The Differential Effects of Stress on the Zebra Finch (Taeniopygia guttata) Brain and Behavior

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ABSTRACT

The detrimental effects of early life stress on brain development and behavior in adulthood are well known, but the effects of acute stressors experienced later in life on brain activation and adult behaviors, such as mate preference, are less understood. It is generally thought that acute stress, unlike chronic stress, leads to adaptive responses like encouraging reproduction over survival. The reverse, promoting survival over reproduction, occurs under chronic stress conditions. Physiological responses to stress are mediated by glucocorticoids receptors (GRs) and DNA methylation has been proposed as a mechanism by which stress, at any point in life, can lead to adaptive and maladaptive changes in the brain and in behavior. Corticosterone, a glucocorticoid steroid hormone, is thought to mediate variation in female
partner preference when environmental conditions are not ideal. Female zebra finches choose their mates based in part on male song and it is unknown if female perception of song or preference for her mate is altered by stress.

We examined the effects of an ecologically relevant stressor (cold exposure) and a physiological stressor (oral corticosterone administration) experienced early in life to determine if they differentially alter HPA axis sensitivity and global DNA methylation in the brains of juvenile and adult zebra finches. We showed that both stressors resulted in blunting of the stress response in juveniles and adults, and in hypomethylation of the brains of juveniles only.

We investigated whether exposure to an acute stressor in adulthood alters female preference for their mate or for mate’s song. We found that acute stress decreased the strength of a female’s preference for her mate and her mate’s song. We also examined whether exposure to an acute stressor would alter a female’s typical pattern of neuronal activation, using an antibody to the immediate early gene ZENK, in auditory brain regions that might be involved in perceptual song processing. In addition, we investigated whether acute stress would increase GR quantity in auditory brain regions that might be involved in perceptual song processing. We demonstrated that acute stress decreased neuronal activation and increased GR immunoreactivity in all brain regions.

INDEX WORDS: Zebra finch, Stress, Corticosterone, Partner preference, ZENK protein, Glucocorticoid receptor
THE DIFFERENTIAL EFFECTS OF STRESS ON THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*) BRAIN AND BEHAVIOR

by

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THE DIFFERENTIAL EFFECTS OF STRESS ON THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*) BRAIN AND BEHAVIOR

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DEDICATION

To my fiancé, Greg Mein, your encouragement, love and strength have kept me afloat, thank you. To my parents, Glenford and Judy Cheesman, thank you for supporting me, loving me and believing in me. To my sisters, Dr. Khadeen Cheesman Were, Cherisse Cheesman and Dr. Asa Cheesman, you inspire me to be my best self every day, thank you.
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LIST OF ABBREVIATIONS

BSTL,  Lateral bed nucleus of the stria terminalis
BSTM,  Medial bed nucleus of the stria terminalis
Cb,  Cerebellum
CMM,  Caudomedial mesopallium
Cort,  Corticosterone
DAB,  3, 3’- diaminobenzidine tetrahydrochloride
GC,  Glucocorticoids
GR,  Glucocorticoid receptor
HP,  Hippocampus
HPA,  Hypothalamic-pituitary-adrenal
IEG,  Immediate early gene
IHC,  Immunohistochemistry
MR,  Mineralocorticoid receptor
NCM,  Caudomedial nidopallium
POM,  Medial preoptic nucleus
PVN,  Paraventricular nucleus
1 GENERAL INTRODUCTION: FEMALE MATE CHOICE, FEMALE PARTNER PREFERENCE AND STRESS IN THE ZEBRA FINCH

1.1 Overview

In many vertebrate species, male quality is an essential component in how females choose their mates (Zann, 1996) and there can be considerable variation in female mate choice (Davis and Leary, 2015; Jennions and Petrie, 2007). When environmental conditions are stable (and therefore not stressful), this variation in mate choice can be accounted for by the action of sex steroids (Gordon and Gerhardt, 2009; Lynch et al., 2006) but when an organism experiences stress, circulating sex steroids do not contribute to this variation (Cotton et al., 2006; Johnson, 2003). Instead, corticosterone, an adrenal steroid hormone involved in the stress response, is thought the mediate the differences in female mate choice (the initial choice of a mate) and female partner preference (maintaining a preference for a pair-bonded mate) under stressful environmental conditions (Wingfield and Sapolsky, 2003).

Many research studies investigating stress have focused on the effects of developmental or early life stress on adult physiology, behavior, reproductive success and survival (Cyr and Romero, 2007; Lindstrom, 1999; Monaghan et al., 2012; Naguib and Nemitz, 2007; Naguib et al., 2006; Paul et al., 2015; Shahbazi et al., 2014). When studies have investigated stress in adulthood, the focus was typically on chronic stress as opposed to acute stress (Breuner et al., 2008; Cyr and Romero, 2007), and in the few instances when the effect of acute stressors was investigated, female initial mate choice and not female partner preference was studied (Kavaliers and Ossenkopp, 2001). Assessing the effect of acute or non-chronic stressors experienced in adulthood on established female partner preferences is important for understanding the
mechanisms underlying pair bonds and monogamous behavior. This will allow researchers to more carefully examine the potential impact of stress and other environmental factors on the resilience of adult social bonding behaviors.

1.2 The Australian zebra finch

Australian zebra finches (*Taeniopygia guttata*) are highly social perching songbirds (order Passeriformes, suborder oscine) of the Estrildidae family (Mello, 2014; Zann, 1996). They are granivores that are endemic to central Australia, the Lesser Sundas islands in eastern Indonesia and tropical and subtropical parts of Africa (Mello, 2014; Zann, 1996). As granivores, zebra finches primarily eat millet seed, and also metabolize much of their water from seed intake (Zann, 1996).

These gregarious birds live in large flocks, breed colonially, form life-long pair-bonds and practice bi-parental care of offspring (Adkins-Regan, 2002; Zann, 1996). Zebra finches are opportunistic breeders when in captivity (and partially so in the wild) so they can breed anytime of the year (Mello, 2014). In the wild, zebra finches breed primarily after significant rainfall (Adkins-Regan, 2002; Zann, 1996). Zebra finches are socially monogamous (Zann, 1996) but they also copulate with extra-pair individuals, a practice that is common in socially monogamous avian species (Birkhead et al., 1988; Zann, 1996).

One of the most easily recognized physical characteristics of a zebra finch is distinct, sexually-dimorphic coloration (Mello, 2014; Zann, 1996) (Figure 1.1). Zebra finch males are colorful with bright orange cheek patches, brilliant red beaks and zebra-like horizontal stripes on their throats and fore necks while zebra finch females lack colored cheek patches, have orange beaks and lack black barring of the throat and fore neck (Mello, 2014; Zann, 1996).
Zebra finch males sing one song used for courtship and females do not sing (Lauay et al., 2004; Zann, 1996). Females use male song as one of the criteria to choose their mates (Nowicki et al., 2002; Zann, 1996), and once a pair bond is established they display a preference for the song of their mate over the song of other non-mate males (Clayton, 1988; Miller, 1979a; Riebel et al., 2002). Young females hear and remember the song of their fathers and familiar brothers (Catchpole and Slater, 2008; Riebel and Smallegange, 2003; Riebel et al., 2002; Zann, 1996), and exposure to familiar conspecific song or father song early in life influences song preferences (Riebel, 2000) and even mate choice (Grant and Grant, 1997) in females.

Zebra finch males are closed ended learners meaning that the final version of their song is crystallized and cannot be altered in any way (Woodgate et al., 2012). Similar to human speech development, vocal learning begins with the sensory stage which is characterized by juvenile males experiencing father/tutor song exposure and forming an auditory template for their own song (Bertram et al., 2014; Deregnaucourt et al., 2013; Glaze and Troyer, 2013; Woodgate et al.,

*Figure 1.1 Picture of male (right) and female (left) zebra finch showing their sexually dimorphic plumage and beak coloration.*

2012; Zann, 1996) (Figure 1.2). The sensory-motor phase follows and overlaps with the sensory phase, and it is characterized by the juvenile singing subsong (very unstructured vocalizations) to obtain the sensory feedback needed for proper song learning (Bertram et al., 2014; Zann, 1996) (Figure 1.2). Simply, the juvenile hears its own vocalizations and makes adaptive modifications to those vocalizations based off the auditory input it receives from father/tutor song (Miller et al., 2010). This subsong, which is similar to the babbling of human infants, is created between post hatch day 28 (P28) and P35 (Bertram et al., 2014; Miller et al., 2010; Zann, 1996). By P50, juveniles sing a plastic song that is characterized by syllables orchestrated in a time sensitive manner (Bertram et al., 2014; Zann, 1996). Once the male bird reaches adulthood at around P90, its final song pattern is fused into motor memory and this song will be produced for the rest of its life (Bertram et al., 2014; Zann, 1996).

![Figure 1.2 Zebra finch developmental timeline.](image)

The earliest sex differences in zebra finches are reported at post hatch day 1 (P1) and the song control system fully formed around P10. Juvenile males and females start learning song from their father or their tutor around P25-P30. Zebra finches are sexually mature around P60, which is around the time that the critical period for song learning ends. Male sing and their song crystallizes around P90 but females do not sing, but instead learn and recognize the song of their mate as compared to novel males.
In oscine passerine songbirds like the zebra finch, song learning and song production are controlled by a song control system that comprises a set of discrete and interconnected, bilateral forebrain telencephalic nuclei (Bertram et al., 2014; Simpson and Vicario, 1990) (Figure 1.3). The four song nuclei are the HVC (acronym is a proper noun), area X, robust nucleus of the arcopallium (RA) and lateral magnocellular nucleus of the anterior nidopallium (LMAN). The circuit that includes projections from area X to LMAN to dorso-lateral thalamus (DLM) is the anterior forebrain pathway which is involved in vocal learning and plasticity (Bertram et al., 2014; Wada et al., 2013). The projections from HVC to RA to tracheosyringeal motoneurons of the hypoglossal nucleus (nXIIIts) form the vocal motor pathway which is responsible for song production and modulation of breathing (Bertram et al., 2014; Margoliash, 1994; Wada et al., 2013). Motor input from the tracheosyringeal motoneurons is then sent to the avian vocal organ, the syrinx. (Simpson and Vicario, 1990). The projections from LMAN to RA completes the connection between the anterior forebrain and the vocal motor pathways (Wada et al., 2013). Song nuclei are present in both males and females, but males have larger song nuclei and only the males can sing (Nottebohm and Arnold, 1976).

The smallest components of adult song are syllables which are also referred to as elements or notes. Syllables are separated by moments of silence called intervals that last anywhere from 5-10 millisec. (Catchpole and Slater, 2008; Zann, 1996). The syllable and its preceding interval are the unit of zebra finch song and each male sings number of different syllables in a specific order to form a song-phrase (Catchpole and Slater, 2008). The song-phrase contains approximately 3-14 syllables (Riebel, 2000; Woodgate et al., 2012). The song-phrase, which is also called a motif or song-unit, lasts about 1-2 sec. (Hauber et al., 2010; Neubauer,
repeated 1-8 times to form a song-bout (Catchpole and Slater, 2008; Riebel, 2000). The song-phrase, courtship song, is the signature unit of song investigation in zebra finches.

Zebra finches are of much interest to scientists because they exhibit many robust sex differences with respect to physical traits including brain morphology and behavior (Nottebohm and Arnold, 1976; Zann, 1996). They have been used most prominently in studies that investigate the neurological bases and mechanisms of vocal learning (Mello, 2014) and they are also frequently used for studying the effects of hormonal modulation on the brain and on behavior (Adkins-Regan, 2011; Buchanan et al., 2004), including sexual differentiation of the brain.

Figure 1.3 A generalized songbird brain illustrating the song control circuit for song learning and production along with the auditory areas and areas implicated in the perceptual processing of song (parasagittal view).

YELLOW: The song control circuit includes the vocal motor pathway (HVC, robust nucleus of the arcopallium (RA) and the tracheosyringeal motoneurons of the hypoglossal nucleus (not shown)) and the anterior forebrain pathway (AFP) which includes area X, dorsolateral thalamus (DLM) and lateral magnocellular nucleus of the anterior nidopallium (LMAN). RED: Auditory areas and areas involved in song memorization include the caudomedial nidopallium (NCM),
1.3 Male quality, mate choice and female preference

1.3.1 Male qualities that influence female mate choice

Female zebra finches choose their mates based on a variety of factors such as beak color and cheek patch color, preferring redder beaks and more orange cheek patches (Simons et al., 2012). Male song is another very important, if not the most important, factor in female zebra finch mate choice (Catchpole and Slater, 2008). These sexual traits are reliable indicators of male quality since they are costly to develop and maintain (Catchpole and Slater, 2008; Hill and McGraw, 2006a). Coloration of beaks and cheek patches in male zebra finches are carotenoid dependent and reflect the overall health of the male (Hill and McGraw, 2006a), while complexity of male song may signal the reproductive fitness of the male (Catchpole and Slater, 2008). Multiple zebra finch studies have shown that both hearing male courtship song and perceiving male sexual traits, like beak and cheek patch color, interact to influence female mate choice (Campbell and Hauber, 2009a; Campbell and Hauber, 2009b). Although beak and plumage coloration and male vocalizations are important in relaying information about male quality in zebra finches and many other songbird species, these factors are not the only ones used to assess male quality in all avian species (Catchpole and Slater, 2008; Hill and McGraw, 2006a).

1.3.2 Mate choice behaviors

Mate choice can be defined as the pattern of mating that arises when organisms are influenced or inclined to mate with other organisms that have certain phenotypes (Andersson and Simmons, 2006; Jennions and Petrie, 2007). Mate choice occurs in both males and females.
(Andersson and Iwasa, 1996) and the term “female mate choice” refers to females choosing to mate with males bearing particular sexual traits (Andersson and Simmons, 2006). There are three classes of mate choice behaviors displayed by female zebra finches. The first is pair bonding (e.g. perching in close proximity to mate over a non-mate male) and breeding/nest building, the second involves social and sexual displays, and the third is preference for particular songs (i.e. mate song over non-mate song, and mate’s song played at a faster rate over mate’s song played at a slower rate; (Hauber et al., 2010; Miller, 1979b; Tomaszycki and Adkins-Regan, 2006; Zann, 1996). These factors also influence a partner’s preference for their own bonded mate. The impact of acute stress on the three behaviors in the examples above will be addressed in this study.

1.3.3 Female partner preference in zebra finches

Zebra finches are a highly sexually dimorphic species and robust sex differences are observed in plumage, behavior, and neuroanatomy. The establishment and maintenance of partner preference is one of the most sexually differentiated behaviors seen, with males aiming pairing behavior toward females, and females aiming pairing behavior toward males (Adkins-Regan, 2002; Adkins-Regan, 2009; Zann, 1996). Sexual partner preference is defined as the propensity or disposition to mate with a particular partner and it differs from mate choice in that choice indicates that an action was taken but preference implies that an action may or may not be taken (Cotton et al., 2006; Heisler et al., 1987). Female partner preference refers to females displaying a proclivity to direct mate choice behaviors toward a particular male (Andersson and Simmons, 2006).
1.3.4 Female song preference in zebra finches

Females have many established preferences for song which can be tested using song playback experiments where the spatial proximity of the female to the source of the playback signifies preference (Campbell and Hauber, 2009a; Miller, 1979b). Above all, females prefer conspecific song over heterospecific song (Clayton and Prove, 1989; Clayton, 1990; Lauay et al., 2004; Riebel, 2000), and they also prefer familiar conspecific song over unfamiliar conspecific song (Miller, 1979b). Females have demonstrated a preference for high rates of song output (Collins et al., 1994; ten Cate and Mug, 1984) and for a greater number of different syllables (Holveck and Riebel, 2007; Vyas et al., 2009). Directed song, a song a male sings directly towards his partner, is typically sung at a faster rate with more introductory syllables and song-phrases per bout, and usually accompanied by a courtship dance (Williams, 2001), is preferred over undirected song (Woolley and Doupe, 2008). Pair bonded females prefer the songs of their mates over the songs of other conspecific males (Miller, 1979b; Woolley and Doupe, 2008). Interestingly, once the pair bond has formed, producing higher rate song and utilizing many different syllables are not required to maintain the bond (Tomaszycki and Adkins-Regan, 2006). Spatial proximity preferences of females for particular song stimuli have been shown to be reliable in a number of studies on domesticated zebra finch behavior (Campbell and Hauber, 2009a; Campbell and Hauber, 2010; Forstmeier and Birkhead, 2004; Tomaszycki and Adkins-Regan, 2005).

1.3.5 Song preference and female zebra finch brain

Songbirds, including the zebra finch, consolidate and store memories for individual songs in the caudomedial nidopallium (NCM) (Figure 2) (Mello et al., 1992; Woolley and Doupe,
10

2008), a region of the telencephalon that is homologous to the superficial layers of mammalian auditory cortex (Adkins-Regan, 2011; Theunissen and Shaevitz, 2006). The NCM plays a role in the perceptual processing of song (Bolhuis and Gahr, 2006; Jarvis et al., 1995; Terpstra et al., 2006). Experiments that have found support for the role of the NCM in song perception, including song discrimination (Hernandez et al., 2008; Tomaszynski and Blaine, 2014), have done so by using immediate early gene (IEG) expression which serves as a marker for neural activation (Hernandez et al., 2008). These studies implicate the NCM and the caudomedial mesopallium (CMM) (Figure 1.3), a structure that is adjacent to the NCM, in the perceptual processing of song (Hernandez et al., 2008).

The IEG ZENK (which is also known as Egr-1, Zif-268, NGFI-A, Krox-24) encodes a transcription factor whose protein product, ZENK, binds to promoter regions on target genes upstream and is widely used as an indicator of neuronal activation (Hernandez et al., 2008; Tischmeyer and Grimm, 1999). Both ZENK gene and protein expression is highest in the NCM and CMM when conspecific song is played to both adult and juvenile females than when heterospecific song is played (Bailey et al., 2002; Bailey and Wade, 2003; Gentner et al., 2001; Mello and Ribeiro, 1998; Mello et al., 1992).

1.4 HPA axis, glucocorticoids and glucocorticoid receptors

1.4.1 Stress, the stress response and the HPA axis

Stress can be defined as intrinsic or extrinsic forces that challenge or threaten the complicated and constantly changing equilibrium (homeostasis) that is necessary to maintain the life of an organism (Chrousos and Gold, 1992; Tsigos and Chrousos, 2002). Stressors like severe temperature changes, food or water shortages, aggressive encounters, noise or social
subordination can disturb physiological homeostasis (Nelson, 2011). In response to this, an organism mounts a stress response which will serve to reestablish homeostasis by utilizing a series of physiological and behavioral counter-actions (Selye, 1950).

There are two endocrine systems that are responsible for the mediation of stress over its course, one involves epinephrine (adrenaline) secreted from the adrenal medulla, and the other involves glucocorticoids (GCs) secreted from the adrenal cortex (Nelson, 2011; Stratakis and Chrousos, 1995). These two systems act on different timescales. The sympathetic nervous system starts to secrete norepinephrine (noradrenaline) and the adrenal medulla starts to secrete epinephrine within seconds of an organism experiencing a stressor (Nelson, 2011). Within minutes of perceiving a stressor, an organism’s adrenal cortices begin to secrete GCs (Nelson, 2011). Both epinephrine and GCs are commonly known as stress hormones although their main endocrine function is in metabolism, and their levels vary with circadian rhythms even in the absence of stress (Becker et al., 2002; Buckingham, 2006; Nelson, 2011). Epinephrine cannot cross the blood-brain barrier, but GCs can and therefore are able to mediate the behavioral effects of stress (Nelson, 2011). GCs are steroid hormones that include corticosterone/cortisol (Becker et al., 2002), and they serve as the final effectors in the HPA (hypothalamic-pituitary-adrenal) axis (Tsigos and Chrousos, 2002).

The common pathway mediating the stress response is activation of the HPA axis (Nelson, 2011) (Figure 1.4) which causes corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoids to be released within minutes of the onset of a stressor (Stratakis and Chrousos, 1995). The paraventricular nucleus (PVN) of the hypothalamus releases the neurohormone CRH in response to a stressor (Tsigos and Chrousos, 2002). CRH acts on the basophilic corticotrope cells of the anterior pituitary gland to stimulate
Figure 1.4 Diagram showing negative control of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis releases hormones in response to stress. Within minutes, the hypothalamus releases corticotropin-releasing hormone (CRH) and other hormones which stimulate (+) adrenocorticotropic hormone (ACTH) release from the anterior pituitary. ACTH stimulates corticosterone (and cortisol) secretion from the adrenal cortex. When the stressor is removed, tissue response to corticosterone ends the stress response by negative feedback control (−), which acts on the hippocampus, hypothalamus and anterior pituitary. Image redrawn from Paul et al, 2015.

The synthesis and release of ACTH into the bloodstream (Tsigos and Chrousos, 2002). ACTH subsequently causes the release of glucocorticoids from the zona fasciculata of the adrenal cortex (Bao et al., 2008; Buckingham, 2006). HPA axis activation results in both immediate and delayed physiological effects (Sapolsky et al., 2000). The immediate effect includes increased cardiovascular tone, stimulation of immune function, energy mobilization, inhibition of reproductive physiology and behavior and loss of appetite (Sapolsky et al., 2000). The delayed effects occur as a result of the synthesis and release of glucocorticoids from the adrenal cortex (Sapolsky et al., 2000). After the stressor or threat is over, tissue response to GCs terminates the
stress response by a negative feedback control, which acts on the levels of the anterior pituitary, hypothalamus and hippocampus (Swaab et al., 2005).

1.4.2 Glucocorticoids and glucocorticoid receptors

Corticosterone is the GC form found in most rodents, birds (including the zebra finch), reptiles and fish, while cortisol is the form found in most primates, large mammals and carnivores (Stratakis and Chrousos, 1995). Some animals like sheep, pigs and dogs produce both corticosterone and cortisol (Buckingham, 2006). The immediate substrate for the biosynthesis of steroid hormones, like corticosterone, is cholesterol which is synthesized primarily in the liver, and transported to the adrenal cortex where ACTH activates the enzymes required for corticosterone synthesis (Norman and Henry, 2015). Corticosterone, like other steroid hormones, cannot be stored in cells and are instead released as they are synthesized (Norman and Henry, 2015). After synthesis and release, about 95% of corticosterone binds with high affinity to corticosteroid-binding globulins (CBGs) (Buckingham, 2006; Norman and Henry, 2015). When corticosterone in the blood plasma is transported by systemic circulation to the target tissues, it enters the cells via diffusion but only if it is unbound from CBGs (Norman and Henry, 2015). Corticosterone exerts its actions mainly via two distinct intracellular (cytoplasmic) receptors, mineralocorticoid receptors and glucocorticoid receptors, which regulate target gene transcription (Buckingham, 2006; Herman et al., 2016). These receptors bind to corticosterone and are translocated to the nucleus where they can function as transcription factors, directly or indirectly, to either induce or suppress DNA transcription, and therefore, protein synthesis (Buckingham, 2006; Herman et al., 2016).
There are any differences between the two receptors for corticosterone. Mineralocorticoid receptors (MR) have a high binding affinity for corticosterone and are found in tissues concerned with Na+/K+ balance (ex. sweat glands, distal convoluted tubule of the kidney, parotid gland and colon) and in brain regions like the limbic system, entorhinal cortex and the hypothalamus (Breen et al., 2004; Buckingham, 2006; Herman et al., 2016). In contrast, glucocorticoid receptors (GR) have a low binding affinity for corticosterone and are found in many tissues of the body (ex. liver, lymphocytes, adipose cells, kidney, anterior pituitary) and in many brain regions (Buckingham, 2006; Herman et al., 2016; Norman and Henry, 2015). MRs are responsible for mediating the effects of the basal circadian rhythm of very low concentrations of corticosterone while GRs are responsible for mediating the effects of high concentrations of corticosterone (Breen et al., 2004; Buckingham, 2006). MR/GR balance is important since one of the MR-mediated effects of corticosterone is the suppression of the activity of the HPA axis (Oitzl et al., 1995), and when imbalances occur (ex. in stress-related disorders), increased activation of the HPA axis is observed (Bao et al., 2008).

1.5 Chronic stress vs acute stress

Acute stress causes short-term activation of the HPA axis and is thought to be adaptive and potentially beneficial (McEwen, 2006; Sapolsky, 2000) since acute stress responses include enhanced cognition, enhanced analgesia and energy mobilization (Becker et al., 2002). In a laboratory setting, restraint protocols are effective at eliciting an acute adrenocortical response in many vertebrates, including zebra finches (Banerjee and Adkins-Regan, 2011; Wingfield, 1995). Multiple studies have found support for another adaptive function of acute stress. Many experiments have found support for the role of acute GC elevation in mediating the tradeoff
between reproduction and survival even though the results of those studies are varied (Breuner et al., 2008).

When the stress response is prolonged or repeated, it can be said that an organism is experiencing chronic stress (Becker et al., 2002; Herman et al., 2016). Chronic stress is thought to be maladaptive and has long-lasting and usually harmful effects on physiology, behavior, reproductive success and survival (Breuner et al., 2008; McEwen, 2006; Nelson, 2011; Sapolsky et al., 2000) which include myopathy, impaired disease resistance and accelerated neural degeneration during aging (Becker et al., 2002). The varied and mostly adverse effects of chronic stress have been widely explored in zebra finches as well as other animals, but the effects of acute stress on reproductive behaviors like partner preference have been mostly under-explored (Banerjee and Adkins-Regan, 2011; LaPlante et al., 2014).

1.6 Dissertation Goals

The overall goal of this dissertation is to address the following questions: 1) does acute stress alter an adult zebra finch female’s preference to be in close proximity to her mate and/or his song? 2) is the mate song induced expression of ZENK protein in the NCM and CMM of an adult zebra finch female brain altered by acute stress? and 3) is the distribution and subcellular localization of GR-immunoreactive neurons in the adult zebra finch female brain altered by acute stress? In chapter 2, I describe research on the effect of an ecologically relevant stressor, cold stress, that is experienced early life on HPA axis sensitivity and global DNA methylation in the brains of juvenile and adult zebra finches of both sexes. Early life stress has been shown to have damaging effects on the brain and behavior later in life. In chapter 3, I tested whether acute stress could alter female preference for her mate using three different two-choice preference paradigms
for proximity, song and song rate preference. Many studies confirm a female’s preference for
being in close proximity to her mate and for being in close proximity to the song of her mate but,
to the best of our knowledge, no studies address the effect that acute stress can have on the
strength of her preference. Acute stress is thought to have beneficial effects but, so far, research
results have not been consistent. In chapter 4, I hypothesized that acute stress would change the
expression of ZENK protein in the NCM and CMM of females that were exposed to their mate’s
song after the stressor. We further studied the effects of acute stress on the presence and quantity
of GR-immunoreactive neurons in regions of the female brain like the hippocampus, NCM,
CMM, and cerebellum. The combined results are then synthesized in chapter 5, and together
these studies help us understand how acute stress affects the strength of female preference in the
adult zebra finch.
2 PHYSIOLOGICAL AND ECOLOGICAL STRESSORS DECREASE GLOBAL METHYLATION LEVELS IN JUVENILES AND ATTENUATE THE ACUTE STRESS RESPONSE IN ADULT ZEBRA FINCHES (TAE NIOPYGIA GUTTATA)

with

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Abstract

Stress in early life can have detrimental effects on brain development and behavior in adulthood. Animals in the wild, unlike animals in a laboratory setting, are vulnerable to a wide array of environmental factors that can induce stress. In endotherms, cold exposure, especially early in life, can be stressful. We examined the effects of an ecologically relevant stressor (cold exposure) and a physiological stressor (oral corticosterone (Cort) administration) experienced early in life to determine if they differentially alter HPA axis sensitivity, and global DNA methylation in the brains of juvenile and adult Australian zebra finches (Taeniopygia gutta). We hypothesized that daily exposure to both stressors during the first two weeks post-hatch will alter HPA axis sensitivity and global brain DNA methylation levels in juveniles and adults. In both juveniles and adults, controls exhibited an elevation in plasma Cort after 15-mins. of restraint stress; however, juveniles and adults that were exposed to cold or fed oral Cort early in life demonstrated a blunted stress response. Adults that were exposed to cold or fed oral Cort showed no significant differences in global DNA methylation levels when compared to their controls and to each other, but juveniles showed significantly lower global DNA methylation levels when compared to their controls. DNA methylation studies suggest that both hypomethylation on a
global scale, and hypermethylation in specific gene sequences can occur after adverse early life events. The attenuated Cort response shown by juveniles and adults in response to early life stress could protect against the noxious effects of prolonged glucocorticoid secretion.

2.1 Introduction

2.1.1 Australian zebra finches

The Australian zebra finch, Taeniopygia guttata, is a passerine songbird endemic to central Australia and the Lesser Sundas islands (Zann, 1996). They are a highly gregarious species and males and females form life-long pair bonds both in wild and in domesticated populations (Perfito et al., 2007; Smiley et al., 2012; Svec et al., 2009). They are opportunistic breeders that nest and mate during periods of rain or wet conditions, and males and females both participate in nest building, brooding and incubation behaviors (Zann, 1996). Once parents start incubating, characteristics of developing embryos can be staged developmentally, and eggs typically hatch after a 14-day incubation period (Zann, 1996).

On the day of hatching, age is denoted as P0 (P: post-hatch, 0: days old). After hatching, zebra finch young are altricial and unable to move, thermoregulate, or feed independently without parental care (Zann, 1996). Zebra finch hatchlings remain in the nest until they fledge around P17-18 (Zann, 1996). Offspring are considered juveniles around P30 and display black beaks and dull gray downy feathers. Sexually dimorphic plumage differences begin to develop around P40, when birds are considered nutritionally independent from their parents (Zann, 1996). By P120 or older, birds are considered adults, male cheek patch and beak coloration are completely mature along with the male’s final crystalized song.
2.1.2 The hypothalamic-pituitary-adrenal axis

The HPA or hypothalamic-pituitary-adrenal axis regulates stress responses and operates via negative feedback (Figure 2.1). Briefly, corticotrophin-releasing hormone (CRH), synthesized in the paraventricular nucleus (PVN) of the hypothalamus, is released into the hypophyseal portal system and acts on receptors in the anterior pituitary to stimulate the release of adrenocorticotropic hormone (ACTH) into circulation (Banerjee et al., 2012; Wright et al., 2013). ACTH stimulates cells in the zona fasciculata of the adrenal cortex to synthesis and secrete glucocorticoids (GCs), such as corticosterone (Cort) or cortisol, into circulation (Banerjee et al., 2012; Wright et al., 2013). Cort is bound to binding globulins, such as corticosteroid binding globulin (CBG), and carried through the blood. CBGs regulate the amount of free circulating Cort available and reduce target tissue interactions, thereby buffering the negative consequences of GCs (Crino et al., 2014).

![Figure 2.1 Schematic of the vertebrate Hypothalamic-Pituitary-Adrenal (HPA) axis.](image)

2.1.3 Corticosterone, glucocorticoid receptor, and negative feedback

The classical mechanism of GC action is via binding to glucocorticoid receptors (GRs) or mineralocorticoid receptors (MRs). Distribution of these receptors in the brain includes,
hippocampus, cerebellum, and hypothalamus (Banerjee et al., 2012; Wada and Breuner, 2010; Wright et al., 2013). The relative affinity differs between GR and MR. MR has a high affinity for GCs and are primarily responsible for maintaining basal GC levels, while GR has relatively lower affinity for GCs (Banerjee et al., 2012; Wada and Breuner, 2010; Wright et al., 2013). Both receptor types are required for the stress response (Wada and Breuner, 2010); however, higher concentrations of GRs are required to initiate negative feedback which suggests that GR is mainly responsible for shutting off the heightened stress response (Wright et al., 2013). This indicates GR levels play an important role in HPA axis sensitivity with more GR resulting in an efficient negative feedback response, but chronically lower GR levels resulting in a diminished or hyposensitive response (Banerjee et al., 2012; Wada and Breuner, 2010).

2.1.4 Early life stress

Song is essential for male reproductive fitness, and the quality of song depends on the development of the forebrain song nuclei (Vyas et al., 2009; Woodgate et al., 2012). During the first month of life, the zebra finch brain undergoes a period of massive neuronal proliferation and pruning as the song nuclei develop. This occurs in parallel with sensory learning, memorization, and song production (Deregnaucourt et al., 2013; Woodgate et al., 2012; Zann, 1996). Environmental and physiological conditions early in life, such as those associated with stress, can alter the song nuclei development and the ability to learn and sing a courtship song. Ultimately, this early exposure to stress can alter reproductive fitness.

2.1.5 Stress exposure in zebra finch

Direct administration of Cort through daily oral dosing or subcutaneous implants has been used with positive results (Buchanan et al., 2004; Shahbazi et al., 2014; Spencer et al.,
Cort dissolved in peanut oil is the least invasive method to increase Cort concentrations in hatchlings for the first few weeks post-hatch.

Nutritional deprivation to mimic food scarcity has also been used to induce stress. This is done by altering the millet seed to husk ratio or by decreasing the amount of food given on a particular day (Buchanan et al., 2003; Spencer et al., 2003; Zimmer et al., 2013). As a result, parents providing care for offspring have to increase their foraging efforts in order to provide food. However, food deprivation does not directly increase Cort levels as demonstrated in 18-day old hatchlings (Spencer et al., 2003). Furthermore, zebra finches metabolize much of their water from seed consumption (the water content of standard seed mixture ranges between 7-10% (Zann, 1996); suggesting that seed deprivation may be disrupting water balance rather than energy balance. Nutritional deprivation may be a more relevant stressor for species with precocial offspring, such as Japanese quail (Coturnix japonica) and domestic chickens (Gallus gallus). In both species, seed removal or reduction did result in significant increases in Cort, possibly due to the need for increased foraging efforts (Zimmer et al., 2013).

Oral Cort administration is a physiological stressor that elevates circulating Cort above baseline for a period of hours to days. Food deprivation, on the other hand, has been described as an ecologically relevant stressor, although it has not been shown to directly increase Cort levels above baseline in species with altricial offspring. Both nutritional deprivation and Cort administration have been shown to alter song nuclei size, song quality, basal Cort concentrations, and body condition and size in songbirds (Buchanan et al., 2004; Crino et al., 2014; Schmidt et al., 2013; Spencer et al., 2003; Spencer and Verhulst, 2007).
2.1.6 Cold stress

While cold stress is not as commonly used to induce a stress response, it is a very potent and relevant ecological stressor and perhaps more appropriate to use in zebra finches due to the fact that it is unlikely to alter water balance. Cold exposure does not alter Cort levels in adult birds (Johnson and Rashotte, 2002), but does result in a decrease in carotenoid concentration in adult male zebra finches, resulting in turn, duller beaks that make them less attractive to females (Eraud et al., 2007). One study has assessed the effect of cold on hatchling Cort levels in eastern bluebird chicks (Lynn and Kern, 2014), which are altricial and require significant amount of parental care. Hatchlings were housed at four temperature conditions; cold (1.8 ± 0.08°C), cool (9.08 ± 0.04°C), ambient (22.0 ± 0.04°C), and brooding (23.3 ± 0.06°C) from P5 to P7. The cold and cool treatments showed significantly higher levels of Cort by P7 than ambient or brooding temperatures.

2.1.7 Changes in the song system associated with early life stress

Studies using Cort administration or food deprivation demonstrated significant changes in both song quality and the size of the forebrain song nuclei (Buchanan et al., 2003; Shahbazi et al., 2014; Spencer et al., 2003; Spencer and Verhulst, 2007; Zimmer et al., 2013). Adult male zebra finches that were chronically stressed as hatchlings, sang with fewer unique syllables, had lower song accuracy scores, and lower peak frequencies (Spencer et al., 2003). Furthermore, these changes were accompanied by reductions in song nuclei size. Song sparrows that were either fed Cort or food deprived until age P18 showed a decrease in RA (Nucleus robustus arcopallii) size in addition to decreased song accuracy scores, song types and syllables (Schmidt et al., 2013). Zebra finch adults and juveniles exhibited a decrease in HVC (acronym is a proper
name; located in the nidopallium) size in both Cort-treated and nutritionally deprived treatment groups (Buchanan et al., 2004; Shahbazi et al., 2014). It is hypothesized that elevated Cort may act in the brain as an oxidative stressor thereby decreasing neuron number in HVC (Shahbazi et al., 2014). GCs have been demonstrated to damage free-radical scavenging enzymes such as superoxide dismutase resulting in decreased antioxidant capabilities in the brain (Shahbazi et al., 2014; Zafir and Banu, 2009). During normal song nuclei development, HVC undergoes the most dramatic change from hatching to adulthood with projection neurons primarily extending towards LMAN (nucleus lateralis magnocellularis nidopallii) during development and RA during adulthood (Bertram et al., 2014; Buchanan et al., 2004; Shahbazi et al., 2014; Zann, 1996).

2.1.8 Physiological and molecular alterations with early life stress

It has been hypothesized that hatchlings undergo a period of hyporesponsiveness similar to the stress hyporesponsive period (SHRP) experienced by neonatal rodent pups. The SHRP is described as a mechanism to protect altricial young from the harmful effects of Cort release in early life (Wada and Breuner, 2010). MR Cort binding capacity was highest in P1-P3 hatchlings and then decreased by P7 suggesting high receptor densities may be needed for the negative feedback response at P1-P3 (Wada and Breuner, 2010). In eastern bluebird nestlings, Cort levels are at their lowest at P4-P5 and increase until P7, and this steady increase in Cort during development was also observed in cold exposed birds with Cort levels higher at P7 compared to earlier ages (Lynn and Kern, 2014). Together, these results suggest a hyposensitive period for Cort release and feedback, and that early life stress potentially has “organizational power” on brain development and functioning (Wada and Breuner, 2010).
A reduction in HPA axis responsiveness resulting from early stress exposure is associated with multiple cognitive and neurological issues, including cognitive deficits and disease (Harris and Seckl, 2011) which often cannot be assessed until animals are older (juveniles or adults). Cort administration and food deprivation early in life both resulted in significantly elevated Cort levels when compared to controls in juvenile finches (Crino et al., 2014; Spencer et al., 2003). While it is evident that the Cort response early in life is responsible for the MR and GR concentrations in the brain in hatchlings (Wada and Breuner, 2010), hatchlings exposed to maternal deprivation during the first two-three days of life, exhibited higher Cort levels after 30 minutes of isolation compared to controls when tested as adults (Banerjee et al., 2012). This effect was accompanied by an overall decrease in MR mRNA in the hypothalamus, hippocampus, and cerebellum and a significant decrease in GR mRNA in the hypothalamus of these maternally deprived offspring (Banerjee et al., 2012).

2.1.9 Epigenetics

The developmental environment can affect the methylation of the genome. CpG islands are a gene’s coding region “on and off” switch and the addition of a methyl group (CH3) to cytosine’s pyrimidine ring on the 5th carbon results in a 5-methylcytosine structure, which serves as a transcription barrier and prevents the transcription of that particular gene (Gryzinska et al., 2013; Murphy et al., 2013; Wright et al., 2013). Conversely, the removal of methyl groups along the CpG islands promotes transcription by allowing transcription factors to bind to the promoter region. Epigenetic mechanisms such as these are a potential mechanism connecting early developmental stress and variations in song parameters and their corresponding nuclei.
2.2 Goal of this study

The goal of this study is to examine how an ecologically-relevant stressor (cold exposure) and a physiological stressor (daily oral Cort administration) alter the adult stress response and global methylation in the brain. The only published studies that have examined the relationship between temperature and stress in zebra finches have focused either on cold stress and metabolism in adults (Jimeno et al., 2017), or on elevated temperatures during nesting and incubation (Andrew et al., 2017). There are no studies investigating the impact of cold on stress responsiveness and brain development. In the wild, zebra finches are accustomed to dry and warm temperatures but will continue to breed at 12°C during the winter months (Zann, 1996), and so, cold exposure is an ecologically relevant but underutilized stressor in zebra finches across all life history stages.

Zebra finches exposed to Cort and to maternal or nutritional deprivation early in life demonstrated high Cort levels and a weakened negative feedback response compared to controls (Crino et al., 2014; Spencer et al., 2003). In this study, we hope to determine if cold exposure or oral Cort administration in early life can alter baseline and restraint Cort levels later in life. We will also examine if early life cold exposure and oral Cort treatment alter epigenetics by assessing global methylation levels among male and female zebra finches in two different age groups: juveniles (P25-30) and adults (over P120). Methylation levels can be transferred across generations; data from similar studies could potentially show how perturbation of the environment may dictate the behavioral and physiological changes across an individual’s life history as well as across generations (Morgan and Bale, 2011).
2.3 Methods

2.3.1 Zebra finch care and breeding

Male and female zebra finches were housed in either communal flight cages (40-60 birds) or individual breeding cages (IBCs; contain one breeding pair plus non-adult offspring) in the Georgia State University (GSU) animal facility. The GSU Institutional Use and Animal Care Committee approved all animal procedures. Aviary temperature was maintained at 23°C and the birds were kept on a 12-hour light: 12-hour dark photoperiod. Flight cages contained 12 nest boxes attached along the periphery. IBCs contained a single nest box situated near the entrance. All birds had ad libitum access to millet seed (standard Kaytee finch seed), water, shell grit, and cuttlefish bone. Birds were given hardboiled eggs and spinach supplement once a week to promote and accelerate egg laying and breeding behaviors and were misted with water from a spray bottle to stimulate breeding conditions.

Established pair bonds were determined by observing adults in their home cages performing allopreening and clumping behaviors. Mated pairs allopreen, in which a male and female pair grooms one another, and maintain close physical contact or “clump” for long periods of time (Smiley et al., 2012). All birds had unique colored leg bands used to identify pair bonds. Further observations and indicators of well-established pair bonds were assessed and included nest building, incubating and brooding behavior.

Twelve mated pairs were transferred from the one of the communal flight cages and into individual breeding cages (IBCs). These pairs were allowed four weeks to mate. One pair failed to yield fertilized eggs and exhibited abnormal breeding behaviors, so they were replaced by the end of the 4-week period with a new breeding pair. All eggs were candeled (shining a bright light behind the egg to visualize blood vessels) approximately one week after laying to determine if
the egg was fertilized. Zebra finch eggs have a 14-day (approximately) incubation period. One of the flight cages housed four additional pair bonded zebra finches whose hatchlings were used in this study. The number of eggs laid, and offspring hatched were recorded Monday through Saturday.

2.3.2 Sexing of hatchlings

Zebra finches do not display sexually dimorphic features until around P50-60 and therefore the sex of birds was determined via PCR following a minimally invasive protocol (Soderstrom et al., 2007). Briefly, DNA was extracted from one drop of blood following a standard protocol and then PCR was conducted using zebra finch specific primers to the W and Z sex chromosomes, W1 primer (5'-GGGTTTTGACTGACTAACTGATT-3’), W2 primer (5’-GTTCAAGCTACATGAATAAACA-3’), Z1 primer (5’-GTGTAGTCCGCTGCTTTTGG-3’), Z2 primer (5’-GTTCGTGGTCTTCCACGTCC-3’). Gel electrophoresis was used to visualize PCR products.

2.3.3 Experimental design and treatment groups

New hatchlings and eggs were checked six days a week (Mon. through Sat.). New hatchlings observed on Mon. were aged ±1 days and new hatchings on other days were aged P0. IBCs were designated as part of oral Cort or cold experimental group and all hatchlings in each clutch (in-clutch design) were randomly assigned as experimental or control. The left middle talon of all experimental birds was marked with blue nail polish to distinguish them from control birds. Hatchings were added to the study until there were 10 males and 10 females for both experimental (cold and oral Cort) and control treatments. Sample sizes were designed to
compensate for possible reduced mortality before birds fledge. Zebra finches have altricial offspring with survivorship between 60-75% at 10 days post hatch. Juvenile (P30) final samples were cold exposure n = 8 (4 male, 4 female), cold control n = 6 (4 male, 2 female), oral Cort n = 7 (3 male, 4 female), and Cort control n = 8 (4 male, 4 female). Adult (P120) final samples sizes were cold exposure n = 7 (4 male, 3 female), cold control n = 6 (3 male, 3 female), oral Cort n = 8 (4 male, 4 female), and Cort control n = 6 (3 male, 3 female).

2.3.4 Cold exposure

Hatchlings were exposed to cold stress treatments for 14 days starting on post-hatch day 3, or P3 (±1 days) until post-hatch day 17, or P17 (±1 days). Cold exposure occurred for 20 mins./day between noon and 2pm. The cold chamber temperature was maintained between 9°C-11°C. Hatchlings were transported from their nest boxes to the behavioral suite. Once the hatchlings were placed in the plastic weigh boat, the chamber was covered for 20 mins, and the temperature was recorded every 2 mins. Due to their inability to thermoregulate, each hatchling was stimulated by a single touch to ensure that they were responsive. Exaggerated movements from the wing, leg, and neck characterized significant amount of movement for each two-minute period. If the hatchling failed to respond to touch, it was immediately removed from the chamber and placed into their home nest. Early removal typically occurred during the first day of cold stress. These birds still received 14 days of cold stress and remained in the study. Control birds were placed in an ice-free cold chamber for 20 mins. following the protocol described above.

2.3.5 Cold stress chamber

Three styrofoam coolers were used as cold chambers with five, equally spaced holes punched in the lid (Figure 2.2). A thermometer was inserted into one hole with the other four
serving as air-ports. A layer of wet ice 2-3 inches deep was added to the floor of the cooler and a plastic weigh boat was suspended from the top approximately two inches above the ice. Once the air inside the chamber reached 9°C the hatching was placed in a plastic weigh-boat suspended over the ice.

![Figure 2.2 Styrofoam cold exposure chamber.](image)

### 2.3.6 Administration of oral corticosterone

Hatchlings within a clutch were randomly placed in the experimental or control group. Experimental animals were fed 0.5 mg/ml of CORT dissolved in 25 µl of sterilized peanut oil with the controls receiving 25 µl peanut oil following a modified version of a tested protocol (Spencer and Verhulst, 2007). All birds received the treatment once daily (between noon and 2pm) beginning 3 days post-hatch (P3) and ending 17 days post-hatch (P17). Feeding consisted
of dropping the peanut oil directly into back of mouth using a pipette (hatchling birds naturally open their mouths with any stimulus targeting the beak).

2.3.7 **Blood collection for radioimmunoassay**

Juvenile or adult experimental and control birds were captured (in under three minutes) from their home cages for blood collection to determine basal Cort levels. Blood was collected from the right alar wing vein using a sterile needle (BD PrecisionGlide™ 26G x 5/8 (0.45mm x 16mm). Approximately 150-µl of blood was collected into multiple Fisherbrand® micro-hematocrit capillaries. Gelfoam® Dental powder was applied to the wound to stop the bleeding. Blood samples were transferred from hematocrit tubes to 0.6 ml Fisherbrand® microcentrifuge tubes and centrifuged in an Eppendorf miniSpin plus centrifuge at RT for 4 mins at 8000 rpm. A minimum of 15 µl of plasma was pipetted out and transferred to a new 0.6 ml Fisherbrand® microcentrifuge tube for Cort RIA. The remaining sample was saved for further DNA analysis. Plasma and blood was stored at -20°C.

2.3.8 **Restraint stress**

Juvenile (P30) and adults (P120) experimental and control birds were housed in their home IBC or flight cage. Birds were captured from their home cage and placed in an opaque cloth bag for 15 mins and held gently. Restraint stress and blood collection was staggered by 8 mins to ensure adequate time for blood to coagulate at the injury to site. Blood collection followed the protocol described above except that blood was drawn from the left alar wing vein instead of the right. All IBC birds were returned to their respective cages and allowed 1.5 to 2 hrs of recovery before brain collection. Flight cage birds were not returned to their home cages but
remained in the holding cage within the aviary and were also allowed 1.5 to 2 hrs of recovery before brain collection.

2.3.9 Radioimmunoassay for Cort

Total plasma Cort concentrations were measured using RIA (Corticosterone 125I RIA kit, catalog # 07 – 120102; MP Biomedicals LLC, Solon, OH, for rat plasma). We previously validated the use of this kit in zebra finches for measuring plasma Cort levels, and this kit has been used in other songbirds as well (Newman, 2010). To validate, we initially ran zebra finch pooled plasma with rat and hamster pooled plasma. The standard curve was matched to the zebra finch Cort concentrations range (by adding 6.25 ng and 12.5 ng through serial dilution of 25 ng standard to increase sensitivity). Once established, we ran a dilution series (1:2 98 %, 1:4 102 %, and 1:6 115 %) of zebra finch plasma to get linearity and obtained the overall recovery. In addition to the two (low and high concentrations of Cort) controls that were provided by the kit, pools of zebra finch, rat (control for assay), and hamster (in house control) plasma were used as extra controls for each assay run. Recovery rate was 99 % and detection limit was 6.25-1000 ng / mL. Intra-assay & inter-assay coefficient variations were 5.3 % and 9.4 %, respectively.

2.3.10 Brain collection

Brains were collected from experimental and control birds to measure global DNA methylation. Prior to sacrifice, all surgical supplies were washed with soap, water, and 70% ethanol in order to prevent DNA contamination. Birds were given an overdose of Isoflurane until the heart stopped beating and then were rapidly decapitated using sharp scissors. The scalp was removed from the base of the neck and pulled towards the beak to expose the skull. An incision
was made around the periphery of the skull, which was carefully lifted to expose the brain. The entire brain (cerebrum, olfactory bulb and cerebellum), was quickly removed and placed into a 10 mL tube and flash frozen on dry ice and then stored at -80°C.

2.3.11 DNA and protein extraction

2.3.11.1 Homogenization and phase separation

TRIzol® Reagent was used to extract DNA and protein (for future analyses) from the brain following a standard protocol. For every 100 mg of brain tissue, 1 mL of TRIzol® Reagent was added and homogenized. Between each sample, the homogenizer was cleaned with three washes: milli-Q water, 1 M NaOH, and milli-Q water. The resulting homogenate was shaken and incubated at room temperature before chloroform was added. Once the samples were centrifuged (Allegra™ 21R Centrifuge) at 5,500 x g for 25 minutes at 4°C, the top aqueous layer containing RNA was pipetted out of the solution and immediately discarded. The interphase and organic layer containing the genomic DNA and protein, respectively, were saved for extraction.

2.3.11.2 DNA precipitation, wash and resuspension

For DNA precipitation, the organic layer was treated with 100% ethanol (0.3 mL 100% ethanol: 1 mL TRIzol® Reagent) and centrifuged 4°C. The protein supernatant was saved for further protein precipitation, wash and resuspension. The remaining DNA pellet was washed with 0.1 M sodium citrate in 10% ethanol (1 mL sodium citrate: 1 mL TRIzol® Reagent) and 75% ethanol (2 mL 75% ethanol: 1 mL TRIzol® Reagent). After the removal of supernatant, the DNA pellet was dried and resuspended in 8 mM NaOH (0.3 mL 8 mM NaOH: 70 mg brain).
2.3.11.3 *MethylFlash colorimetric methylation quantification*

To measure global methylation DNA in whole brain we used the MethylFlash™ Methylated DNA Quantification Kit (Colormetric) kit. In short, approximately 200 ng of DNA was bound to high affinity strip wells for capture and detection antibodies to specifically bind to methylated portions of DNA (5-mC). Quantification was determined colorimetrically through a microplate spectrophotometer with OD intensity directly proportional to the amount of methylated DNA. To determine the percent and amount of methylation, a standard curve was produced based on various positive control concentrations. The slope of the standard curve was used to determine the absolute amount of methylated DNA in the sample based on the formula below:

\[
5\text{-mC }% = \left[ \frac{(\text{Sample OD} - \text{ME3 OD})}{\text{S}} \times \frac{\text{ME4 OD} - \text{ME3 OD}}{2} \times \frac{\text{P}}{\text{S}} \times 100\%ight]
\]

2.3.12 *Statistics*

All data were analyzed using IBM SPSS Statistics for Windows, version 25.0 (SPSS Inc, Chicago, IL). Parametric statistical tests were used for all data since assumptions were not violated. Statistical significance was accepted at \( p < 0.05 \) for all tests. The average plasma Cort concentration for baseline and restraint, as well as the average brain methylation levels in males and females were compared using paired samples t-tests. No significant differences were found between the sexes in either the cold stress or oral Cort conditions, so all analyses were conducted on pooled plasma Cort concentrations and pooled brain methylation levels for males and females.

A mixed-design ANOVA was used to compare the main effects of treatment and time (of Cort measurement), and the interaction effect between treatment and time on plasma Cort
concentration in juveniles. Another mixed-design ANOVA was used to compare the main effects of treatment and time, and the interaction effect between treatment and time on plasma Cort concentration in adults. Treatment (cold exposed, cold control, Cort fed, Cort control) was the between-subjects factor and time (baseline, restraint) was the within-subjects factor. A one-way ANOVA was used to compare the brain methylation levels in cold exposed, cold control, Cort fed and Cort control juveniles. Another one-way ANOVA was used to compare the brain methylation levels in cold exposed, cold control, Cort fed and Cort control adults.

2.4 Results

2.4.1 The effects of early life cold stress and Cort administration on Cort response after 15-minutes of restraint stress in juveniles

A mixed-design ANOVA was used to examine the effect of treatment and time on plasma Cort concentration. There was a statistically significant interaction between the effects of treatment and time on plasma Cort concentration ($F(3, 25) = 22.1, p < 0.001$), and there was a main effect of both treatment ($p < 0.001$) and time ($p < 0.001$) on Cort concentration (Figure 2.3).

There was a significant difference between baseline and restraint Cort concentrations in both cold control and Cort control juveniles ($p < 0.001$, $p < 0.001$, respectively), but there was no significant difference between baseline and restraint Cort concentrations in cold exposed and Cort fed juveniles ($p < 0.221$, $p < 0.113$, respectively). Juveniles stressed early in life had a blunted stress response to 15 mins. of restraint, but juveniles that did not experience early life stress had higher Cort concentrations (typical stress response) after 15 mins. of restraint.
Figure 2.3 Baseline and restraint Cort concentrations (mean +/- SEM) for cold (n = 8), cold control (n = 6), Cort, (n = 7) and Cort control (n = 8) juvenile (P30) birds exposed to either cold or oral Cort in early life.

Cort levels were significantly different between baseline and restraint levels in cold exposed, cold control birds and Cort-treated birds (p < 0.05).

Baseline Cort concentration in cold exposed and Cort fed juveniles were significantly different from their controls (p < 0.001, p < 0.001, respectively) and from each other (p < 0.001).

Baseline Cort concentration in cold control and Cort control juveniles were not significantly different from each other (p < 0.568). Restraint Cort concentration in cold exposed juveniles were significantly different when compared to their controls (p = 0.001). Cort fed juveniles had no significant differences in restraint Cort concentration when compared to their controls.

Juveniles that experienced cold stress or oral Cort administration early in life had elevated baseline Cort concentrations, but juveniles that did not experience early life stress had low
baseline Cort concentrations. Restraint Cort concentration in response to 15 mins. of restraint was lower in cold exposed juveniles than its controls.

2.4.2 The effects of early life cold stress and Cort administration on Cort response after 15 minutes of restraint stress in adults

A mixed-design ANOVA was used to examine the effect of treatment and time on plasma Cort concentration. There was a statistically significant interaction between the effects of treatment and time on plasma Cort concentration ($F(3, 23) = 33.22, p < 0.001$), and there was a main effect of both treatment ($p < 0.001$) and time ($p < 0.001$) on Cort concentration (Figure 2.4).

![Figure 2.4](image)

*Figure 2.4* Baseline and restraint Cort concentrations (mean +/- SEM) for cold (n = 7), cold control (n = 6), Cort, (n = 8) and Cort control (n = 6) adult (P120) birds exposed to either cold or oral Cort in early life. Cort levels were significantly different between baseline and restraint levels in cold control birds and baseline and restraint Cort-treated birds ($p < 0.05$).
There was a significant difference between baseline and restraint Cort concentrations in both cold control and Cort control adults ($p < 0.001$, $p < 0.001$, respectively), but there was no significant difference between baseline and restraint Cort concentrations in cold exposed and Cort fed adults ($p < 0.221$, $p < 0.113$, respectively). Adults stressed early in life had a blunted stress response to 15 mins. of restraint, but adults that did not experience early life stress had higher Cort concentrations (typical in a stress response) after 15 mins. of restraint.

Baseline Cort concentration in cold exposed and Cort fed adults were significantly different from their controls ($p < 0.001$, $p < 0.001$, respectively) and from each other ($p < 0.001$). Baseline Cort concentration in cold control and Cort control adults were not significantly different from each other ($p < 0.568$). Restraint Cort concentration in cold exposed adults were significantly different when compared to their controls ($p = 0.001$). Cort fed adults had no significant differences in restraint Cort concentration when compared to their controls. Adults that experienced cold stress or oral Cort administration early in life had elevated baseline Cort concentrations, but adults that did not experience early life stress had low baseline Cort concentrations. Restraint Cort concentration in response to 15 mins. of restraint was lower in cold exposed adults than its control.

2.4.3 The effects of early life stress and Cort administration on global brain DNA methylation levels after 15 minutes of restraint stress

A one-way ANOVA was used to examine the effect of treatment on global brain DNA methylation levels. There were statistically significant differences between treatments in global brain DNA methylation levels in juveniles, ($F(3, 25) = 13.47$, $p < 0.001$). Post hoc analysis using
the Bonferroni test revealed that early life stress resulted in decreased methylation levels (hypomethylation) in both cold exposed and Cort fed juveniles when compared to controls ($p < 0.001$, $p = 0.010$, respectively; Figure 2.5). Methylation levels in cold exposed and Cort fed juveniles were not significantly different from each other ($p = 1.000$).

![Figure 2.5 Percent global methylation in juvenile birds (P30) after early life cold or Cort treatment.](image)

Cold exposed and Cort-treated birds had significantly reduced 5mC levels as compared to controls ($p < 0.05$).

2.4.4 The effects of early life stress and Cort administration on global brain DNA methylation levels after 15 minutes of restraint stress in adults

A one-way ANOVA was used to examine the effect of treatment on global brain DNA methylation levels. There were statistically significant differences between treatments in global brain DNA methylation levels in adults, ($F(3, 22) = 3.77$, $p = 0.025$). Post hoc analysis using the Bonferroni test revealed that there was a significant difference in methylation levels in Cort fed
adults when compared to cold control adults ($p = 0.028$; Figure 2.6). There were no other significant differences. Early life stress did not alter methylation levels in cold exposed or Cort fed adults when compared to their controls. Methylation levels in cold exposed and Cort fed juveniles were not significantly different from each other ($p = 1.000$).

![Figure 2.6 Percent global methylation in adult birds (P120) after early life cold or Cort treatment. Cold exposed birds had significantly reduced 5mC levels as compared to controls ($p < 0.05$).](image)

**Figure 2.6 Percent global methylation in adult birds (P120) after early life cold or Cort treatment.** Cold exposed birds had significantly reduced 5mC levels as compared to controls ($p < 0.05$).

### 2.5 Discussion

The aim of this study was to determine if early life cold exposure affects the sensitivity of the zebra finch HPA axis, and to evaluate if this ecologically relevant manipulation can alter global brain DNA methylation in comparison to a physiological stressor (oral Cort administration). Cold exposure has never been used as an early life stressor like nutritional deprivation, which is a commonly administered early life stressor. However, cold exposure is an
ecologically relevant stressor that developing hatchlings can be exposed to in early life under natural conditions. Even among these studies, the effect of cold exposure was only determined within one specific age group and not across developmental stages. For our experimental design, birds were exposed to acute bouts of cold (20 mins./day for 14 days) early in life and then exposed to an acute stressor as juveniles or adults. While laboratory manipulation may not completely mirror environmental patterns, experimentally induced cold conditions and cold conditions in the wild have both been shown to increase Cort levels. Altricial (such as zebra finches) and precocial nestlings (such as Japanese quail) exhibit significant increases in Cort not only in laboratory manipulations but also immediately after deteriorating weather conditions characterized by cold temperatures, heavy wind and torrential downpours (Bize et al., 2010; Lynn and Kern, 2014). Cort release promotes offspring adaptation to deteriorating and life-threatening situations (Mujahid, 2010).

In our study, both cold exposure and oral Cort administration in juvenile and adult birds altered HPA responsiveness. In both experimental groups for both ages we observed heightened baseline plasma Cort levels as compared to control baseline levels, suggesting that cold or Cort-treatment may re-program birds to maintain higher homeostatic Cort levels. While there was significant difference in plasma Cort levels after restraint in cold-exposed juveniles there was not in oral Cort treated juveniles or adults from either treatment group. The lack of a response to restraint may suggest that these birds are mainlining higher baseline levels to begin with or that they have an attenuated stress response. This suggests that juveniles and adults may be maintaining higher homeostatic baseline levels compared to control birds. As expected, Cort concentrations in control juvenile and adult birds in both experimental groups were significantly
higher after restraint stress compared to baseline and are consistent with previous studies (Banerjee and Adkins-Regan, 2011).

Many studies support the idea that early life stress programs a hyper-responsive and prolonged HPA axis response, which has been attributed to low MR and GR binding leading to a reduced negative feedback response (Banerjee and Adkins-Regan, 2011). Interestingly, neither the adult cold-exposed or oral Cort-treated birds demonstrated a significant spike in Cort after restraint stress but instead had non-significant slight decrease in Cort after restraint. Similarly, studies conducted on white crowned sparrow (altricial) and Japanese quail (precocial) exhibited a hyporesponsive response among pre-hatch Cort and post-hatch Cort fed birds with a corresponding increase in GR:MR ratios and receptor abundance (Wada and Breuner, 2010; Zimmer et al., 2013; Zimmer and Spencer, 2014). High GR binding capacity accelerates the negative feedback response resulting in a blunted Cort response after stress.

Furthermore, similar findings were observed in precocial chicken hatchlings (Gallus gallus), which were placed in a small, mesh box for 22 days as a form of early life stress. These birds experienced handling, social isolation, food and water deprivation, and a 10°C drop in ambient temperature for up to 3 hrs/day (Goerlich et al., 2012). Interestingly, the resulting adult offspring and the offspring of early life stressed specimens also exhibited the same blunted Cort response, which suggests that this programmed response has the capacity to cross one, and possibly multiple generations. Mice exposed to early life stress during adolescence also exhibited a dampened Cort response after acute restraint stress (Xu et al., 2011). In our study, the restraint Cort response in juveniles from both experimental groups did not significantly differ between treatments. Juveniles exposed to Cort have been shown to have significantly higher baseline and
restraint plasma Cort concentrations but our juvenile (and adult) birds showed no such difference (Crino et al., 2014).

Excess prenatal and postnatal GC secretion during development has been associated with increased cardiovascular, metabolic, and neuroendocrine risks in adulthood (Harris and Seckl, 2011). These effects could be detrimental in the developing brain and have also been connected to psychological disorders from anxiety to autism. Excess GC exposure in brain tissue and other cells has been shown to increase oxidative stress through an increase in reactive oxygen species (ROS) (Ramirez et al., 2003; Zafir and Banu, 2009). Adult female zebra finches and chickens injected with Cort exhibited higher levels of telomere loss, which predisposes cells to apoptosis (Ramirez et al., 2003). Furthermore, Cort has been shown to reduce the actions of free-radical scavenging enzymes (e.g. superoxide dismutase and glutathione reductase) (Zafir and Banu, 2009). Cort administration early in life has been implicated to decrease neuron numbers in areas like the HVC and it could be through the damaging properties of ROS and the telomere loss in DNA that this occurs (Ramirez et al., 2003; Shahbazi et al., 2014). A dampened Cort response to stress could possibly be a way to protect the developing offspring from the noxious effects of GCs in order to adapt to a harsh and changing environment and prevent systemic tissue damage (Goerlich et al., 2012; Wada and Breuner, 2010). This early developmental profile may program and organize the brain in a way that allows the offspring to survive and thrive in hazardous conditions. Environment-matching studies have shown that the combination of prenatal and postnatal stressors attenuates the stress response later in life, after an acute stressor (Merrill and Grindstaff, 2015; Zimmer et al., 2013). For example, prior to the shelling of their first clutch and/or newly born offspring, female zebra finches were treated with an antigen that activates helper-T cells, which synthesize ACTH thereby promoting Cort release from the adrenal glands
(Merrill and Grindstaff, 2015). There was a significant effect on Cort response with the antigen, and the effect was more potent when the antigen was given both prenatally through the pregnant mother and also postnatally to the offspring (Merrill and Grindstaff, 2015). Our study focused on postnatal stress exposure rather than prenatal stress. Additional studies need to be conducted to determine how prenatal and postnatal stressors affect the developing HPA axis.

In both juveniles and adults, the Cort levels of cold-exposed or oral Cort fed birds were elevated suggesting that these birds operate at higher homeostatic limits than control birds. MR, the receptor responsible for maintaining baseline Cort levels, undergoes the most change during development with higher binding affinities at P1-P3 and lower binding affinities as hatchlings age (Wada and Breuner, 2010). After a significant weather event (i.e. wind, cold and rain), altricial alpine swifts exhibited higher levels of baseline Cort (Bize et al., 2010). Furthermore, MR abundance was shown to significantly decline in the hypothalamus, cerebellum, and hippocampus of maternally deprived zebra finches (Banerjee and Adkins-Regan, 2011). It is possible that the heightened baseline levels may be attributed to changes in MR abundance in the brain, which has been shown to change during development and be the most affected receptor later in life.

The differences between early life stress versus control suggest a strong organizational power at the genomic level that allows an organism to adapt to their environment. One mechanism by which stress in early life can lead to adaptive changes is via DNA methylation. Few studies have assessed the effect of early life stress on global DNA methylation in birds, particularly zebra finches. Early life stress and early life conditions have been shown to exert lifelong changes in the individual through epigenetic mechanisms and across generations (Goerlich et al., 2012; Morgan and Bale, 2011). Juveniles exposed to both treatments
demonstrated significant hypomethylation in DNA as compared to controls, but neither treatment resulted in a change in global methylation in adults. Our results contradict previous research, many of which observed hypomethylation in adulthood rather than in juveniles.

The juvenile period is a critical period for song learning and production. During this time, juveniles are in the middle of the sensory phase and are hearing their father’s or another male tutor’s song. In addition, these birds are also beginning their sensory motor phase when they learn and practice their song (Zann, 1996). It is not until adulthood (P120) that these birds form their final crystalized song that is resistant to change or the addition of syllables (Zann, 1996). In between juvenile and adult, neural circuits are being remodeled. The connection from LMAN to RA, which is a dominant connection among juveniles, is rewired to a stronger connection between RA and HVC in adulthood (Bertram et al., 2014; Zann, 1996). Global DNA hypomethylation may be one way in which these changes can occur, by allowing transcription factors to access promoter regions in genes required for axon and neuron growth. By adulthood, the critical period for learning is closed and the song is fully crystallized, which means all unnecessary neurons are pruned away.

In rodents, maternal deprivation in early life results in significant decreases in global methylation levels in adulthood (Anier et al., 2014). Our results are similar but only in juvenile bird with adults showing no significant differences between controls. Global hypomethylation could be attributed to specific hypomethylation at certain genes (Anier et al., 2014). Accompanying global hypomethylation, gene specific hypomethylation occurs in the CRH promoter and the Avp enhancer in rats that were stressed early in life (Chen et al., 2012; Murgatroyd et al., 2009). Hypomethylation of the CRH promoter was shown to increase Cort levels in maternally deprived rats. Furthermore, Avp prolongs and sustains the activity of the
HPA axis by promoting CRH expression. Avp hypomethylation can be another avenue to sustain a potent stress response; however, upregulation of DNMT3a, a protein responsible for de novo DNA methylation, as well as identification of a trend towards DNA hypermethylation in the PP1C and A2AR promoter regions in the nucleus accumbens was found (Anier et al., 2014). PP1C and A2AR, the genes for neuroplasticity, were found to be significantly hypermethylated particularly in adulthood. Upregulation of DNMT3 suggests these proteins are necessary in early stressed birds to maintain methylation levels in a constantly changing environment. Taken together, hypermethylation of DNMT and genes for neuroplasticity could possibly be the dominating epigenetic change in adulthood.

2.6 Conclusions and Future Studies

In conclusion, these results demonstrate that early stress exposure, either from cold or Cort treatment, alters stress responsiveness differently across an animal’s life history. Both ages showed a heightened baseline plasma Cort response accompanied by a blunted response to restraint stress in cold-exposed birds. Juveniles demonstrated the most significant difference in methylation with cold-exposed birds exhibiting global hypomethylation. Changes in methylation levels could have occurred as the bird developed its final song and unnecessary neurons were pruned away. However, global DNA methylation does not address what is happening in the brain during stress because our methylation studies did not focus on genes associated with the HPA axis. However, hypomethylation of the CRH promoter (Chen et al., 2012) and Avp promoter (Murgatroyd et al., 2009) may be one possible way that stress and methylation could be connected and could be attributed to heightened baseline levels in our birds.
Additional studies are needed to determine the cumulative effects of prenatal and postnatal stressors, which have been shown to also greatly affect HPA axis response. One possible direction can be to look at methylation pattern differences in GR, MR or other HPA axis related receptors. We have started this process by performing a bisulfite conversion on genomic DNA from brains of cold-exposed and control juvenile and adults. This procedure converts cytosines to uracils, without affecting 5-methylcytosine. This results in changes in a DNA sequence that reflects the methylation status of that sequence, and we are currently designing the appropriate GR and MR primers to use. These studies may yield information on the DNA methylation of the GR and MR promoters. By specifically examining these receptors, we can directly determine if there is an epigenetic role in early life stress specifically in the HPA axis negative feedback response. Following a targeted, gene specific approach, future studies, such as Western blot analysis, are needed to determine GR and MR abundance in the brain, which may address how these proteins are altered during development and in adulthood.

Furthermore, early life stress has both physiological and behavioral consequences particularly in the forebrain song nuclei. Future studies need to address both global methylation and gene specific methylation in areas particularly sensitive to development such as the HVC (Buchanan et al., 2004; Shahbazi et al., 2014). Furthermore, synaptic connections between the song control system undergoes significant change during development with connections between LMAN and RA in the juvenile stage, and connection between HVC and RA during adulthood (Bertram et al., 2014; Zann, 1996). Song is considered as an honest signal for male quality, and song has been the most affected factor in early life stress (Spencer et al., 2003). Studies have shown that breeding conditions can affect song learning with Cort exposed birds reared in small clutches, in individual breeding cages, having the highest song learning scores compared to birds.
reared in crowded housing conditions (Shahbazi et al., 2014). Cort exposure or any form of early life stress could possibly shift life history timing in these birds resulting in poor learning and copying of song. Housing conditions should be an additional factor to consider when comparing Cort response and global DNA methylation levels. Aberrant DNA methylation could be source of abnormal physiological, psychological and behavioral phenotypes. In all, these studies contribute to our understanding of the sensitivity of the brain and nervous system to early life experience.
3 EFFECTS OF ACUTE STRESS ON PREFERENCE BEHAVIOR IN PAIR BONDED ADULT FEMALE ZEBRA FINCHES

Abstract

Female zebra finches demonstrate a strong preference for their mate including their mate’s idiosyncratic song. We investigated whether exposure to an acute stressor (15 minutes in duration designed to elevate baseline corticosterone) would alter female preference for their mate or for mate’s song. We hypothesized that restraint stress will alter female preference behavior (time spent perching near her mate) for her mate and the song of her mate versus a non-mate male. We also hypothesized that the perch zone a female visits has an effect on time spent perching. We tested these two hypotheses in three different scenarios. We looked at proximity preference (females preferring to be in close proximity to their mate), preference for a mate’s song, and preference for their mate’s own song played back at a higher rate. We measured the amount of time adult females spent perched in the zone closest to a mate (mate zone), the zone closest to a non-mate male (non-mate zone) and the space between these two zones (neutral zone). Using a two-way repeated measures ANOVA, we found that there was a significant interaction between stress and zone on time spent perching for song and song rate preference tests, but not for proximity preference.

3.1 Introduction

3.1.1 Factors that affect female mate choice

Female zebra finches, like many other songbirds, choose their mates (partners) by assessing phenotypic cues like male beak and cheek patch color, and song rate and quality (Hill
and McGraw, 2006a; Zann, 1996). These cues, especially song, serve as an honest indicator of male quality because they are costly to develop and maintain (Catchpole and Slater, 2008; Spencer et al., 2003). Females do not sing, and they choose their mates based in part on male song quality (Hauber et al., 2010). They recognize the song their father and the song of their mate, and they prefer those songs over the song of an unfamiliar male (Hauber et al., 2010; Miller, 1979a; Miller, 1979b).

Female zebra finches, like most female song birds, prefer song over no song, and more songs over fewer songs (Catchpole and Slater, 2008; Searcy and Yasukawa, 1996). Most male songbirds increase song output by reducing the inter-song interval which is also referred to as increasing song rate (Catchpole and Slater, 2008; Nolan and Hill, 2004). Another characteristic of song that female birds display a preference for is increased song complexity, which is an indicator of song quality (Reid et al., 2004). Song complexity includes, but is not limited to, repertoire size, number of syllables, and average song phrase duration (Airey et al., 2000; Buchanan and Catchpole, 2000; Catchpole and Slater, 2008; Neubauer, 1999; Spencer et al., 2005). These examples all provide support for a female’s ability to display preference for song.

Other indicators of male quality include beak and plumage coloration. The color intensity of both are dependent on carotenoids, which are only available through dietary intake, and are linked to the male’s foraging ability and overall health (Hill and McGraw, 2006a; Toomey and McGraw, 2012). Carotenoids can enhance immune responsiveness and antioxidant protection in both males and females and so convey information about bird phenotypic quality (Simons and Verhulst, 2011; Toomey and McGraw, 2012). In male zebra finches, carotenoids confer a bright red hue to the beaks and an orange hue to the cheek patches (Hill and McGraw, 2006b). It is well documented that females of many species, especially songbirds, prefer more
intense carotenoid-based pigmentation in males (Hill and McGraw, 2006b; Toomey and McGraw, 2012). Some experiments have demonstrated that beak redness was correlated with song rate (Collins et al., 1994), but more recent experiments have demonstrated that females’ preference for males with redder beaks was not solely due to its correlation with high song rate (Simons and Verhulst, 2011).

Interestingly, female zebra finches also prefer males banded with red leg identification bands as shown in experiments that compared the pairing and breeding rates of males wearing red or pink leg bands versus males wearing light green leg bands (Burley et al., 1982). Further experiments showed that females paired with males wearing red leg bands spent more time closer to those males, and produced more sons than females paired with males wearing green leg bands (Burley et al., 1982).

An additional indicator of male quality and health is immune profile. Across vertebrate taxa, stress-induced changes in white blood cell profiles are well documented (Davis et al., 2008). In passerines, the relative proportion of heterophils to lymphocytes (H : L ratio) increased in response to a wide variety of stressors like parasitic infection (Davis et al., 2004) and long-distance migration (Owen and Moore, 2006). High H : L ratios indicate high stress and were found in pied flycatcher nestlings with reduced growth (Moreno et al., 2002), while low H : L ratios indicate low stress and were found in song sparrows with large repertoires (Pfaff et al., 2007). The use of hematological analysis in stress studies complements the use of hormone assays, and is becoming more common (Davis et al., 2008).
3.1.2 Stress and female partner preference

Proximity preference experiments using a 2-choice paradigm, with both male and female zebra finches choosing, showed that a low dose of corticosterone significantly increased time spent next to an opposite-sex individual over a same-sex individual in males only and that this low dose did not alter preference for the opposite-sex individual in females (LaPlante et al., 2014). This same experiment showed that both male and female zebra finches prefer to spend time next to a same-sex group over an opposite-sex individual, and a low dose of corticosterone reverses that preference (LaPlante et al., 2014).

Unlike chronic stressors, acute stressors are thought to be beneficial (Breuner et al., 2008), but studies of the effect of acute stress on partner preference have yielded mixed results. In humans, exposure to an acute stressor increased males’ attraction to females (Dutton and Aron, 2013). In prairie voles, the effect of corticosterone on partner preference was sexually dimorphic with males’ preference for females increasing, and females’ preference for male decreasing (DeVries et al., 1995; DeVries et al., 1996). Female mice administered exogenous corticosterone showed a marked dose dependent decrease in their preference for male odors (Kavaliers and Ossenkopp, 2001). Experiments using the green treefrog demonstrated that higher corticosterone doses decreased the strength of female preference for male calls produced at higher rates (Davis and Leary, 2015). Research on the effects of acute stress on female partner preference is limited and requires further investigation.

We hypothesized that an acute stressor would alter female partner preference in mated adult zebra finches. We tested the effect of restraint stress on the time females spent perched in the two choice zones and the one neutral zone when tested with three different 2-choice partner preference paradigms. Time spent perched in the three zones was quantified for the proximity,
song and song rate partner preference paradigms. We also hypothesized that the zone in which females preferred to perch would be altered by restraint stress.

3.2 Methods

3.2.1 Animals

Male and female zebra finches were housed in the Carruth breeding colony at Georgia State University. The Georgia State University Institutional Use and Animal Care Committee granted approval for all animal procedures. Birds were housed in individual breeding cages and the aviary temperature was maintained at 23°C. Birds were kept on a 12-hr. light: 12-hr. dark photoperiod. All birds had ad libitum access to seed, water, shell grit and cuttlefish bone. Each individual flight cage had one mated pair and their offspring that were younger than P120 (120 days post-hatch). After P120, offspring were considered adults and were removed from the individual breeding cage. Only pairs with at least two successful clutches were used in these experiments.

3.2.2 Coloration of males

Pictures of all experimental male’s beak and cheek patches were taken in a controlled light environment using a Canon EOS Digital Rebel XT camera (Canon, Tokyo, Japan). The digital images were processed using Image J, version 1.51v (National Institutes of Health, Bethesda, MD) to extract the RBG color values. Male cheek patch and beak coloration was determined using MATLAB, version R2017b (MathWorks, Natick, MA). Using MATLAB, the distribution of red color values for each bird were shown on histograms with saturation on the x-axis and frequency count on the y-axis. The range of red color values were all between 0-255, and where the distribution fell from 0-255, determined redness of the beak or cheek patch. In
other words, redness was preserved using this methodology, so a higher red value meant a brighter or deeper red.

3.2.3 Immune profile with Wright-Giesma stain

Immune profile was determined using white blood cell counts. The left alar vein of each male was punctured with a 26-gauge sterile needle (Becton, Dickinson & Company, Franklin Lakes, NJ) and blood was then collected using heparinized micro-hematocrit capillary tubes (Fisher Scientific International, Pittsburgh, PA). A blood smear was made by placing a small drop of blood close to the end of a clean microscope slide using a micro-hematocrit capillary tube. A spreader slide was then positioned at a 45° in the center of the blood drop until the blood had run across its bottom edge. The spreader slide was gently moved backward to the edge of the slide then forward to create an even blood smear. The blood smear was immediately fixed in absolute methanol for 7 mins.

Staining procedure is outlined in Clinical Avian Medicine, volume 2 (Harrison and Lightfoot, 2006). Briefly, slides with fixed blood smears were immersed in Wright-Giemsa stain for 3 minutes an equal amount of Sorensen’s pH 6.5-6.8 buffer was added and mixed gently by blowing using a glass pipet until a metallic green sheen appeared. Slides stayed in the stain for 6 min. then they were rinsed in buffer and allowed to stand for differentiation for 1 min. After washing copiously with buffer, they were placed in slide racks to for drying. Heterophil to lymphocyte ratio was determined by using light microscopy to identify and count leukocytes.
3.2.4 **Restraint stress**

Females were captured in under 1 min., from their home cages and placed in an opaque cloth bag and held gently for 15 mins. (without applying pressure on the thorax). During this time, the female could hear other birds, but could not see them. Each female bird was used as its own control, so each female experienced both restraint stress and the control conditions (no restraint) before the start of preference testing, in random order. Fifteen mins. of restraint has been determined to be sufficient in studies conducted by our lab and others (Wada et al, 2008).

3.2.5 **Blood collection**

Before restraint stress, blood samples were collected within 3 mins. of capture by piercing the left alar vein of each female bird with a 26-gauge sterile needle (Becton, Dickinson & Company, Franklin Lakes, NJ). Blood was then collected using heparinized micro-hematocrit capillary tubes (Fisher Scientific International, Pittsburgh, PA). To halt bleeding, GelFoam sponge (Pfizer, Inc., New York City, NY) was used to staunch the puncture wound prior to starting the 15 min. of restraint stress began. A blood sample was collected again immediately after restraint stress from the right alar vein. Samples were centrifuged at 12000 rpm and plasma was stored at -80°C.

3.2.6 **Radioimmunoassay (RIA)**

Prior to conducting preference behavior testing, RIA was used to measure plasma corticosterone concentrations in male and female zebra finches before and after 15 min of restraint stress using a Corticosterone $^{125}$I RIA kit (catalog # 07-120102; MP Biomedicals LLC, Solon, OH, for mice and rats). This kit has been validated for measuring plasma corticosterone
concentrations in zebra finches (Shahbazi et al., 2014) and has also been used in previous studies with song sparrows (Newman, 2010). Recovery rate was 99% and the detection limit was 6.25-1000 ng/ml. Intra-assay & inter-assay coefficient variations were 1.4% and 9.0% respectively.

3.2.7 Song recording

Complete songs from 11 adult male zebra finches between 150 and 365 days of age were recorded in a sound attenuated room using Audacity 2.1.2 via a microphone (Dynex USB microphone) and a desktop computer (Apple iMac). Directed songs (song sung towards female mate) of each bird were recorded for 20 min. on several different days and at different times throughout the day. The song sample for each male was selected after determining that the same directed song was sung on different days. The recorded song was noise reduced using Goldwave software (Goldwave, Inc., St. John’s, NL, Canada). Only songs from 10 pair bonded males were used because, at the time of testing, the male 11 and his mate did not meet the requirement of having at least two successful clutches.

3.2.8 Preference tests and Analysis

3.2.8.1 Experiment 1

Proximity preference tests consisted of calculating the time a female spent perched in the mate zone, neutral zone, or non-mate zone. Three individual breeding cages measuring 16”L x 22”W x 16”H were setup in a row with the two outside cages immediately next to either side of the middle cage. A perch measuring 22” was divided into 3 equal length of 7.3” and was placed in the center cage (Figure 3.1). The center cage contained the female while the cages on either
side housed her mate or a non-mate male during 10 min of proximity preference testing. For proximity preference tests, 8 trials were conducted, and to account for side bias, the mate and non-mate bird location was balanced across the trials.

Two painted white, rectangular pieces of plywood were placed in between the center cage and the outer cages to ensure that the birds did not interact prior to testing. Testing began when the pieces of plywood were removed, and the researcher exited the room. All sessions were video recorded using a camcorder (JVC Everio, model GZ-MG330) and tripod and time spent in the different perch zones was determined by scoring each session after the completion of the experiment.

Figure 3.1 The two-choice proximity preference testing paradigm.

3.2.8.2 Experiment 2

Song preference tests consisted of calculating the time a female spent perched in the mate zone, neutral zone or non-mate zone. The setup was similar to experiment 1 except that the outer cages were empty and on the side of each outer cage was a wireless Bluetooth speaker that played either the mate’s song or a non-mate song (Figure 3.2). Similar to experiment 1a, 8-10 min. trials were conducted, and the location of the speakers playing mate and non-mate song were balanced across trials.
During song preference testing, mate song was played 3 consecutive times in a 30 sec. period from the 1st speaker and was followed by a 30 sec. period of silence. Non-mate song was played 3 consecutive times in a 30 sec. period from the 2nd speaker and was followed by a 30 sec. period of silence. Songs from each player were staggered so that when song came from one of the speakers, silence came from the other. Time spent perched in each zone was calculated in the same manner as in experiment 1.

![Two-choice song preference testing paradigm](image)

*Figure 3.2 The two-choice song preference testing paradigm.*

### 3.2.8.3 Experiment 3

The setup was identical to experiment 2 except that mate song was played from both speakers (Figure 3.3). Mate song was played 4 times in 1 min. with a 5 second period of silence between each song. After 1 min. of song, a 1 min. period of silence was observed. Mate song was played 3 times in 1 min. with a 10 sec period of silence between each song. After 1 min. of song, a 1 min. period of silence was observed. Songs from each player were staggered like in experiment b and time spent perched in each zone was calculated in the same manner as in experiment 1.
3.2.9 **Statistical Analysis**

All data were analyzed using IBM SPSS Statistics for Mac, version 25.0 (SPSS Inc, Chicago, IL). Parametric statistical tests were used for all data since assumptions were not violated. Statistical significance was accepted at $p < 0.05$ for all tests. A paired samples t-test was used to compare mean plasma corticosterone concentration in females before restraint vs after restraint. A two-way repeated measures (within-within-subjects) ANOVA was conducted to compare the main effects of stress and zone, and the interaction effect between stress and zone on time females spent perched. Stress (no restraint, restraint) and zone (mate, neutral, non-mate) were both within-subjects factors for proximity and song preference testing while stress (no restraint, restraint) and zone (faster rate, neutral, slower rate) were within-subjects factors for song rate preference testing.

3.3 **Results**

3.3.1 **Analysis of male quality: coloration and immune profile**

There were minor and not significant coloration intensity differences between the males’ beaks and cheek patches, but none were marked enough to allow for classification of the males as either low quality or high quality (Figure 3.4). All experimental and control males displayed
similar coloration patterns and red color intensities. All males analyzed were adults, P120 and above, but it is possible that the minor coloration differences were due to age. The precise ages of all the males (n = 10) were not known so we could not further investigate these minor coloration differences.

Leukocyte analysis indicated very low white blood cell counts and identifying enough leukocytes to develop a heterophil to lymphocyte ratio was an issue with all of the blood smears made from the males’ blood, because both cell types were not always present in each smear. Blood smears made from females were also made and observed via light microscopy, and the results were the same. In both males (n = 7) and females (n = 4) leukocyte numbers were too low to develop a heterophil to lymphocyte ratio. Essentially, there were no indicators of illness or infection in any birds based on the low leukocyte cell counts.

All the males were either reared or lived in individual breeding cages for at least 1 year, and experienced similar rearing or living conditions. This may have contributed to the lack of differences in coloration and immune profile due to the healthy rearing conditions of all birds.
Figure 3.4 Pictures of the beaks and cheek patches of 4 pair-bonded males (A, B, C and D) that were used to determine coloration differences between experimental males using Image J and R software (see Methods; n=10).

E and G are histograms showing the distribution of red color values for the beaks of males pictured in A and B, respectively. The average red color value for the beaks of the males in pictures A and B was 205 and 237, respectively. F and H are histograms showing the distribution of red color values for the cheek patches of males pictured in A and B, respectively. The average red color value for the cheek patches of the males in pictures A and B was 178 and 187, respectively.
The mortality rate of the birds in the Carruth breeding colony is extremely low, less than 6 natural deaths in adult birds per year in a colony the fluctuates between 80-150 birds (approximately 5%), and our low white blood cell count data aligns with the results of our male coloration and immune profile analysis. In comparison, zebra finches experience high mortality rates in the wild (Zann, 1996). The average life span in the wild can be as low as 2-4 months, and in one population, the mean annual survivorship to 12 months was 4% (Zann and Runciman, 1994). The mortality rate of zebra finches in captive breeding colonies is lower than in the wild, but still higher than the rate in the Carruth colony. The colony housed at UCLA as part of Dr. Art Arnold’s lab had a yearly sub-adult and adult mortality rate of 18-25% from 1998-2004.

3.3.2 The effects of restraint on plasma corticosterone in females

Blood corticosterone concentration in females before and after restraint was measured using RIA. Females who experienced restraint demonstrated significantly elevated plasma corticosterone concentrations as compared to pre-restraint concentrations (Figure 3.5). Since there was a significant difference between prior-restraint (n = 7) vs. after restraint (n = 7) plasma corticosterone concentrations in females ($t(6) = -4.701, p = 0.003$), the null hypothesis of equal plasma corticosterone concentration means was rejected. Thus, restraint stress for 15 min significantly elevated plasma corticosterone concentration in females when compared to the females’ baseline plasma corticosterone concentration.
Figure 3.5 Plasma corticosterone concentration after 15 min of restraint stress. Blood was collected from female zebra finches before and after restraint (n = 7). Plasma corticosterone concentration was measured using a radioimmunoassay (see Methods). Females had a significantly higher mean plasma corticosterone concentration ($t(6) = -4.701, p = 0.003$) after restraint (orange bar) than before restraint (blue bar). Data represents mean values with error bars that show the 95% confidence interval. Asterisks highlight significant differences between groups (*$p < 0.05$).

3.3.3 The effects of acute stress on female proximity preference behavior

A two-way repeated measures ANOVA was used to examine the effect of stress and zone on time spent perched. There was a statistically significant interaction between the effects of stress and zone on time spent perched ($F(2, 18) = 140.609, p < 0.001$), and there was a main effect of stress ($p < 0.001$) on time spent perched. There was no main effect of zone ($p = 0.146$) on time spent perched (Figure 3.6).
Figure 3.6 The effects of restraint stress on female zebra finch partner preference using a two-choice proximity preference paradigm.

Females (n = 10) were tested before and after 15 min of restraint. The average time spent in each of the 3 zones during 8 trials was calculated. There was a significant interaction between the effect of stress and zone on time females spent perched. There was also a main effect of stress, but not zone, on time females spent perched. Females spent significantly less time perched next to their mates and the non-mate males when stressed vs not stressed (p < 0.001 and p < 0.001) and significantly more time perched in the neutral zone when stressed vs not stressed (p < 0.001). When females were not stressed, time spent perched in the neutral zone was significantly less than time spent in both the mate zone (p < 0.001) and non-mate zone (p < 0.001). When females were stressed, time spent perched in the neutral zone was significantly more than time spent in both the mate zone (p < 0.001) and non-mate zone (p < 0.001). Data represents mean values with error bars that show the 95% confidence interval. Asterisks highlight significant differences within the not stressed group and within the stressed group (*p < 0.05). Hashtags highlight significant differences between the not stressed zones and the stressed zones (#p < 0.05).

Time females spent perched in the mate zone, neutral zone and non-mate zone after restraint was significantly different (p < 0.001, p < 0.001 and p < 0.001, respectively) from time spent perched in those zones when not exposed to restraint stress. These results mean that females spent significantly less time perched in the mate zone and non-mate zone when they were stressed before proximity preference testing, but significantly more time perched in the neutral zone when they were stressed before proximity preference testing. Acute stress
significantly increased time spent perched in the neutral zone, and significantly reduced time spent in both the mate and non-mate zones.

When females were not stressed before proximity preference testing, they spent significantly less time perched in the neutral zone than both the mate zone \((p < 0.001)\) and the non-mate zone \((p < 0.001)\). Time spent perched in the mate vs non-mate zones were not significantly different \((p = 0.111)\). Females showed no preference for a single zone but preferred to perch closer to both their mates and the non-mate males than in the neutral zone when not stressed.

When females were stressed before proximity preference testing, females spent significantly more time perched in the neutral zone than in both the mate zone \((p < 0.001)\) and the non-mate zone \((p < 0.001)\). Time spent perched in the mate vs non-mate zones were not significantly different \((p = 1.000)\). Females preferred to perch in the neutral zone when stressed.

### 3.3.4 The effects of acute stress on female song preference behavior

A two-way repeated measures ANOVA was conducted that examined the effect of stress and zone on time spent perched. There was a statistically significant interaction between the effects of stress and zone on time spent perched \((F(2, 18) = 386.969, p < 0.001)\), and there was a main effect of both stress and zone on time spent perched; \(p < 0.001\) and \(p < 0.001\) respectively (Figure 3.7).
Figure 3.7 The effects of restraint stress on female zebra finch partner preference using a two-choice song preference paradigm.

Females (n = 10) were tested before and after 15 min of restraint. The average time spent in each of the 3 zones during 8 trials was calculated. There was a significant interaction between the effect of stress and zone on time females spent perched. There was also a main effect of both stress and zone on time females spent perched. Females spent significantly less time perched next to their mates and the non-mate males when stressed vs not stressed (p < 0.001 and p < 0.001), and significantly more time perched in the neutral zone when stressed vs not stressed (p < 0.001). When females were not stressed, time spent perched in the neutral zone was significantly less than time spent in both the mate zone (p < 0.001) and non-mate zone (p < 0.001), and time spent in the mate zone was significantly more than time spent in the non-mate zone (p < 0.001). When females were stressed, time spent perched in the neutral zone was significantly more than time spent in both the mate zone (p < 0.001) and non-mate zone (p < 0.001), and time spent in the mate zone was significantly more than in the non-mate zone (p = 0.019). Data represents mean values with error bars that show the 95% confidence interval. Asterisks highlight significant differences within the not stressed group and within the stressed group (*p < 0.05). Hashtags highlight significant differences between the not stressed zones and the stressed zones (#p < 0.05).

The time females spent perched in the mate zone, neutral zone and non-mate zone after restraint was significantly different (p < 0.001, p < 0.001 and p < 0.001, respectively) from time
spent perched in those zones when not restrained. These results suggest that females spent significantly less time perched in the mate zone and non-mate zone when they were stressed before song preference testing, but significantly more time perched in the neutral zone when they were stressed before song preference testing. Acute stress significantly increased time spent perched in the neutral zone, and significantly reduced time spent in the mate and non-mate zones.

When females were not stressed before song preference testing, females spent significantly more time perched in the mate zone than in both the neutral zone \((p < 0.001)\) and the non-mate zone \((p < 0.001)\). Females also spent significantly more time perched in the non-mate zone than in the neutral zone \((p < 0.001)\). Females preferred to perch closer to the song of their mate when not stressed, but also showed a preference for perching closer to the non-mate male than in the neutral zone.

When females were stressed before song preference testing, females spent significantly more time perched in the neutral zone than in both the mate zone \((p < 0.001)\) and the non-mate zone \((p < 0.001)\). Females also spent significantly more time perched in the mate zone than in the non-mate zone \((p = 0.019)\). Females preferred to perch in the neutral zone when stressed, but also showed a preference for perching closer to their mates than non-mate males.

### 3.3.5 The effects of acute stress on female song rate preference behavior

A two-way repeated measures ANOVA was conducted that examined the effect of stress and zone on time spent perched. There was a statistically significant interaction between the effects of stress and zone on time spent perched \((F(2, 18) = 653.971, p < 0.001)\), and there was a
main effect of both stress and zone on time spent perched; \( p = 0.046 \) and \( p < 0.001 \) respectively (Figure 3.8).

Figure 3.8 The effects of restraint stress on female zebra finch partner preference using a two-choice song rate preference paradigm.

Females \( (n = 10) \) were tested before and after 15 min of restraint. The average time spent in each of the 3 zones during 8 trials was calculated. There was a significant interaction between the effect of stress and zone on time females spent perched. There was also a main effect of both stress and zone on time females spent perched. Females spent significantly less time perched in the faster rate (FR) zone and slower rate (SR) zone when stressed vs not stressed \( (p < 0.001 \) and \( p < 0.001 \)), and significantly more time perched in the neutral zone when stressed vs not stressed \( (p < 0.001) \). When females were not stressed, time spent perched in the neutral zone was significantly less than time spent in both the FR zone \( (p < 0.001) \) and SR zone \( (p < 0.001) \), and time spent in the FR zone was significantly more than time spent in the SR zone \( (p < 0.001) \). When females were stressed, time spent perched in the neutral zone was significantly more than time spent in both the FR zone \( (p < 0.001) \) and SR zone \( (p < 0.001) \), and time spent in the FR zone was significantly more than time spent in the SR zone \( (p < 0.001) \). Data represents mean values with error bars that show the 95% confidence interval. Asterisks highlight significant differences within the not stressed group and within the stressed group \( (*p < 0.05) \). Hashtags highlight significant differences between the not stressed zones and the stressed zones \( (#p < 0.05) \).
The time females spent perched in the faster rate (FR) zone, neutral zone and slower rate (SR) zone after restraint was significantly different ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively) from time spent perched in those zones when not restrained. These results indicate that females spent significantly less time perched in the FR zone and SR zone when they were stressed before song rate preference testing, and significantly more time perched in the neutral zone when they were stressed before song rate preference testing. Acute stress significantly increased time spent perched in the neutral zone, and significantly reduced time spent in the FR and SR zones.

When females were not stressed before song rate preference testing, females spent significantly more time perched in the FR zone than in both the neutral zone ($p < 0.001$) and SR zone ($p < 0.001$). Females also spent significantly more time perched in the SR zone than in the neutral zone ($p < 0.001$). Females preferred to perch closer to the speaker playing the song of their mate played back at a faster rate when not stressed, but also showed a preference for perching closer to the song of their mate that was played back at a slower rate than in the neutral zone.

When females were stressed before song rate preference testing, females spent significantly more time perched in the neutral zone than in both the FR zone ($p < 0.001$) and SR zone ($p < 0.001$). Females also spent significantly more time perched in the FR zone than in the SR zone ($p < 0.001$). Females preferred to perch in the neutral zone when stressed, but also showed a preference for the perching closer to the song of their mate that was played back at a faster rate than the song of their mate that was played back at a slower rate.
3.4 Discussion

This research demonstrates that when beak and cheek patch coloration intensity scores of pair bonded males were analyzed, no significant differences intensity were found. We could not classify our males as low-quality or high-quality based on beak or cheek patch coloration. We used digital photography which relies heavily on each photograph being taken in the same ambient lighting conditions as well as on the quality of the camera if male coloration is to be analyzed accurately (Hill and McGraw, 2006b). Color swatches are normally pictured in the photographs to improve accuracy, but since we had standardized our ambient lighting conditions and had used the same digital camera we did not include color swatches in the photographs. This photography method is common and has been done to determine the rank order of colors in birds with reliable accuracy (Alonso-Alvarez et al., 2004; Dale, 2000; Fitze and Richner, 2002; Tschirren et al., 2003). Our findings of no significant differences in carotenoid-based coloration between males does not cause us to question our method since rearing, living and social conditions as well as diets of all 10 males were very similar. It is common to find no significant differences in carotenoid-based coloration in groups of captive zebra finches of both sexes that have not been exposed to experimental manipulation (Blount et al., 2003; McGraw et al., 2011).

In order to classify our males as low-quality or high-quality based on white blood cell count, we measured H : L ratios from prepared blood smear slides. We could not determine male quality using this methodology since for most blood smears heterophils only or lymphocytes were visible and for all blood smears both the heterophils and lymphocytes were present at extremely low numbers. All of these issues prevented us from calculating the H : L ratios of all 10 males. Heterophils and lymphocytes together compose about 80% of the white blood cells in birds (Rupley, 1997) so it was unusual that we could not identify enough to form a H : L ratio.
Multiple studies have demonstrated that H : L ratios can be easily calculated in domestic chickens (Branton et al., 1997), wild great tits (Hauptmanova et al., 2002; Ots et al., 1998), wild zebra finches (Naguib et al., 2004) and many other wild avian species (Davis et al., 2008). We speculate that the captive conditions of our zebra finches were housed in resulted in low levels of illness and parasitic load resulting reduced white blood cell counts.

We found that when compared to baseline, 15 mins. of restraint stress significantly elevated plasma corticosterone our females. Similar 15 mins. restraint stress protocols were previously shown to significantly elevate plasma corticosterone in zebra finches (Wada et al., 2008), and even 10 mins. restraint stress protocols have been shown to significantly elevate plasma corticosterone (Banerjee and Adkins-Regan, 2011; McGraw et al., 2011). We have not seen a significant elevation of plasma corticosterone after 10 mins. of restraint in our past studies, but we see a trend toward significance for this restraint time period.

The effects of an acute stressor on female partner preference has been limited but two studies have demonstrated that it has an inhibitory effect on the development of partner preference in prairie voles (DeVries et al., 1995; DeVries et al., 1996), and on the strength of preference for high call rate in green treefrogs (Davis and Leary, 2015). We found that restraint altered female partner preference for all three of preference testing paradigms.

Acute stress altered preferences in the proximity preference paradigm. We found that when females were not stressed they showed no preference for spending more or less time perched near to their mate or near to the non-mate male. Females spent the least amount of time perched in the neutral zone. When these females were stressed, they preferred to spend more time perched in the neutral zone and less time perched near their mate or the non-mate male. Again, females showed no preference for spending more time perched near to their mate or near
to the non-mate male. These findings are consistent with previous studies that show a reduction in the strength of female preference when the animal is acutely stressed (Davis and Leary, 2015; DeVries et al., 1995; DeVries et al., 1996). Our females did not prefer to spend more time perched next to their mate than the non-mate male when not stressed which is a different result than was expected since a female’s preference for being in close proximity to her mate is very well-documented (Banerjee and Adkins-Regan, 2011; Clayton, 1990; Zann, 1996).

Acute stress weakened a female’s preference for the song of her mate in the song preference paradigm. We found that when females were not stressed they preferred to spend more time perched near to their mate than perched near to the non-mate male or in the neutral zone, with the least time spent perched in the neutral zone. When these females were stressed, they preferred to spend more time perched in the neutral zone and less time perched near to their mate and the non-mate male, with the least time spent perched near to the non-mate male. Our findings are consistent with previous studies that have found that female zebra finches prefer the song of their mate over the song of another male (Miller, 1979b; Woolley and Doupe, 2008).

Acute stress weakened a female’s preference for the song of her mate played at a faster rate in the song rate preference paradigm. Our findings showed that when females were not stressed they preferred to spend more time perched near to the speaker playing their mate’s song at a faster rate than perched near to the speaker playing their mate’s song at a slower rate or neutral zone, with the least time spent perched in the neutral zone. When these females were stressed, they preferred to spend more time perched in the neutral zone and less time perched near to the speaker playing their mate’s song at a faster rate and the speaker playing their mate’s song at a slower rate, with the least time spent perched near to the speaker playing their mate’s song at a slower rate. Our findings are consistent with previous studies that have found that many
female songbirds, including zebra finches, prefer the song of their mate sung at a faster rate over the song of their mate sung at a slower rate (Catchpole and Slater, 2008; Nolan and Hill, 2004).

The initial goal of this research was to explore how female zebra finches exposed to acute respond to high or low-quality males as determined by plumage and song. Since all our experimental males (and males in our breeding colony) can be likely classified as high quality, we focused our experiments on assessing the effect of acute stress on females’ preference for their own mate. Our findings demonstrate that acute stress alters females’ preference for their mate, and in two of our three partner preference paradigms, acute stress decreased a female’s preference for her mate. Our proximity partner preference paradigm demonstrated that acute stress significantly decreased the amount of time females spent perched next to their mate even though the females did not display a preference for their mate. These results are similar to the recent findings of Davis and Leary (2015) that showed a decrease in the strength of female preference for the more attractive higher call rates of male treefrogs.

Zebra finches are widely known to be socially monogamous, however, they also engage in extra-pair copulations both in the wild and laboratory (Birkhead et al., 1988; Houtman, 1992; Zann, 1996). It is unknown whether these extra-pair copulations are solely opportunistic or if they also occur under stressful conditions. Extra-pair copulations are biased towards males who are more attractive (have a higher song rate) than the mate (Houtman, 1992) and investigating how stress affects this interaction is a possible future direction. Overall, these results demonstrate that an acute stressor can significantly decrease females’ preference for their mate.
4 ACUTE STRESS ALTERS SONG-INDUCED ZENK AND GLUCOCORTICOID RECEPTOR IMMUNOREACTIVITY IN THE ADULT FEMALE ZEBRA FINCH BRAIN

Abstract

Female perception of male song is essential for female mate choice. Displaying a preference for the song of one male over another has implications for male reproductive success. We investigated whether 15 minutes of exposure to an acute stressor that was designed to elevate baseline corticosterone, would alter a female’s typical pattern of neuronal activation in auditory brain regions (caudomedial nidopallium (NCM), caudomedial mesopallium (CMM) and Field L) as well as other regions that might be involved in perceptual song processing. We hypothesized that females that experienced restraint stress will have altered ZENK, an immediate early gene (IEG), immunoreactivity in brain regions involved in auditory perception when compared to control females that did not experience restraint stress. We also hypothesized that experiencing restraint stress will increase glucocorticoid receptor (GR) immunoreactivity in a female’s auditory brain regions as well as other regions that might be involved in perceptual song processing. We looked at ZENK and GR immunoreactivity changes after 30 minutes of mate-song playback for females that were restrained before testing, and for females that were not restrained before testing. We measured the total number of immunoreactive (ir) neurons, the number of neurons with nuclear labeling, and the number of neurons with only cytoplasmic labeling in each of the brain regions of interest for both ZENK and GR. Using independent samples t-tests, we found that acute stress significantly decreased the quantity and subcellular
localization of ZENK-ir neurons in almost all brain regions analyzed, and significantly increased the quantity and subcellular localization of GR-ir neurons in almost all brain regions analyzed.

4.1 Introduction

4.1.1 The role of ZENK and the NCM in the perceptual processing of male song in songbirds

The Immediate Early Gene (IEG), ZENK, is the most well-known and well-utilized short-term neuronal activation marker in the songbird brain. The ZENK gene encodes a zinc finger transcriptional regulator protein called ZENK (Christy and Nathans, 1989) that controls expression levels of downstream genes that have promoters that bind ZENK (Gupta et al., 1991). ZENK protein is translated in the cytoplasm then enters the nucleus where it activates transcription of target genes that produce protein products (Tischmeyer and Grimm, 1999) which are required for many functions including, long-term memory formation (Jones et al., 2001; Mokin and Keifer, 2005). ZENK protein can be observed in neurons as early as 15 minutes after the start of an auditory stimulus but peak ZENK protein levels are reached in 1-2 hours after stimulus onset (Mello and Ribeiro, 1998). ZENK protein returns to basal levels by 6 hours after stimulus onset, and songbirds that have not been exposed to a relevant auditory stimulus show very little ZENK protein expression in auditory brain regions (Mello, 2002; Mello and Ribeiro, 1998). The NCM is a secondary auditory cortical region that consolidates and stores memories for individual songs (Lampen et al., 2017; Mello et al., 1992; Theunissen and Shaevitz, 2006; Woolley and Doupe, 2008), but its responsiveness to song is highly variable. Responsiveness to song in the NCM (or other auditory and non-auditory brain regions of interest) can be measured by visualizing and quantifying ZENK-ir neurons with immunohistochemistry.
As shown by experiments that use ZENK protein and gene expression as markers of neuronal activation in the NCM, ZENK expression is highest when adult and juvenile female zebra finches hear the song of conspecific males, moderate when they hear the song of heterospecific males and low/absent when they hear non-song auditory stimuli or silence (Bailey et al., 2002; Bailey and Wade, 2003; Gentner et al., 2001; Mello et al., 1995; Mello et al., 1992). Conspecific songs of high quality elicit a higher number of ZENK-ir neurons than conspecific songs of low quality (Leitner et al., 2005; Tomaszyci et al., 2006), and in both male and females, the NCM is more responsive during unfamiliar song than during familiar song (Terpstra et al., 2006; Woolley and Doupe, 2008). After temporary deactivation of the NCM, female zebra finches that previously spent significantly more time perched next to males that sang normal song rather than distorted song, spent a similar amount of time perched next to males singing normal song and males singing distorted song (Tomaszycki and Blaine, 2014). This study highlighted the role of the NCM in song perception.

ZENK activation attenuates with repeated presentation of a particular song but activation can be restored after introduction of a novel song (Mello et al., 1995). This phenomenon was also seen with electrophysiological studies that showed with repeated daily presentation of the same conspecific song, a prolonged habituation (about 48 hours) occurs (Chew et al., 1995; Chew et al., 1996a; Chew et al., 1996b). Habituation lasted the longest in response to repeated conspecific song, was shorter with repeated heterospecific song, and did not occur with repeated non-song stimuli (Chew et al., 1995). All of these results suggest that the NCM is highly responsive to more complex and more behaviorally/biologically relevant auditory stimuli; however, the more familiar the song stimulus and the less novel the context of its presentation, the less responsive (or non-responsive) the NCM becomes.
4.1.2  *Auditory and non-auditory brain regions that may play a role in perceptual processing of song in female songbirds*

The CMM is another secondary auditory cortical region that is highly activated in response to conspecific song versus heterospecific song and non-song stimuli (Mello et al., 1995; Scully et al., 2017), and to more complex, higher quality songs (Gentner et al., 2001; Leitner et al., 2005). Unlike the NCM which is responds more robustly to unfamiliar versus familiar song, the CMM responds more robustly to directed versus undirected song (Woolley and Doupe, 2008). In female zebra finches, CMM lesions significantly reduced courtship solicitation displays (CSDs) toward conspecific males and increased CSDs toward heterospecific males (MacDougall-Shackleton et al., 1998). In females without CMM lesions, CSDs toward conspecific males was significantly higher than CSDs to heterospecific males (MacDougall-Shackleton et al., 1998).

Field L is the primary auditory forebrain area and it is analogous to mammalian primary auditory cortex (Theunissen et al., 2004). In both male and female zebra finches, Field L is activated by hearing natural sounds and it displays conspecific song selectivity but not as strongly as NCM or CMM (Hauber et al., 2007; Hauber et al., 2013; Mello, 2002; Theunissen et al., 2004). NCM and CMM both receive direct inputs from Field L (Theunissen et al., 2004) so for these reasons, it was included as a region of interest in our experiments. The hippocampus (HP) was also included as a region of interest since it shares a common pattern of neuronal activation, that is only seen in females, in response to conspecific song as the NCM (Bailey et al., 2002). Just like in mammals, The HP is essential for the consolidation of spatial memories (Ash et al., 2012; Bailey et al., 2009). Both the lateral bed nucleus of the stria terminalis (BSTL)
and medial bed nucleus of the stria terminalis (BSTM) play a role in modulating social behavior (Vicario et al., 2017). The BSTL is specifically involved in modulating the anxiety/fear response, motivation and pain (Bupesh et al., 2011; Vicario et al., 2014) while the BSTM is involved in promoting preferences for familiar mates instead of unfamiliar neighbors, and large groups instead of small ones (Goodson, 2013; Kelly and Goodson, 2013). Ideally, we would have investigated both regions but only the BSTL could be reliably identified. It is common knowledge that the cerebellum (Cb) is required for the coordination of complex motor function, but its role in cognition is less acknowledged (Gordon, 2007). Cerebellar lesions in adult female zebra finches resulted in deficits in motor and cognitive function during a spatial working memory task (Spence et al., 2009). We included the Cb as a region of interest because little is known about the role of the Cb in songbird behavior, and because it is implicated in cognitive performance and memory (Andreescu et al., 2007; Gandhi et al., 2000; Gordon, 2007).

4.1.3 Stress and perceptual song processing

To the best of our knowledge, no studies have been conducted on the impact of acute stress on female perceptual song processing in primary or secondary auditory brain regions. One study was conducted on adult male zebra finches to investigate the effect of testing conditions (acute isolation for 1 hour, and acute isolation experienced simultaneously with restraint for 1 hour) on the specificity of ZENK gene expression in the NCM and CMM (Park and Clayton, 2002). An interesting aspect of this study was that the stressors started 1 hour before song playback began and remained in place until song playback ended. This study showed that males under combined conditions of isolation and restraint show little selectivity for conspecific song. Another study has shown that the NCM in female rats seems to be resistant to chronic
corticosterone elevation as evidenced by low levels of calbindin, which increases in response to neurotoxic insult, in the neurons of the NCM compared to high levels in other brain regions (Ash et al., 2012). Yet another study has shown that chronic corticosterone exposure elevates the expression of the memory-related IEGs, including Egr-1 (a mammalian ortholog of ZENK), in the amygdala of rats (Monsey et al., 2014). It is well-know that glucocorticoids can have protective effects as well as harmful effect on the auditory system, but these studies have focused on the prevention or exacerbation of hearing loss, so the focus has been on the inner ear and not the auditory brain regions (Meltser and Canlon, 2011; Singer et al., 2018). Chronic stress has also been shown to cause dendritic atrophy in the inferior colliculus and thalamic medial geniculate nucleus of rats, which are located in the auditory mesencephalon, not the auditory cortex (Dagnino-Subiabre et al., 2009).

4.1.4 Auditory and non-auditory brain regions that may experience elevated GR immunoreactivity in response to acute stress

Previous research in our lab has mapped GR neuronal immunoreactivity distribution and subcellular localization in the juvenile and adult male zebra finch brain (Shahbazi et al., 2011). We included some of the regions that exhibited high intensity GR labeling in males in our analysis of females. These brain regions include the medial preoptic nucleus (POM), the paraventricular nucleus (PVN), the BSTL and the Cb. The POM plays a role in regulating male sexual behavior (Alger and Ritters, 2006), and is a sexually dimorphic brain region (Balthazart et al., 1996). The PVN of the hypothalamus is one of the most important autonomic control centers in the brain, and it contains neurons that are involved in controlling stress (Ferguson et al., 2008). Some brain regions were selected because they offered a more complete picture of GR
immunoreactivity across the parts of the brain (telencephalon, diencephalon and metencephalon), and others were included because they were auditory brain regions (CMM, NCM and Field L) or non-auditory brain regions (HP, BSTL) that may be involved in the perceptual processing of male song in females. There was overlap among many of these selection criteria.

### 4.2 Methods

#### 4.2.1 Animals

All adult female zebra finches used in these experiments were housed in the Carruth breeding colony at Georgia State University. Approval for all animal procedures was granted by the Georgia State University Institutional Use and Animal Care Committee. Birds were housed in individual breeding cages and the aviary temperature was maintained at 23°C. Birds were kept on a 12-hour light: 12-hour dark photoperiod. All birds had *ad libitum* access to seed, water, shell grit and cuttlefish bone. Each individual flight cage had one mated pair and their offspring that were younger than P120 (120 days post-hatch). After P120, offspring were considered adults and were removed from the individual breeding cage.

#### 4.2.2 Restraint stress and mate-song playback

Females were captured in under 1 minute from their cages and placed in an opaque cloth bag and held gently for 15 minutes (without applying pressure on the thorax). During this time, the female could hear other birds, but could not see them. Females either experienced restraint (*n* = 5) or the control conditions (no restraint; *n* = 6) before exposure to 30 minutes of mate-song playback. Fifteen mins. of restraint has been determined to be sufficient in studies conducted by our lab and others (Wada et al, 2008).
After restraint (or after capture for controls), females were immediately placed in the testing cage and the mate-song playback experiments began (Figure 4.1). For each female, her mate’s song was played 3 consecutive times in a 30 second period and was followed by 30 seconds of silence. This song and silence sequence was repeated for 30 minutes (Figure 4.2). After mate-song exposure ended, females were left in the testing cage for 1 hour. Based on previous song-induced ZENK studies, a 1 hour wait time after song playback is necessary for peak ZENK expression (Bailey et al., 2002; Scully et al., 2017). Conducting ZENK experiments in the dark is common when using males since it reduces motor activities, like vocalizing, that can increase ZENK expression (Jarvis et al., 2000; Lampen et al., 2014; Mello and Ribeiro, 1998). Our experiments were conducted in a dark room, the lights were turned off immediately before the start of mate-song playback and turned back on after the 1 hour wait period. Female zebra finches do not sing but they do make calls, and it is more common for ZENK expression studies to be conducted in the dark unless bird behavior is being recorded (Jarvis et al., 2000; Lampen et al., 2014). Fifteen to 24 hours before mate-song playback experiments started, females were moved to a different room, so they would not hear their mate’s song before testing began. Most experiments isolate the females from all conspecific song for at least 48 hours, and more commonly for weeks, before song playback begins. This was not an option with our aviary setup; however, ZENK protein reach basal levels within 6 hours after the last stimulus onset.

Figure 4.1 Diagram illustrating experimental setup for mate-song playback.
4.2.3 Song recording

Complete songs from 11 adult male zebra finches between 150 and 365 days of age were previously recorded and used in these experiments. Songs were recorded in a sound attenuated room using Audacity 2.1.2 via a microphone (Dynex USB microphone) and a desktop computer (Apple iMac). Directed songs (song sung towards female mate) of each bird were recorded for 20 min on several different days and at different times throughout the day. The song sample for each male was selected after determining that the same directed song was sung on different days. The recorded song was noise reduced using Goldwave software (Goldwave, Inc., St. John’s, NL, Canada).

Figure 4.2 Diagram showing the pattern of mate-song playback. Adapted from Bailey et al., 2002.
4.2.4 Immunohistochemistry

4.2.4.1 Brain collection and histological preparation

To determine neuronal cell counts and labeling intensity of ZENK-immunoreactive (ZENK-ir) and GR-ir neurons, immunohistochemistry (IHC) was performed on the brains of adult females who were restrained or not restrained before exposure to their mate’s song. When the 1 hour wait time after song playback ended, an overdose of Isoflurane, an inhalational anesthetic, was administered. Females were decapitated, and skin and feathers were removed from the head. The brains were removed from the skull and placed in 4% paraformaldehyde for 48h. After fixation, brains were placed in a 15% sucrose solution until they sank. Next, brains were placed in a 30% sucrose solution until they sank. Brains were then embedded in optimal cutting temperature (OCT) medium, a cryoprotectant, and stored at -80°C until use. Brains were moved to -20°C for 30 minutes before being coronally sectioned at 40µm using a cryostat for frozen sections. Every first section was used to examine ZENK immunoreactivity and every second section was used as a negative control. Every third section was used to examine GR immunoreactivity and every fourth section was used as a negative control. Sections were placed on gelatin-subbed microscope slides that were subbed twice and stored at -20°C until use.

Standard IHC protocols were followed (Duncan et al., 2011; Shahbazi et al., 2011). Immediately before IHC, slides were kept at room temperature for 20-30 minutes then rinsed 3 times for 3 minutes each in Tris-buffered saline (0.5M TBS, pH 7.6). All solutions were made with 0.5M TBS. Next, slides were washed with a detergent, 0.2% Triton X-100 (Sigma chemical Co.), for 3 minutes and treated with trypsin (porcine trypsin type II, 1mg/1mL deionized water) (Sigma-Aldrich, St. Louis, MO) for 3 minutes. Slides were then incubated with 3% hydrogen peroxide for 15 minutes to eliminate endogenous peroxidase activity. Slides were rinsed 3 times
in TBS for 3 minutes each, and before incubation with primary antibody, slides were treated with hot 0.01M citrate buffer (pH 6.0) for 5 minutes to expose antigenic sites. The steps listed above were followed for both ZENK and GR IHC.

4.2.4.2 **ZENK IHC**

Sections were then incubated with primary antibody made in goat against ZENK (antibody labeled as EGR-1; polyclonal, concentration of 1:250, cat. no. AF2818; Novus Biologicals, Littleton, CO) for 48 hours at 4°C and covered with Parafilm (American National, Greenwich, CT) to prevent drying. A humidity chamber was used to house slides after primary antibody application. After 48 hours, slides were rinsed 3 times for 5, 10 and 10 minutes in TBS then incubated with biotinylated rabbit anti-goat IgG secondary antibody (SeraCare Life Sciences, Milford, MA) for 90 minutes at room temperature. Slides were rinsed 3 times for 5 minutes each then incubated with peroxidase labeled streptavidin solution (SeraCare Life Sciences, Milford, MA) for 1 hour at room temperature. After 2 rinses in TBS for 5 minutes each, immunoreactivity was visualized when the tissue was incubated with DAB (3, 3'-diaminobenzidine tetrahydrochloride) solution (Vector Laboratories, Inc., Burlingame, CA) for 25 minutes. After rinsing the slides 1 time in distilled water then 2 times in TBS for 5 minutes each, the slides were put through serial dehydration with increasing concentrations of ethanol. A tissue clearing agent, SafeClear (Fisher Scientific, Fair Lawn, NJ) was used before the slides were coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ), a mounting medium. After coverslipping, the slides were allowed to dry for 24 hours under a fume hood. Control slides received 10% normal rabbit serum (SeraCare Life Sciences, Milford, MA) in lieu of primary antibody.
4.2.4.3 **GR IHC**

Sections were then incubated with primary antibody made in rabbit against GR (polyclonal, concentration of 1:125, cat. no. PA1-511A; ThermoFisher Scientific, Waltham, MA) for 48 hours at 4°C and covered with Parafilm (American National, Greenwich, CT) to prevent drying. A humidity chamber was used to house slides after primary antibody application. After 48 hours, slides were rinsed 3 times for 5, 10 and 10 minutes in TBS then incubated with biotinylated goat anti-rabbit IgG secondary antibody (SeraCare Life Sciences, Milford, MA) for 90 minutes at room temperature. Slides were rinsed 3 times for 5 minutes each then incubated with peroxidase labeled streptavidin solution (SeraCare Life Sciences, Milford, MA) for 1 hour at room temperature. After 2 rinses in TBS for 5 minutes each, immunoreactivity was visualized when the tissue was incubated with DAB solution (Vector Laboratories, Inc., Burlingame, CA) for 25 minutes. After rinsing the slides 1 time in distilled water then 2 times in TBS for 5 minutes each, the slides were put through serial dehydration with increasing concentrations of ethanol. A tissue clearing agent, SafeClear (Fisher Scientific, Fair Lawn, NJ) was used before the slides were coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ), a mounting medium. After coverslipping, slides were allowed to dry for 24 hours under a fume hood. Control slides received 10% normal goat serum (SeraCare Life Sciences, Milford, MA) in lieu of primary antibody.

4.2.5 **Quantification of ZENK-ir and GR-ir neurons using DAB IHC**

ZENK immunoreactivity was quantified for 6 brain regions: hippocampus (HP), CMM, NCM, Field L, lateral bed nucleus of the stria terminalis (BSTL) and cerebellum (Cb) (Figure 4.3). Counting was accomplished using a light microscope with an ocular grid (16mm²/sq) under
the 40X objective. Sections representing each of the 6 brain regions were subjectively chosen, and all ZENK-ir neurons within the ocular grid were counted. For each brain region, the total number of ZENK-ir neurons (T; i.e. neurons with either nuclear or cytoplasmic labeling, or both) was determined. Next, the number of ZENK-ir neurons with nuclear labeling (N; this included neurons with both nuclear and cytoplasmic staining, and neurons with nuclear staining only) was determined. The number of ZENK-ir neurons with cytoplasmic labeling only (C) was calculated by subtracting N from T.

Representative photos for all brain regions for experimental females that were restrained (n = 4; One brain was lost during the IHC protocol, so 4 brains were used for ZENK and GR quantification in the restrained group) and for females that were not restrained (n = 4; Two brains were badly damaged during the IHC protocol, so 4 brains were used for ZENK and GR quantification in the not restrained group), were taken under the 40X objective with a camera coupled to the light microscope. Images were captured using SPOT basic software (Diagnostic Instruments Inc., Sterling Heights, MI).

GR immunoreactivity was quantified for 8 brain regions: HP, CMM, NCM, Field L, BSTL, preoptic medial nucleus (POM), paraventricular nucleus (PVN) and Cb (Figure 4.2). Quantifying GR-ir neurons and obtaining representative images of the 8 brain regions was done using the same methodology as described for ZENK-ir neurons above.
Figure 4.3 Diagrams showing the location of regions where ZENK-ir neurons and GR-ir neurons were counted.

A shows Field L (L; top square;) and caudomedial nidopallium (Nc; bottom square). B shows the hippocampus (HP; top) and caudomedial mesopallium (M; bottom). C shows the location of the lateral bed nucleus of the stria terminalis (BSTL) and D shows the location of the cerebellum (Cb). Both sides (right and left) of preoptic medial nucleus (POM; top) and paraventricular nucleus (PVN; bottom) are shown in E. All diagrams are adapted from Nixdorf-Bergweiler and Bischof, 2007. A, B, C, D and E are transverse plates 31, 29, 19, 34 and 28, respectively.

4.2.6 Statistical Analysis

All data were analyzed using IBM SPSS Statistics for Mac, version 25.0 (SPSS Inc, Chicago IL). Parametric statistical tests were used for all data since assumptions were not violated. Statistical significance was accepted at $p < 0.05$ for all tests. The average number of cells in the right and left hemispheres of each bird were compared using a paired samples t-test.
No significant differences were found between hemispheres, so all analyses were conducted on the average pooled cell counts for ZENK and GR labeled neurons.

Independent samples t-tests were used to compare the mean number of ZENK-ir neurons in each brain region (NCM, CMM, Field L, HP, BSTL, Cb) of females who were stressed vs not stressed before mate-song playback experiments. Specifically, means of (i) the total number of ZENK-ir neurons (T), (ii) the number of ZENK-ir neurons containing nuclear labeling (N), and (iii) the number of ZENK-ir neurons containing cytoplasmic labeling only (C) in each brain region of stressed and not stressed females were analyzed.

Independent samples t-tests were used to compare the mean number of GR-ir neurons in each brain region (NCM, CMM, Field L, HP, BSTL, POM, PVN, Cb) of females who were stressed vs not stressed before mate-song playback experiments. Specifically, means of (i) the total number of GR-ir neurons (T), (ii) the number of GR-ir neurons containing nuclear labeling (N), and (iii) the number of GR-ir neurons containing cytoplasmic labeling only (C) in each brain region of stressed and not stressed females were analyzed.

### 4.3 Results

#### 4.3.1 ZENK expression and labeling intensity in the female zebra finch brain

DAB IHC was used to determine ZENK-ir neuronal distribution in six specific brain regions that were located across the telencephalon (HP, CMM, NCM, Field L and BSTL) and metencephalon (Cb). Three of those telencephalic regions were auditory brain regions (NCM, CMM and Field L). The HP was of interest since it might have a potential role in certain facets of song perception (Bailey et al., 2009), and the BSTL was included since it is involved in modulating social behavior (Vicario et al., 2017). High intensity labeling of ZENK-ir neurons
was only observed in the NCM (Figure 4.3). Moderate intensity labeling of ZENK-ir neurons was seen in the CMM, HP, BSTL and Cb (Figure 4.4), while light intensity labeling was observed in Field L (Figure 4.4). ZENK-ir labeling was only present in the brains of females that were not stressed before mate-song playback. Females that were stressed before mate-song playback had no ZENK-ir labeling in any of the six brain regions of interest.

Figure 4.4 Photomicrographs of ZENK immunoreactivity in two auditory brain regions in the adult female zebra finch brain using DAB IHC. Brain regions with GR labeling are A, D and G which show the caudomedial nidopallium (NCM), caudomedial mesopallium (CMM), and hippocampus (HP) respectively in stressed females. B, E and H show those same auditory regions in not stressed females. C, F and I show those regions in not stressed controls (negative control that did not receive antibody). All images were taken at 40X. Scale bars = 20µm.
4.3.2 GR expression and labeling intensity in the female zebra finch brain

DAB IHC was used to determine GR-ir neuronal distribution in eight specific brain regions that were located across the telencephalon (HP, CMM, NCM, Field L and BSTL), diencephalon (POM and PVN) and metencephalon (Cb). Previous studies in our lab and others support the wide expression of GR-ir neurons seen across the telencephalic brain regions, and in specific diencephalic and metencephalic brain structures (Dickens et al., 2009; Shahbazi et al., 2011). High intensity labeling of GR-ir neurons was observed in many brain regions including, HP, CMM, POM and PVN (Figure 4.5). Moderate intensity labeling of GR-ir neurons was only seen in the Cb while light intensity labeling of GR-ir neurons was seen the NCM, Field L and BSTL (Figure 4.5). GR-ir labeling was only present in the brains of females who were stressed before mate-song playback. There was no GR-ir labeling in the eight brain regions of females that were not stressed before mate-song playback. Females that were stressed before mate-song playback had no GR-ir labeling in any of the eight brain regions of interest.

4.3.3 ZENK-ir neuronal cell number and subcellular immunoreactivity pattern

To test the hypothesis that stressed females and not stressed females had statistically significant different means for the total number of ZENK-ir neurons in each brain region, an independent samples t-test was performed. The independent samples t-test was associated with a statistically significant effect for the HP ($t(3) = -11.81, p = .001$), CMM ($t(3) = -21.35, p < .001$), NCM ($t(6) = -29.89, p < .001$), Field L ($t(6) = -17.59, p < .001$), BSTL ($t(3) = -8.95, p = .003$) and Cb ($t(3) = -13.05, p = .001$). Thus, stressing females before mate-song playback significantly decreased the total number of ZENK-ir neurons in all brain regions when compared to females that were not stressed before mate-song playback (Figure 4.6). Acute stress
significantly reduced the total number of ZENK labeled neurons in the HP, CMM, NCM, Field L, BSTL and Cb.

![Image of photomicrographs](Image)

**Figure 4.5 Photomicrographs of GR immunoreactivity in three auditory brain regions in the adult female zebra finch brain using DAB IHC.**

Brain regions with GR labeling are A, D and G which show the caudomedial nidopallium (NCM), caudomedial mesopallium (CMM), and Field L respectively in stressed females. B, E and H show those same auditory regions in not stressed females. C, F and I show those regions in stressed controls (negative control that did not receive antibody). All images were taken at 40X. Scale bars = 20µm.

To test the hypothesis that stressed females and not stressed females had statistically significant different means for the number of ZENK-ir neurons with nuclear labeling in each brain region, an independent samples t-test was performed. The independent samples t-test was associated with a statistically significant effect for the HP ($t(6) = -11.31$, $p < .001$), CMM ($t(6) = -17.59$, $p < .001$), NCM ($t(3) = -14.14$, $p = .001$), BSTL ($t(3) = -6.35$, $p = .008$) and Cb ($t(3) = -$
10.91, \( p = .002 \). Thus, stressing females before mate-song playback significantly decreased the number of ZENK-ir neurons with nuclear labeling in all brain regions, except Field L, when compared to females that were not stressed before mate-song playback (Figure 4.7). The independent samples t-test was not associated with a statistically significant effect for Field L \( (t(6) = -2.44, p = .050) \) (Figure 4.7). Acute stress significantly reduced the number of ZENK-ir neurons with nuclear labeling in the HP, CMM, NCM, BSTL and Cb, but did not significantly reduce the number of ZENK-ir neurons with nuclear labeling in Field L.

To test the hypothesis that stressed females and not stressed females had statistically significant different means for the number of ZENK-ir neurons with only cytoplasmic labeling in each brain region, an independent samples t-test was performed. The independent samples t-test was associated with a statistically significant effect for the HP \( (t(6) = -5.43, p = .002) \), CMM \( (t(3) = -8.95, p = .003) \), NCM \( (t(3) = -6.85, p = .006) \), BSTL \( (t(6) = -10.73, p < .001) \) and Cb \( (t(3) = -11.26, p = .002) \). Thus, stressing females before mate-song playback significantly decreased the number of ZENK-ir neurons with only cytoplasmic labeling in all brain regions, except Field L, when compared to females that were not stressed before mate-song playback (Figure 4.8). The independent samples t-test was not associated with a statistically significant effect for Field L \( (t(6) = -2.44, p = .050) \) (Figure 4.8). Acute stress significantly reduced the number of ZENK-ir neurons with only cytoplasmic labeling in the HP, CMM, NCM, BSTL and Cb, but did not significantly reduce the number of ZENK-ir neurons with only cytoplasmic labeling in the Field L.
Figure 4.6 The effects of restraint stress before mate-song playback on the total number of ZENK labeled neurons in the female zebra finch brain using IHC. Females either experienced restraint stress (n = 5) or experienced no stress (n = 6; control conditions) immediately before mate-song playback experiments. The average of the total number of ZENK-ir neurons in each of the brain regions was determined for stressed females and not stressed females. Stressed females had a significantly decreased total number of ZENK-ir neurons in the HP (p = .001), CMM (p < .001), NCM (p < .001), Field L (p < .001), BSTL (p = .003) and Cb (p = .001) when compared to not stressed females. Data represents mean values ± SEM. Asterisks highlight significant differences between the stressed group and the not stressed group (*p < 0.05).
Figure 4.7 The effects of restraint stress before mate-song playback on the number of ZENK-ir neurons with nuclear labeling in the female zebra finch brain using IHC.

Females either experienced restraint stress (n = 5) or experienced no stress (n = 6; control conditions) immediately before mate-song playback experiments. The average number of ZENK-ir neurons with nuclear labeling in each of the brain regions was determined for stressed females and not stressed females. Stressed females had a significantly decreased number of ZENK-ir neurons with nuclear labeling in the HP (p < .002), CMM (p < .001), NCM (p = .001), BSTL (p = .008) and Cb (p = .002) when compared to not stressed females. The number of ZENK-ir neurons with nuclear labeling in Field L of stressed females was not significantly different when compared to not stressed females (p = .050). Data represents mean values ± SEM. Asterisks highlight significant differences between the stressed group and the not stressed group (*p < 0.05).
Figure 4.8 The effects of restraint stress before mate-song playback on the number of ZENK-ir neurons with only cytoplasmic labeling in the female zebra finch brain using IHC.

Females either experienced restraint stress (n = 5) or experienced no stress (n = 6; control conditions) immediately before mate-song playback experiments. The average number of ZENK-ir neurons with only cytoplasmic labeling in each of the brain regions was determined for stressed females and not stressed females. Stressed females had a significantly decreased number of ZENK-ir neurons with only cytoplasmic labeling in the HP (p = .002), CMM (p = .003), NCM (p = .006), BSTL (p < .001) and Cb (p = .002) when compared to not stressed females. The number of ZENK-ir neurons with only cytoplasmic labeling in Field L of stressed females was not significantly different when compared to not stressed females (p = .050). Data represents mean values ± SEM. Asterisks highlight significant differences between the stressed group and the not stressed group (*p < 0.05).

4.3.4 GR-it neuronal cell number and subcellular immunoreactivity pattern

To test the hypothesis that stressed females and not stressed females had statistically significant different means for the total number of GR-ir neurons in each brain region, an independent samples t-test was performed. The independent samples t-test was associated with a statistically significant effect for the HP (t(3) = 16.30, p = .001), CMM (t(3) = 17.69, p < .001), NCM (t(3) = 8.65, p = .003), Field L (t(3) = 24.01, p < .001), BSTL (t(3) = 5.87, p = .010),
POM ($t(6) = 24.58, p < .001$), PVN ($t(6) = 8.40, p < .001$) and Cb ($t(3) = 16.79, p < .001$).

Thus, stressing females before mate-song playback significantly increased the total number of GR-ir neurons in all brain regions when compared to females that were not stressed before mate-song playback (Figure 4.9). Acute stress significantly increased the total number of GR labeled neurons in the HP, CMM, NCM, Field L, BSTL, POM, PVN and Cb.

To test the hypothesis that stressed females and not stressed females had statistically significant different means for the number of GR-ir neurons with nuclear labeling in each brain region, an independent samples t-test was performed. The independent samples t-test was associated with a statistically significant effect for the HP ($t(3) = 9.66, p = .002$), CMM ($t(3) = 9.56, p = .002$), POM ($t(3) = 6.18, p = .009$), PVN ($t(3) = 7.55, p = .005$) and Cb ($t(6) = 17.88, p < .001$). Thus, stressing females before mate-song playback significantly increased the number of GR-ir neurons with nuclear labeling in all brain regions except, NCM, Field L and BSTL, when compared to females that were not stressed before mate-song playback experiments (Figure 4.10). The independent samples t-test was not associated with a statistically significant effect for BSTL ($t(3) = 2.04, p = .133$), and a t-statistic could not be computed for NCM and Field L since the standard deviations for both groups were zero (Figure 4.10). Acute stress significantly increased the number of GR-ir neurons with nuclear labeling in the HP, CMM, POM, PVN and Cb, but did not significantly increase the number of GR-ir neurons with nuclear labeling in the BSTL.

To test the hypothesis that stressed females and not stressed females had statistically significant different means for the number of GR-ir neurons with only cytoplasmic labeling in each brain region, an independent samples t-test was performed. The independent samples t-test was associated with a statistically significant effect for the HP ($t(3) = 10.97, p = .002$), CMM
(t(3) = 9.60, p = .002), NCM (t(3) = 13.19, p = .001), Field L (t(3) = 10.33, p = .002), BSTL (t(6) = 517.96, p < .001), POM (t(3) = 19.71, p < .001), PVN (t(3) = 20.15, p < .001) and Cb (t(3) = 24.30, p < .001). Thus, stressing females before mate-song playback significantly increased the number of GR-ir neurons with only cytoplasmic labeling in all brain regions when compared to females that were not stressed before mate-song playback (Figure 4.11). Acute stress significantly increased the number of GR-ir neurons with only cytoplasmic labeling in the HP, CMM, NCM, Field L, BSTL, POM, PVN and Cb.

**Figure 4.9** The effects of restraint stress before mate-song playback on the total number of GR labeled neurons in the female zebra finch brain using IHC. Females either experienced restraint stress (n = 5) or experienced no stress (n = 6; control conditions) immediately before mate-song playback experiments. The average of the total number of GR-ir neurons in each of the brain regions was determined for stressed females and not stressed females. Stressed females had a significantly increased total number of GR-ir neurons in the HP (p = .001), CMM (p < .001), NCM (p = .003), Field L (p < .001), BSTL (p = .010), POM (p < .001), PVN (p < .001) and Cb (p = .001) when compared to not stressed females. Data represents mean values ± SEM. Asterisks highlight significant differences between the stressed group and the not stressed group (*p < 0.05).
Figure 4.10 The effects of restraint stress before mate-song playback on the number of GR-ir neurons with nuclear labeling in the female zebra finch brain using IHC.
Females either experienced restraint stress (n = 5) or experienced no stress (n = 6; control conditions) immediately before mate-song playback experiments. The average number of GR-ir neurons with nuclear labeling in each of the brain regions was determined for stressed females and not stressed females. Stressed females had a significantly increased number of GR-ir neurons with nuclear labeling in the HP (p = .002), CMM (p = .002), POM (p = .009), PVN (p = .005) and Cb (p < .001) when compared to not stressed females. The number of GR-ir neurons with nuclear labeling in the BSTL of stressed females was not significantly different when compared to not stressed females (p = .133). Data represents mean values ± SEM. Asterisks highlight significant differences between the stressed group and the not stressed group (*p < 0.05).
Figure 4.11 The effects of restraint stress before mate-song playback on the number of GR-ir neurons with only cytoplasmic labeling in the female zebra finch brain using IHC. Females either experienced restraint stress (n = 5) or experienced no stress (n = 6; control conditions) immediately before mate-song playback experiments. The average number of GR-ir neurons with only cytoplasmic labeling in each of the brain regions was determined for stressed females and not stressed females. Stressed females had a significantly increased number of GR-ir neurons with only cytoplasmic labeling in the HP (p = .002), CMM (p = .002), NCM (p = .001), Field L (p = .002), BSTL (p < .001), POM (p < .001), PVN (p < .001) and Cb (p < .001) when compared to not stressed females. Data represents mean values ± SEM. Asterisks highlight significant differences between the stressed group and the not stressed group (*p < 0.05).

4.4 Discussion

Acute stress experienced before mate-song playback decreased neuronal activation in all auditory and non-auditory brain regions that we examined that likely play a role in perceptual song processing. We found that when females were not stressed before mate-song playback, ZENK labeled neurons were present in the HP, CMM, NCM, Field L, BSTL and Cb. When females were stressed before mate-song playback, few to no ZENK labeled neurons were present
in the HP, CMM, NCM, Field L, BSTL and Cb. When ZENK-ir neurons were analyzed based on subcellular localization of their labeling, all brain regions in not stressed females had ZENK-ir neurons with nuclear labeling, along with ZENK-ir neurons with only cytoplasmic labeling.

It was not surprising to see ZENK-ir neurons in the auditory brain regions of not stressed females. Previous studies have shown that the NCM, CMM and Field L are activated in response to conspecific song, and the NCM and CMM are often investigated in ZENK protein and gene expression studies aimed at understanding how female songbirds perceive male song (Bailey et al., 2002; Bailey and Wade, 2003; Bolhuis et al., 2012; Gentner et al., 2001; Hauber et al., 2007; Leitner et al., 2005; Mello et al., 1995; Mello et al., 1992; Scully et al., 2017; Terpstra et al., 2006; Woolley and Doupe, 2008). Non-auditory brain regions were selected because of the likelihood that their functions (HP, consolidation of spatial memories; BSTL, modulation of social behavior; Cb, cognition and memory) could be useful in the perceptual processing of song. Because of this, it was also not surprising to see that ZENK-ir neurons were present in all of these non-auditory brain regions.

The NCM was the only brain region that exhibited high intensity labeling of ZENK-ir neurons, while Field L was the only region that exhibited light intensity labeling of ZENK-ir neurons. The HP, CMM, BSTL and Cb all had moderate intensity labeling of ZENK-ir neurons. Given the role of the NCM as the principal site for the consolidation and storage of individual song memories (Lampen et al., 2017; Mello et al., 1992), and the role of Field L in processing the less complex elements of conspecific song (Hauber et al., 2007; Mello, 2002; Theunissen et al., 2004), their respective labeling intensities are to be expected.

Acute stress experienced before mate-song playback increased GR immunoreactivity in all auditory and non-auditory brain regions that likely play a role in perceptual song processing.
We found that when females were not stressed before mate-song playback, no GR labeled neurons were present in the HP, CMM, NCM, Field L, BSTL, POM, PVN and Cb. When females were stressed before mate-song playback, GR labeled neurons were present in the HP, CMM, NCM, Field L, BSTL, POM, PVN and Cb. Unbound GR reside in the cytoplasmic subcellular compartment and when bound by ligand are then translocated into the nucleus when they facilitate transcription and the stress response (Breen et al., 2004; Buckingham, 2006). Our antibody recognizes bound and unbound GR (Shahbazi et al., 2011). When GR-ir neurons were analyzed based on subcellular localization of their labeling, all brain regions (except NCM and Field L) in stressed females had GR-ir neurons with nuclear labeling indicating ligand binding, along with GR-ir neurons with only cytoplasmic labeling. Field L and NCM were the only brain regions where all GR labeling was cytoplasmic.

Previous research findings in our lab have shown that male zebra finches express GR-ir neurons in many brain regions including, HP, Field L, BSTL, POM, PVN and Cb (Shahbazi et al., 2011). It was not surprising to see that our stressed females showed GR-ir neurons in those same auditory and non-auditory brain regions. High intensity labeling of GR-ir neurons was seen in the HP, CMM, POM and PVN. The POM is responsible for regulating male sexual behavior (Alger and Riters, 2006) so it was unexpected to see high intensity GR-ir labeling in this region. Moderate intensity labeling of GR-ir neurons was only seen in the Cb, and light intensity labeling of GR-ir neurons was seen in the NCM, Field L and BSTL. The presence of GR-ir neurons in some auditory brain regions is not unusual in female songbirds since in situ hybridization experiments have shown that GR was expressed in the NCM and CMM of female Bengalese finches (Suzuki et al., 2011). These results taken together show that the avian primary (Field L) and secondary auditory cortices (NCM, Field L) can be influenced by stressful events.
Our findings demonstrate that acute stress experienced before mate-song playback, significantly decreased neuronal activation and significantly increased GR immunoreactivity in auditory and non-auditory brain regions that potentially play a role in the perceptual processing of song. GR is a low affinity receptor that is only activated by high corticosterone concentrations, while mineralocorticoid receptor (MR) is a high affinity receptor that responds to basal concentrations of corticosterone (Breen et al., 2004; Buckingham, 2006; Suzuki et al., 2011). We analyzed GR immunoreactivity but did not investigate MR immunoreactivity, which might have yielded more striking results in our not stressed females. To date, our lab has not been able to identify an antibody to MR that gives consistent staining and therefore we have been unable to explore this further. Determining how acute stress affects MR immunoreactivity will contribute more to our understanding of how acute stress affects neuronal activation and song processing in the avian brain.
5 GENERAL DISCUSSION

This series of studies addressed the effects of early life stress, both physiological and ecological, and acute stress in adulthood on brain and behavior, using multiple levels of biological analysis. These levels include behavioral analysis (partner preference), circulating hormone levels (plasma corticosterone concentration), neuroanatomical analysis (ZENK protein and glucocorticoid receptor immunoreactivity) and epigenetic analysis (global DNA methylation levels). We have demonstrated that chronic stress experienced in early life can have lasting effects on stress responsiveness and global methylation in the brain, and that acute stress in adulthood is potent enough to influence female partner preference and brain activation in brain regions involved in social affiliative behaviors.

5.1 The impact of early life stress on the juvenile and adult stress response and global DNA methylation in the brain

Early life conditions, especially stressful ones, have been shown to exert lifelong changes via epigenetic mechanisms that are persistent and transgenerational (Goerlich et al., 2012; Morgan and Bale, 2011). DNA methylation is one proposed mechanism by which stress in early life leads to adaptive changes later in development (Goerlich et al., 2012; Morgan and Bale, 2011). Early in life stress exposure can cause a reduction in HPA axis responsiveness, which is associated with multiple cognitive and neurological issues including, cognitive deficits and disease which often cannot be assessed until animals are older (juveniles or adults) (Harris and Seckl, 2011).
Cold stress is not commonly used to induce stress in a laboratory setting but it is a very potent and relevant ecological stressor (Eraud et al., 2007; Johnson and Rashotte, 2002). A cold stress study in eastern bluebird chicks showed that hatchlings housed in cold or cool temperatures had significantly higher levels of Cort by P7 than hatchlings housed in ambient or brooding temperatures (Lynn and Kern, 2014). Oral Cort administration is a physiological stressor and Cort dissolved in peanut oil is the least invasive method to increase Cort concentrations in hatchlings for the first few weeks post-hatch (Shahbazi et al., 2014; Spencer et al., 2003; Spencer and Verhulst, 2007; Zimmer et al., 2013). Cort administration early in life resulted in significantly elevated Cort levels when compared to controls in juvenile zebra finches (Crino et al., 2014; Spencer et al., 2003). We have demonstrated that early life stress alters hypothalamic pituitary adrenal (HPA) axis sensitivity in both juveniles and adults, and global DNA methylation levels in juveniles but not adults.

Chapter 2 addressed two questions about whether an ecologically-relevant stressor (cold exposure) and a physiological stressor (daily oral Cort administration) could alter the stress response in juveniles and adults, and whether cold exposure and daily oral Cort administration could alter global methylation in the brains of juveniles and adults. We hypothesized that daily exposure to both stressors during the first two weeks post-hatch will alter HPA axis sensitivity and global brain DNA methylation levels in juveniles and adults. A variety of environmental factors can induce stress but is unknown if cold stress (ecological) has the same impact as Cort dosing (physiological) on HPA axis sensitivity and global DNA methylation in juveniles and adults. Our study demonstrated that both cold exposed and Cort fed juveniles and adults exhibited heightened baseline plasma Cort concentrations when compared to controls. In both cold exposed and Cort fed controls, there was a significant difference between baseline and
restraint plasma Cort concentration in both juveniles and adults, but there was no significant difference between baseline and restraint plasma Cort concentrations in either cold exposed or Cort fed juvenile and adult birds. Our research also demonstrated that both cold exposed and Cort fed juveniles exhibited significantly lower global DNA methylation levels when compared to controls. Cold exposed and Cort fed adults did not exhibit significant differences in global DNA methylation levels when compared to controls.

While we recognize that our laboratory cold manipulation may not completely mirror environmental experiences, we did see significant effects from this treatment as well as oral Cort administration. The juvenile period is a critical period for song learning and production and during this period neural circuits are being remodeled (Bertram et al., 2014; Zann, 1996). Global DNA hypomethylation may be one way in which these changes can occur, by allowing transcription factors to access promoter regions in genes required for axon and neuron growth (Chen et al., 2012; Murgatroyd et al., 2009).

Regarding DNA methylation, future research should include investigating stress-induced methylation of genes of interest including, GR, MR, and CRH. Studies have suggested that hypomethylation of the CRH promoter (Chen et al., 2012) and Avp promoter (Murgatroyd et al., 2009) may be one possible way that stress and methylation could be connected, and could be attributed to heightened baseline levels in our birds.

5.2 Acute stress and its effect on the strength female mate preference

Responses to acute stress include enhanced cognition, enhanced analgesia and energy mobilization (Becker et al., 2002), and therefore are thought to be adaptive and potentially beneficial (McEwen, 2006; Sapolsky et al., 2000). It is also thought that an increase in acute GC
secretion (in response to an acute stressor) is responsible for mediating the tradeoff between reproduction and survival although those studies have inconsistent results (Breuner et al., 2008). Female zebra finches exhibit a strong preference for the closeness of their mate and their mate’s distinctive song (Catchpole and Slater, 2008; Zann, 1996). In both male and females zebra finches, restraint stress (an acute stressor) successfully elicits an acute adrenocortical response (Banerjee and Adkins-Regan, 2011; Wingfield, 1995), and studies on mice and green treefrogs have shown that acute stress weakens unmated female preference for qualities that affect female mate choice, such as male odor and higher call rates (Davis and Leary, 2015; Kavaliers and Ossenkopp, 2001). We have demonstrated that acute stress alters female preference for an established partner or mate.

Chapter 3 addressed the question whether acute stress alters and adult zebra finch female’s preference to be in close proximity to her mate and/or his song. We hypothesized that restraint stress will alter female preference behavior (time spent perching near her mate) for her mate and the song of her mate versus a non-mate male. We also hypothesized that the perch zone a pair-bonded female visits has an effect on time spent perching. Several studies consistently support a female’s preference for being in close proximity to her mate and for being in close proximity to the song of her mate (Catchpole and Slater, 2008; Miller, 1979b; Nolan and Hill, 2004; Woolley and Doupe, 2008) but, to the best of our knowledge, no studies address the effect that acute stress can have on the strength of her preference. Our results demonstrated that acute stress altered female preferences in the all preference paradigms. Acute stress decreased a female’s preference for the song of her mate versus the song of a non-mate in the song preference paradigm, and also decreased a female’s preference for the song of her mate sung at a faster rate versus a slower rate in the song rate preference paradigm. In the song and song rate preference paradigms, females
displayed a preference for their mate’s song and the song of their mate played at a faster rate when not stressed. Our proximity partner preference paradigm demonstrated that acute stress decreased the amount of time females spent perched next to their mate even though the females did not display a preference for their mate when not stressed. Overall, these results demonstrate that an acute stressor can significantly decrease females’ preference for their mate.

Zebra finches are widely considered socially monogamous, but it is widely known that they also engage in extra-pair copulations both in the field and laboratory settings (Birkhead et al., 1988; Houtman, 1992; Zann, 1996). Extra-pair copulations may be opportunistic, but it is also possible that their occurrence is impacted by stressful conditions. Interestingly, extra-pair copulations are biased towards males who are more attractive (have a higher song rate) than the mate (Houtman, 1992) and investigating how stress affects this interaction is a possible future direction.

5.3 Changes in ZENK protein expression and glucocorticoid receptor distribution in response to acute stress

The NCM is a secondary auditory cortical region that consolidates and stores memories for individual songs and so, may play a role in the perceptual processing of song (Lampen et al., 2017; Mello et al., 1992; Theunissen and Shaevitz, 2006; Tomaszycki and Blaine, 2014; Woolley and Doupe, 2008). It is also thought that the NCM is involved in female mate choice and female partner (mate) preference since females choose mates on the basis of song quality (Tomaszycki and Adkins-Regan, 2005). After temporary deactivation of the NCM, female zebra finches that previously spent significantly more time perched next to males that sang normal song rather than distorted song, spent a similar amount of time perched next to males singing
normal song and males singing distorted song (Tomaszycki and Blaine, 2014). Responsiveness to song in the NCM can be measured by visualizing and quantifying ZENK immunoreactive (ir) neurons. Responsiveness to song in other auditory brain regions (CMM, Field L) as well as non-auditory brain regions (HP, BSTL, Cb) can also be measured by quantifying ZENK-ir neurons. We investigated ZENK immunoreactivity of non-auditory brain regions because their functions (HP, consolidation of spatial memories; BSTL, modulation of social behavior; Cb, cognitive performance and memory) make them feasible candidates for being directly and/or indirectly involved in perceptual song processing (Andreescu et al., 2007; Ash et al., 2012; Bailey et al., 2009; Bupesh et al., 2011; Gandhi et al., 2000; Gordon, 2007; Vicario et al., 2014). It is well-known that GCs can have protective effects as well as harmful effect on the auditory system, but these studies only examined inner ear structures, not brain regions (Meltser and Canlon, 2011; Singer et al., 2018). If auditory and non-auditory brain regions are sensitive to acute stress, the presence of GRs will indicate that the potential effects of acute stress may be mediated by GRs. We have demonstrated that acute stress alters both ZENK and GR immunoreactivity in primary and secondary auditory brain regions as well as non-auditory brain regions.

Chapter 4 addressed two questions about whether mate-song induced expression of ZENK protein in the NCM and CMM of an adult zebra finch female brain is altered by acute stress, and whether the distribution and subcellular localization of GR-ir neurons in the adult female zebra finch brain is altered by acute stress experienced before mate-song playback. We hypothesized the restraint stress experienced before mate-song playback will alter the quantity of ZENK-ir and GR-ir neurons in the auditory and non-auditory brain regions that may play a role in the perceptual processing of song. Our results demonstrated that acute stress experienced before mate-song playback altered neuronal activation and GR immunoreactivity in all observed brain
regions. Acute stress decreased the total number of ZENK-ir neurons in all auditory and non-auditory brain regions that we examined that likely play a role in perceptual song processing. We observed ZENK-ir neurons with nuclear labeling (which includes nuclear only and both nuclear and cytoplasmic labeling), and ZENK-ir neurons with only cytoplasmic labeling in all brain regions. Acute stress increased the total number of GR-ir neurons in all auditory and non-auditory brain regions that likely play a role in perceptual song processing. We observed GR-ir neurons with nuclear labeling and GR-ir neurons with only cytoplasmic labeling in most brain regions. Field L and NCM were the only brain regions where all GR labeling was cytoplasmic. When the labeling intensity was examined in non-stressed females, we found that the NCM was the only brain region with high intensity labeling of ZENK-ir neurons, and Field L was the only brain region with light intensity labeling of ZENK-ir neurons. This was expected since the NCM is the main site of individual song consolidation and storage (Lampen et al., 2017; Mello et al., 1992), while Field L processes natural sounds and the less complex elements of conspecific song (Hauber et al., 2007; Mello, 2002; Theunissen et al., 2004). The POM is responsible for regulating male sexual behavior (Alger and Riters, 2006), so it was unexpected to see high intensity labeling of GR-ir neurons in this region in stressed females. We are still trying to understand the relevance of this result in females.

GR is a low affinity receptor that responds to high corticosterone concentrations, while mineralocorticoid receptor (MR) is a high affinity receptor that responds to low (basal) concentrations of corticosterone (Breen et al., 2004; Buckingham, 2006; Suzuki et al., 2011). Exploring MR immunoreactivity is important but to date, our lab has not been able to identify an antibody to MR that gives consistent staining. Determining how acute stress affects MR
immunoreactivity will contribute more to our understanding of how acute stress affects neuronal activation and song processing in the avian brain.

5.4 Conclusions

In summary, chapter 2 demonstrated that juveniles and adults that either experienced an ecological stressor or a physiological stressor early in life displayed a muted stress response in reaction to restraint stress, while juveniles and adults that did not experience either of these stressors displayed a typical stress response in reaction to restraint stress. These results suggest that cold exposure and Cort treatment may reprogram birds to maintain higher homeostatic Cort levels. This concept is supported by the research that showed 4 day old chicks acquired tolerance for a cold environment (which was not present at one day old), and that this tolerance was in response to elevated circulating plasma Cort levels which enabled the chicks to maintain body temperature in the midst of deteriorating environmental conditions (Mujahid, 2010). Chapter 2 also demonstrated that juveniles that experienced either an ecological stressor or a physiological stressor early in life exhibited hypomethylation in the brain, and that adults that experienced an ecological stressor early in life exhibited hypermethylation in the brain. Adults that experienced a physiological stressor early in life showed no difference in methylation. Our results contradict previous research, many of which observed hypomethylation in adulthood rather than in juveniles (Anier et al., 2014; Goerlich et al., 2012; Morgan and Bale, 2011). This study examined the effects of an ecological (cold) and physiological stressor (oral Cort) on the stress response and the brain at two timepoints, P30 and P120, later in life. Together, the results of these two experiments suggest that the brain and nervous system are highly sensitive to early life challenges.
In summary, chapter 3 demonstrated that experiencing acute stress diminishes a female’s preference behavior towards her mate. Restraint decreased the strength of female preference which resulted in females no longer preferring to perch closer to their mate’s song instead of a non-mate’s song, and no longer preferring to perch closer to the song of their mate played at a faster rate instead of at a slower rate. Even in the proximity preference paradigm where females did not display a preference for their mate when not restrained, restraint decreased the strength of female preference for her mate even further. A female’s preference for being in close proximity to her mate (proximity preference) is very well-documented (Banerjee and Adkins-Regan, 2011; Clayton, 1990; Zann, 1996); however, our females did not prefer to spend more time perched next to their mate than the non-mate male when they did not experience restraint. Despite this anomaly, all our findings are consistent with previous studies that show a reduction in the strength of female preference when the animal is acutely stressed (Davis and Leary, 2015; DeVries et al., 1995; DeVries et al., 1996). To the best of our knowledge, this study is the first to show the effect of acute stress experienced in adulthood on the resilience of female mate preference in established mated pairs.

In summary, chapter 4 demonstrated that acute stress experienced before mate-song playback decreased neuronal activation and increased GR immunoreactivity in primary and secondary auditory brain regions as well as non-auditory brain regions that may play a role in the perceptual processing of song. When staining intensity was analyzed, the NCM and Field L were the only auditory regions to have light intensity labeling of GR-ir neurons, but this does not diminish our findings that show the avian primary and secondary cortical regions can be influenced by stressful events. Our results are also supported by *in situ hybridization* experiments with Bengalese finches that have shown that GR can be expressed in the NCM and CMM of
females (Suzuki et al., 2011). After an extensive literature search, we found that there have been no studies investigating the impact of acute stress on auditory cortical areas and non-auditory cortical areas that may be involved in the processing of male song by females.
REFERENCES


Murphy, T.M., N. Mullins, M. Ryan, T. Foster, C. Kelly, R. McClelland, J. O'Grady, E.
Corcoran, J. Brady, M. Reilly, A. Jeffers, K. Brown, A. Maher, N. Bannan, A. Casement,
D. Lynch, S. Bolger, A. Buckley, L. Quinlivan, L. Daly, C. Kelleher, and K.M. Malone.
2013. Genetic variation in DNMT3B and increased global DNA methylation is associated
Naguib, M., and A. Nemitz. 2007. Living with the past: nutritional stress in juvenile males has
immediate effects on their plumage ornaments and on adult attractiveness in zebra
Naguib, M., K. Riebel, A. Marzal, and D. Gil. 2004. Nestling immunocompetence and
Publishers, Sunderland, MA, USA.
Neubauer, R.L. 1999. Super-normal length song preferences of female zebra finches
2010. Corticosterone and dehydroepiandrosterone have opposing effects on adult


