Social isolation in avian species

Stressful effects of social separation and isolation have been observed in other avian species. When pair-housed domestic chicks were subjected to a social separation paradigm of 7 minutes, both male and female chicks showed an elevation of corticosterone suggesting that the manipulation was stressful for these birds (Jones and Williams, 1992). Independent of season, a gregarious non-monogamous avian species, starlings showed an increase in corticosterone levels when they were isolated from their social groups (Apfelbeck and Raess, 2008). Greylag geese are a long-term monogamous, female-bonded avian species consisting of primary families comprising fledged geese and their parents as well as secondary families consisting of parents and offspring that failed to leave to group in the following breeding season. Adult and sub-adult individuals of both primary and secondary families secrete lower levels of corticosterone in challenging situations and fair better in antagonistic interactions as

compared to geese that did not belong to families (Scheiber et al., 2005; Scheiber et al., 2009). When pair-housed roosters were separated and housed in single cages, a significant elevation of corticosterone was observed 2 days hours after the relocation of the birds after which levels returned to baseline. The authors suggest that after 2 days of single housing, the roosters were habituated (Carlsson et al., 2009). In our experiment, corticosterone levels of paired zebra finches that were isolated in a novel environment away from their partners and flocks, corticosterone levels returned to baseline within 30 minutes. This might be adaptive strategy as in the absence of any clear adverse stimuli, it would not be beneficial to maintain high circulating levels of corticosterone due to the gamut of wide-ranging harmful effects exerted by this hormone over a long duration of time that include inhibition of reproduction, immune system suppression, neuronal cell death, inhibition of growth, interference with cell signaling cascades. Therefore although in the short term, elevated glucocorticoids may aid in survival, there is a high cost to the organism if these levels remain elevated over long durations (McEwen and Stellar, 1993; McEwen, 1998; Sapolsky et al., 2000; Wingfield and Sapolsky, 2003).

Social organization, relationships, individual status and social buffering

Social buffering has been observed in a number of mammalian species including non-human primates, humans and rodents during infancy as well as adulthood (Kikusui et al., 2006; Hennessy et al., 2009). These studies led us to predict that the adrenocortical activation observed when zebra finches are isolated from their partners would be mitigated or reduced if they were isolated along with their pair-bonded partners. In a study performed in zebra finches it was observed that 24-hour isolation and pair separation resulted in elevated corticosterone levels. Hormone levels returned to baseline when the separated birds were reunited with their partners. In addition, even if the birds that were separated from their partners along with a same-sex bird, an

elevation of corticosterone levels was observed after a 24-hour period (Remage-Healey et al., 2003). Our results are interesting in this light, suggesting that at least the initial stress response in response to 10 minutes of isolation is not buffered by even the presence of the female partner (or any other bird) in male zebra finches and after 30 minutes, corticosterone levels in paired birds are no longer significantly different from baseline. The profile of changes in corticosterone levels is similar in unpaired and paired birds suggesting that separation from the home aviary is an important reason underlying the increase in corticosterone levels in both unpaired and paired birds. It is possible that there is a time course of changes in corticosterone levels in response to isolation and separation in zebra finches. Therefore although at thirty minutes corticosterone levels are no different from baseline, a longer duration of isolation such as 24 hours would result in elevated levels of corticosterone in separated birds as observed by Remage-Healey et al. (2003).

It might be the case that the social system of a species dictates the stress buffering effects by conspecifics. Studies in primates have shown the effect of social organization on social buffering. Squirrel monkeys (*Saimiri sciureus*) and titi monkeys (*Callicebus cupreus*) are new world primates of the Cebidae family and bear strong similarities of diet, behavior and extent of range. But they are different in social organization. Squirrel monkeys are social primates that live in large mixed sex and age-graded groups composed of 10-50 individuals. Adult monkeys primarily associate with individuals of their own age and sex resulting in a sexually segregated social structure (Mendoza et al., 1991; Hennessy et al., 2009). Titi monkeys in contrast, are socially monogamous mammals and pair-bonded individuals spend copious amounts of time together. They live in small groups of 2-5 animals and the nucleus of the group is a stable male-female pair (Hoffman et al., 1995; Fernandez Duque et al., 1997). Interestingly, when male-female pairs of both species were exposed to novelty, it was

observed that the cortisol response of the squirrel monkey pair was not buffered by the presence of the partner whereas the presence of the partner reduced the HPA response of Titi monkeys (Hennessy et al., 1995). When adult squirrel monkeys were exposed to snakes, once again the presence of multiple companions from the social group successfully dampened the stress response of the subject (Coe et al., 1978; Stanton et al., 1985). Callitrichid primates such as marmosets (*Callithrix kuhli*) also have long-term male-female pair bonds characterized by high rates of affiliative behaviors. In this species too, the presence of the partner in a novel environment dampened the stress response as measured through cortisol levels and stress-related behaviors (Smith et al., 1998). In contrast to squirrel monkeys, social buffering in pair-housed rhesus monkeys on exposure to novelty or was provided by the presence of a single companion (Winslow et al., 2003). These experiments underscore the importance of social organization in the social buffering abilities of conspecifics.

The importance of social relationships and stress buffering can be gleaned from a large body of work concerning social buffering in guinea pigs (*Cavia porcellus*). Guinea pigs are a highly gregarious South American rodent species. Domestic guinea pigs form harem groups consist of a few females and a male. The males direct most of their socio-sexual behaviors towards females of their harems. Therefore males are bonded to some females and are familiar with other females in their colony (but a different harem group). When an adult male was exposed to a novel environment with a female from its harem, the cortisol elevation was mild compared to those males that were exposed to a novel stimulus alone. When the harem female was replaced by an unfamiliar female from a different colony or a familiar female from another harem, dampening of the stress response was not observed. Similarly for female guinea pigs, the presence of the male partner during exposure to novelty reduced the rise of cortisol levels. The presence of a familiar male from the same social group did not have a

buffering effect (Hennessy, 1999; Graves and Hennessy, 2000; Hennessy et al., 2000; Adrian et al., 2008; Hennessy et al., 2008; Hennessy et al., 2009; Maken and Hennessy, 2009). In our experiments, the presence of either a female partner, a familiar female or a familiar male did not have any social buffering effects on the HPA axis stress response when male zebra finches were separated from their home aviary with any one of the above classes of bird. As zebra finches are a highly social species it is possible that the presence of a single conspecific is not adequate to prevent a rise in corticosterone. Once again, these results might underscore the importance of social organization and the buffering abilities of conspecifics. It is also possible that a longer duration of isolation would reflect the role of different conspecifics and their social buffering effects. It is possible that the first 10 minutes of isolation is a rapid HPA axis response that occurs regardless of the presence of conspecific, irrespective of the relationship of the conspecific and the subject.

Another factor that might play a role in the regulation of the HPA response of the subject in isolation along with a conspecific, is the status and behavior of the conspecific. In a highly social species, it is critical to account for the dominant-subordinate relations, aggression as well as sexual interaction (Kiyokawa et al., 2004; Kikusui et al., 2006). A recent study examined the social buffering effect and its relation the stress status of a conspecific. The authors found that when rats were subjected to hyperthermia with unstressed partners, the subjects displayed attenuated stress-induced hyperthermia and fearful behaviors. In comparison, rat partners that were shocked prior to the hyperthermia were not as good in preventing these stress – related behaviors in the subjects (Kiyokawa et al., 2004). In our experiment, we did not observe the interactions of the subjects with their familiar conspecifics during 10 and 30 minutes of isolation and therefore cannot conclude if any aggressive or sexual

behaviors played a role in either enhancing or mitigating a stress response in a particular subject.

Conclusions

In summary, our experiments have shown that periods of isolation and separation as short as 10 minutes can result in the activation of the HPA axis of zebra finches and are reflected by elevated corticosterone levels in isolated birds. Levels of corticosterone are no longer significantly different from baseline after 30 minutes of isolation. It would be interesting to know if the removal of a bird from one aviary into another aviary with a similar social composition as its home aviary, would be perceived as stressful by zebra finches. In addition would the isolation of an individual from a territorial species be perceived as stressful? Birds like song sparrows likely spend periods of time when they are the only bird their territory in the absence of conspecifics (Akçay et al., 2009). Therefore it is likely that they are habituated to periods of 'alone time' and therefore might display a stress response when isolated for short periods of time. Finally, the presence of a conspecific does not act as a social buffer against the stress response of separated and isolated birds at least during the first 10 minutes of isolation. Our results suggest that as in many mammalian species (Kikusui et al., 2006; Hennessy et al., 2009), social organization plays an important role in the buffering effects of conspecifics in a species.

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CHAPTER 5

EARLY FEMALE DEPRIVATION HAS LONG-LASTING CONSEQUENCES ON CORTICOSTERONE RESPONSES TO ISOLATION AND CORTICOSTERONE RECEPTOR mRNA LEVELS

Abstract

Early-life stress caused by the deprivation of maternal care has been shown to have long-lasting effects on physiology and behavior of offspring in a number of mammalian species such as rats, mice and rhesus monkeys. The hypothalamic-pituitary-adrenal axis of such offspring is hyperresponsive to stressors in adulthood. Does the deprivation of maternal care alter stress responsiveness of offspring of biparental species or can fathers compensate for the absence of mothers? We sought to address this question using a monogamous and biparental avian species- the zebra finch, *Taeniopygia guttata*. Zebra finches form life-long pair bonds and both male and female birds contribute to raising their offspring. To test the effect of deprivation of maternal care, our experiment consisted of one group of birds that was raised by both male and female parents (control) and another raised by males alone (female-deprived). When the control and female-deprived offspring reached adulthood, they were subjected to restraint and isolation, and their corticosterone levels were measured. We also measured the baseline levels of the two corticosterone receptors; glucocorticoid receptor and mineralocorticoid receptor in specific areas of the brain. Our results suggest female-deprived offspring are hyperresponsive to stressors like isolation in comparison to controls. Furthermore, mRNA levels of both GR and MR receptors are altered in female-deprived offspring in comparison to controls, a region specific manner.

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis mediates the response to stressful events in vertebrates. The parvocellular cells of the paraventricular nucleus of the hypothalamus act as the primary players for the initiation of the neuroendocrine cascade in response to physical and psychological stresses. Input from the limbic system as well as the brain stem triggers release of corticotrophin releasing hormone (CRH) and arginine-vasopressin (AVP). These peptides are secreted into the hypophyseal portal system by which they are transported into the anterior pituitary where they stimulate the release of adrenocorticotrophic hormone (ACTH) by the corticotroph cells into the circulatory system. Once in circulation, ACTH is transported to the adrenal cortex where it stimulates the release of glucocorticoids (McEwen et al., 1995; Lightman, 2008). Glucocorticoids have a multitude of acute effects some of which are enhancing metabolism and channeling energy to muscles, stimulation of the immune system, inhibiting reproductive behaviors, enhancing cognitive abilities and cerebral glucose utilization and decreasing appetite. These effects aid in the survival of an organism. Chronically elevated glucocorticoid levels in an organism on the other hand have highly deleterious effects in the long term (Plotsky et al., 1998; Sapolsky et al., 2000).

In mammals and birds, there are two major glucocorticoid receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). In mammals, the highest density of GR expression is in the paraventricular nucleus of the hypothalamus and the hippocampus whereas MR is expressed in the lateral septum and hippocampus (Reul and Dekloet, 1985). Recent studies have shown that both receptors are expressed in the avian brain. In situ hybridization studies in the zebra finch brain have shown that GR mRNA transcripts are concentrated in the hippocampus, hypothalamic paraventricular nucleus, ventromedial hypothalamus,

tuberohypothalamus, optic tract, nidopallium and the cerebellum. MR mRNA was detected in fewer areas with highest concentration in the hippocampus (Hodgson et al., 2007; Dickens et al., 2009). Glucocorticoid receptors are selective for naturally occurring as well as synthetic glucocorticoids and have a lower affinity for them as compared to the mineralocorticoid receptors. In addition to mediating the effects of glucocorticoids in the nervous system and the rest of the body, GR and MR play a key role in the regulation of the HPA axis via both negative and positive feedback. Corticosterone binding to GR in the hippocampus is an important mechanism of negative feedback of the HPA axis. The balance of expression of both the GR and MR is thought to be critical to maintain homeostasis within an organism (de Kloet, 2000; de Kloet et al., 2005; de Kloet et al., 2008).

Manipulations of the early environment have been known to enhance or dampen the HPA axis response to different stressors. In uniparental mammals, an increase or decrease in maternal care has been shown to affect the regulation of the stress response along with altered GR and CRH gene expression in the hippocampus and hypothalamus respectively. In rats where females are the sole providers of parental care, periods of deprivation of maternal care enhances the stress responsiveness of the HPA axis of rat offspring. This effect lasts well into adulthood. In response to a stressor such as restraint, ACTH and corticosterone levels in maternally-deprived rats remain elevated for a longer duration of time as compared to control animals (Lippmann et al., 2007). In addition, maternal deprivation has anxiogenic effects as these offspring are highly fearful in adulthood, performing poorly in behavioral tests of novelty that involve feeding or exploring a novel environment such as an elevated plus-maze. Studies have also shown that levels of GR expression in the hippocampus of maternally deprived offspring in adulthood are lower than that of control offspring. CRF levels in the paraventricular nucleus of maternally deprived offspring are higher

when compared to control offspring. These altered expression patterns of GR and CRF contribute to hyperresponsive HPA axis in maternally deprived offspring (Plotsky and Meaney, 1993; Anisman et al., 1998; Francis and Meaney, 1999; Meaney, 2001).

Little is known about the effects of maternal or paternal deprivation in biparental species where both parents contribute to raising offspring. In biparental species, it is possible that the parent who remains can compensate for the absence of its partner and thereby shield its offspring from any adverse effects. In the present study, we sought to address the effect of maternal deprivation on the HPA axis of offspring of a lifelong monogamous and biparental avian species- the zebra finch, *Taeniopygia guttata*. Zebra finch offspring were raised by their fathers (female-deprived) in complete absence of their mothers from an early stage or by both parents (control). When these offspring reached adulthood, their corticosterone levels were measured in response to restraint and isolation. In addition, levels of GR and MR mRNA were measured in specific brain areas – hippocampus and hypothalamus as they are components of the HPA axis and the cerebellum as a control region, to address the possibility that any differences in corticosterone levels between female-deprived offspring and control offspring in response to stressors might be mediated by changes in corticosterone receptor expression.

Materials and Methods

Breeding colonies, female deprivation manipulation, and subsequent housing

Male and female zebra finches that were raised in the laboratory in the same colony and had prior breeding experience were allowed to pair in aviaries. All blood sampling was performed between 9am-12pm. Birds were housed on a 14:10 hour light /dark cycle. Humidity ranged from 30-70% and room temperatures were maintained at 22°C. Each breeding aviary (0.94 X 0.76 X 0.94 m) was divided into two halves. In one half, both male and female birds raised their young (control offspring) whereas in

the other half, male birds raised the young alone (female-deprived offspring). Adult females were removed from this half of the aviary when the chicks were between 2-12 days old. Chicks were not provided with supplemental crop feeding and the adult males raised them to independence. Although the female-deprived offspring did not have any tactile contact with adult females after the manipulation, they had visual and auditory contact with adult females in the adjacent half of the aviary, once they had fledged. At 45-55 days of age, the offspring from both groups were moved into unisex aviaries. At this age, zebra finches are no longer dependent on their parents for food and can be sexed based on their plumage coloration (Zann, 1996b). The unisex male and female aviaries were in the same room and therefore birds could see and hear individuals of the opposite sex. All animal procedures conformed to Federal and State regulations and were approved by the Cornell University IACUC.

Corticosterone levels in control and female-deprived birds that were subjected to isolation or restraint

Adult control and female-deprived offspring of both sexes were sampled between 100-150 days of age. Birds were isolated from their peers by placing them in single cages (45 X 25 X 23 cm) in a room that had no other birds in it for either 10 minutes ('isolation 10') or 30 minutes ('isolation 30'). Birds were subjected to restraint by placing them in brown paper bags in a lit room for either 10 minutes ('restraint 10') or 30 minutes ('restraint 30'). On one day, 1 control and 1 female-deprived bird were captured from their aviaries and bled in less than 3 minutes to measure baseline levels ('baseline') of corticosterone. Following that, a control and female-deprived bird was subject to 10 minutes of isolation or restraint. Another pair of control and female-deprived birds were exposed to 30 minutes of isolation or restraint. Immediately after capture for baseline levels or after isolation or restraint, a blood sample of

approximately 100ul was obtained under 3 minutes, by piercing the alar vein of the left wing using a 26-gauge sterile needle (used once for each bird) and gathering the welling blood using heparinized capillary tubes. Samples were centrifuged at 12,000 rpm and plasma was stored at -80°C.

This paradigm was carried out over several days such that n=12 for control baseline group, n=12 for control isolation 10 minutes group, n=12 for control isolation 30 minutes group, n=11 for female-deprived baseline group, n=13 for female-deprived isolation 10 minutes group, n=12 for female-deprived isolation 30 minutes group, n=13 for control baseline group, n=13 for control restraint 10 minutes group, n=13 for control restraint 30 minutes group, n=13 for female-deprived baseline group, n=13 for female-deprived restraint 10 minutes group, n=14 for female-deprived restraint 30 minutes group. This was not a repeated measures design experiment and birds were randomly assigned to treatment groups. Within the isolation experiment, a single bird could belong to any one of the 'baseline', 'isolation 10' or 'isolation 30' groups. Within the restraint experiment, a single bird could belong to any one of the 'baseline', 'restraint 10' or 'restraint 30' groups. Although the same pool of birds was subjected to isolation and restraint with a month between the two stress paradigms, it was not necessarily the case that a bird that was subject to 10 minutes of isolation was exposed to 10 minutes of restraint a month later.

Enzyme-immunoassay to measure corticosterone

We used corticosterone EIA kits (Cayman Chemical, cat.no.500651). We ran samples in duplicate at a dilution of 1:10 in buffer and took the average of both of readings to obtain the final value of each sample. The intra- and interassay variances were 8.13% and 9.6% respectively.

Tissue collection and absolute- quantitative real-time PCR (qPCR) to measure GR and MR mRNA levels

Male and female birds that were subjected to isolation and restraint were sacrificed in a CO₂ chamber. The time interval between the stress paradigms and sacrifice was 2-4 months. Immediately after sacrifice, brains were dissected out and placed on dry ice until frozen then stored at -80°C.

Brains were then sectioned in a cryostat at 200um thickness. A Palkovitz punch set was used to punch out three brain areas of interest (hippocampus, hypothalamus and cerebellum) and these were stored in eppendorf tubes. RNA was isolated from these brain regions using Trizol (Invitrogen, Carlsbad, CA). Genomic DNA contamination was removed by DNase I treatment (Invitrogen, Carlsbad, CA) followed by reverse transcription using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA).

Absolute qPCR methods were adapted from a previous study (Arterbery et al.). Absolute qPCR was carried out on cDNA from different brain regions using gene-specific primer pairs for the glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and the house-keeping gene Beta-actin (BA). All primer pairs amplified products 150 nucleotides in length, isolated a single product with no dimer pairs, and had a standard efficiency of no less than 99%. The following primer pairs were designed based on published zebra finch nucleotide gene sequences. Glucocorticoid receptor (GR GeneID: 100008583) forward primer: 5' TGA AGA GCC AGT CCC TGT TCG AG, GR reverse primer: 5'CAA CCA CAT CAT GCA TAG AGT CCA GCA, Mineralocorticoid receptor (MR GeneID: 751776) forward primer: 5'AAG AGT CGG CCA AAC ATC CTT GTT CT, MR reverse primer: 5'AAG AAA CGG GTG GTC CTA AAA TCC CAG, Beta-actin (BA GeneID: 751978) forward primer:

5'TCA TCA CCA TTG GCA ATG AGA GGT TCA G, BA reverse primer: 5'GCA TAC AGG TCC TTA CGG ATG TCC A.

All real-time reactions were run in triplicate along with no template controls and contained the following: 10 ul of 2x Power SYBR Green PCR Master Mix (Applied Biosystems), 2 ul of forward and reverse primer at a concentration of 100 nM, 4 ul of H₂O, and 2 ul of the appropriate cDNA. Reactions were run on an Applied Biosystems 7900 HT Sequence Detection System at the Cornell University Life Sciences Core Laboratory Center under the default manufacturer's conditions (SDS 2.1 software) using 60°C as a melting temperature. Gene copy number was determined for each tissue sampled from each individual using standard curve analysis for all gene primer sets, including housekeeping genes. The standards covered a linear range of 5x10⁶ to 100 copy/ul. Briefly, the raw Ct values were converted to copy number with the standard curve produced using SDS 2.1 (Applied Biosystems). Each target gene copy number was normalized using the beta actin copy number from the same tissue sample. Normalized data are reported as a ratio of copy numbers of GR or MR to BA.

Statistics

SPSS version 17.0 was used for statistical analysis. Corticosterone values were analyzed using univariate ANOVA. GR and MR values were analyzed using mixed linear model analysis. Receptor data were ln transformed to meet assumptions for normality and equality of variances. Bonferroni tests were performed to correct for multiple comparisons. Significance levels were set at p<0.05 for all tests.

Results

Corticosterone levels in control and female-deprived offspring subjected to 10 or 30 minutes of restraint

Overall, there was no significant interaction between treatment and time ($F_{2,72}=2.310$, $p=0.107$) or main effect of treatment ($F_{1,72}=0.776$, $p=0.381$). There was a significant main effect of time ($F_{2,72}=38.767$, $p<0.001$). Within the control group, baseline corticosterone levels were significantly lower than the birds exposed to 30 minutes of restraint ($p<0.001$) but not 10 minutes of restraint ($p=0.09$). Corticosterone levels of birds exposed to 10 minutes of restraint were significantly lower than the birds exposed to 30 minutes of restraint ($p=0.048$). Within the female-deprived group, baseline corticosterone levels were significantly lower than the birds exposed to 10 minutes of restraint ($p<0.001$) and 30 minutes of restraint ($p<0.001$). Corticosterone levels of the birds exposed to 10 minutes of restraint were significantly lower than the birds exposed to 30 minutes of restraint ($p<0.001$). There were no significant differences between control and female-deprived groups for baseline levels as well as 10 minutes or 30 minutes of restraint.

Corticosterone levels in control and female-deprived offspring subjected to 10 or 30 minutes of isolation

Overall, there was a significant interaction between treatment (control or female-deprived) and duration of isolation ($F_{2,67}=4.795$, $p=0.011$). There was no significant main effect of treatment ($F_{1,67}=3.535$, $p=0.064$) and a significant main effect of time ($F_{2,67}=18.59$, $p<0.001$). Within the control group, baseline levels were significantly lower from those of birds subjected to 10 minutes of isolation ($p<0.001$). The corticosterone levels of the group subjected to 10 minutes of isolation were significantly higher from the group subjected to 30 minutes of isolation ($p=0.003$).

Within the female-deprived group, baseline corticosterone levels were significantly lower than the group exposed to 10 minutes ($p < 0.001$) or 30 minutes of isolation ($p < 0.001$). The corticosterone levels of the groups exposed to 10 or 30 minutes of isolation were not significantly different from one another ($p = 0.284$). Between the control and female-deprived groups, corticosterone levels in the control group exposed to 30 minutes of isolation were significantly lower than the female-deprived group exposed to 30 minutes of isolation ($p = 0.001$).

Glucocorticoid receptor (GR) mRNA levels in the hippocampus, hypothalamus and cerebellum of control and female-deprived birds

Overall there was no significant interaction between treatment group (control or female-deprived) and brain region (hippocampus, hypothalamus and cerebellum) ($F_{2,53.49} = 1.495$, $p = 0.233$). There was a significant main effect of treatment ($F_{1,28.18} = 6.78$, $p = 0.014$) and region ($F_{2,53.49} = 46.59$, $p < 0.001$). GR mRNA levels did not differ between control and female-deprived groups within the hippocampus ($p = 0.681$) and cerebellum ($p = 0.165$). Within the hypothalamus, GR mRNA levels were significantly lower in the female-deprived group as compared to the control group ($p = 0.036$).

Mineralocorticoid receptor (MR) mRNA levels in the hippocampus, hypothalamus and cerebellum of control and female-deprived birds

Overall there was no significant interaction between treatment group (control or female-deprived) and brain region (hippocampus, hypothalamus and cerebellum) ($F_{2,52.79} = 0.926$, $p = 0.402$). There was a significant main effect of treatment ($F_{1,27.97} = 24.38$, $p < 0.001$), but not of region ($F_{2,52.79} = 2.33$, $p = 0.107$). MR mRNA levels of the female-deprived group were significantly lower than control group within the hippocampus ($p = 0.042$), hypothalamus ($p < 0.001$) and cerebellum ($p < 0.001$).

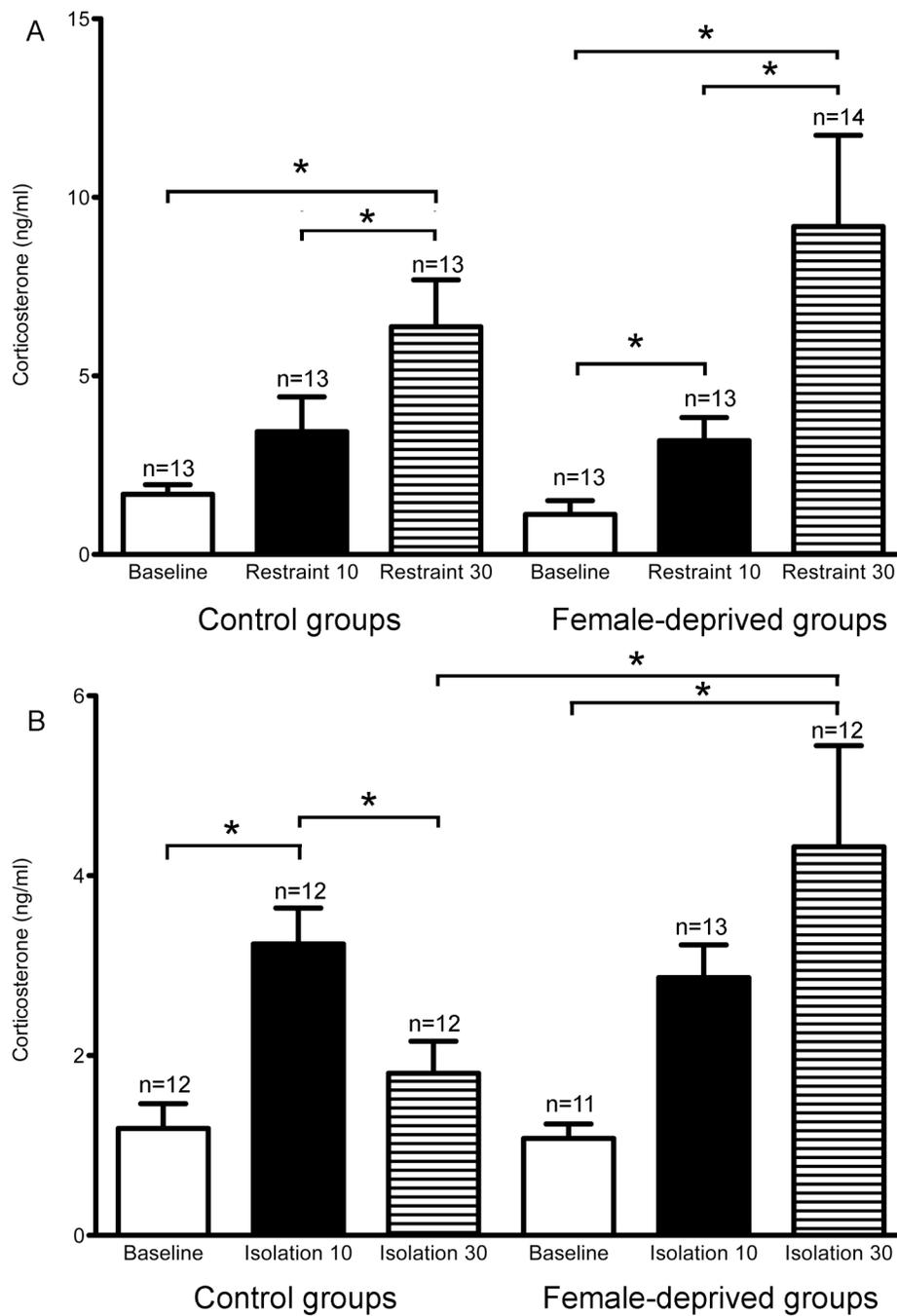


Figure 5.1. Corticosterone levels (mean \pm SEM) in control and female-deprived birds of both sexes in (A) baseline, restraint 10 and restraint 30 groups and (B) baseline, isolation 10 and isolation 30 groups, * $P < 0.05$.

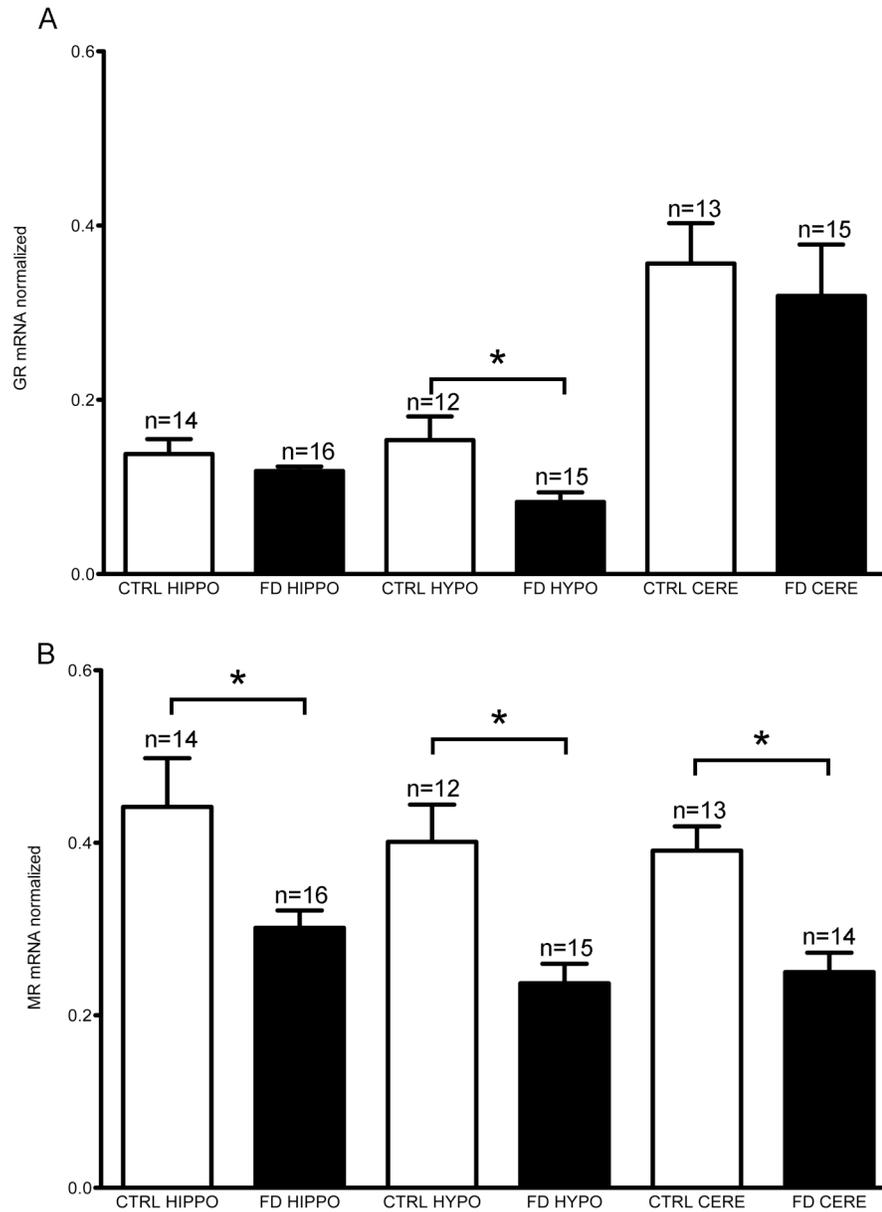


Figure 5.2. GR mRNA (A) and MR mRNA (B) levels (mean \pm SEM) in control (CTRL) and female-deprived (FD) adult zebra finch offspring in the hippocampus (HIPPO), hypothalamus (HYPO) and cerebellum (CERE), * $P < 0.05$.

Discussion

The experiments presented have shown that corticosterone levels in both control and female-deprived adult offspring exposed to 10 minutes of isolation were higher than corticosterone levels of undisturbed birds (baseline) belonging to control and female-deprived groups respectively. Corticosterone levels in adult offspring of the control group exposed to 30 minutes of isolation were no longer significantly different from levels in undisturbed birds of the control group. In contrast, corticosterone levels in adult offspring of the female-deprived group exposed to 30 minutes of isolation were significantly higher than levels in the undisturbed birds (baseline) of the female-deprived group. Additionally, corticosterone levels of adult control offspring exposed to 30 minutes of isolation were significantly lower than corticosterone levels of female-deprived offspring subjected to the same treatment. In response to restraint, there were no significant differences in the levels of corticosterone in control versus female-deprived zebra finches at either the 10 minute or the 30 minute time point. In both control and female-deprived groups, corticosterone levels of birds exposed to 30 minutes of stress were significantly higher than birds that were undisturbed. Levels of corticosterone in birds belonging to female-deprived group that were exposed to 10 minutes of restraint were significantly higher than baseline and significantly lower than the birds exposed to 30 minutes of restraint. Similarly, levels of corticosterone in birds belonging to control group that were exposed to 10 minutes of restraint, were significantly lower than the levels of birds exposed to 30 minutes of restraint but were not significantly higher than levels in birds that were undisturbed. Additionally, mRNA levels of GR were lower in the hypothalamus of female-deprived males in comparison to control birds and mRNA levels of MR of female-deprived birds are lower than mRNA levels of control birds specifically in the hypothalamus, hippocampus and the cerebellum. These results suggest that the absence of mothers

and adult females during development in zebra finches, affects HPA axis development in offspring, leading to HPA axis hyperresponsiveness in adulthood. It is possible that both mothers and unrelated adult females influence HPA axis development through adult offspring interactions as zebra finches are a highly social species and in large aviaries it is difficult to rule out the role of one over the other. To our knowledge, the effects of perturbations of the early social environment on the HPA axis of birds have not been explored thus far.

The maternal deprivation literature from mammals has shown that periods of maternal deprivation during the stress hypo-responsive period can lead to life-long changes in the responsiveness of the HPA axis. The stress hypo-responsive period lasts from postnatal day 2 to day 14 in rat pups and it is a period when the HPA axis response to stressors is minimal. This period protects the nervous system of the developing pup from the deleterious effects of high levels of corticosterone (Sapolsky and Meaney, 1986). If rat pups are subjected to more than 3 hours of maternal separation or deprivation per day during this period, their HPA axis has been shown to be hyperresponsive to stressors in adulthood. When adult maternally deprived rat offspring were subjected to restraint, their serum ACTH and corticosterone levels were higher than those of control offspring. In addition, mRNA levels of CRH have been shown to be higher in the paraventricular nucleus of the hypothalamus in maternally deprived rats in adulthood along with lower levels of GR in the hippocampus. Such molecular changes are indicative of a hyperresponsive HPA axis as enhanced CRH synthesis would stimulate higher levels of corticosterone release from the adrenal cortex and lower levels of GR in the hippocampus would indicate decreased GR mediated negative feedback into the HPA axis by the hippocampus (Plotsky and Meaney, 1993; Anisman et al., 1998; Francis and Meaney, 1999; Lippmann et al., 2007).

It is likely that birds also have a stress hyporesponsive period. A study using Florida scrub-jay, an avian species with altricial young, showed that nestlings had a lower corticosterone response that lasted for a shorter duration, to capture and handling when compared to older birds (Rensel et al., 2010). Similarly, when newly hatched wild starlings were subjected to restraint, their corticosterone levels were not significantly higher than the control group whereas corticosterone levels in 10 day old starlings subjected to restraint were higher than controls (Schmidt et al., 2009). Similar observations were made in nestlings of mocking birds and white-crowned sparrows (Sims and Holberton, 2000; Wada et al., 2007). In zebra finches, an adult-like stress response was observed 16 days after hatching but not prior to that (Wada et al., 2009). As it is likely that birds also have a stress hyporesponsive period during development, as in mammals, perturbing their early social environment would alter the responsiveness of their HPA axis.

Although the effect of developmental manipulations on GR and MR expression has not been explored, studies have found interesting effects of adult manipulations on levels of GR and MR mRNA expression in the avian brain. Hodgson and colleagues have shown that in a line of zebra finches selected for a high corticosterone response to an acute stressor, MR but NOT GR mRNA levels were decreased in the hippocampus (Hodgson et al., 2007). In another study, the authors showed that when wild-caught adult starlings were exposed to chronic unpredictable stress over a period of sixteen days, levels of MR mRNA but not GR mRNA levels were lower in the hippocampus and GR mRNA expression levels were lower in the hypothalamus when compared to control birds (Dickens et al., 2009). Our results are consistent with these studies, as we also observed lower levels of MR mRNA in the hippocampus and lower levels of GR mRNA in the hypothalamus in female-deprived birds lending support to our results that female-deprived birds are hyperresponsive to stressors in adulthood.

Most studies to tease out the role of GR and MR have been performed in mammals but it is clear that the brains of birds express both receptors in similar regions and do have similar negative feedback mechanisms (Kovacs et al., 1989; Wingfield and Kitaysky, 2002; Vandenborne et al., 2005b; Vandenborne et al., 2005a; Hazard et al., 2007).

Experiments have shown that GR is important for mediating glucocorticoid feedback after a stressor activates the HPA axis and MR is necessary for regulation basal HPA tone. The GR has 5-10 nm affinity for glucocorticoids and is bound only when levels are intermediate to high such as during the peak of a circadian response or after the initiation of a stress response. In contrast, the affinity of MR for glucocorticoids is 5-10 fold higher than GR so that it is occupied even during periods of basal secretion. Glucocorticoids promote emotion, cognition, motivation and aid in memory storage of sensory stimuli. In addition, they increase metabolism and arousal. Under conditions where levels of these hormones remain chronically elevated, they can have damaging effects on cognition and physiology. In a healthy organism, glucocorticoids have a dual mode of action, the first being positive feedback on the HPA axis to allow for the permissive actions of these hormones and the second being negative feedback on stress induced activation after the passing of a stressor or adverse situations, both these actions being mediated via the GR and MR receptors (de Kloet et al., 2008).

Hippocampal MR preferentially binds to corticosterone and, due to its high affinity, is always bound to a large extent. Recent data suggest that MR activation is essential to maintain a stable excitatory tone in the hippocampus. The excitatory signals via glutamate are carried by neurons from the hippocampus that then synapse with inhibitory neurons in the hippocampus which in turn synapse on CRH producing cells in the paraventricular nucleus of the hypothalamus. Therefore glucocorticoid binding to the MR receptor results in an inhibitory tone on basal and stress-induced

HPA axis activation (de Kloet, 2000; Joels et al., 2008; de Kloet et al., 2009).

Therefore decreased MR in the hippocampus would indicate that the inhibitory tone in the hypothalamus was decreased potentially resulting in a hyper responsive HPA axis in female-deprived birds.

GR receptors in the paraventricular hypothalamus are expressed by CRF synthesizing neurons. When levels of corticosterone are high, the binding of these hormones to the GR receptors in the hypothalamus, acts as an inhibitory switch and the negative feedback loop is turned on. Thus the decrease in GR levels in the hypothalamus in female-deprived birds might be indicative of a less efficient negative feedback pathway via this region (De Kloet et al., 1998; Conrad et al., 2009; Kageyama and Suda, 2009). This might be one of the reasons that corticosterone levels are significantly higher than baseline after 30 minutes of isolation in female-deprived birds in contrast to control birds.

Although MR mRNA expression and binding sites have been reported in the cerebellum of non-human primate species, it is unclear what role this receptor plays in this region (Brooke et al., 1994; Meyer et al., 1998; Patel et al., 2000). The cerebellum has not been implicated in the regulation of the HPA axis and therefore it is unclear what effects lower MR mRNA in the cerebellum may have in female-deprived birds.

Studies in rats have shown that pups of mothers that demonstrate low levels of maternal care have decreased levels of GR in the hippocampus. This effect is mediated through the epigenetic changes in the pups. Offspring of low care-giving dams have higher levels of methylation at the GR promoter sites and therefore lower levels of transcription of the GR gene in comparison to pups of mothers with high levels of maternal care (Champagne et al., 2003; Weaver et al., 2004; Szyf et al., 2005; Champagne and Curley, 2009). Female zebra finches clump and allopreen their offspring. Perhaps the absence of these tactile behaviors in a female-deprived

environment leads to epigenetic changes in offspring that result in altered gene expression. It is possible that in the absence of their partners, male zebra finches can compensate by increasing their efforts to provide nutrition and ensure survival of offspring but due to increased energy demands it is not possible for them to provide tactile contact as well.

On a final note, it is important to debate the consequences of a hyperresponsive HPA axis in that one that remains activated for a longer duration of time in female-deprived birds as opposed to control birds. Corticosterone is critical for the survival of an organism as it is responsible for mobilizing glycogen stored in the liver and therefore releasing glucose necessary for any heightened physical activity not only under an emergency but also when environmental demands are increased such as during nest guarding or providing food for offspring. Chronically elevated levels of corticosterone are known to have harmful events on physiology, neuron morphology and cognition in mammals. Hippocampal neurons are highly vulnerable to prolonged corticosterone secretion and undergo dendritic atrophy as well as cell death under such conditions (Jacobson and Sapolsky, 1991; Sapolsky et al., 2000). It is possible that female-deprived birds are at a disadvantage if indeed elevated corticosterone mediated damage has occurred. On the other hand, it is possible that via epigenetic changes in gene expression in the female-deprived offspring, they are better prepared to cope with their environment. Offspring of low levels of maternal care rat mothers, in contrast to those of high maternal care mothers, displayed significantly impaired LTP under basal conditions but surprisingly a significantly enhanced LTP in response to high corticosterone *in vitro*. Adult rat offspring of mothers that display low levels of maternal care displayed enhanced memory relative to high maternal care offspring when tested in a hippocampus-dependent, contextual fear-conditioning paradigm (Champagne et al., 2008). These results suggest that the early social environment may

modulate optimal cognitive functioning in different environments such that offspring of high and low care mothers showing learn best under contexts of low and high stress, respectively. Data from rats suggest that through maternal care, the endocrine and cognitive systems of offspring can be ‘programmed’ to be increasingly sensitive and receptive to stressful or adverse situations. This might ensure the survival of offspring in environments where dangers are imminent. Although these effects do come at the cost of rendering on organism more susceptible to stress related pathology in the long term, they also potentially ensure survival in risky environments (Cameron et al., 2005; Zhang et al., 2006; Meaney et al., 2007; Zhang and Meaney, 2010).

Further studies will be required to know the effects of female-deprivation on cognitive abilities of zebra finch offspring as well its effects on song and song nuclei.

In conclusion, our study has shed light on the effects of altering the social environment of a socially monogamous and biparental species by the removal of all mothers and females. Such a manipulation altered HPA axis responsiveness and glucocorticoid receptor expression in female-deprived offspring in comparison to control offspring. To the best of our knowledge this is the first study to address the effects of such a manipulation in a biparental species.

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CHAPTER 6

EARLY LIFE FEMALE-DEPRIVATION HAS LONG-TERM CONSEQUENCES FOR BEHAVIOR AND FOS EXPRESSION IN MONOGAMOUS AND BIPARENTAL ZEBRA FINCHES

Abstract

An important component of mate choice is the sex of the potential mate, yet little is known about the factors that influence an individual to pair with the opposite sex. Here we addressed the role of exposure to adult females during development in adult mate choice, by raising zebra finch offspring in the absence of all adult females including their mothers. When tested as adults, most males that had been raised without females paired with other males raised in a similar environment. In contrast, male offspring that were raised in the presence of adults of both sexes paired with females in adulthood. The most likely interpretation of this dramatic effect on pairing outcome is imprinting on the father in the absence of the mother. These results thus suggest that sexual imprinting or some other form of social learning is a key developmental process for choice of sex of partner in addition to species and individual characteristics. In addition to addressing the effects of female-deprivation on behaviors and sexual partner preference we investigated their effects on immediate early gene Fos expression in male offspring that were exposed to male or female stimuli during adulthood. Our findings suggest that neural activity in the medial mesopallium and nidopallium is altered in female-deprived males in response to female stimuli, suggesting that this developmental manipulation has long-lasting behavioral and neural consequences.

Introduction

The choice of a mate is critical in species that are socially or genetically monogamous, biparental, and form long term pair bonds, as an individual's fitness depends on its mate (Black and Hulme, 1996). It is therefore important that a bird learn to recognize individuals of its own species. Previous studies have shown that birds accrue information about their social environment through their parents and siblings during a sensitive period early in development. These acquired preferences are then consolidated as they reach reproductive maturity and guide their choice of mates. This phenomenon is known as sexual imprinting (Immelmann, 1972; Kruijt, 1985; Bischof and Rollenhagen, 1999).

In addition to species recognition, it is also critical that birds recognize individuals of the opposite sex as mates. Little is known about how birds learn to prefer individuals of the opposite sex within their own species. Most of what is known about the development of sexual partner preference in socially monogamous species comes from studies of zebra finches (Adkins-Regan 2002). Zebra finches, a monogamous and biparental avian species, pair when they reach reproductive maturity and form life-long pair bonds in the wild (Zann 1996). Within this species, preferences for species and color morph have been shown to result from sexual imprinting (Immelmann, 1969; Vos et al., 1993; Witte and Caspers, 2006).

Although we know that zebra finches imprint on their parents' traits like beak color and novel ornaments, whether the birds also learn sexual partner preferences through visual and social cues of their parents' is unknown. In this species in particular, it is known that there are two stages of sexual imprinting. The acquisition phase lasts for the first 40 days of life and is when a specific partner preference is acquired by zebra finch offspring based on features of their parents. The consolidation

phase extends from day 60-100 and is when acquired preferences are cemented by exposure to adult birds of the same species (Bischof and Clayton, 1991; Kruijt and Meeuwissen, 1991; Oetting et al., 1995; Bischof, 2003).

Previous studies have demonstrated that both hormones and early social environment influence the development of sexual partner preference in this species. Females treated with estradiol benzoate during the first two weeks after hatching preferred to pair with other females, but only if they had been housed in all female aviaries as juveniles, suggesting that sexual partner preference was masculinized by estradiol treatment (Mansukhani et al., 1996; Adkins-Regan, 2002). When zebra finches were raised without adult males in the aviaries (by removing those males when the chicks were still in the nest), both sexes failed to show the normal sexual partner preference, instead pairing with both sexes equally often (Adkins-Regan and Krakauer, 2000). The effect of uniparental rearing by males alone (by removing adult females) has not been determined before.

Taken together, the effect of adult male removal and the research on sexual imprinting raise the possibility that sexual partner preference itself might be acquired in part through imprinting on the opposite sex parent or some other form of social learning. Here we addressed the role played by exposure to adult females during early development in adult pairing preference by removing all adult females from breeding aviaries when chicks were 2-12 days old.

In addition to addressing the role of adult females in the development of sexual partner preference in zebra finch offspring, we investigated the effect of such a manipulation on neuronal activation in specific brain areas associated with the phenomenon of sexual imprinting. If the absence of adult females in the early developing environment could affect sexual partner preference, it is possible that one might observe differences in the immediate early gene Fos expression in response to

male or female cues. To test our hypothesis, we raised zebra finch offspring in a biparental as well as a female-deprived environment. Once the control and female-deprived male offspring reached adulthood they were exposed to novel adult males or novel adult females or no stimulus at all. We then counted Fos-immunoreactive (Fos-IR) cells in areas of the brain implicated in the consolidation of acquired preferences, namely the mesopallium and nidopallium, in addition to limbic areas such as the lateral septum, bed nucleus of stria terminalis (BNST) and the ventromedial hypothalamus (VMH) that play a role in socio-sexual behaviors (Goodson et al., 2005).

Materials and methods

Breeding colonies, female deprivation manipulation, and subsequent housing

Male and female zebra finches that were raised in the laboratory in the same colony and had prior breeding experience were allowed to pair in aviaries. Each breeding aviary (0.94 X 0.76 X 0.94 m) was divided into two halves. In one half, both male and female birds raised their young (control offspring) whereas in the other half, male birds raised the young alone (female-deprived offspring). Adult females were removed from this half of the aviary when the chicks were between 2-12 days old. It was not necessary to provide the chicks with supplemental crop feeding and the adult males successfully raised the young to independence. Although the female-deprived offspring did not have any tactile contact with adult females after the manipulation, they had visual and auditory contact with adult females in the adjacent half of the aviary, once they had fledged. Three such breeding cycles were carried out successively with different breeding pairs for each cycle to generate adequate numbers of male and female offspring (n=24 control females, n=24 female deprived females, n=21 control males, n=21 female-deprived males). At 45-55 days of age, the offspring

from both groups were moved into unisex aviaries. At this age, zebra finches are no longer dependent on their parents for food and can be sexed based on their plumage coloration (Zann, 1996b). The unisex male and female aviaries were in the same room and therefore birds could see and hear individuals of the opposite sex. Male zebra finches that were used in the immediate early gene expression experiment were moved into individual cages (and not unisex aviaries) that were stacked beside each other between 45-55 days of age. Therefore, the birds were exposed to visual, auditory and social cues from other male birds but they could not physically interact with one another. All animal procedures conformed to Federal and State regulations and were approved by the Cornell University IACUC.

Offspring growth

Offspring were weighed, and tarsus length was measured, every 5-7 days from hatching until they were at least 40 days old, to determine any difference between chicks raised by two parents versus one. In addition, the number of mortalities was noted in both groups.

Behavioral tests

Zebra finches reach reproductive maturity between 60 and 90 days (Zann, 1996b). The partner preference and mate choice tests were performed when the birds were at least 100 days of age. The tests lasted for 2 weeks, wherein days 1-5 were the early pairing phase and days 10-14 were the late pairing phase. The testing cages were aviaries (0.94 X 0.76 X 0.94m) containing nest boxes and nest material. Each aviary contained a biased sex ratio of birds consisting of 2:1 males: females for male subjects and 2:1 females: males for female subjects. This was to ensure that subjects would have to compete for their preferred partners. Hence if the treatment had simply

affected the overall attractiveness of a bird, it would likely remain unpaired in these tests. Each testing aviary either had 3 control males, 3 female-deprived males and 3 stimulus females, or 3 control females, 3 female-deprived females and 3 stimulus males. Stimulus males and females were sexually experienced birds that were unrelated to any of the control or experimental birds and that had been housed in unisex aviaries so that they were currently unpaired. All birds were observed for 15 minutes per day during the early (days 1-5) and late (days 10-14) pairing phases to determine pairing status. The behaviors of most birds were scored (males; control n=21, female deprived n=21; females; control n=18, female-deprived n=18).

The following behaviors were recorded during each observation period by an observer blind to the subject's group using a computerized event coder (Event Coder 1.0). All behaviors have been well established as indicators of courtship, aggression, or pairing (Zann, 1996b).

Directed song bouts: a salient male courtship behavior in which the male sings to a target bird while perched close to the target. Males produce several repetitions of the song before stopping, marking the end of the song bout. Only males sing in this species.

Undirected song bouts: singing that is not obviously directed at another bird.

Aggression: zebra finches are gregarious colonial breeders. Most aggression in this species occurs in two contexts: (1) during the pairing process, as birds defend their chosen partner from pairing attempts by others, and (2) nest box defense against intrusions by others. In our experiment, we scored the number of bouts of aggression in 15 minutes of observation. Each bout consisted of one bird attacking another.

Clumping: perching in direct physical contact with another bird. In this species, clumping by adults of both sexes is only seen in paired birds or birds in the process of

pairing. This behavior was coded as duration in seconds during a 15 minute period of observation.

Allopreening: this behavior is usually performed while two birds clump with each other and involves preening another bird. It is performed by both sexes and only seen in paired birds or birds in the process of pairing. This behavior was coded as duration in seconds during a 15 minute period of observation.

In nest box: birds in the process of pairing spend substantial time in the chosen nest box together. Time (duration in seconds) in a nest box was recorded separately depending on whether the subject was in the box alone, with a male, or with a female.

Pair status: at the end of 14 days, birds that frequently spent time in a nest box together were categorized as paired.

Fos expression in response to male or female stimuli

Males that were housed individually in cages were used in these trials. Three subjects were run every day between 9 and 12am. Each subject was removed from its cage and placed in a different cage located in a room devoid of any other cages. This bird was then exposed to an unfamiliar male or female bird (placed in the same cage as the subject) or no stimulus at all for 90 minutes. The treatment groups were control male alone (n=5), control male with male (n=5), control male with female (n=5), female-deprived male alone (n=5), female-deprived male with male (n=5) and female-deprived male with female (n=5). Birds were observed for the 15 minutes of the trials.

Tissue processing

At the end of 90 minutes, the subject was deeply anesthetized using Nembutal and transcardially perfused using saline (0.9%) and paraformaldehyde solution (4% in 0.1M PBS, pH 7.4). Brains were removed and postfixed overnight followed by 30%

sucrose solution for 2 days. They were then embedded in a gelatin-sucrose medium and stored in a 30% sucrose and 10% paraformaldehyde solution until time of sectioning.

Brains were sectioned using a cryostat into 40um thick sections. These sections were then placed in 0.1M PBS buffer and stored at 4°C. Following this, immunohistochemistry to label Fos positive neurons was performed. Briefly, sections were rinsed in PBS for 20 minutes, followed by 0.5% hydrogen peroxide for 5 minutes and then a 20 minute PBS wash. The sections were then transferred into a Normal goat serum (NGS) and TritonX-100 blocking solution for 60 minutes after which they were placed in a solution containing the primary antibody (1:18000, c-Fos antibody, sc253, Santa Cruz) for 48 hours at 4°C. Tissue was then rinsed for 20 minutes in PBS buffer, and incubated with a 2% NGS secondary antibody solution (1:250 goat antirabbit; Vector Laboratories) for 90 min. Tissue was rinsed again, incubated in AB solution (Vector ABC kit; Vector Laboratories) for 1 hour, rinsed, and the avidin-biotin complex was visualized using diaminobenzine (Sigma). Sections were then mounted on gel-coated slides, dehydrated, and coverslipped. Each round of immunohistochemistry consisted of 6 wells of each brain from all 6 treatment groups (6 brains in total) to eliminate the possibility of group differences due to inconsistent immuno-staining between rounds.

Quantification

Fos counting was performed manually using an ocular grid 1cm X 1cm using an Olympus microscope at 40X magnification. Counting was done in every section that contained a given brain area (about 3-6 sections for each area).

Statistical procedures

Data from days 1-5 were averaged to summarize the early pairing phase and data from days 10-14 were averaged to summarize the late pairing phase. Mann-Whitney *U*-tests and Fisher's exact probability tests were used to analyze the behaviors recorded, using GraphPad Prism for Macintosh (version 5.0). Mixed-effects logistic regression and Fisher's exact probability test taking into account breeding cycle and nest as well as the day of the behavioral test were used to analyze the effect of female deprivation on pairing outcome using STATA for Windows (release 10). Mixed linear model analysis was used to analyze the effect of sex and treatment on offspring mass over time taking into account nest and breeding cycle using SPSS for Windows, version 16.0. Fos counts from each brain area (for each bird) were averaged taken into account the number of sections and number of Fos positive cells counted. These numbers were then averaged across all the birds for each brain area and then analyzed using mixed linear model analysis using SPSS for Macintosh, version 18.0. Fos data were natural log transformed to correct for normality and unequal variances. Bonferroni tests were performed to correct for multiple comparisons. The significance level was set at 0.05 for all tests.

Results

Pair status

We observed that the removal of adult females from the early rearing environment of zebra finches affected partner preference in adulthood. Adult males that were raised in the absence of adult females paired with males that were raised under similar conditions. We observed that 76.19% or 16 out of 21 control males had paired with female birds whereas only 19.04% or 4 out of 21 female-deprived males had paired with female birds (Figure 6.1.A, $\chi^2 = 11.92$, $p = 0.0006$). Of the 21 female-deprived males, 57.14% or 12 out of 21 individuals paired with each other.

Figure 6.1. Pairing status of control and female-deprived male (A) and female (B) zebra finches at the end of two weeks. * Significantly different at the 0.05 level.

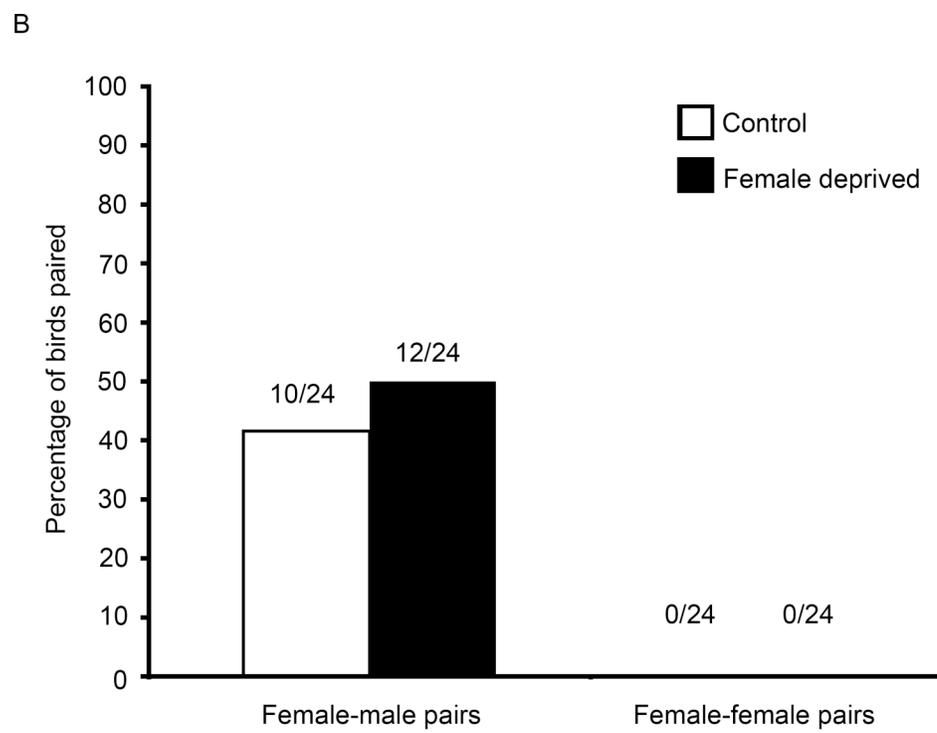
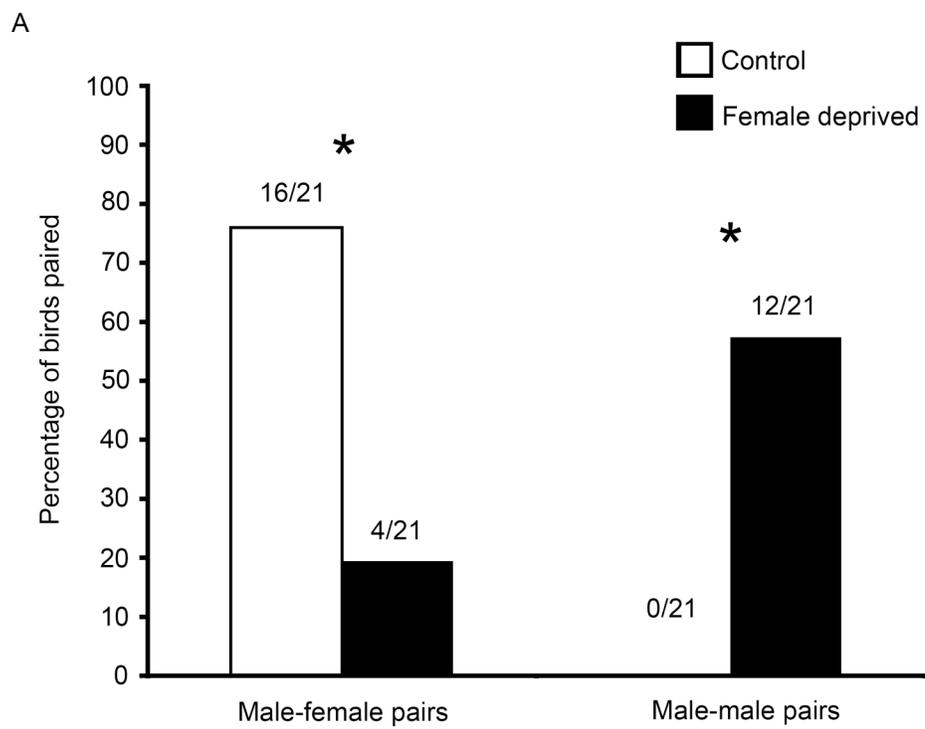


Figure 6.2. Frequency (mean \pm SEM) of courtship and aggressive behavior during the early pairing phase (A) and the late pairing phase (B) observed in adult male offspring. Shown are means across 5 days for 15 minute daily observation periods.

* Significantly different at the 0.05 level.

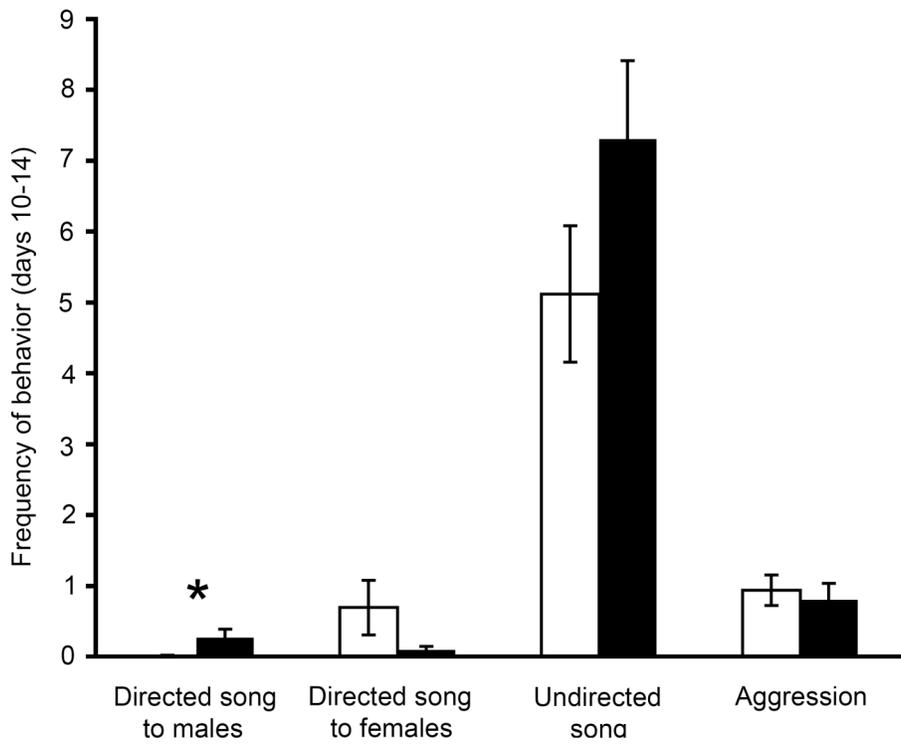
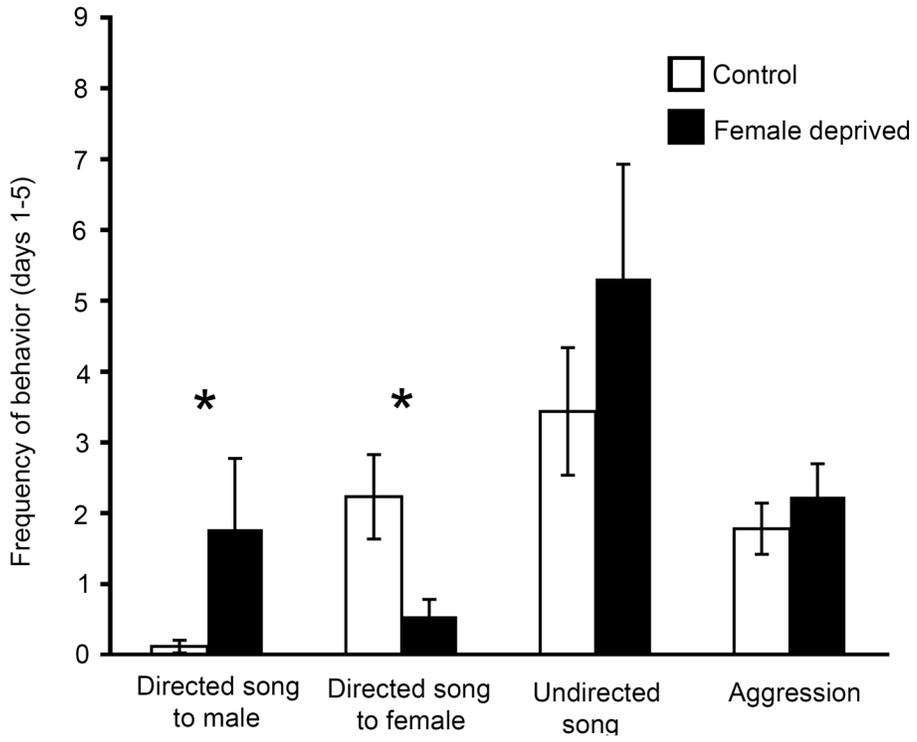
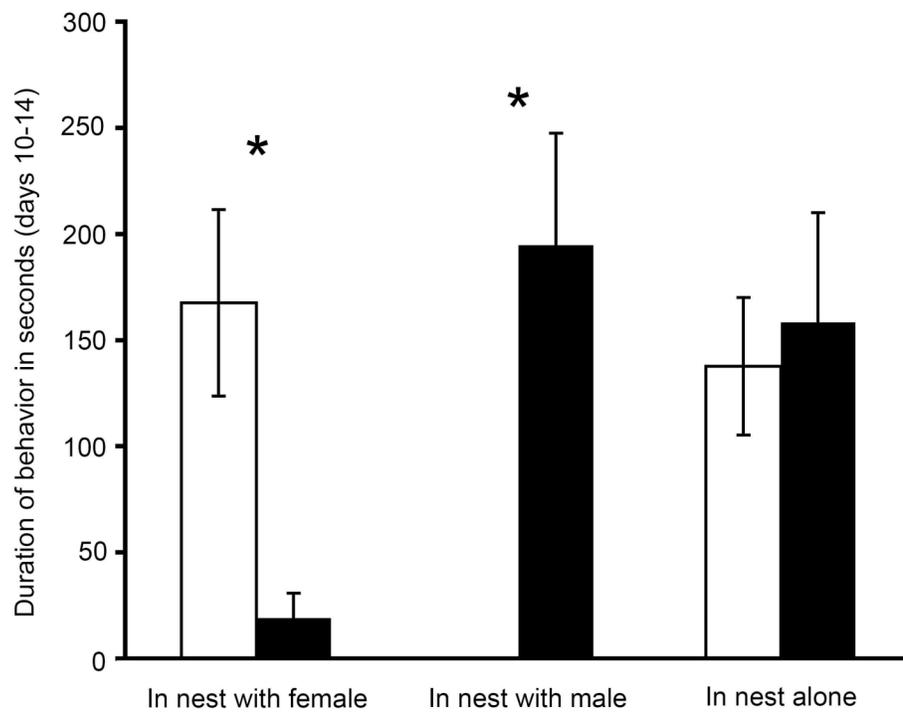
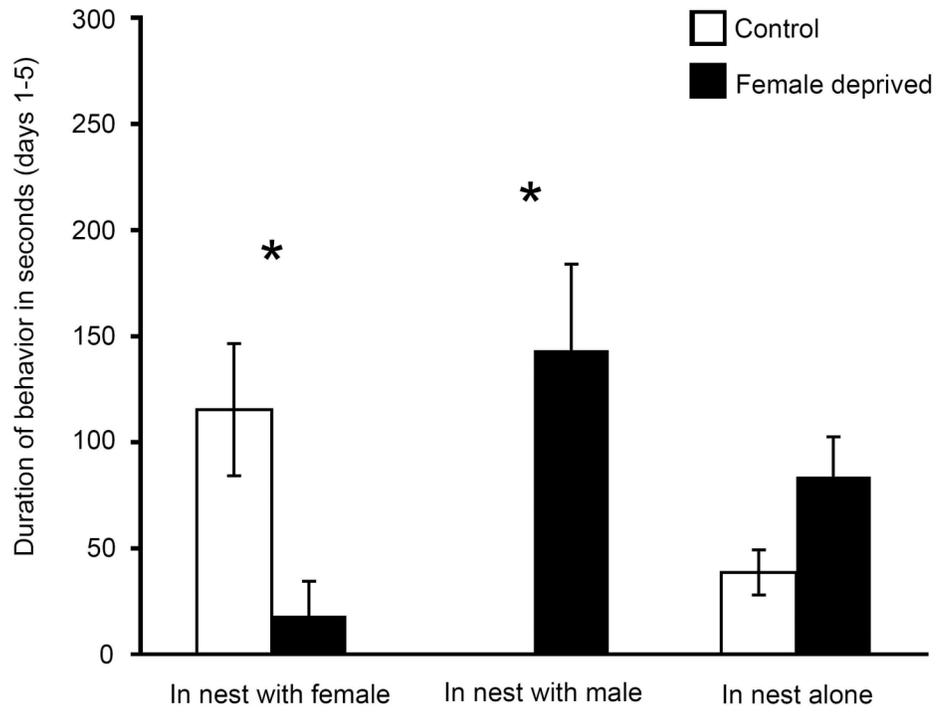


Figure 6.3. Duration of pairing behavior (mean \pm SEM) during the early pairing phase (days 1-5) (A) and the late pairing phase (days 10-14) (B) observed in male offspring. Shown are means across 5 days for 15 minute daily observation periods. * Significantly different at the 0.05 level.



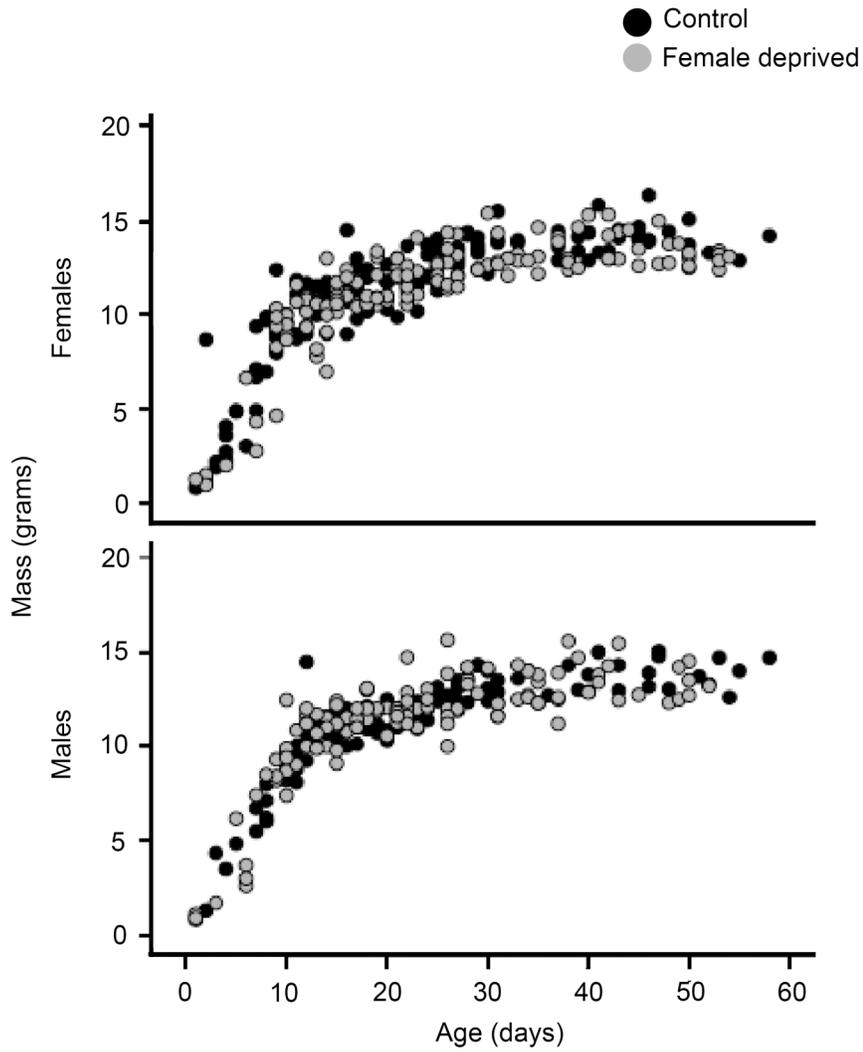


Figure 6.4. Mass (grams) of control (black circles) and female-deprived (grey circles) offspring over time (days). Birds were weighed every 5-7 days.

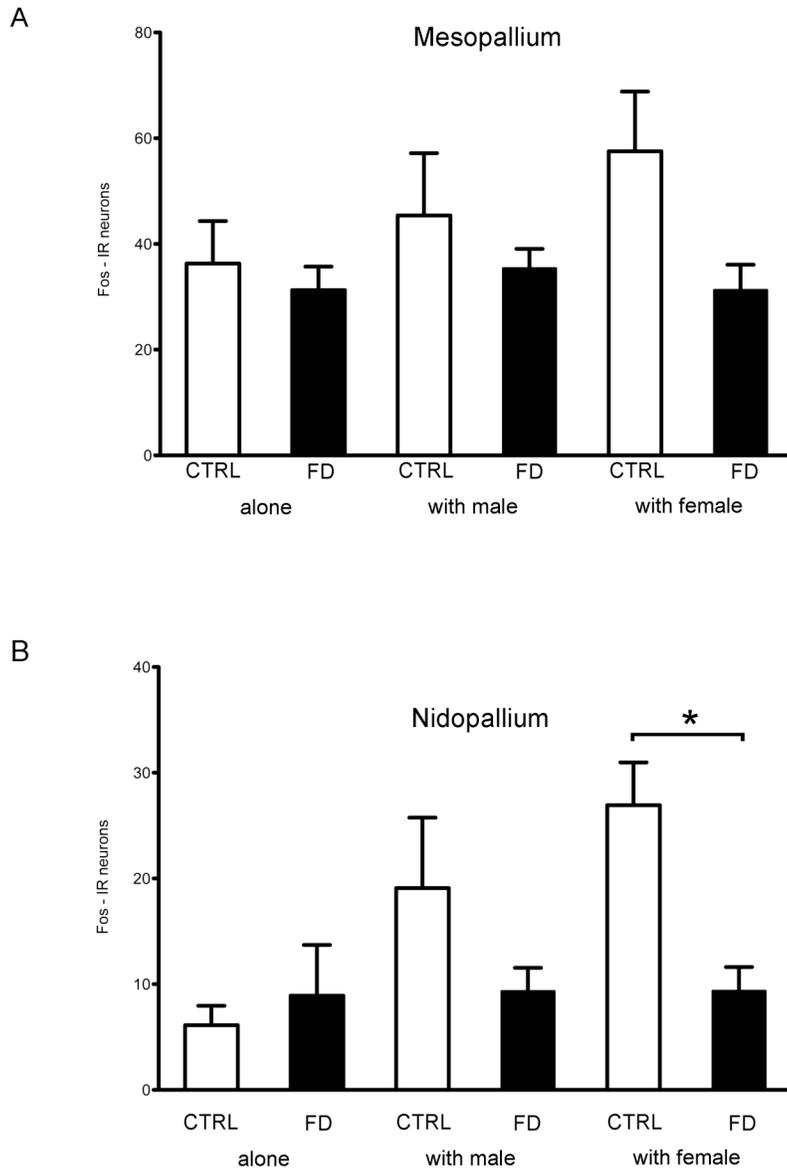


Figure 6.5. Numbers of Fos-immunoreactive (Fos-IR) neurons (mean \pm SEM) in control (CTRL) and female-deprived (FD) birds in (A) mesopallium and (B) nidopallium in response to no stimulus (alone), male or female stimuli. *P<0.01.

Table 6.1. Courtship and pairing behavior of females

Early pairing phase (mean \pm SE)

| Behavior | Control | Female deprived | <i>U</i> | <i>P</i> |
|-----------------------------|-------------------|-------------------|----------|----------|
| Aggression | 3.740 \pm 0.92 | 6.52 \pm 1.7 | 154 | 0.8019 |
| Clump with male partner | 0.979 \pm 0.93 | 10.3 \pm 9.15 | 151 | 0.5807 |
| Allopreen with male partner | 0.979 \pm 0.93 | 9.25 \pm 8.13 | 151 | 0.1892 |
| In nest with male | 43.37 \pm 16.86 | 58.30 \pm 26.88 | 159.0 | 0.9267 |
| In nest alone | 66.66 \pm 31.26 | 84.12 \pm 26.03 | 142.5 | 0.5380 |

Late pairing phase (mean \pm SE)

| Behavior | Control | Female deprived | <i>U</i> | <i>P</i> |
|-----------------------------|-------------------|-------------------|----------|----------|
| Aggression | 2.18 \pm 0.68 | 2.23 \pm 0.77 | 152.5 | 0.7654 |
| Clump with male partner | 25.62 \pm 20.52 | 4.02 \pm 2.49 | 148.5 | 0.5720 |
| Allopreen with male partner | 22.68 \pm 20.14 | 3.06 \pm 2.44 | 153 | 0.6971 |
| In nest with male | 45.36 \pm 19.81 | 109.7 \pm 39.20 | 129 | 0.2656 |
| In nest alone | 59.06 \pm 35.45 | 108.2 \pm 35.20 | 129 | 0.2508 |

Therefore 6 same sex pairs were formed between female-deprived males. In stark contrast, control males did not form any same-sex pairs (Figure 6.1, Fisher's exact test, $p < 0.0001$). There was no effect of female deprivation on the pairing status of the female offspring (Figure 6.1.B, as 10 out of 24 or 41.6% of control female birds paired with other males whereas 12 out of 24 or 50% of female-deprived female birds paired with male birds (Figure 1B, $\chi^2 = 0.33$, $p = 0.5628$). There were no same-sex pairs observed among female offspring. Thus the effect of female deprivation on partner preference was specific to male offspring as females raised in a female-deprived environment displayed a preference similar to that of controls and paired with males.

Behaviors

In addition to a difference in the pairing status of control and female-deprived male offspring, we also observed differences in the courtship and pairing behaviors of these birds in adulthood. During the early pairing phase that lasted from days 1-5 (Figure 6.2.A), female-deprived male offspring sang more bouts of directed song to males than control birds did ($U = 143.5$, $p = 0.0273$), whereas control males sang more bouts of directed song to females than female-deprived males did ($U = 117$, $p = 0.011$). There was no difference in the number of bouts of undirected song between the groups ($U = 192.5$, $p = 0.6566$). During this phase, the number of bouts of aggression displayed by the two groups did not differ from each other ($U = 207.5$, $p = 0.9582$). The two groups of males also differed in the measures of pairing during the early phase (Figure 6.3.A). Female-deprived males spent more time in nests with other males than control birds did ($U = 110$, $p = 0.0005$) whereas control males spent more time in nests with females than female-deprived birds did ($U = 116$, $p = 0.0064$). There was no significant difference between the two groups with regard to the amount of time spent alone in a nest ($U = 156$, $p = 0.1607$). Clumping and allopreening were observed infrequently in both groups and did not differ significantly between them (data not shown).

During the late pairing phase that extended from days 10-14, female-deprived males sang more bouts of directed song to males than control males (Figure 6.2.B, $U=138$, $p=0.01$). There were no differences in the number of bouts of directed song to females (Figure 6.2.B, $U=164.5$, $p=0.155$), undirected song (Figure 6.2.B, $U=182.5$, $p=0.4807$) or aggressive behavior (Figure 3A, $U=174$, $p=0.3365$) between the two groups. However, there were significant differences in pairing behaviors between the two groups (Figure 6.3.B). Female-deprived male offspring spent more time in the nest with males ($U=73.50$, $p<0.0001$). In contrast, control offspring spent more time in the nest with females ($U=59.50$, $p<0.0001$). There was no difference in the time spent in a nest alone between groups ($U=187$, $p=0.5563$). Once again, clumping and allopreening were observed infrequently and did not differ between the two groups (data not shown). With regard to females, there were no significant differences between the two groups for any measure of pairing or aggression in either the early or late pairing phase (Table 6.1).

Body mass

To ensure that the female-deprived offspring were being adequately fed by the male zebra finches in the absence of their female partners, offspring were weighed every 5-7 days. We observed that female-deprived chicks of both sexes ($n=21$ males, $n=24$ females), had weights similar to control chicks that were reared by both male and female parents ($n=21$ males, $n=24$ females). Weights of the chicks did not differ significantly over time between the control and female-deprived groups (Figure 6.4). There was no significant interaction between treatment, sex and age, ($F_{2,475.85}=0.592$, $p=0.554$). There was no effect of sex ($F_{1,488.46}=0.474$, $p=0.491$) or treatment ($F_{1,41.43}=0.002$, $p=0.968$) on weight of chicks over time controlling for breeding cycle (replicate) ($n=3$) and nest ($n=22$). A different set of adult birds was used in each breeding cycle to generate control and female-deprived offspring. Few chicks died

(control=4, female-deprived=3). These data are important in that it is unlikely that the mate choice of female-deprived males was different from that of controls because they were unable to compete with control male offspring due to a deficit in body mass.

Fos-IR neurons

Within the no stimulus (subjects tested alone) group, there were no differences between the control and female-deprived males in numbers of Fos neurons in the lateral septum ($F_{1,55.88}=0.030$, $p=0.864$), the mesopallium, ($F_{1,55.88}=0.073$, $p=0.789$) and the nidopallium ($F_{1,55.88}=0.006$, $p=0.941$) (Figure 6.5). Similarly, there were no significant differences in numbers of Fos expressing neurons between the control and female-deprived offspring when they were exposed to male stimuli in any of these regions; lateral septum, ($F_{1,55.88}$, $p=0.509$), mesopallium, ($F_{1,55.88}$, $p=0.237$), nidopallium, ($F_{1,55.88}$, $p=0.180$). However, in the birds that were exposed to female stimuli, there was a trend towards fewer Fos-IR neurons in the mesopallium, ($F_{1,55.88}=3.24$, $p=0.077$), and significantly fewer Fos-IR neurons in the nidopallium, ($F_{1,55.88}=8.85$, $p=0.004$) of female-deprived males when compared to controls (Fig.6.5). No significant differences were observed in the lateral septum ($F_{1,55.88}=0.999$, $p=0.322$).

Discussion

The experiments presented have shown that the absence of adult females from the early social environment affects the sexual partner preference of male zebra finch offspring. A significantly higher percentage of adult female-deprived male zebra finch offspring as compared to control male offspring preferred to pair with males. Adult female-deprived female offspring paired with males, similar to control adult female-deprived female offspring. In an independent experiment, fewer numbers of cells expressing Fos were detected in the nidopallium of adult female-deprived males when compared to controls, in response to presentation of female stimuli.

It is possible that in our experiment, zebra finches that were raised in the absence of adult females imprinted on the adult males in their environment. Many studies across several vertebrate species suggest that sexual imprinting on the parent of the opposite sex shapes the mate preferences of offspring. Sexual imprinting is often studied by cross-fostering offspring of one species to another, and has been shown to be important for the formation of partner preferences in avian species like pigeons, doves, ducks, geese, galliformes and estrildid finches as well as other vertebrate species (Immelmann, 1975; ten Cate and Vos, 1999). In the wild, cross fostering of offspring between two species of tits, the blue tit, *Parus caeruleus* and the great tit, *Parus major*, resulted in heterospecific pairing between them (Slagsvold et al., 2002). In fish, mate choice in female adult guppies was influenced by the phenotype of the male they were raised with during development, as females that were raised with colorful males had higher choice scores for colorful males in mate choice tests (Breden et al., 1995). In humans too, there is evidence for opposite sex imprinting between daughters and fathers as well as mothers and sons. One study has demonstrated that women who were adopted chose partners who resembled their adoptive fathers, suggesting that they may have imprinted on their fathers' physical features. Moreover in another study of women, there was a strong relationship between proportions of their fathers' face and those of a face they found attractive, when the subjects rated their early relations with their fathers highly. Men too seem to imprint on their mothers, as judges found a significant degree of similarity between husbands' mothers and husbands' wives (Berezkei et al., 2002; Berezkei et al., 2004; Wiszewska et al., 2007).

Studies have shown that zebra finch offspring imprint on individuals belonging to the species that raised them. Male zebra finch offspring that were cross fostered to Bengalese finches preferred to court female Bengalese finches when given a choice between a zebra finch female and a Bengalese finch female (Immelmann, 1969; Kruijt

and Meeuwissen, 1991). In addition, within the same species, zebra finch offspring imprint on certain novel or exaggerated parental traits. Female and not male zebra finches that were raised with parents that had a red feather ornament showed a preference for mates that possessed the same red ornament. In a different experiment, zebra finch females imprinted on a novel blue feather ornament when raised by males and not females that had the novel blue ornament. As adults, these females preferred males with the blue feather ornament to unadorned males in mate choice tests. This study also suggests that females learn characteristics of the parent of the opposite sex (Witte and Caspers, 2006). In another experiment, when zebra finch offspring were raised with adult birds that had artificial crests, unlike in previously described experiments, both male and female offspring developed a preference for a mate of the opposite sex that bore the same crest (Burley, 2006). Another example of opposite sex imprinting is provided by a study in which male zebra finches were raised by males and females that had white plumage and only differed in their beak color, ranging from red to orange. Male offspring that were raised by such individuals preferred females that had beaks that were of a more extreme color than their mothers' beak color, suggesting that sexual imprinting in males during development can result in a preference for exaggerated physical characteristics (ten Cate et al., 2006). Thus it is possible that female-deprived male zebra finches imprinted on adult males and used their acquired preferences to pair with other males in adulthood.

The current study is the second in our research program to demonstrate that a change in the early social environment can alter zebra finch choice of partner sex in adulthood possibly via sexual imprinting. A previous study from the laboratory has shown that the removal of adult males from the early rearing environment led to an increased incidence of same sex partner preference in both male and female zebra finch offspring as opposed to a sex-specific effect (Adkins-Regan and Krakauer,

2000). This treatment might have affected both sexes for the following reasons. Male deprived male offspring did not learn normal songs due to absence of adult males during development. Therefore these males may have been less attractive to females so that few could form pairs with females. Male-deprived females, on the other hand, may have preferred to pair with other females because they had imprinted on adult females during development. In sum, the absence of adult males during development resulted in same-sex pairs in offspring of both sexes whereas the absence of adult females during development selectively increased same-sex pairing in male offspring.

Previous studies in zebra finches have shown that there are two stages in sexual imprinting. The first stage, the acquisition phase, extends from when the offspring have opened their eyes at about 5-10 days old until they are about 30-40 days old, while the second stage, the consolidation phase, lasts from days 60-100 after hatching (Bischof and Clayton, 1991; Immelmann et al., 1991; Kruijt and Meeuwissen, 1991; Oetting et al., 1995; Oetting and Bischof, 1996). It seems plausible that in our experiments, both male and female zebra finch offspring imprinted and acquired a preference for males in the absence of adult females in the aviary. As all offspring were moved into single sex aviaries after they had reached independence, the male offspring could only interact with other males thus leading to consolidation of their preferences that were acquired in the earlier acquisition phase. Although both control and female-deprived female offspring were placed in all-female aviaries after independence, they preferred males as mates during adulthood. This observation supports the idea that the social environment during the acquisition phase early in development strongly influences the development of partner preferences in zebra finches.

A study by Kruijt et al. suggests that siblings can influence the development of sexual preference (Kruijt et al., 1983). The authors showed that if male zebra finches

are raised by Bengalese finches along with 2-4 zebra finch siblings, then as adults the male birds sing to zebra finch females at least 5% of the time. In our study, nearly 20% (4 out of 21 birds) of female-deprived males did pair with adult females (Figure 6.1A). As female-deprived offspring could interact with both female siblings and unrelated female offspring during development until at least 45 days of age, it is possible that their sexual partner preference was influenced by the presence of young females in their environment. Therefore a few female-deprived birds might have developed a preference for females despite the absence of adult females during development.

Studies in fishes, mammals and birds have shown that there are sex differences in the learning of mate choice by offspring. Studies in Lake Victoria cichlids suggest that mate preferences in female fish are influenced by their mothers' phenotype. Females of one species of cichlid that were cross-fostered with a different species of cichlid, displayed more to males of their mothers' species. A similar experiment performed with male cichlids suggests that mate choice in males is not influenced by the phenotype of their mothers (Verzijden et al., 2008). In contrast to certain species of fish, male offspring of mammalian species such as goats and sheep are more likely to sexually imprint on the adult females. A study by Kendrick et al. has shown that when goats and sheep were cross-fostered, male offspring preferred to mate and socialize with females of the maternal species whereas this effect was much weaker and reversible in female offspring (Kendrick et al., 1998). In zebra finches, although both sexes are known to imprint on their parents, there are differences in the features that they imprint on. For example, male zebra finches that are raised by wild type or white morphs or mixed morph pairs prefer females of their mothers' morph in a mate choice test. In addition, males from mixed morph parents preferred males of their mother's morph to females of their father's morph, suggesting that male zebra finches acquire

preferences based on morphological features of their mothers. In the absence of female morphs that are similar to their mothers, they prefer to pair with male morphs that bear similarity with their mothers (Vos, 1994). Female zebra finches, on the other hand, preferred males in a mate choice experiment regardless of whether or not the males had features similar to their fathers (Vos, 1995). In our experiments, male offspring appeared to imprint on adult males, and despite being given a choice between males and females in adulthood, they chose to pair with males possibly due to the physical similarity to the adult males that raised them.

Although the phenomenon of sexual imprinting seems like the most likely explanation for our results, there are certainly other possible, but less likely interpretations. The first is that perhaps offspring raised by single males were unable to compete with females due to a lack of attractiveness because of insufficient nutrition during development. It is known that both male and female zebra finches contribute to raising their offspring and performing parental duties such as feeding their young [4]. We put forth a few arguments against this claim. First, our data from behavioral observations in female-deprived female offspring show that these offspring had similar pairing success with males as compared to control females, suggesting that female-deprived female offspring were probably equally attractive to males as control females. Furthermore, female-deprived males sang bouts of directed song to other males and displayed pairing behaviors towards other males during the early pairing phase, suggesting that they were actively courting other males. Furthermore, a study has shown that female zebra finches given a choice between males raised by females alone versus males raised by both parents, chose to spend more time with males raised by single females (Royle et al., 2002), suggesting that females find males raised by a single parent more attractive. Taken together, these data suggest that it is unlikely that female-deprived males were rejected by potential female suitors and thereby were

forced to pair with other males. Instead it is likely that the female-deprived male offspring preferred to pair with other males.

Another interpretation of our results would be that female-deprived males were more fearful of adult females (neophobic), because they had been raised in the absence of adult females, and therefore chose the familiar sex (males). This is an unlikely explanation, however. During development, although female-deprived birds did not have tactile contact with adult females in the same aviary, they had both visual and auditory contact with adult females from the control group that was in an adjacent compartment, separated by wire mesh. In addition, they were raised along with their female siblings until they were 45-55 days old, when males and females can be distinguished quite clearly on the basis of plumage markings. After being moved to single sex aviaries on reaching independence, birds of both sexes could see and hear each other, as both male and female single sex aviaries were placed adjacent to each other. In sum, a lack of overall attractiveness of female-deprived male offspring and neophobia seem to be unlikely explanations for our observations. Although we have not proven that sexual imprinting is the only explanation for our results, it certainly seems to be the most likely one.

In addition to behavioral effects of female-deprivation in male zebra finches, in a separate experiment, we investigated immediate early gene *fos* expression in control and female-deprived male offspring during adulthood in a first courtship paradigm similar in some respects to one performed by Sadananda and Bischof wherein birds were raised in individual cages from independence till adulthood (Sadananda and Bischof, 2002). Unlike the paradigm used by the authors, the birds in our experiment were not raised in visual isolation, as they could see and interact with each other through their wire mesh cages. As adults, male control and female-deprived offspring were exposed to male or female stimuli or no stimulus within their cage. Amongst the

birds that were exposed to female stimuli, fewer numbers of Fos-immunoreactive neurons were observed in the mesopallium (trend) and nidopallium of female-deprived birds as compared to control birds. These data suggest that neuronal activity in these areas in response to female stimuli, but not male stimuli or no social stimulus, is potentially altered by the absence of females during offspring development.

Collectively, the pallium is thought to be homologous to the mammalian neocortex involved in higher order sensory processing (Reiner et al., 2004; Reiner, 2005; Reiner et al., 2005; Medina and Abellan, 2009). It has been shown that the lateral nidopallium receives input from a large number of telencephalic brain areas and therefore plays a key role in integration of information from various pathways (Sadananda et al., 2007). Previous studies have shown that neural activity and plasticity in the lateral areas of mesopallium and lateral nidopallium (formerly known as lateral neo/hyperstriatum ventrale) are implicated during the consolidation phase of sexual imprinting. Spine density of neurons in these regions has been shown to be irreversibly decreased during this phase. In addition uptake of radioactive 2-deoxyglucose is enhanced during first courtship, suggesting that these areas have increased neuronal activity. In contrast, the more medial areas of the nidopallium and mesopallium do not show increased uptake of 2-deoxyglucose during first courtship, although earlier studies have shown that these areas are active in birds that actively court females after a long period of isolation (Bischof and Herrmann, 1986, 1988; Sadananda and Bischof, 2002; Lieshoff et al., 2004). In our study, Fos-IR cells were counted in the medial areas of the nidopallium and mesopallium. Therefore it is unlikely that the lower numbers of Fos-immunoreactive neurons in comparison to controls were due to a conflict of sensory information acquired during development (when the female-deprived offspring learned features of their male parents) versus the first courtship (when their stimulus was female instead of male) when consolidation of

acquired information takes place. Also, we did not observe higher numbers of Fos-IR neurons in pallial regions of female-deprived males that were exposed to males, suggesting that this effect might not be related to consolidation of developmentally acquired information.

There is evidence for the pallial regions displaying enhanced neuronal activation during the processing of sensory cues within birds. For example, in response to auditory components of courtship in zebra finches, higher levels of immediate early gene *zenk* were observed in the auditory region of the caudomedial mesopallium (CMM) (Avey et al., 2005). One explanation of our results would be that sensory cues from females were not as salient to female-deprived males as they were to control birds due to their lack of exposure to adult females during development, and therefore the levels of neuronal activation in regions that process sensory information, namely the pallial areas, were less in female-deprived versus control birds. On the other hand, both control and female-deprived offspring were exposed to adult male stimuli during development, and therefore it is possible that the pallial neurons processed sensory information from adult males in a similar fashion within both control and female-deprived groups. The possibility also exists that female-deprived males did not court females, and so numbers of Fos-IR cells were fewer in these regions when compared to control males that did court females.

Previous studies have shown that Fos expression in limbic areas such as the BNST and lateral septum are associated with socio-sexual behaviors (Goodson and Evans, 2004; Goodson et al., 2009a). We did not observe any differences in numbers of Fos expressing neurons in the lateral septum, suggesting that the lack of adult female stimuli during development could have resulted in altered function of pallial areas involved in perception but not in that of limbic areas involved in aggression. Behavioral observations are consistent with this, as female-deprived males and

females display similar levels of aggression, courtship and nesting behaviors as control birds.

To conclude, our experiment suggests that male zebra finches acquire a preference for individuals of the opposite sex during development. This preference could be acquired based on imprinting on the morphological features of adult females early in life. It is not yet known whether the presence of the mothers specifically is required or simply adult females in the group. The processes involved in guiding an individual offspring of a biparental species to learn or imprint on the features of an adult of the opposite sex given the presence of both parents remain to be understood. Most studies that have been performed previously have either cross-fostered offspring of one species to another or modified certain physical characteristics of parents to assess the contribution of either parent on the development of mate choice by the process of imprinting. Our study has shown that sexual imprinting or some other form of social learning during development plays an important role in the acquisition of a preference for sex of partner. In addition, patterns of Fos expression in the mesopallium and nidopallium of control and female-deprived males suggest that the absence of females from the early social environment of male offspring could affect the processing of sensory cues from adult females even after these offspring have reached adulthood.

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CHAPTER 7

SUMMARY AND FUTURE DIRECTIONS

The experiments presented in chapters 2 and 3 suggest that mesolimbic dopaminergic pathway is involved in pair formation in zebra finches. Neuronal activity in dopaminergic cells in the midbrain ventral tegmental area (VTA) was greater in male and female birds that were paired. Additionally, intracellular dopamine levels in the medial striatum that contains the projection area of the ventral tegmental area were higher in paired birds versus unpaired birds. Contrary to our hypothesis, blockade of D1 and D2 dopamine receptors both via systemic treatment with antagonists and direct administration into the medial striatum did not disrupt pair bond formation. Although males that were treated with receptor blockers did sing fewer bouts of directed song than males that were treated with saline, pair bonding itself was not affected after five days of treatment. Studies in monogamous prairie voles have shown that the administration of a D1/D2 antagonist and D2-receptor antagonist prevents partner preference formation in this species (Gingrich et al., 2000; Aragona et al., 2006). It is possible that a dopamine D2 receptor selective antagonist would affect pairing behaviors, given that such an antagonist does disrupt partner preference formation in prairie voles. Perhaps as mating seems to be important for pairing in prairie voles, dopaminergic drugs alter motivational pathways involved in sexual behaviors that then impact pairing in this species. It is not known what role mating plays in zebra finch pair bonding but if indeed copulation does not play as important a role, altering motivation for procreation would not affect pairing in this species. Another reason why we did not observe in changes in pairing status with dopamine antagonists is that perhaps there lies a great deal of redundancy within the dopaminergic system as well as interacting neuromodulator systems. Therefore, blocking one or two receptors of a specific neuromodulator does not alter a behavior

that is fundamental to the reproductive success of this species. Another possibility is that circuits underlying pairing behaviors are organized early in development.

Therefore developmental manipulations such as selective ablation of dopaminergic input into the nucleus accumbens or the use of dopamine receptor agonists and antagonists would be necessary to alter a bird's ability and desire to pair in adulthood. Regardless of the results from the pharmacological experiments, one could infer that the mesolimbic dopaminergic pathway is recruited during pairing and the expression of pairing behaviors. Questions remain on the role of other neuromodulators that interact with dopamine such as opioids, glutamate, GABA monoamines such as serotonin and norepinephrine and neuropeptides such as vasotocin and mesotocin, in courtship and pairing behaviors in this species. Given that most of these neuromodulators have been implicated in affiliative behaviors in mammals (Young et al., 2001; Wang and Aragona, 2004), it is possible that they do play a role in avian species as well.

Transitioning from the neural mechanisms of pair bond formation to the effects of social stressors on corticosterone, it was observed that isolating zebra finches resulted in higher levels of corticosterone in the isolated birds compared to birds that were not subjected to any such manipulation. Interestingly, such an effect was only observed after 10 minutes of isolation and not after 30 minutes of isolation suggesting that a negative feedback loop is turned on that terminates activation of the hypothalamic-pituitary-adrenal (HPA) axis within 30 minutes. In addition this suggests that corticosterone in circulation is rapidly metabolized with 30 minutes. Studies in species such as guinea pigs have shown that when male guinea pigs are separated from their social groups, the presence of a familiar female can prevent the rise in corticosterone that would occur in the absence of a conspecific (Hennessy et al., 2009; Maken and Hennessy, 2009). The experiments presented in chapter 4 suggest that the initial

increase of corticosterone in response to 10 minutes of isolation is not prevented by the presence of another bird regardless of whether it is partner or another familiar bird. This could be due to the nature of social organization of these species. As zebra finches normally inhabit large flocks, the removal of a bird from its flock would lead to alarm and activation of the HPA axis regardless of the presence of another bird. Another factor that could play a role in the absence of social buffering by conspecifics is that the subjects in our experiments were paired and had nests containing eggs. Therefore perhaps the separation from nest and eggs contributed to the HPA activation despite the presence of a conspecific. It would be interesting to know the effect of social buffering in birds that were unpaired and did not have nests. Finally, performing a similar set of experiments in birds that are territorial and zealously guard their territories in the wild, would throw light upon the importance of social organization in the corticosterone responses to isolation as well as the social buffering effects of conspecifics. It is possible that the isolation of a song sparrow would not lead to the initial high levels of corticosterone as observed in zebra finches in response to isolation and the presence of a conspecific might actually lead to an increase in hormone levels.

In mammals, perturbations in early life maternal care through periods of deprivation of maternal care, alters the responsiveness of the HPA axis to stressors. Offspring of rat pups that are subjected to periods of maternal deprivation during the stress hyporesponsive period are hyperresponsive to stressors such as restraint in adulthood. This effect is thought to be mediated via a decrease in expression of corticosterone receptor GR in the hippocampus, a key player in the negative feedback loop of the HPA axis, resulting in decreased sensitivity to corticosterone, leading to a hyperresponsive stress axis (Plotsky and Meaney, 1993; Plotsky et al., 1998; Francis and Meaney, 1999). Data presented in chapter 5 showed that a similar manipulation in

biparental zebra finches wherein all adult females including mothers were removed from breeding aviaries when zebra finch offspring were 2-12 days old, resulted in offspring being hyperresponsive to isolation in adulthood. This suggests that early life perturbations of parental care can affect the HPA axis regardless of whether a species is uniparental or biparental. Corticosterone receptor expression was decreased in the hypothalamus, hippocampus and cerebellum in birds that were raised in the absence of mothers in comparison to those that were raised with biparental care. It is not known if an alteration of the levels of GR and MR receptors played a role in the HPA axis effects in the female-deprived birds. It has been shown that the quality and intensity of maternal care in rats can alter gene expression via epigenetic modifications (Weaver et al., 2004; Szyf et al., 2005; McEwen, 2008). It is possible that the absence of maternal care in zebra finches during the developmental stage results in epigenetic modifications not only in the DNA sequences that code for corticosterone receptors but also other genes that are recruited in cognition and reproductive behaviors. The results presented in chapter 6 showed that a majority of female-deprived male offspring chose to pair with other males, and also showed an altered pattern of Fos expression when exposed to males and females in comparison to their male counterparts raised with biparental care. Therefore it is possible that epigenetic changes, perhaps occurring as a result of the absence of maternal care, lead to differential expression in genes involved in processing of sensory cues in addition to genes involved in the regulation of the HPA axis.

In summary, the experiments presented in this thesis have explored the interaction of neural circuitry, early social environment and adult behavior. Although our experiments suggested that dopamine is involved in pairing behaviors in zebra finches in adulthood, blockade of dopaminergic neurotransmission did not inhibit pair formation. The absence of mothers from the early social environment altered

responsiveness of the HPA axis to isolation in adulthood, mate choice in adult male zebra finches as well as Fos expression in response to male and female stimuli in these birds. These experiments are but a first step in the exploration of mechanisms involved in affiliative behaviors in this social species. Given their complex behavioral associations and monogamous and biparental social system, zebra finches are an excellent model system to explore the neural, genetic and epigenetic bases of affiliative behaviors.

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