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THE EFFECTS OF EARLY CORTICOSTERONE TREATMENT ON THE DEVELOPMENT OF THE AVIAN SONG CONTROL SYSTEM

by

MAHIN SHAHBAZI

Under the Direction of Laura L. Carruth

ABSTRACT

Stress has long lasting effects on physiology, development, behavior, reproductive success and survival. These effects are mediated by glucocorticoids, such as corticosterone (Cort), via glucocorticoid receptors (GR), though the exact mechanisms underlying these effects are unknown. Early developmental stress affects the size of the avian song control nuclei (particularly HVC; proper name) and song quality in many songbirds, suggesting a direct link between brain and behavior. HVC is required for song learning and production. The complexity of the male zebra finch (*Taeniopygia guttata*) courtship song is important in female mate choice. Although the mechanisms behind the effects of developmental stress on song nuclei size and song quality

are unknown, it is likely that elevated levels of Cort via GR within brain song nuclei play a significant role.

We investigated the distribution, quantity, and subcellular-localization of GR- immunoreactive (GR-ir) neurons in the brains of male zebra finches 10 days post-hatch and in adulthood using immunohistochemistry. There was wide distribution of GR-ir neurons including two song nuclei HVC and robust nucleus of the arcopallium (RA). Distribution did not vary between the two ages but there were significant differences in the overall number of GR-ir neurons and their subcellular localization.

We hypothesized that early Cort treatment would reduce song quality and HVC size in adult males. We inserted Cort implants in males at four days post-hatch and quantified the effects of early Cort treatment on adult song quality. Early Cort treatment decreased song similarity between the tutor and tutee's songs and resulted in poorer copies of tutor song, but did not alter mean amplitude or song duration.

Early Cort treatment reduced the HVC size in both juvenile and adult birds. This result suggests that the effect of developmental stress on the HVC size may be mediated through Cort via activation of GR within HVC as a mechanism by which HVC size and song quality are altered in developmentally stressed birds. These results suggest a potential role for Cort in mediating adverse effects of developmental stress in adult male zebra finches and highlight the developmental plasticity of the zebra finch brain.

INDEX WORDS: Glucocorticoid receptor, Corticosterone, Stress, Brain song nuclei, Song quality, Songbird

THE EFFECTS OF EARLY CORTICOSTERONE TREATMENT ON THE DEVELOPMENT OF THE AVIAN SONG CONTROL SYSTEM

by

MAHIN SHAHBAZI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2012

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August 2012

DEDICATION

To my daughter, Christina, thank you so much for your love and understanding.

To my parents, brothers, and sisters, I cannot thank you enough for your incredible love, encouragement, and support through all these years.

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CHAPTER 1: GENERAL INTRODUCTION: THE HPA AXIS, GLUCOCORTICOID RECEPTORS AND THE AVIAN BRAIN

1.1. Overview

Experiencing early developmental stress can have long-lasting effects on the physiology, behavior, reproductive success, and survival (Lindstrom, 1999; Naguib et al., 2006; Naguib and Nemitz, 2007). The mechanisms underlying the effect of early stress on neuronal development are not well understood and it is important to identify these mechanisms in order to examine the impact of stress on brain and function.

Exposure to early chronic stress or elevated levels of glucocorticoids (GC) can result in stress-related mental disorders and brain abnormalities (Sapolsky, 2000 and 2001) and glucocorticoid receptors (GR) mediate the adverse effects of GC on brain and its function (Sapolsky, 2000 and 2001; Krugers et al., 2006). The Australian zebra finch (*Taeniopygia guttata*), a songbird, is a powerful model for studying stress-induced brain plasticity because reduction of the size of song control nucleus HVC (proper name) following lesion or exposure to an early chronic stress, such as food restriction or corticosterone (Cort) administration, results in a reduction of song complexity in zebra finches, swamp sparrows, and song sparrows (Spencer et al., 2003; Buchanan et al., 2004; Nowicki et al., 2002; MacDonald et al., 2006), suggesting a direct link between brain plasticity and behavior in songbirds (Pfaff et al., 2007). HVC is required for learning and production of song (Nottebohm et al., 1976; Vu et al., 1994; Yu and Margoliash, 1996; Hohnloser et al., 2002), and song complexity is important for mate choice (Searcy and Marler 1981; Baker et al., 1986; Catchpole et al., 1984; Clayton and Prove 1989; Neubauer 1999).

There is a wide variation between songbirds in their song complexity. Song complexity is usually measured as song repertoire size (the number of different song types) and the number of different syllable types per song type. Song repertoire size can vary from one (e.g. zebra finches) to hundreds (e.g. mocking bird, *Mimns polyglottis;* DeVoogd et al., 1993; Nowicki et al., 2002). In zebra finches, song complexity was measured using average song phrase duration, the total number of syllables, and the number of syllable types (Boogert et al., 2008; Spencer et al., 2003; Brumm et al., 2009). The mechanisms underlying these effects are unknown. The food-restricted song sparrows have elevated baseline Cort (MacDonald et al., 2006; Kempster et al., 2007). These findings suggest that Cort may mediate the effects of early stress, although Cort is only one of a several hormones released during stress response.

GR play a role in stress and activation of GR within HVC, may be a specific mechanism by which Cort acts to decrease HVC size and reduce song complexity in stressed birds. Therefore, it is critical to determine whether GR play a role in the stress-induced reduction of HVC size and song complexity. Thus, the study of GR role in stress-induced reduction of HVC size and song complexity will increase our understanding of how early stress affects adult human brain function.The present review summarizes findings on the effects of early Cort treatment on HVC size and song complexity with a focus on the distribution of GR in brain and song system of male zebra finches.

1.2. The HPA axis, stress response, and hormones involved in stress

When homeostasis is disrupted by a stressor, such as temperature extremes, food or water deprivation, aggressive interactions, or social subordination, (Nelson, 2005), an individual displays a stress response, the series of physiological and behavioral responses that help to reestab-

lish homeostasis (Selye, 1950). On the other hand, prolonged activation of the hypothalamic-anterior pituitary-adrenal (HPA; see Figure 1.1.) axis can harm the body by increasing the risk of mental disorders, obesity, heart disease and other illnesses (McEwen, 2007). The common pathway mediating the stress response is activation of the HPA axis, resulting in both immediate and delayed effects (Sapolsky et al., 2000). Some examples of immediate physiological responses of stress axis activation include: increased cardiovascular tone, immune system activation and energy mobilization whereas delayed effects typically involve secretion of glucocorticoids from the adrenal cortex (Sapolsky et al., 2000). The paraventricular nucleus (PVH) of the hypothalamus releases corticotropin-releasing hormone (CRH) in response to a stressor (Nelson, 2005). CRH acts on the basophilic corticotrope cells of the anterior pituitary gland to stimulate the synthesis and release of adrenocorticotropin (ACTH) into the bloodstream (Tsigos and Chrousos, 2002). ACTH subsequently causes the release of GC (including Cort and cortisol) from the zona fasciculata of the adrenal cortex (Nelson, 2005; Bao et al., 2007). After the stressor or threat is over, tissue response to GC 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). GC are involved in carbohydrate metabolism and are usually released in response to stressful stimuli. In addition to the release of GC, CRH, and ACTH, other hormones are released during stress including vasopressin (VP), prolactin, cathecholamines and β-endorphins (de Kloet et al., 1991; Tsigos and Chrousos, 2002). By contrast, gonadothropins and gonadal steroids are decreased during stress response (Tsigos and Chrousos, 2002).

1.3. Synthesis and metabolism of glucocorticoids

The immediate substrate for biosynthesis of steroid hormones is cholesterol, made from circulating lipoproteins and synthesized primarily in the liver, the intestine and to a lesser extent in other tissues. Cholesterol is transported to the adrenal cortex, where ACTH activates the enzymes required for biosynthesis of glucocorticoids and the mineralocorticoid aldosterone (Norman and Litwack, 1997; see Figure 1.2.). Cholesterol is first converted to the C21 steroid pregnenolone, a progestin that is the precursor to all other steroid hormones. Pregnenolone is converted to progesterone and via an enzymatic pathway into different corticoids. Like other steroid hormones, corticoids cannot be stored in cells and are released as they are synthesized. The C21 corticosteroids include the GC (such as Cort and cortisol) and mineralocorticoids (synthesized in the adrenal zona glomerulosa) such as aldosterone. Cort and cortisol bind with high affinity to corticosteroid-binding globulin (CBG or transcortin) in the blood plasma for transportation via the circulation to target tissues. GC circulate in blood mainly bound to CBG, however, it is the free steroid that enters the target cell by diffusion (Norman and Litwack, 1997).

Most animals make either Cort or cortisol, and it is not common to produce both of them in large amounts. Cort is the primary GC in reptiles, birds, and some mammals (most rodents), whereas cortisol is the primary GC in fishes and primates including humans (Nelson, 2005). Cort is converted to 18-hydroxycorticosterone and then into aldosterone, the primary mineralocorticoid in all terrestrial vertebrates, which is critical in ion exchange and water balance (Nelson, 2005).

In birds, like in mammals, the major site of GC breakdown is the liver and to some extent the kidney. GC metabolites, mainly conjugated as sulfates or glucuronides, are excreted via the bile into the gut and via the kidney into the urine. The metabolism of GC generally includes 5α

or 5β reduction, hydroxylation, or reduction of functional groups or side-chain cleavage in the case of 17α-hydroxylated metabolites (Norman and Litwack, 1997; Mostl et al., 2005)

1.4. Glucocorticoid receptors

GC regulate the function of many organ systems, including the brain, liver, pancreas, and fat tissue. The extent of the effects of GC on target tissues depends to some extent on the quantity and distribution of receptors that bind these hormones. Although GC act on many tissues of the body to provide a response to stress (especially long-term stress), the liver is one major target organ because it contains a large number of GR in hepatocytes. Other organs such as lymphocytes including thymus cells, adipose cells, kidney, anterior pituitary, and especially various parts of the brain are as well important targets for GC (Norman and Litwack, 1997).

Rapid effects of GC are mediated by the activation of membrane-associated receptors independent of cytosolic (also known as intracellular or cytoplasmic) GR. Delayed effects of GC are mediated by activation of cytosolic receptors. Cytosolic receptors can be classified as corticosteroid type I, or MR, and corticosteroid type II, or GR. These receptors bind to their ligand, and are translocated to the nucleus. In the nucleus, they bind to a GC response element sequence in the promoter region of different glucocorticoid-regulated genes, and activate or suppress transcription (Rozeboom et al., 2007).

In response to a stressful event, GR act as sensors and their activation mediates anxiety-related behaviors and affects learning and memory. GR upregulation prevents further activation of the HPA axis by negative feedback mechanisms (Breen et al., 2004; Swaab et al., 2005). GR have low affinity for GC and are primarily activated when plasma GC levels are high (Breen et al., 2004). The negative feedback control of GC can be disrupted in stress-related mental disor-

ders (Mizoguchi et al., 2003). In the absence of chronic stress, overexpression of GR in forebrain of transgenic mice leads to an 'age-like phenotype' (increased anxiety, depressant like behavior and cognitive dysfunction) in young animals (Wei et al., 2007). By contrast, blockade of GR in rat hippocampus restores hippocampal synaptic plasticity during stress (Krugers et al., 2006).

MR regulate the basal circadian rhythm of Cort levels and, in contrast to GR, have a high affinity for Cort and are heavily occupied and activated at basal conditions (Breen et al., 2004). MR are important for the sensitivity and feedback of neuroendocrine responses. MR/GR imbalances can occur and the blockade of MR by a specific antagonist increases circulating levels of Cort under basal and stress condition (Ratka et al., 1989). This finding suggests that one of the MR-mediated effects of Cort is the suppression of the activity of the HPA axis (Oitzl et al., 1995). According to the MR/GR balance hypothesis (de Kloet, 1991) an increase in the amount of MR relative to GR in rats will result in a reduced responsiveness of the HPA axis to stress (Oitzl et al., 1995). The increased activation of the HPA axis in many stress-related disorders is due to a MR/GR imbalance (Bao et al., 2007).

Rapid physiological and behavioral effects of GC are mediated by actions of membrane-associated GR. Such rapid actions are mediated through membrane-associated G protein-coupled receptors and activation of downstream signaling cascades (Tasker et al., 2006; Breuner and Orchinik, 2001). There is a rapid suppression of the HPA axis activation during stress response which may be mediated through a rapid mechanism independent of gene transcription (Tasker et al., 2006). Despite additional roles for MR and membrane-associated GR, the involvement of GR during stress might be a specific mechanism by which HVC size and song complexity decrease.

1.5. Actions of glucocorticoids

Unbound GR and MR are present in the cytoplasm and unoccupied GR forms a complex oligomer containing a dimer of hsp 90, a monomer of immunophilin p52, and hsp 70. The confirmation of this oligomeric form of the GR allows binding of the ligand, whereas the monomer of the free receptor does not rebind the steroid well after the ligand has dissociated. In addition to producing the steroid binding form of the receptor, the oligomer may also function to anchor the receptor to cytoplasmic skeletal element such as tubulin or actin. After the steroidal hormone binds to the receptor in oligomeric form, the oligomeric complex dissociates and releases free receptor monomer. This process is called cytoplasmic activation or transformation and the receptor becomes hyperphosphorylated. The activated receptor can enter the nucleopore as a monomer or homodimer and is transported to the nucleoplasm where it gains access to DNA. There it binds to DNA as a homodimer and assumes a configuration that is favorable for the entry of the other transactivating proteins including the transcriptional complex (Mckenna and O'Malley, 2002). When the transcriptional complex is in place, RNA polymerase II transcribes the open reading frame of the activated gene. Finally, the steroid-receptor complex dissociates from the DNA and, after dephosphorylation, the receptor is transported back into the cytoplasm. There, the receptor is reassembled into the inactive oligomeric form and is ready for another event. The receptor may be degraded instead of entering the reassembly process. The dissociated steroid is metabolized to an inactive form in tissues like liver (Mangelsdorf et al., 1995; Mckenna and O'Malley, 2002; Norman and Litwack, 1997).

1.6. The zebra finch as a model system

Zebra finches (*Taenopygia guttata*) are a powerful model for investigating stress-induced brain plasticity. In male zebra finches, early stress decreases the size of HVC (proper name), a song nucleus, and alters song complexity, suggesting a direct link between brain and behavior in songbirds including swamp sparrows, canaries, and song sparrows (Nowicki et al., 2002; Spencer et al., 2005; MacDonald et al., 2006). Although the mechanisms behind this effect are unknown, it is likely that the elevated levels of Cort and/or changes in the sensitivity of GR play a significant role. GR may play a critical role in mediating the effects of GC on the regions of the brain involved with singing behavior and song complexity. Unlike other songbirds, in zebra finches, the size of song control nuclei and singing do not change seasonally and they sing only one song throughout their lives. Juvenile male zebra finches learn their song from their father and their song is very similar to the song of their father. Therefore, it is more appropriate to study the effects of early stress on HVC size and song complexity in zebra finches than any other songbirds. MR and membrane-associated GR may have potential roles in stress-induced reduction of HVC size and song complexity.

1.7. Developmental timeline

The earliest sex differences in zebra finch brain are reported at post hatch day 1(P1; Duncan and Carruth, 2007) and the song control system is fully formed around P10. Juvenile zebra finches start learning song from their father or their tutor around P25-P30 and they are sexually mature around P60 and their critical period for song learning is closed around this time. The song is crystallized around P90 (Immelmann 1969; Price 1979; Doupe, 1993; Williams and Mehta 1999). See Figure 1.3. for a complete developmental timeline.

1.8. Song control system

The song control system (SCS; see Figure 1.4.) is composed of a set of discrete and interconnected telencephalic nuclei, the song control nuclei, and includes HVC (acronym is a proper noun, previously named as the hyperstriatum ventrale, pars caudale, or the high vocal center), area X, robust nucleus of the arcopallium (RA), and lateral magnocellular nucleus of the anterior nidopallium (LMAN). The circuit including projections from area X to LMAN to dorsolateral thalamus (DLM) is the anterior forebrain pathway (AFP) and is involved in vocal learning and plasticity. The projections from HVC to RA to the tracheosyringeal motoneurons of the hypoglossal nucleus (nXIIts) form the motor pathway, which is involved in song production. Motor input from the tracheosyringeal motoneurons then is sent to the syrinx (avian vocal organ). The projection from LMAN to RA completes the connection between the AFP and the motor pathway. Together the song nuclei control the learning and production of song in male zebra finches (Nottebohm et al., 1976; Doupe, 1993; Williams and Mehta, 1999). Only male zebra finches sing and they have larger song nuclei compared with females (Nottebohm and Arnold, 1976).

1.9. Songs and closed-ended critical period learners

Birdsong is a form of conspecific communication (Williams, 2004). The smallest components of song are elements (syllables, notes). Elements are separated from each other by intervals, or gaps of silence that range from 5-10 ms in duration. The element and its preceding silent interval are the unit of zebra finch song. Each male sings a number of different elements in a set order and together these form the song-phrase (song-unit, motif, song; Catchpole and Slater, 2008; Zann, 1996). Within one performance, males may repeat song-phrase several times to form a song (bout). Several identical elements usually precede the first song-phrase and are referred to

as introductory notes which can be included in the song-phrase itself. The song-phrase is a natural unit of song investigation in zebra finches. Most researchers use song-phrase rather than song because the number of song-phrases sung per song depends on motivation and varies with directed and undirected song (Zann, 1996). Song syntax involves the timing and tempo and sequence of song features (Williams, 2004). Each male sings its song elements in a fixed order and the pattern is somewhat constant among zebra finches (Zann, 1996).

Male zebra finches learn their song from an adult male tutor, usually their father. Song learning consists of several stages (Marler, 1981; Williams, 2004). First is an auditory or sensory learning stage, which young (fledgling) males listen to and memorize the tutor's song. Second is a 'subsong' or sensory-motor stage, which young males produce sounds and listen to the results (like practicing the song). The third stage is plastic song, during which males alter and adjust their song closely to the memorized tutor song. The last stage is song crystallization, in which song becomes fixed in its adult form and the components of the song and the order which they are sung become stereotyped (Williams, 2004). These stages may be distinct or overlap. Zebra finches are non-seasonal breeders and complete all of the stages of song learning during a short period. Zebra finches learn and sing only one courtship song which is crystallized when they reach sexual maturity around 90 days old. After this time the song is not altered. Auditory feedback is required to maintain the song quality which declines after deafening (Konishi,1964; Marler and Sherman,1982). Therefore, zebra finches (*Taeniopygia guttata*) are considered to be critical period, closed-ended learners which indicate their song learning ability is age-limited and requires a critical time period (Immelmann, 1969). In closed-ended learners such as zebra finches, the stages of song learning overlap. Their sensory stage lasts from 10 to about 60 days of age. At the end of this period, the sensitive window for song learning closes. The sensorimotor stage

(including subsong and plastic song stages) lasts from about 25 days into adulthood at around 90 days. At about 90 days, song becomes crystallized (Immelmann, 1969; Price 1979; Williams and Mehta, 1998).

1.10. The underlying possible mechanisms controlling stress-induced reduction of HVC size and song complexity

There is more evidence in favor of the delayed effects of hormones released during stress than there is for the immediate physiological effects of stress response on brain abnormalities. Of the hormones released during stress, GC, CRH, ACTH, and VP are most implicated in stress-induced brain abnormalities (Bao et al., 2007; Swaab et al., 2005). Despite the potential influence of multiple factors in the HPA axis, a primary role for GC in stress-induced brain abnormalities has been demonstrated by multiple lines of research. First, GC are the primary end product of activation of the HPA axis and prolonged increased levels of GC decrease hippocampal volume and cognitive function in Cushing syndrome and some stress-related mental disorders (Swaab et al., 2005; Bao et al., 2007; Sapolsky, 2000 and 2001; Radley and Morrison, 2005). In fact, in Cushing syndrome (associated with chronic exposure to high level of cortisol), there is a strong negative correlation between the plasma cortisol level, the size of hippocampus, and cognitive dysfunction and these effects are to some extent reversible after correction of hypercortisolism (Wolkowitz et al., 1999). Second, increased GC levels are associated with depression. Patients with Cushing syndrome and patients receiving GC therapy mostly develop depression (Sapolsky, 2000, 2001; Mitchell and O'Keane, 1998). In addition, GR antagonists and inhibitors of GC production are used as antidepressants (Belanoff et al., 2002; Wolkowitz et al., 1999). Third, prolonged increased levels of GC impair learning and memory processes in human, rats,

and zebra finches (Sapolsky, 2000, 2001; Roozendaal et al., 2001; Hodgson et al., 2007). Fourth, chronic increased levels of GC reduce neurogenesis and neuronal proliferation and alter apoptosis (Woolley et al., 1999; Yu et al., 2004; Lau et al., 2007).

We cannot fail to recognize potential roles of other factors such as CRH, VP and ACTH in stress-induced reduction of HVC size and song complexity in developmentally stressed birds. Some evidence supporting the potential roles of CRH, VP and ACTH in stress-induced brain abnormalities are as follows: 1) CRH neuron numbers and CRH mRNA are increased in the hypothalamic paraventricular nucleus (PVN) during depression (Swaab et al., 2005; Bao et al., 2007). CRH receptor antagonists are used as antidepressants (O'Brien et al., 2001; Keck and Holsboer, 2001). 2) VP neuron numbers and VP plasma levels are increased in depression (Swaab et al., 2005; Bao et al., 2007). In a rat model of psychopathology, the levels of VP decrease with antidepressant therapy (Keck et al., 2003). 3) Abnormalities in ACTH control of the HPA axis have been reported in patients with Alzheimer's disease (AD). Patients with mild to moderate AD show temporal lobe atrophy, low plasma ACTH levels and low cognitive function (Nasman et al., 1996). Future studies/aims are necessary to test the roles of these hormones in stress-induced reduction of HVC size and song complexity in stressed birds.

Prolonged increased levels of GC are strongly associated with decreases in hippocampal volume, reduction in cognitive function, development of depression, suppression of neurogenesis and proliferation and alteration of cell apoptosis. Although the potential roles of CRH, VP and ACTH are not mutually exclusive, multiple lines of evidence point to GC as the critical factor in stressed-induced brain abnormalities. Therefore, this dissertation will focus on the possible role of GC (the elevated level of Cort) and their receptors (changes in the number or sensitivity of the receptors) in reduction of HVC size and song quality in early Cort-treated male zebra finches.

1.11. Dissertation goals

The overall goal of this dissertation is to address the following questions: 1) what is the distribution and subcellular localization of glucocorticoid receptor-immunoreactive (GR-ir) neurons in the developing and adult male zebra finch brain? 2) what are the effects of early Cort treatment on learned features of song and stress-related biological markers in adult male zebra finches? and 3) what are the effects of early Cort treatment on HVC and RA size of juvenile (post-hatch day 30) and adult male zebra finches? In chapter 2, I describe research on the subcellular localization and distribution of GR-ir neurons in the male zebra finch brain at two ages. If GR-ir neurons are involved in reduction of HVC size and song quality in developmentally stressed birds, then they should be present in the song nuclei at P10 (post-hatch day 10) and in adulthood. The presence of GR-ir neurons in the regions of the brain where the song control nuclei develop or in the song nuclei themselves indicates that these brain regions are stress sensitive. In chapter 3, I tested whether early Cort treatment would reduce song quality and the learned features of song in adult male zebra finches by comparing the similarity and accuracy of the song to the tutor song. We further studied the effects of early Cort treatment on stressed-related biological markers such as body weight, tarsus length, wing length, and body condition. In chapter 4, I hypothesized that early Cort treatment would decrease the size of HVC in juvenile and adult male zebra finches. If zebra finches are exposed to Cort early, then only HVC size should be decreased significantly. The combined results are then synthesized in chapter 5, and together these studies help us understand how early Cort treatment affects the development of the song system in male zebra finches.

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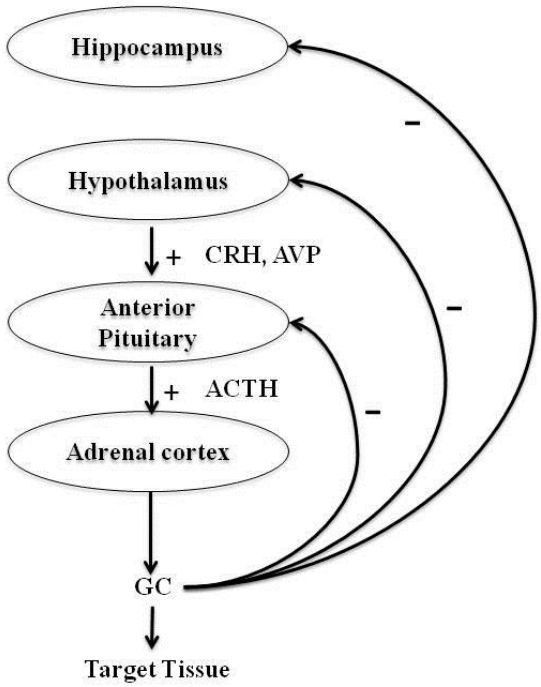

Figure 1.1. Negative control of the hypothalamic-pituitary-adrenal (HPA) axis.
In response to a stressor, the hypothalamus releases corticotropin-releasing hormone (CRH) and other hormones. CRH and Vasopressin (Arginine vasopressin; AVP) acts on the anterior pituitary gland to stimulate (+) the synthesis and release of adrenocorticotropin (ACTH) into the bloodstream. ACTH subsequently stimulates the release of GC (including Cort and cortisol; +) from the adrenal cortex. After the stressor or threat is over, tissue response to GC terminates the stress response by a negative feedback control (-), which acts at the levels of the anterior pituitary, hypothalamus and hippocampus.

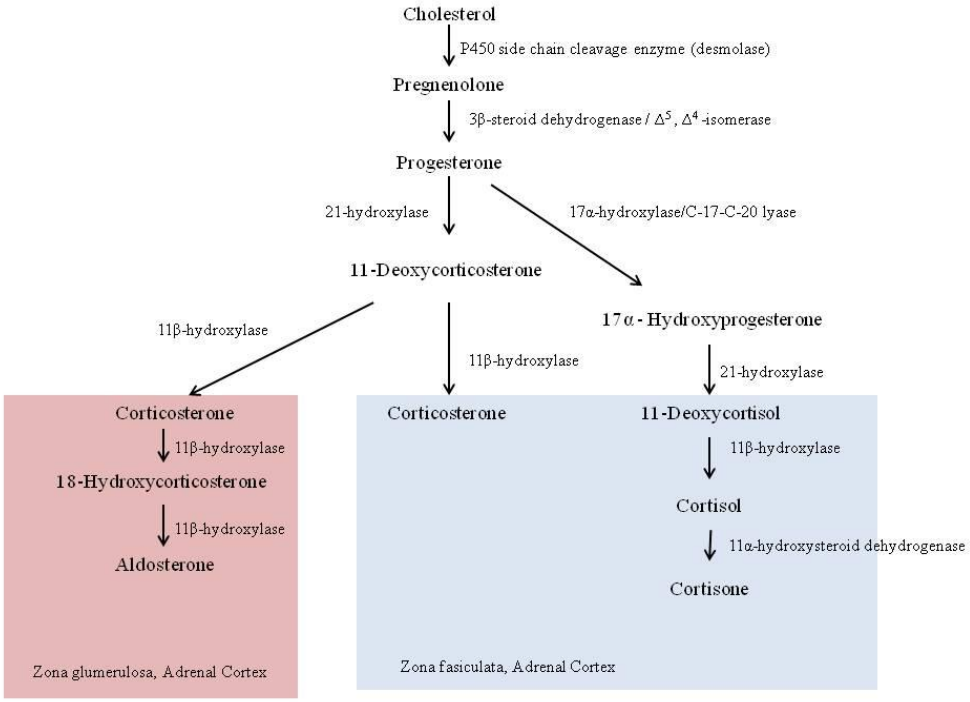

Figure 1.2. Pathways of glucocorticoid and mineralocorticoid biosynthesis in adrenal cortex. The enzyme desmolase cleaves the chain of carbons from the top of the cholesterol molecule to form pregnenolone, a C21 steroid that is the precursor to all other steroids. Arrows indicate the pathways of biosynthesis of corticosterone, cortisol, and aldosterone, though there is no single exclusive pathway (Nelson, 2005; Norman and Litwack, 1997).

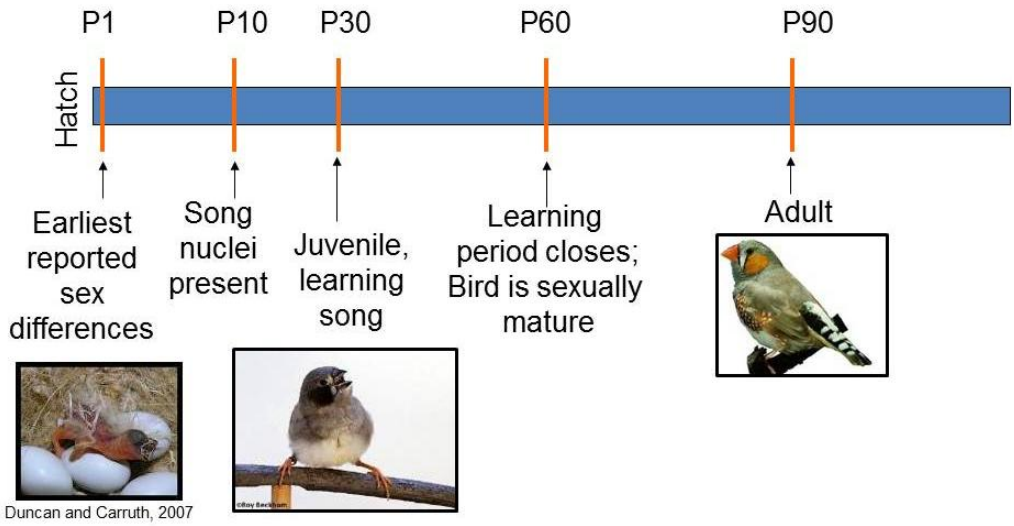

Figure 1.3. Zebra finch developmental timeline. The earliest sex differences in zebra finches are reported at post hatch day 1(P1) and the song control system is fully formed around P10. Juvenile zebra finches start learning song from their father or their tutor around P25-P30 and they are sexually mature around P60 and their critical period for song learning is closed around this time. The song of zebra finches is crystallized around P90.

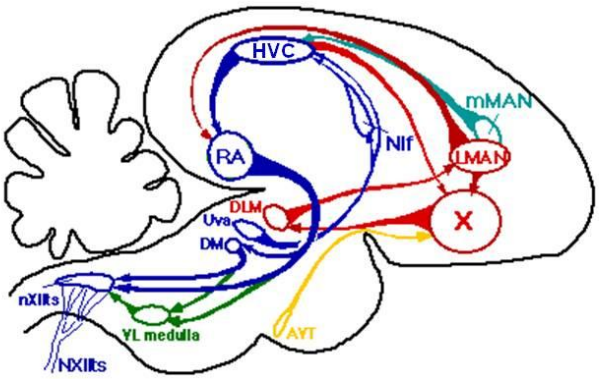

Figure 1.4. Song control circuit for song learning and production. A parasagittal view of a generalized songbird brain, showing the circuits known to be involved in learning and singing song. BLUE: The motor pathway **(**HVC → RA → DM → nXIIts**)** controls vocal muscle activity as well as breathing. RED: The anterior forebrain pathway (AFP) mediates song learning through an indirect loop between the HVC and RA (via area X**,** DLM**,** and LMAN**).** Image from: http://www.williams.edu/Biology/Faculty_Staff/hwilliams/Finches/circuits.html

CHAPTER 2: DISTRIBUTION AND SUBCELLULAR LOCALIZATION OF GLUCOCORTICOID RECEPTOR-IMMUNOREACTIVE NEURONS IN THE DEVELOPING AND ADULT MALE ZEBRA FINCH BRAIN.

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Abstract

Stress has long lasting effects on physiology, development, behavior, reproductive success and the survival of an individual. These effects are mediated by glucocorticoids, such as corticosterone, via glucocorticoid receptors (GR), though the exact mechanisms underlying these effects are unknown. GR have been widely studied in mammals but little is known about GR in other vertebrate groups, especially songbirds. We investigated the distribution, quantity, and subcellular-localization of GR-immunoreactivity (GR-ir) neurons in the brains of male zebra finches on P10 (post-hatch day 10, song nuclei formed), and in adulthood (post-hatch day 90 or older) using immunohistochemistry. GR-ir neurons were widely distributed in the brains of male zebra finches including two song nuclei HVC (acronym is a proper name) and RA (robust nucleus of the arcopallium) and brain regions including HP (hippocampal formation), BSTl (lateral part of the bed nucleus of the stria terminalis), POM (nucleus preopticus medialis), PVN (nucleus paraventricularis magnocelluaris), TeO (optic tectum), S (nucleus of the solitary tract), LoC (Locus coeruleus). Distribution did not vary at the two age points examined, though there were

significant differences in staining intensity. Subcellular GR-immunoreactivity patterns were classified as cytoplasmic, nuclear, or both (cytoplasmic and nuclear) and there were significant differences in the overall number of GR-ir neurons and neurons with both nuclear and cytoplasmic staining in P10 and adult brains. Though, there were no significant differences in the percentage of subcellular GR immunoreactivity patterns between P10 and adults. Our study of GR-ir neuronal distribution in the zebra finch brain may contribute towards understanding of the complex and adverse effects of stress on brain during two different stages of life history.

Keywords: Glucocorticoid receptor (GR), Stress, Song control system (SCS), Songbird, Corticosterone, Immunohistochemistry

2.1. Introduction

Glucocorticoids (GC) play an important role in the homeostasis of many biological systems including stress responsiveness, energy metabolism, and immune and inflammatory responses (De Kloet et al., 2005). During the stress response, GC are secreted from the adrenal cortex after the hypothalamic-pituitary-adrenal (HPA) axis activation (Bao et al., 2008). After the stressor is reduced, tissue response to GC terminates the stress response via negative feedback control acting on the levels of the hippocampus, hypothalamus, and anterior pituitary (Swaab et al., 2005). Prolonged or chronic activation of HPA axis during stress can harm the body by increasing the risk of mental disorders, obesity, heart disease and other illnesses (McEwen, 2007).

GC, such as corticosterone, regulate the function of many organ systems, including the brain, liver, pancreas, and muscle, via glucocorticoid receptors (GR). In the brain, GR play a critical role in mediating the adverse effects of GC on neuronal functioning (Krugers et al., 2006; Sapolsky 2000, 2001). Prolonged GR activation in the brain via either overexpression of GR or

high level of GC mediates anxiety behavior and affects learning and memory (Hodgson et al., 2007; Roozendaal et al., 200; Sapolsky 2000, 2001; Wei et al., 2007).

A genomic response to GC is mediated by activation of cytoplasmic receptors and subsequent transcriptional regulation in the nucleus. Cytoplasmic receptors that can bind GC include the corticosteroid type I or mineralocorticoid receptor (MR), and corticosteroid type II, or GR. Following ligand binding, cytoplasmic GR are translocated to the nucleus (Rozeboom, et al., 2007). In the nucleus, they bind a GR response element sequence in the promoter region of different glucocorticoid-regulated genes, and activate or suppress transcription (Rozeboom, et al., 2007). GR have low affinity for GC and are primarily activated when plasma GC levels are high (Breen et al., 2004). GR activation is regulated by negative feedback of the HPA axis (Breen et al., 2004; Swaab et al., 2005) and the negative feedback control of GC is disrupted in stress-related mental disorders (Mizoguchi et al., 2003).

Experiencing early developmental stress can have long-lasting effects on the physiology, behavior, reproductive success and survival of an individual (Lindstrom, 1999; Naguib and Nemitz, 2007, 2006). We have been studying a powerful model of stress-induced brain plasticity in male zebra finches (*Taniopygia guttata*), an opportunistically breeding songbird in which only males sing. In zebra finches, food restriction or corticosterone, the major avian glucocorticoid, administration during early development results in reduction of HVC (acronym is a proper name) size and song complexity (Buchanan, et al., 2004; MacDonald et al., 2006; Nowicki et al., 2002), suggesting a direct link between brain and behavior. Although the mechanisms behind this effect are unknown, it is likely that the elevated levels of corticosterone and/or changes in the sensitivity of GR play a significant role. Moreover, GR play a major role in regulating the increased levels of corticosterone during stress through a negative feedback mechanism (Bachmann et al.,

2003; Mizoguchi et al., 2003). Therefore, understanding the distribution of GR in the telencephalon, including the song control system (SCS, including HVC and RA) in male zebra finches at different life stages will increase our understanding of the mechanisms of plasticity underlying stress-related effects on neuronal development. The distribution of GR protein in the brain is known for some vertebrate species (Ahima and Harlan, 1990; Carruth et al., 2000; Kovacs et al., 1989; van Eekelen et al., 1987; Yao et al., 2008), though only the GR mRNA distribution has been examined in the songbird brain (Dickens et al., 2009) and the presence of GR protein in the SCS has yet to be published.

The goal of this study was to investigate GR-immunoreactivity in the SCS and other brain regions in the male zebra finch song nuclei during one time point prior to fledging the nest (post-hatch day 10 or P10) and one time point during adulthood using immunohistochemistry and confocal imaging. The presence of these receptors in the regions of the brain where the song control nuclei develop or in the song nuclei themselves indicates that these brain regions are stress sensitive.

2.2. Methods

2.2.1. Animals

We used male zebra finches from our breeding colony at Georgia State University. The Georgia State University Institutional Use and Animal Care Committee granted approval for all animal procedures. Male birds age P10 (10 days post-hatch) and over age P90 (90 days post-hatch) were used. Sex of P10 birds was determined via PCR following the protocol of Soderstrom et al. (2007) and by visual examination of the gonads at the time of tissue collection.

2.2.2. Immunohistochemistry

To determine distribution, neuronal cell counts, and labeling intensity of GR-immunoreactive (GR-ir) neurons, immunohistochemistry (IHC) was performed on the brains of P10 (n = 5) and adult male (n = 6) birds. After an overdose of Isoflurane (an inhalational anesthetic), P10 and adult birds were decapitated as previously described (Duncan et al., 2011). Brains from adult birds were dissected from the skull and placed in Bouin's fixative for 24 h. For P10 birds, after removing the skin and feathers, the entire skull was placed in Bouin's fixative for 24 h after which time the brain was removed from the skull. Fixation was followed by three 12 h rinses in 70% ethanol for both P10 and adult tissue. Adult and P10 brains were then ethanol dehydrated, and cleared in CitriSolv Hybrid (Fisher Scientific, King of Prussia, PA) after which the brains from both age points were then embedded in Paraplast (Oxford Labware, St. Louis, MO), sectioned at 10 µm with alternating coronal sections placed on gelatin-subbed microscope slides, and stored at room temperature until use.

Standard IHC protocols (Duncan et al., 2011) were followed for P10 and adult tissue. Prior to beginning the IHC procedure, tissue was cleared with xylene (Fisher Scientific, Fair Lawn, NJ), rehydrated, and then washed in detergent (0.2% Triton X-100; Sigma Chemical Co.). All solutions were made with Tris-buffered saline (0.5 M TBS, PH 7.6). Slides were incubated in trypsin (porcine trypsin type II, 1mg/1ml deionized water; Sigma-Aldrich, St. Louis, MO) solution for 3 min at room temperature and then were rinsed in 0.5 M TBS. Before incubation with the primary antibody, slides were placed in a hot 0.01M citrate buffer (PH 6.0) wash for 5 min as an antigen retrieval step. IHC was performed in a humidity chamber with an anti-glucocorticoid receptor polyclonal antibody from rabbit as primary antibody (concentration of 1:250; Thermo Scientific/Pierce Antibodies, PA1-510A or PA1-511A). Because little is known about GR im-

munoreactivity in the songbird brain, we used two primary polyclonal antibodies, PA1-510A and PA1-511A (both from Thermo Scientific/Pierce Antibodies, PA). The PA1-510A antibody has been used successfully in Bouin's fixed, paraffin embedded Pacific salmon (*Oncorhychuss nerka kennerlyi*) brain tissue (Carruth et al., 2000) and paraformaldehyde fixed tissue and recognizes a 17 a.a. sequence corresponding to amino acid residues 151-168 from human GR (accession: P04150.1) in the N-terminal region. The PA1-511A antibody detects GR from human, mouse, and rat. PA1-511A antibody corresponds to amino acid residues 346-367 from human GR (accession: P04150.1). These two peptides (PA1-510A and PA1-511A) are present in rat GR (accession: AF 455050.1 or AAL 78956.1) with 88% and 95% identity. Both antibodies gave the same pattern of GR immunoreactivity in Bouin's-fixed tissue. All images are from PA1-510A as the primary antibody.

Slides with P10 and adult sections were incubated with primary antibody (PA1-510A or PA1-511A) at 4°C for 48 h and were covered with strips of parafilm (American National, Greenwich, CT) to make certain that the tissue was fully covered with antibody and to prevent drying. Slides were incubated with a biotinylated antibody (goat anti-rabbit IgG; HistoMark kit; KPL, Gaithersburg, MD) at room temperature for 1 h. Slides were rinsed 3 x 5 min in TBS and then placed in peroxidase labeled streptavidin solution (HistoMark kit; KPL, Gaithersburg, MD) for 30min at room temperature. After incubation, slides were rinsed 3 x 5 min in TBS and were then placed in a DAB solution (Vector Laboratories, Inc., Burlingame, CA) containing DAB, hydrogen peroxidase, and nickel sulfate to visualize the reaction. Slides were then rinsed in distilled water. Slides were coverslipped after serial dilutions in ethanol and xylene. Normal goat serum served as a negative control for PA1-510A and was applied instead of the primary antibody on control slides. The PA1-511A neutralizing peptide (Catalog: PEP-001; Thermo Scien-

tific/Pierce Antibodies; synthetic peptide, amino acids 346-367) was used as the control for PA1-511A. A 10:1 fold dilution of the neutralizing peptide and antibody was applied in place of the antibody on control slides.

2.2.3. Fluorescence immunohistochemistry

A subset of adult male brains (n= 3) were used for fluorescence immunohistochemistry to determine subcellular localization of GR-immunoreactivity. Birds were anesthetized with an overdose of Isoflurane and the brains were perfused with 4% paraformaldehyde (PFA) in 0.1M SPB (Sorensen phosphate buffer, PH 7.4). After decapitation, the brains of these animals were removed from the skull and placed in 4% PFA for at least 24 hours at 4°C. After fixation, the specimens were rinsed in 0.1M SPB and brains were embedded in gelatin (15% in 0.1M SPB; Sigma type A, 90-110 bloom) and cut on ice on a Vibrating microtome (VT 1000 S: Leica, Wetzlar, Germany) in 50-µm thick horizontal sections as previously described (Schmidt, 1997, 2007; Schmidt et al., 2006). Male rat hippocampus was used as a positive control and processed in an identical manner as the zebra finch tissue. Free-floating sections of zebra finch brain and rat brain were degelatinized on a warm water bath. All steps were performed at room temperature on mildly agitated free-floating sections. Tissue sections were rinsed 3 x 10 min in 0.1 M SPB and then incubated for 2 h in a blocking agent containing 5% goat serum and 3% bovine serum albumin in 0.1 M TSPB (SPB + 0.3% Triton X-100 + 0.02% sodium azide). The blocking agent was also used as antibody diluent. Tissue sections were incubated overnight in the primary antibody (PA1-510 A, 1:2000 dilution) and were then rinsed 4 x 20 min in 0.1 M TSPB. Tissue sections were incubated 4 h in the secondary antibody (CY3 - labeled goat anti-rabbit IgG; Jackson ImmunoResearch, West Grove, PA) diluted 1:400 in 0.1 M TSPB and then rinsed 4 x 20 min

in 0.1 M SPB. In the second to the last wash, the sections were incubated 20 min in Hoechst 33258 (Sigma) diluted 1:150 in 0.1 M SPB from a stock solution of 1mg/1ml to counterstain nuclei. After the final rinse in 0.1 M SPB, tissue sections were mounted on slides and coverslipped in (1:1) glycerol/ SPB containing 5% DABCO (diazabicyclol [2.2.2.]octane: Sigma) to prevent bleaching and stored at 4°C until imaging.

2.2.4. Confocal microscopy and image processing

The fluorescently labeled sections of adult male zebra finch brain tissue or rat hippocampus were viewed and imaged on a confocal microscope (LSM 700, Zeiss, Jena, Germany) to generate micrographs using the associated software package (Zen 2009 Light Edition) as previously described (Schmidt, 1997, 2007). To excite and visualize the CY3-labeled secondary antibodies and Hoechst 33258, solid state laser lines of 555-nm and 405-nm were used, respectively. Stacks of 0.9 µm-thick to 2.0-µm-thick optical sections covering the section thickness of 50 µm were collected. Sub-stacks of these optical sections were collapsed to produce single two-dimensional images. All digital images were processed (filtering of high frequency noise, adjustments of brightness and contrast) by a graphics program (Image Pro Plus, Media Cybernetics Inc., Bethesda, MD and Adobe Photoshop CS5, San Jose, CA, USA) before they were arranged to the final figures using an illustration program (Adobe Illustrator CS5, San Jose, CA).

2.2.5. Tissue preparation for western blot analysis

Western blot analysis was conducted to verify the specificity of the primary antibody, PA1-510A. Brains from adult male zebra finches were rapidly dissected and the telencephalon and rest of the brain (all brain tissue except for the telencephalon) were removed. Brain tissue

was homogenized in homogenization buffer (100 mM KCL, 25 mM EDTA, 10 mM Tris solution, 1% (v/v) Triton X-100, 1% (v/v) IGEPAL (Sigma), supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) (Duncan and Carruth, 2007; Duncan et al., 2011; Mielke et al., 2005). Tissue was then centrifuged at 4°C at top speed (15,000-16,000g) for 30 min and placed on ice. Supernatant was rapidly removed and stored in -80°C until use. To determine sample concentrations, protein was quantified by DC (Detergent Compatible) protein assay (Bio-Rad, Hercules, CA).

2.2.6. Western blot analysis for antibody confirmation

As previously described (Duncan et al., 2011), thirty micrograms of each sample (n=2, adult male) was run on 10 % Tris-HCL gels (Bio-Rad, Hercules, CA). Protein was transferred to a PVDF (Polyvinylidene fluoride) membrane (Bio-Rad, Hercules, CA). Membranes were blocked in 5% non-dairy milk (Bio-Rad, Hercules, CA) at room temperature for 1 h with continuous shaking. Membranes were then incubated with primary antibody overnight at 4°C. The primary antibody (PA1-510A) and anti-β-actin (Novus Biologicals, Littleton, CO) were applied at the concentration of 1:1000 and 1:5000, respectively. Goat anti-rabbit secondary antibody (Cell signaling Technology Inc, Danvers, MA) were used at 1:12000 and incubated at room temperature for 1 h. Membranes were washed in Tris-buffered saline 3 x 10 min. The reaction was then visualized with an enhanced chemiluminescence (Millipore) Immobilon Western Kit (Billerica, MA) on Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ).

2.2.7. Quantifying GR-ir neurons using DAB immunohistochemistry

An ocular grid (mm^2) was used with the 40X objective to determine the density of GR-ir neuronal cell bodies in various brain areas. Random sections of P10 and adult male brain including HVC, RA (robust nucleus of the arcopallium), and TeO (optic tectum; Nucleus isthmi, pars parvocellularis of optic tectum was selected as representative of TeO) were subjectively chosen, and all GR-ir neurons within the ocular grid were counted. In the same brain area (section), the total number of neurons with GR immunoreativity was determined regardless of subcellular distribution of GR (total number of GR-ir neurons). Then, number of neurons containing both nuclear and cytoplasmic (N&C) GR labeling and number of neurons with only nuclear (N) GR labeling were counted (total number of neurons with both N&C plus neurons with only N labeling). The number of neurons with only cytoplasmic (C) GR labeling was then calculated by subtracting the total number of neurons with both N&C plus neurons with only N labeling from the total number of GR-ir neurons. All GR-ir counts were corrected for the brain size. The areas of HVC, RA and TeO per representative brain sections were measured for adult and P10 and then the ratio (adult brain area /P10 brain area) was calculated.

2.2.8. Statistical analysis

All data were analyzed using SPSS for Windows, version 17.0 (SPSS Inc, Chicago, IL). First, data were examined for assumptions of parametric statistical test. When assumptions were violated, a non-parametric alternative was used. Statistical significance was accepted at $P < 0.05$. All measures for experiment 3 were examined using mixed-design 2x3 ANOVAs with brain area (HVC, RA, TeO) as within-subjects factor and age (P10, Adult) as between-subjects factor. Independent-samples t- test was used to compare total number of GR-ir neurons, number of neu-

rons with both nuclear and cytoplasmic GR-ir, neurons with cytoplasmic GR-ir in each brain area of P10 versus adult male zebra finch. Mann-Whitney U test was used to compare the mean percentage of neurons with both nuclear and cytoplasmic and neurons with cytoplasmic GR immunoreactivity within each brain area, P10 versus adult male zebra finch.

2.3. Results

2.3.1. Validation of the primary antibody

In sections of rat hippocampus, using PA1-510A, GR was mostly localized in the cytoplasmic compartment similar to GR immunoreactivity visualized with other GR antibodies used in rat CA1 hippocampus (Sarabdjitsingh et al., 2012; before glucocorticoid stimulation; Figure 2.1A). PA1-511A did not give consistent staining pattern with PFA-fixed tissue.

Analysis of antibody specificity using Western blotting with PA1-510A determined that protein is present in the zebra finch brain with a similar molecular weight to the mammalian GR (≈97 KDa; Figure 2.1B), but the results using PA1-511A were inconclusive. One possible explanation for this may be that this antibody is more sensitive to the changes occurring in the protein epitopes during the WB process compared with PA1-510A.

2.3.2. GR-ir neuronal distribution in the P10 and adult zebra finch brain

DAB IHC was used to determine GR-ir neuronal distribution using PA1-510A. Both antibodies gave the same pattern of GR immunoreactivity in Bouin's-fixed tissue. All the distribution data, neuronal cell counts and images are from PA1-510A as the primary antibody. Regions of the zebra finch brain that exhibited the highest intensity of GR-ir labeling corresponded to homologous regions of the mammalian brain that contain GR, but GR-ir neurons also were pre-

sent in several additional areas in the zebra finch brain (see Table 2. 1, Figures 2.2 and 2.3). High intensity labeling of GR-ir neurons were observed in the telencephalon, diencephalon, mesencephalon, metencephalon and brainstem. Telencephalic regions exhibiting high intensity GR-ir labeling, HA (hyperpallium apicale), N (nidopallium), HD (hyperpallium densocellulare), Tn (nucleus taeniae amygdale), BSTl (lateral part of the bed nucleus of the stria terminalis). Telencephalic regions exhibiting moderate intensity labeling of GR-ir neurons included, HP (hippocampal formation), HVC, and Tu (nucleus tuberis). Telencephalic regions exhibiting low intensity labeling of GR-ir neurons included, RA (robust nucleus of the arcopallium), L (field or area L), and Ac (nucleus accumbens). In the diencephalon, POM (nucleus preopticus medialis), PVN (nucleus paraventricularis magnocelluaris), and Tu (tuberoinfundibular hypothalamus; homologous to the mammalian arcuate nucleus) exhibited high intensity GR-ir labeling neurons. In the diencephalon, DLM (nucleus dorsolateralis anterior thalami, pars medialis) exhibited moderate intensity GR-ir labeling neurons. The mesencephalon contained two regions with high intensity GR-ir labeling neurons: Rt (nucleus rotundus) and TeO (optic tectum). Metencephalic regions exhibiting high intensity GR-ir labeling neurons included Cb (cerebellum), cerebellar cortex, cytoplasm of Purkinje cells and stratum molecular. In the brainstem, LoC (Locus coeruleus) and S (nucleus of the solitary tract) exhibited high intensity GR-ir labeling neurons. In the brainstem, Nuclei of oculomotor nerves exhibited moderate intensity GR-ir labeling neurons and DRN (dorsal Raphe nucleus) exhibited low intensity GR-ir labeling neurons.

2.3.3. GR-ir neuronal cell number and subcellular GR immunoreactivity pattern

Fluorescence IHC and confocal microscopy were used to demonstrate subcellular GR immunoreactivity patterns. Figures 2.4A, 2.4B, and 2.4C demonstrate neurons with cytoplasmic

GR immunoreactivity in the telencephalon region. Figure D with E inset demonstrates neurons with both nuclear and cytoplasmic GR immunoreactivity in the metencephalon region (Cb). DAB IHC was used to quantify total number of neurons, number of neurons with both nuclear and cytoplasmic GR immunoreactivity, and number of neurons with only cytoplasmic GR immunoreactivity.

The total number of GR-ir neurons in adult HVC was significantly higher compared with P10 HVC. There were main effects of brain area ($F(2, 8) = 18.532$, $P < 0.001$) and age ($F(1, 4) = 15.236$, $P = 0.017$) in total number of GR-ir neurons in representative brain sections; though the interaction was not significant ($F(2, 8) = 4.108$, $P = 0.059$). Because previous reports (Buchanan et al., 2004; MacDonald et al., 2006; Nowicki et al., 2002) indicate that developmental stress affects size of HVC (a song control nucleus required for learning and production of song) and song complexity, a series of T-tests were performed to compare the total number of neurons in HVC, RA and TeO between P10 and adult male zebra finch. Total number of GR-ir neurons in HVC of adult/designated area was significantly higher than total number of GR-ir neurons in HVC of P10/designated area ($t(4) = -3.627$, $P = 0.022$). There were no significant differences in total number of GR-ir neurons of RA and TeO between two age groups ($t(4) = 0.440$, $P = 0.682$ and $t(4) = -1.862$, $P = 0.136$, respectively; Figure 2.5A).

The number of neurons with both nuclear and cytoplasmic GR immunoreactivity in adult HVC compared with P10 HVC was significantly higher. There was a main effect of brain area $F(2, 8) = 28.033$, $P < 0.001$) but there was not a main effect of age on number of neurons with both cytoplasmic and nuclear GR immunoreactivity ($F(1, 4) = 5.331$, $P = 0.082$); and the interaction was not significant ($F(2, 8) = 1.299$, $P = 0.325$). As determined by a T-test, the mean number of neurons with both cytoplasmic and nuclear GR immunoreactivity in HVC of adult male zebra

finch was significantly greater than the P10 HVC ($t(4) = -3.174$, $P = 0.034$). There were no significant differences in the mean number of neurons with both cytoplasmic and nuclear GR immunoreactivity in RA and TeO between two age groups ($t(4) = -1.178$, $P = 0.304$ and $t(4) = -1.634$, $P = 0.178$, respectively; Figure 2.5B).

No significant differences were observed in number of neurons with cytoplasmic GR immunoreactivity in brain areas between adult vs. P10. There was a main effect of brain area $F(2, 8) = 61.295$, $P < 0.001$) but there was not a main effect of age on the number of neurons with cytoplasmic GR immunoreactivity ($F(1, 4) = 3.378$, $P = 0.140$) and the interaction was not significant ($F(2, 8) = 4.375$, $P = 0.052$). There were no significant differences in mean number of neurons with cytoplasmic GR immunoreactivity in HVC, RA and TeO between two age groups ($t(4) = -2.314$, $P = 0.082$; $t(4) = 1.794$, $P = 0.147$; $t(4) = -1.344$, $P = 0.250$, respectively; Figure 2.5C).

The mean percentage of neurons with both nuclear and cytoplasmic GR immunoreactivity in HVC, RA, and TeO were not significantly different between the two age groups ($U = 3.000$, $Z = -0.655$, $P = 0.513$; $U = 1.000$, $Z = -1.528$, $P = 0.127$; $U = 2.000$, $Z = -1.091$, $P = 0.275$, respectively; Table 2). The mean percentage of neurons with cytoplasmic GR immunoreactivity in HVC, RA, and TeO were not significantly different between two age groups ($U = 4.000$, $Z = -0.218$, $P = 0.513$, $U = 1.000$, $Z = -1.528$, $P = 0.127$ and $U = 2.000$, $Z = -1.091$, $P = 0.275$, respectively; Table 2.2). Because the number of neurons with only nuclear GR immunoreactivity was small, no statistical analyses were performed.

2.4. Discussion

GR-ir neurons were present in zebra finch brain in regions homologous to regions of the mammalian, quail, frog, and fish brain where GR-ir neurons are distributed (Ahima and Harlan,

1990; Carruth et al., 2000; Kovacs et al., 1989; van Eekelen, 1987; Yao et al., 2008). Brain regions that contained GR-ir neurons included HP, BSTl, POM, PVN, TeO, S, LoC and others. This distribution supports previous studies of GR mRNA distribution in the European starling (*Sturnus vulgaris*) brain in which GR was widely expressed throughout the telencephalon including the hippocampus and PVH (Dickens, et al., 2009). In addition, in the SCS system GR-ir neurons were present in two of the four main song control nuclei, HVC and RA. GR-ir neurons are found in some mammalian brain regions (Ahima and Harlan, 1990) not immunoreactive in zebra finch brain (i.e. granule cells of cerebellum had low labeling in zebra finch vs. those of the mammalian cerebellum). Significance of these differences is not clear at present but may be a result of IHC methodology or functional and anatomical differences between the mammalian and avian cerebellum.

Distribution of GR-ir neurons did not vary at either age point, P10 or adult, though there were differences in staining intensity. Nucleus taeniae amygdale (in the telencephalon) and nucleus of solitary tract (in the brainstem) of P10s had higher GR-ir labeling intensity as compared with adult male brains. Nevertheless, the nucleus of the cranial nerve III (nervus oculomotorius) of P10 had lower GR-ir labeling intensity compared with that of adult brains. Significance of these differences is unknown, however, there may be differences between adult and P10 birds eye movements. In adult pigeons, the nucleus of the oculomotor nerver along with the raphe complex send differential signals to the neurons of the nucleus abducens (VI) to generate discharge patterns altering the oscillation components of horizontal saccade (Yang et al., 2008) and these movements may still be maturing in the P10 birds.

Rat hippocampal neurons, used for antibody validation, exhibited cytoplasmic GR immunoreactivity. This finding is consistent with reports that rat hippocampal CA1 neurons show GR-

ir in cytoplasmic compartment (with no nuclear GR-ir) before glucocorticoid stimulation using different antibodies (Sarabdjitsingh, et al., 2010). In our study, in most areas of the zebra finch brain, there were mainly two groups of neurons with GR immunoreactivity. One group had both cytoplasmic and nuclear GR immunoreactivity and the other group of neurons had only cytoplasmic GR immunoreactivity (i.e., HVC of P10 and adult had about 50% of each). Very few areas of zebra finch brain had predominantly nuclear staining, though one example is the POM which may highlight the interaction between the stress and reproductive axes. Wide distribution of GR-ir neurons in rat brain was reported with mostly nuclear or both nuclear and cytoplasmic staining (Ahima and Harlan, 1990). Most quail brain areas showed weak to moderate GR immunoreactivity in nuclei of neurons using a mouse monoclonal antibody (Mab49/1; Kovacs et al., 1989). Stain intensity of GR-ir neurons reported in these studies are not consistent to our study. Inconsistency in protein expression pattern in published results may be explained by a combination of the type of antibody, epitope accessibility, type of fixative, fixative delivery procedures and as well as local cellular context (Sarabdjitsingh, et al., 2010).

There were significant differences in total GR-ir neuronal numbers and number of neurons with both nuclear and cytoplasmic GR immunoreactivity between the HVC representative section of P10 and adult. These findings are supported by the reports of HVC size reduction and subsequent decrease in song complexity in developmentally stressed zebra finches (Buchanan et al., 2004; MacDonald et al., 2006; Nowicki et al., 2002) and the fact that HVC is required for song learning and production. Though, there were no significant differences in total number of GR-ir neurons and number of neurons with both nuclear and cytoplasmic GR immunoreactivity in the RA and TeO representative sections of P10 and adult. The GR-ir neurons in the TeO were counted as a control because this brain region has no direct influence on song control nuclei and

the subcellular distribution of GR-ir neurons in TeO was different than in HVC and RA. The number of neurons with cytoplasmic GR immunoreactivity was not significantly different between two age groups in the HVC, RA and TeO. This finding may be explained by the fact that nuclear staining indicates GR activity. There were no significant differences in mean percentage of neurons with both nuclear and cytoplasmic and neurons with only cytoplasmic GR immunoreactivity of P10 and adult male zebra finch. It should be taken into consideration that the total GR-ir neuronal numbers, number of neurons with both cytoplasmic and nuclear GR immunoreactivity, number of neurons with cytoplasmic GR immunoreactivity were done on a representative section of each HVC, RA and TeO and the count for each area is not actual total number of GR-ir neurons in these areas.

In conclusion, the GR-ir neurons are widely distributed throughout the zebra finch brain. Protein for GR is not only expressed in limbic and stress-related regions but also in two song control nuclei, HVC and RA. Whereas all of the diverse functions of GR in the brain are yet to be fully identified, determining the distribution of GR-ir neurons in zebra finch brain will contribute towards our understanding of the complex and adverse effects of glucocorticoids on neuronal functioning in vertebrates.

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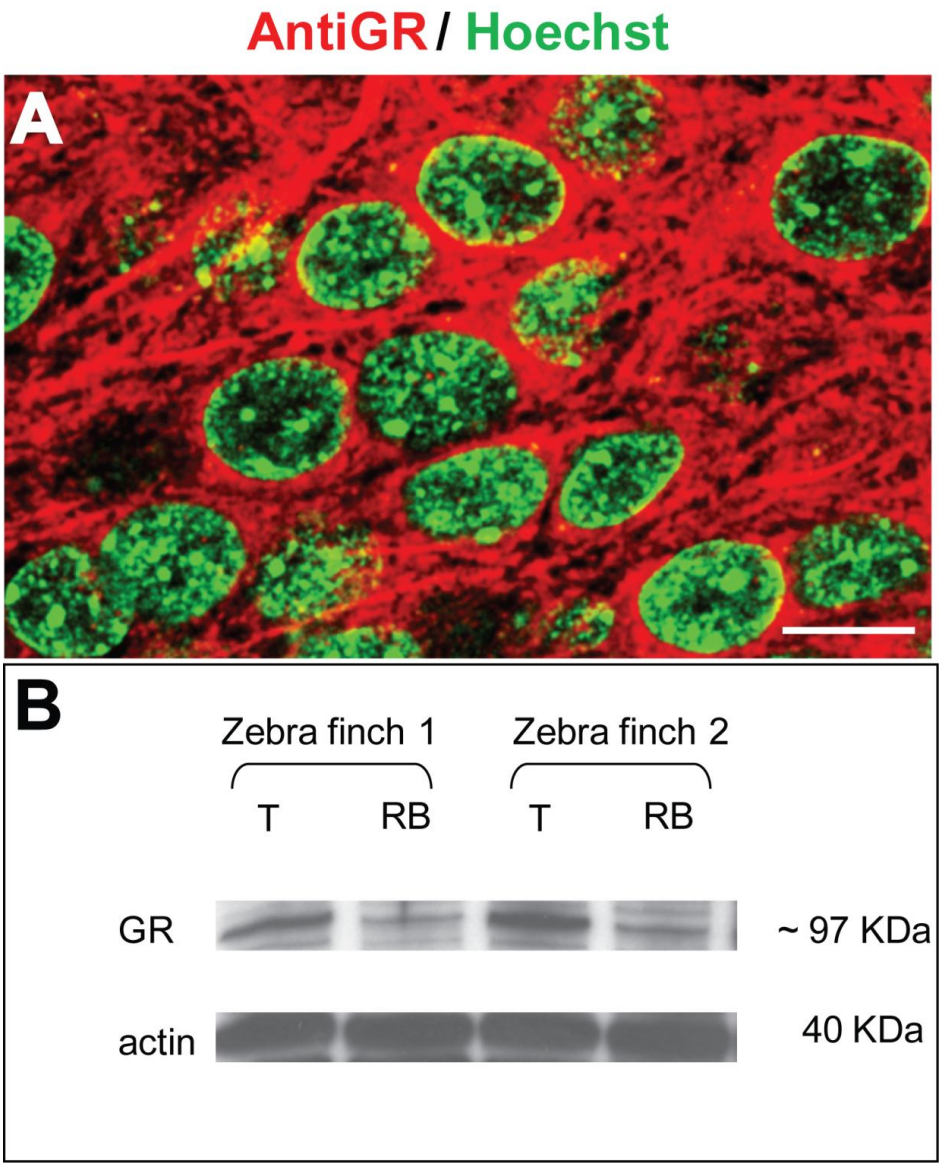

Figure 2.1. Validation of the antibody using fluorescence-IHC and WB. (**A**) A micrograph of GR-like immunoreactive neurons (GR-ir) in the hippocampus of the adult male rat. Scale bar = 10um. (**B**) The blot is representative of western blot analysis on protein extracted from telencephalon (Tel) and rest of the brain (RB, all brain tissue except for tel.) of adult male zebra finch. The primary GR antibody, PA1-510A, detects a ~97 KDa protein representing GR.

Table 2.1. Distribution of GR-like immunoreactive neurons in the brain of P10 and adult male zebra finch. Labeling intensity for GR-ir neurons in the brain of P10 and adult male zebra finch using DAB ICC. Symbols are as follows: low labeling (+), moderate labeling (++), high labeling (+++). Abbreviations for Figs. 2 through 5 are embedded in the table.

Brain region	P10	Adult
Telencephalon		
HA (Hyperpallium apicale)	+++	+++
N (Nidopallium)	+++	+++
HD (Hyperpallium densocellulare)	+++	+++
HP (Hippocampal formation)	++	++
HVC (A proper name)	++	++
RA (Robust nucleus of the arcopallium)	+	+
Tn (Nucleus taeniae amygdale)	+++	++
L (Field or area L)	+	+
Ac (Nucleus accumbens)	+	+
BSTl (Lateral part of the bed nucleus of the stria terminalis)	+++	+++
FLP (Lateral forebrain bundle, cells intermingled in FLP)	+	+
Tu (Nucleus tuberis)	++	++
Diencephalon		
POM (Nucleus preopticus medialis)	+++	+++
PVN (Nucleus paraventricularis magnocelluaris)	+++	+++
DLM (Nucleus dorsolateralis anterior thalami, pars medialis)	++	++
Tu (Tuberoinfundibular hypothalamus; homologous to Arcuate nucleus)	+++	+++
Mesencephalon (midbrain)		
Rt (Nucleus rotundus)	+++	+++
TeO (Optic tectum)	+++	+++
Metencephalon		
Cb (Cerebellum)	+++	+++
Cerebellar cortex	+++	+++
Cytoplasm of Purkinje cells	+++	+++
Stratum molecular	+++	+++
Brainstem		
LoC (Locus coeruleus)	+++	+++
DRN (Dorsal Raphe nucleus)	+	+
Nuclei of oculomotor nerves	++	+
S (Nucleus of the solitary tract)	+++	++

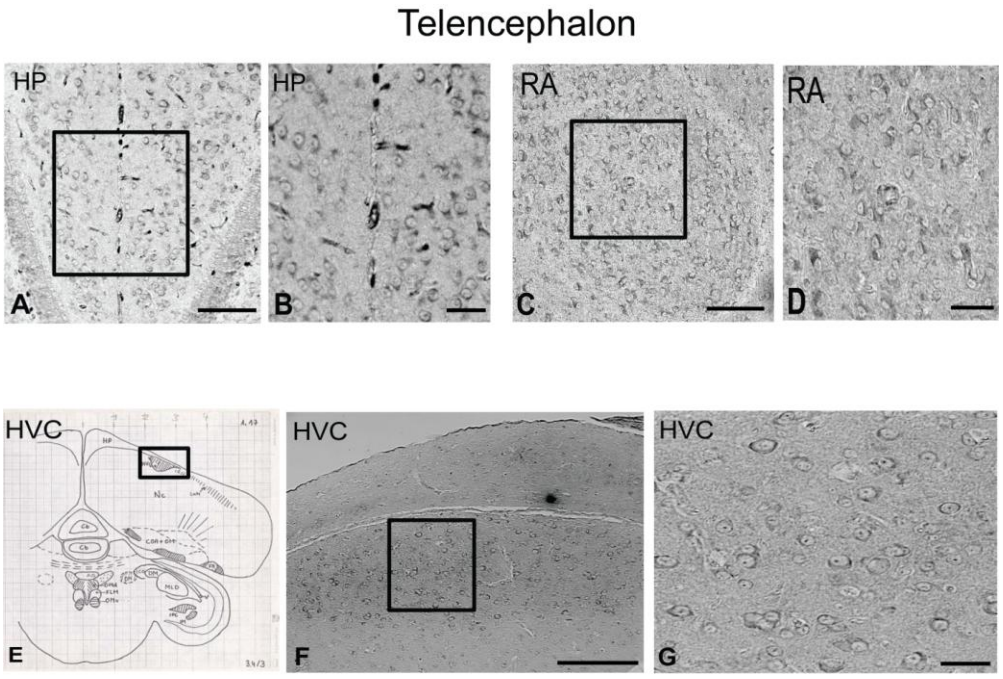

Figure 2.2. Photomicrographs of GR-ir neurons in the brain (Telencephalon) of P10 and adult male zebra finch using DAB-IHC. **A** with **B** inset and **C** with **D** inset showing GR-ir neurons in the Hippocampal formation (HF) and Robust nucleus of the arcopallium (RA) of the P10 male brain, respectively. **E** is a transverse plate 33 (Nixdorf-Bergweiler & Bischof, 2007) indicating HVC (a proper name) in the adult brain. **F** with **G** inset showing GR-ir neurons in the HVC of the adult male brain. Scale bars = 20µm in **B**, **D**, and **G**; 50µm in **A**, C; 100µm in **F**.

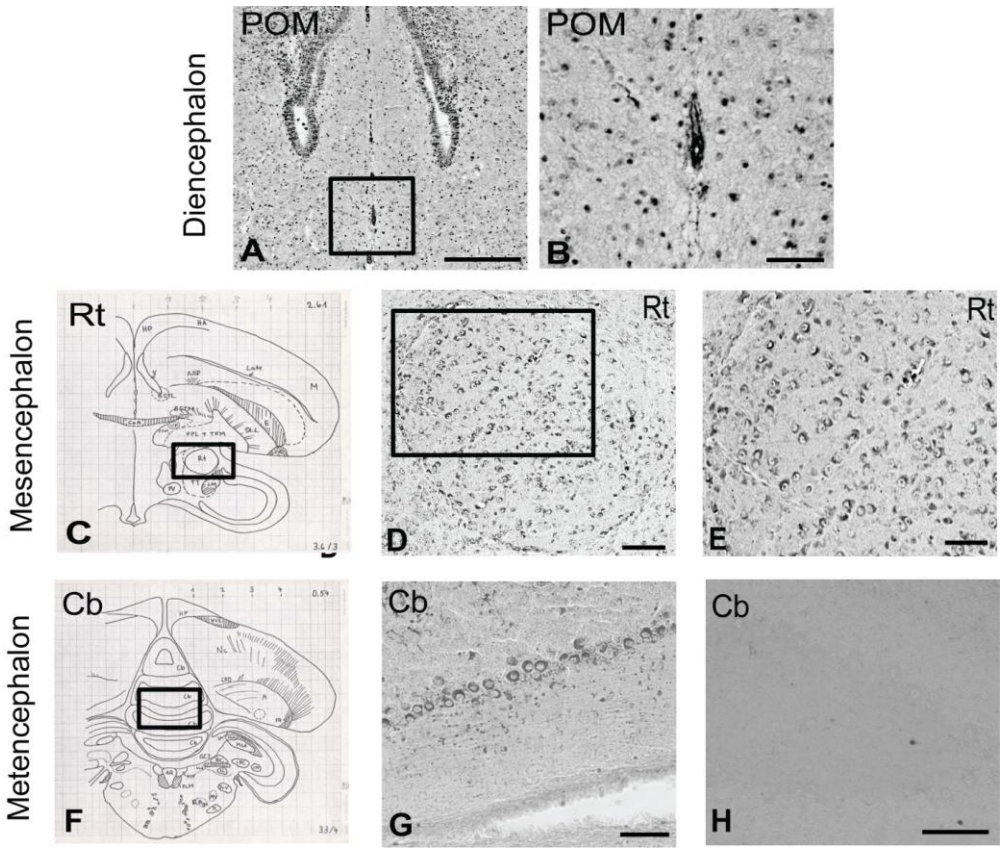

Figure 2.3. Photomicrographs of GR-ir neurons in the brain (Diencephalon, Mesencephalon and Metencephalon) of P10 and adult male zebra finch using DAB-IHC. **A** with **B** inset showing GR-ir neurons in the Nucleus preopticus medialis (POM) of the P10 male brain. **C** and **F** are transverse plates 23 and 34 (Nixdorf-Bergweiler & Bischof, 2007) indicating Nucleus rotundus (RT) and Cerebellum (Cb) in the adult brain, respectively. **D** with **E** inset showing GR-ir neurons in the RT of the adult male brain. **G** (+) showing GR-ir neurons in the Cb of the adult male brain and **H** (-) is a negative control for Cb (no primary antibody was used. Scale bars = 20µm in **B**, **E**; 50µm in **D**, **H**; 100µm in **A**, **G**.

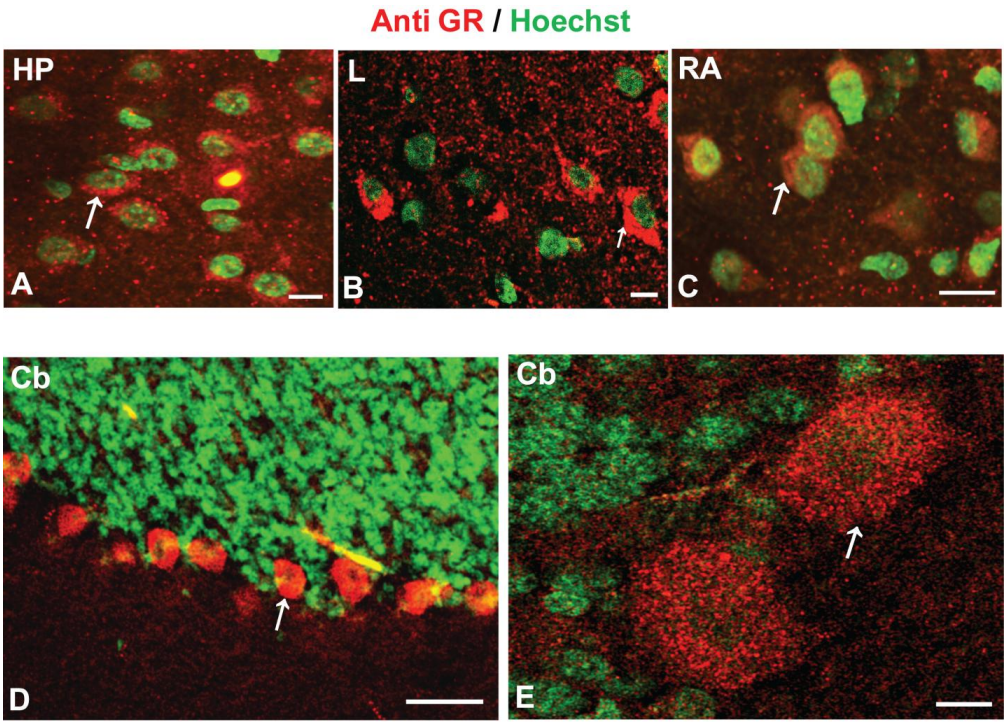

Figure 2.4. Photomicrographs of neurons with cytoplasmic GR immunoreactivity and neurons with both nuclear and cytoplasmic GR immunoreactivity in the brain of adult male zebra finch using Fluorescence-IHC. (**A**) Hippocampal formation (HP), (**B**) Area L, and (**C**) Robust nucleus of the arcopallium (RA) in the telencephalon region, demonstrating cytoplasmic GR immunoreactivity (white arrow). (**D** with **E** inset) Cerebellum (Cb) area in the metencephalon of an adult male zebra finch, demonstrating neurons with both nuclear and cytoplasmic GR immunoreactivity (white arrows). Scale bars = 10µm in **A** and **C**; 50µm in **B**.

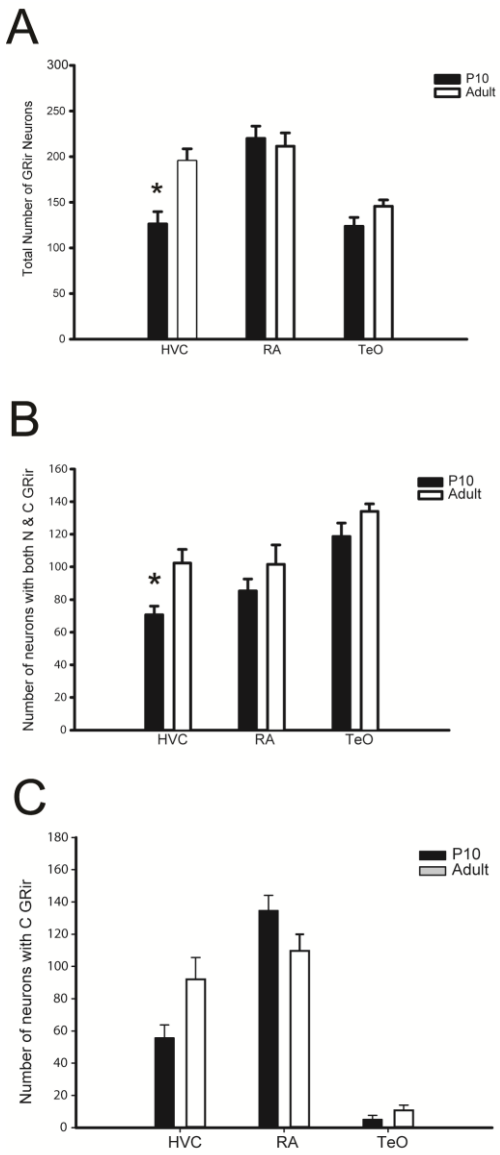

Figure 2.5. Mean ± SE GR-ir neuronal numbers in specific brain regions per representative brain section of P10 and adult male zebra finch. (**A**) Means total number of GR-ir neurons in HVC area of P10 with an asterisk (*) differ significantly from those of HVC area of adult male zebra finch at the P = 0.022 level of probability (Independent samples t-test). There were no significant differences in mean total number of GR-ir neurons in RA and TeO of P10 and adult (P = 0.682 and P = 0.136, respectively). (**B**) Mean number of neurons with both cytoplasmic (**C**) and nuclear (N) GR immunoreactivity of HVC area of P10 with an asterisk (*) differ significantly from those of HVC area of adult male zebra finch at the P = 0.034 level of probability (Independent samples t-test). There were no significant differences in means number of neurons with both cytoplasmic and nuclear GR immunoreactivity in RA and TeO of P10 and adult (P = 0.304 and P = 0.178, respectively). (**C**) Mean number of neurons with cytoplasmic GR immunoreactivity of HVC, RA and TeO areas of P10 do not differ significantly from adult male zebra finch brain areas (P = 0.082, P = 0.147, P = 0.250, respectively). Values are mean ± SEM for 3 animals per age groups. For abbreviations, see **Table 2.1**.

Table 2.2. Mean percentages of neurons with both nuclear and cytoplasmic GR immunoreactivity and neurons with cytoplasmic GR immunoreactivity in specific brain regions per representative brain section of P10 and adult male zebra finch. The mean percentages of neurons with both nuclear and cytoplasmic GR immunoreactivity and neurons with cytoplasmic GR immunoreactivity in HVC, RA, and TeO of P10 and adult male zebra finch. Data shown from neurons with both nuclear and cytoplasmic GR immunoreactivity (second column), neurons with cytoplasmic GR immunoreactivity (third column), and neurons with only nuclear GR immunoreactivity (last column) in HVC, RA and TeO of P10 and adult male zebra finch. There were no significant differences (Mann -Whitney U test; P = 0.513, P = 0.127 and P = 0.275, respectively). Values are mean ± SEM for 3 animals per age groups. For abbreviations of brain areas, see **Table 2.1.**

Brain area	% N/C	% C	% N
HVC (P10-M)	56.3 ± 1.98	43.67 ± 1.98	0
HVC (adult –M)	52.8 ± 5.18	46.6 ± 5.02	0.6 ± 0.33
RA (P10-M)	38.6 ± 2.17	61.4 ± 2.28	0
RA (adult-M)	48.0 ± 4.22	52.0 ± 4.22	0
TeO (P10-M)	96.1 ± 1.96	3.9 ± 1.96	0
TeO (adult- M)	92.0 ± 1.90	7.0 ± 1.92	1.0 ± 0

CHAPTER 3: EARLY CORTICOSTERONE TREATMENT AFFECTS SONG QUALITY AND ACCURACY OF LEARNED SONG FEATURES IN ADULT MALE ZEBRA FINCHES

This chapter is in preparation for submission*:*

Shahbazi, M., Jimenez, P., and Carruth L.L. 2012. Early Corticosterone Treatment Affects Song Quality and Accuracy of Learned song features in adult male zebra finches. In preparation for submission *to Hormones and Behavior.*

Abstract

Male zebra finches (*Taeniopygia guttata*) sing a courtship song that females cannot sing and song complexity (repertoire size) is important for mate choice. Complex songs evolved as a result of sexual selection and the quality and accuracy of learned song features can indicate male quality. Male zebra finches learn their song from their father or an adult male tutor. Early developmental stress in male zebra finches affects learned features of song in adult birds. Although the mechanisms behind this effect are unknown, it is likely that the elevated levels of corticosterone (Cort) play a significant role. We hypothesized that early Cort treatment would reduce song quality and accuracy of learned song features in adult male zebra finches. We tested this hypothesis by treating male zebra finches with Cort at age P4 (post-hatch day 4) and housing them under two different conditions (flight cages and individual breeding cages). We then quantified the effects of early Cort treatment on song quality by analyzing song of adult birds. We found that early Cort treatment decreased song similarity and resulted in poorer copies of father (tutor) song,

nevertheless the treatment did not alter mean amplitude, song duration or repertoire size. In addition, there were no significant differences in stress-related biological markers in Cort treated birds compared with control birds.

3.1. Introduction

Male zebra finches sing a courtship song that females cannot sing and females use complexity of this song (repertoire size) as an important factor for mate choice. Females of many songbird species prefer males with more complex songs (Catchpole and Slater, 1995; Searcy and Yasukawa, 1996; MacDougall-Shackelton, 1997). Complex songs evolved as a result of sexual selection (Searcy and Yasukawa, 1996) and it has been suggested that in order for complex songs to function as an indicator of male quality, they must also be costly to maintain or develop (Anderson, 1994, Spencer et al., 2003). Females typically prefer males that spend more time singing which correlates with other fitness benefits such as territory quality, food availability and increased parental care (Alatalo, et al., 1990; Greig-Smith, 1982).

According to the "Nutritional Stress Hypothesis" (Nowicki et al., 1998; Nowicki et al., 2002, Spencer et al., 2003), song complexity (previously described in chapter 1) is a valuable indicator of male quality. The neuronal pathways controlling song learning and production develop in early life and during a period of time when young birds are likely to experience nutritional stress. Developmentally food restricted male zebra finches sing a song with a shorter song motif duration and with reduced song complexity (Spencer et al., 2003). Songbirds learn their song during first few months of life whereas their song control pathway is still developing (Mooney, 1999; Nottebohm, 1999; Brainard and Doupe, 2002).

Male zebra finches learn their song from their father or an adult male tutor. Song learning starts with an auditory or sensory learning phase during which fledgling birds listen to and memorize the tutor's song (Hultsch and Todt, 2008). Memorization of song is followed by a 'subsong' or sensory-motor phase during which males produce vocalizations and practice their song. After that is "plastic song" when juvenile males refine their song to align and approximate the memorized tutor song. This stage begins with practicing the songs memorized during the sensory learning stage and continues with the production of more stereotyped vocalizations and an increase in vocal amplitude (Hultsch and Todt, 2008). The last stage is song crystallization in adulthood when song is fixed into its final form and it contains a single repeated motif with a stable number of syllables (Immelmann, 1969; Williams, 2004; Spencer et al., 2003).

Female zebra finches prefer males who sing faster rate songs (Houtman, 1992) and longer and more complex songs (Clayton and Prove, 1989; Collins, 1999). Food restricted male zebra finches had reduced song complexity (songs with reduced number of syllables and shorter duration; Spencer et al., 2003). Although the exact mechanisms behind this effect are unknown, it is likely that the elevated levels of corticosterone (Cort) and/or changes in the sensitivity of glucocorticoid receptors play a significant role. Exposure to chronic stress or elevated Cort in animals (particularly primates) results in hippocampal atrophy and cognitive dysfunction and glucocorticoid receptors mediate the adverse effects of glucocorticoids (such as Cort and cortisol) on hippocampal function during prolonged stress (Ferguson and Sapolsky, 2007; Krugers et al., 2006).

Therefore, the zebra finch is a powerful model species to study the possible mechanisms underlying the effects of early chronic stress on song quality and accuracy. Cort may play a potential role in mediating the effects of early chronic stress on the neuronal development required for learning and production of song. Although food restricted zebra finches had no elevation in

plasma Cort concentration, both food-restricted and Cort-fed birds had shorter songs (Spencer et al., 2003). By contrast the food-restricted song sparrows had elevated baseline Cort (MacDonald et al., 2006; Kempster et al., 2007). These findings suggest that Cort may mediate the effects of early stress, although Cort is only one of a several hormones released during stress response.

We hypothesized that early Cort treatment reduces song quality and accuracy of learned song features in adult male zebra finches. We tested the potential role of Cort in reduction of song quality and accuracy by using Cort implants in male zebra finches at age P4±1 in two different housing conditions and then quantified the effects of early Cort treatment on song quality by analyzing song of adult birds.

3.2. Methods

3.2.1. Animals

We used male zebra finches from our breeding colony at Georgia State University. The Georgia State University Institutional Use and Animal Care Committee granted approval for all animal procedures. Male birds age P4±1 (4 days post-hatch) and adult (>90) were used. Sex of birds was determined via PCR following the protocol of Soderstrom et al., 2007 and by visual examination of the testes at the time of tissue collection for P30 (30 days post-hatch) birds. Subjects were housed in nest boxes (with nesting material) in individual breeding cages (IBC; 16″Lx 22″W x16″H) with their parents and nestlings or in flight cages (FC; 60″L x 30″W x 72.5″H) with multiple families and mixed age cohorts. Air temperature was held constant at 20-22°C and relative humidity maintained at 30-50 %. The light cycle was 12:12 h light / dark. The cages were sprayed with water once a day every day to imitate rainfall. As opportunistic breeders zebra finches nest during rainy periods. The birds were fed *ad libitum* diet of standard finch millet

seed. In addition, they were given a mixture of fresh greens (spinach) and a protein supplement (boiled eggs) once a week. Fresh drinking water (changed daily), oyster shell grit and cuttlebone were provided.

Cort implants were made of Dow Corning silastic tubing (0.76 mm ID x 1.19 mm OD) in three lengths (5mm, 10 mm and 15 mm) and packed with crystalline Cort (catalog C2505-500MG, Sigma-Aldrich, St. Louis, MO) and sealed at the two ends with silicone paste. For Cort treatment, the effective lengths of 10-15mm have been used in adult songbirds to elevate Cort concentration moderately above baseline level (Astheimer et al., 2000; Breuner and Hahn, 2003; Martin et al., 2005; Newman et al., 2010). We used an effective length of 10mm for adult zebra finches and 5mm for P4±1 zebra finches. We used 5mm, 10mm, and 15mm Cort implants for validation of Cort implants in saline. In previous studies, Cort release from silastic implants was facilitated by making a small hole in the implant (Silverin, 1998; Astheimer et al., 2000; Newman et al., 2010). Using a 26-gauge needle a single hole was made near the end of the tubing (but not through the silicone plug) in each Cort implant used for P4±1 and adult birds in our study. The implants used for validation of Cort release in saline were identical except they lacked the hole.

3.2.2. Validation of Cort implants in vitro

The first experiment was performed to validate Cort implants *in vitro*. To investigate the release of Cort from silastic implants over a three-week period, we incubated the three different sized implants in 2ml saline (0.75%) over three weeks at room temperature. The implants included 5mm empty (control), 5 mm Cort, 10 mm Cort, and 15 mm Cort in four different testing tubes. At the end of week one, week two, and week three, an aliquot of 200µl sample from each

test tube was transferred into a fresh tube for Cort measurements (four samples/each implant). To compensate for the removal of the aliquot, 200µl fresh saline was added back to each tube containing the implants each time we removed a 200µl sample. At the end of 3 weeks, we had 3 samples (or aliquots) from each of the implants. Samples from the first, second, and third week were used for Cort measurement. Samples were stored at -20°C until Cort extraction (from saline) and Cort measurement. Similar *in vitro* experiments of validation of Cort implants were performed previously (Newman et al., 2010).

3.2.3. Validation of Cort implants in vivo

The second experiment was performed to validate Cort implants *in vivo*. Adult male zebra finches were implanted subcutaneously above flight muscle (or along the chest) with either 10 mm Cort (n = 5) or 10 mm empty (n = 4) implants. Two days before implantation (day-2), baseline blood samples were collected. Blood samples were also collected on day 1 (one day after implantation), day 3 (three days after implantation) and day 7 (seven days after implantation). Blood samples were collected from the alar wing vein by puncturing the vein using a 26-gauge needle and blood was then collected into heparinized microhematocrit tubes. Blood collection occurred within 3 minutes of capture at the same time each day (around 11am) for each bird. Blood samples were kept on ice until centrifuged. Then, after centrifuging blood, plasma was collected and stored at -20◦C until analysis. One of the birds from Cort treated group died on day 3 after implantation and the samples were not used for the analysis.

3.2.4. Plasma Cort concentration in early Cort treated juvenile birds

Male zebra finches at age P4±1 were divided into 3 groups. Group 1 and 2 received subcutaneous implants (above flight muscle) of either 5mm Cort (n = 6) or empty implants (n = 3), respectively. Group 3 (n = 3) did not receive an implant and were raised normally. After an overdose of Isoflurane (an inhalational anesthetic), the birds were decapitated as juveniles (at age P30±1) and their brains collected for Nissl stain (See Chapter 4) and trunk blood collected for measuring plasma Cort concentration as described above.

3.2.5. Hormone assays

Radioimmunoassay (RIA) was used to measure plasma Cort concentration in juvenile and adult birds. RIA was also used to measure Cort concentration in saline. Saline samples containing Cort were first subjected to an extraction protocol (Newman et al., 2009) prior to conducting RIA. Corticosterone ^{125}I RIA kit (catalog # 07 – 120102; MP Biomedicals LLC, Solon, OH) was used to measure plasma and saline Cort concentrations. Not only did we validate the use of this kit for measuring plasma Cort concentration in zebra finches, but the kit has been used in previous studies in zebra finches as well (Newman et al., 2010). Recovery rate was 98% and detection limit was 6.25-1000 ng / ml. Intra-assay & inter-assay coefficient variations were 4.4% and 14.6%, respectively. Saline samples collected at the end of 3 weeks had very high Cort levels and further dilution was needed, though for economic reasons, we only included the samples from week one and week two.

3.2.6. Song recording and analysis

Male zebra finches at age P4±1were divided into three groups. Group 1, total n = 17 including n = 8 from flight cages (FC; each FC contains 40-50 mixed-sex and mix-aged birds) and n = 9 from individual breeding cages (IBC; contains a male-female pair with a single or double clutch of offspring), received subcutaneous crystalline Cort implants (5 mm) above the flight muscle. Group 2 (total n = 8 including n = 4 FC and n = 4 for IBC), were control for Group 1 and received empty implants. Group 3 (n = 8 including n = 2 FC and n = 6 for IBC) were also control (for the treatment) and received no implants. Birds were then raised in the two different housing conditions, FCs or IBCs. By the time the birds reached adulthood (P>90), 3 birds had died (1 Cort-implanted and 2 empty-implanted birds), therefore, the final analysis involved n = 7 Cort treated male birds in FCs and 9 in IBC, and n = 3 empty implanted birds each for FC and IBC. Songs of early Cort treated and control male birds were recorded in adulthood between 150 and 210 days of age in a sound attenuated room using Raven lite 1.0 via a microphone (Dynex USB microphone) and a laptop computer (Dell Inspiron). Directed songs (song sung toward mate as part of courtship) of each bird were recorded for at least 3-5 times for 15 min on different days. Song was recorded as soon as a female was introduced. The recorded songs were analyzed blind using Sound Pro Analysis (song from different days for each bird were compared to make sure that they sing the same directed song on different days) and then two random songs from two different days were analyzed for each bird. The recorded song (motif) was selected and noise reduced using GoldWave software (GoldWave, Inc., St. John's, NL, Canada). Then, the song of each bird (copy) was compared to the father song (model or template) and analyzed via automated procedures (Sound Analysis Prousing asymmetric comparison; Tchernnichovski et al., 2000). Song parameters including song similarity, accuracy, sequential match, total score, duration, av-

erage pitch, mean amplitude and mean frequency were analyzed. Total number of syllables and number of syllable types in the motif (each analyzed song) were determined for each bird. Song similarity is the percentage of tutor's song included in final sections (tutee's song). Song accuracy is the average local (for each syllable) similarity across final sections. Sequential match is sorting the final sections according to their temporal order in reference to tutor's song. Total score is the global similarity score that takes both percent similarity and accuracy into consideration (the product of song accuracy and percent similarity; Tchernnichovski et al., 2000).

3.2.7. Assessing the biological markers in Cort treated and control birds

In order to determine whether the Cort treatment affected overall body condition, three biological makers for stress were measured: metatarsal (tarsus) leg bone length, body weight, and body condition. Wing length was also measured in adults. All juvenile birds from each treatment groups (n = 5 from Cort and No-Cort groups) were weighed at day 30 ± 1 and the length of their tarsi were measured. The average of left and right tarsus was used in the statistical analysis. All adult male birds from different treatment groups (n=16 Cort and n=14 No-Cort groups) were weighted at day 210 (after recording their song) and the length of their tarsi and wings were measured. The average of left and right values for both tarsi and wing was used in the statistical analysis. To obtain body condition for juvenile and adult birds, body weight was divided by tarsus length. Body condition is a relative weight at a given size which is an indication of individual fitness.

3.2.8. Statistical analysis

All data were analyzed using IBM SPSS Statistics for Windows, version 19.0 (SPSS Inc, Chicago, IL). First, data were examined for assumptions of parametric statistical tests. When assumptions were violated, a non-parametric alternative was used. Statistical significance was accepted at $P < 0.05$. Independent Samples t-tests were used to compare plasma Cort concentration between empty and no implanted birds. Mann-Whitney U tests were used to compare the plasma Cort concentration between Cort treated and control (No-Cort treated) P30 birds. One-Way ANOVA was used to compare plasma Cort concentration on different days after treatment in adult birds. Tukey tests were used as Post-hoc tests as needed. Mann-Whitney U tests were used to compare stress-related biological markers between Cort treated and control birds. In addition Mann-Whitney U tests were used to compare song parameters between Cort and control birds. Pearson correlation test was used to obtain the correlation between plasma Cort concentration and body condition in juvenile birds.

3.3. Results

3.3.1. Validation of Cort implants in vitro

Cort was released in physiological saline from 5mm, 10mm and 15 mm silastic implants during a two-week validation period. The saline Cort concentration was very high at the end of three weeks and further dilution was needed for measurement. At the end of 3 weeks incubation, all implants in saline still contained Cort. Table 3.1 demonstrates Cort concentration released in saline during two weeks.

3.3.2. Validation of Cort implants in vivo

There was a significant difference in plasma Cort concentration in different days after implantation in Cort-treated birds ($F(3, 14) = 5.859$, $P = 0.008$; Figure 3.1). To find out which groups differed, post-hoc analysis (Tukey) was used. Cort implanted birds had significantly higher plasma Cort concentration on day1 (one day after surgery) compared with day -2 (two days before surgery) and day 7 (seven days after surgery; $P = 0.032$, $P = 0.011$, respectively; Figure 3.1). There were no significant differences in plasma Cort Concentration between day 1 and day 3 (three days after surgery; $P = 0.670$; Figure 3.1) in Cort implanted birds. No significant differences were observed in plasma Cort concentration between day 3 with day -2, day 1, and day 7 ($P = 0.307$, $P = 0.670$, $P = 0.116$, respectively; Figure 3.1). There was no significant difference in plasma Cort concentration between day -2 and day 7 ($P = 0.875$; Figure 3.1). Our results are consistent with those of other researchers using silastic implants in song sparrows (Newman et al., 2010). Previous studies using silastic Cort implants in adult song sparrows and Black-legged kittiwakes indicated that plasma Cort levels were higher on day1 and day 2 after implantation and plasma Cort levels were not significantly higher on subsequent days (Newman et al., 2010; Angelier et al., 2007). In our study, plasma Cort levels were high on day1 and maybe day 2 after implantation as well.

There were no significant differences in plasma Cort concentration in different days after implantation in adult birds that received empty implants. There were no significant differences in day -2, day 1, day3, and day 7 for empty-implanted birds ($F(3, 12) = 0.694$, $P = 0.573$; Figure 3.1).

3.3.3. Plasma Cort concentration in early Cort treated juvenile birds

Blood Cort concentration was measured to validate the implants using RIA. We found that Cort-treated juvenile male zebra finches had significantly lower plasma Cort than control (No-Cort treated) birds. Figure 3.2 demonstrates plasma Cort concentration in Cort treated juvenile male zebra finches. There was a significant difference between Cort (n = 6) vs. control (n = 6) juvenile birds (U = 3.000, Z = -2.191, P = 0.028). There was no significant difference between empty (n = 3) and no-implanted (n = 3) juvenile birds (t (4) = 0.269, P = 0.801), therefore, both groups were combined into one control (No-Cort treated) group.

3.3.4. The effects of early Cort treatment on stress-related biological markers in juvenile birds

There were no significant differences in the stress-related biological markers between Cort and control juvenile birds. There were no significant differences in body weight, tarsus length, and body condition between Cort (n = 5) and control (n = 5) juvenile birds (U = 12.000, Z = -0.104, P = 0.918; U = 7.000, Z = -1.149, P = 0.252; U = 12.000, Z = -0.104, P = 0.918, respectively; Figure 3.3; Table 3.2).

3.3.5. Correlation between plasma Cort concentration and body condition

There was a significant correlation between plasma Cort concentration and body condition in juvenile male zebra finches regardless of treatment group (r = - 0.660, P = 0.038; Figure 3.4). Juvenile male birds with higher plasma Cort concentration had lower body condition.

3.3.6. The effects of early Cort treatment on song parameters

We found that Cort treated adult male birds had significantly lower song similarity and total score than control birds. Figure 3.15 (A-C) demonstrates example sonograms from control (No-Cort treated birds; Figure 3.15A) and Cort treated birds (Figures 3.15B and 3.15C) from FCs. Cort treated adult male birds from FCs (n = 7) had significantly lower song similarity and total score than control (No-Cort; combined empty and no implants; n = 14) adult birds (U = 18.500, Z = -2.283, P = 0.022;; U = 13.000, Z = -2.686, P = 0.008; respectively; Figure 3.5). There were no significant differences in song accuracy and song sequential match between Cort treated birds from FCs and control birds (U= 24.000, Z= -1.866, P= 0.062; U= 34.000, Z= -1.121, P=0.262; accordingly; Figure 3.5).

There were no significant differences in song similarity, accuracy, sequential match, and total score between empty and no implanted birds (U = 23.000, Z = -0.130, P = 0.976; U = 14.000, Z = -1.292, P = 0.196; U = 18.500, Z = -0.711, P = 0.478; U = 16.000, Z = -1.033, P = 0.302; respectively), therefore, they were combined as one No-Cort group (control).

In the IBCs, there were no significant differences in song similarity, accuracy, sequential match, and total score between Cort treated (n = 9) and control (n = 14) birds (U = 61.000, Z = -0.126, P = 0.900; U = 56.500, Z = -0.410, P = 0.682; U = 56.500, Z = -0.412, P = 0.682; U = 61.000, Z= -0.126, P = 0.900; respectively; Figure 3.6). Figures 3.16A and 3.16B demonstrate example sonograms from control and Cort treated birds from IBCs, respectively.

We found that Cort treated birds in IBC #2 had significantly lower song similarity, song accuracy, and total score compared with control adult birds. This breeding pair had a clutch of 7 whereas the other ICBs averaged 2-3 nestlings. These results are consistent with the results from the FCs (see above). Figures 3.17A and 3.17B demonstrate example sonograms from control and

Cort treated birds from IBC #2. Cort treated birds (n = 3) in IBC #2 had lower similarity, accuracy and total score in comparison with control (siblings; n = 4 including n = 1 empty and n = 3 no implanted) birds (U = 0.000, Z = -2.141, P = 0.032; U = 0.000, Z = -2.121, P = 0.034; U = 0.000, Z = -2.121, P = 0.034, respectively; Figure 3.7). As was the case for birds from the FCs, song sequential match was not different between Cort and control birds from IBC #2 (U = 5.000, Z = -0.367, P = 0.714; Figure 3.7).

There were no significant differences in duration, average pitch, mean amplitude, and mean frequency of song between Cort treated and control birds from FCs (U = 44.000, Z = -0.373, P = 0.710; U = 35.000, Z = -1.044, P = 0.296; U = 27.000, Z = -1.641, P = 0.102; U = 26.000, Z = -1.716, P = 0.086; Figure 3.8A-D, respectively).

We found that Cort treated birds in IBCs had higher average pitch and mean frequency than control birds (U = 34.000, Z = -2.738, P = 0.006; U = 45.00, Z = -2.241, P = 0.026; Figures 3.9B and 3.9D, respectively). There were no significant differences in duration and mean amplitude between Cort treated and control birds from IBCs (U = 79.000, Z = -0.702, P = 0.484; U = 61.000, Z = -1.516, P = 0.130; Figures 3.9A and 3.9C, accordingly).

We found that Cort (n=3) treated birds in IBC #2 (similar to all other IBCs) had higher average pitch than control (n=4) siblings (U = 0.000, Z = -2.121, P = 0.034; Figure 3.10B). There were no significant differences in duration, mean amplitude, and mean frequency between Cort treated and control birds from IBC #2 (U = 2.000, Z = -1.414, P = 0.158; U = 4.000, Z = -0.707, P = 0.480; U = 5.000, Z = -0.354, P = 0.724; Figures 3.10A, 3.10C, and 3.10D, accordingly).

There were no significant differences in the total number of syllables and number of syllable types between Cort treated and control birds from FCs (U = 31.000, Z = -1.354, P = 0.176; U = 30.000, Z = -1.432, P = 0.152; respectively; Figures 3.11A and 3.11B, respectively).

We found that the total number of syllables was significantly higher in Cort treated birds from all IBCs and IBC#2 than control birds (U = 44.000, Z = -2.308, P = 0.022; U = 0.000, Z = -2.181, P = 0.030; Figures 3.12A and 3.13A, respectively). Though, there were no significant differences in the number of syllable types between Cort treated and control birds from all IBCs and IBC #2 (U = 33.500, Z = -1.877, P = 0.062; U = 1.000, Z = -1.834, P = 0.068; Figures 3.12B and 3.13B, accordingly).

3.3.7. The effects of early Cort treatment on stress-related biological markers in adult birds

There were no significant differences in stress-related biological markers including body weight, tarsus length, wing length, and body condition between Cort treated (n = 16) and control (n = 14) adults (all birds from both FCs and IBCs; U = 67.000, Z = -1.871, P = 0.062; U = 75.000, Z = -1.538, P = 0.124; U = 70.000, Z = -1.755, P = 0.080; U = 87.000, Z = -1.039, P = 0.300; Figure 3.14A-D, respectively; see Table 3.2).

There were no significant differences in body weight, tarsus length, wing length, and body condition between empty implanted birds (n = 6) and no-implanted birds (n = 8), therefore they were merged into one group as control (No-Cort treated) birds (U = 21.000, Z = -0.387, P = 0.698; U = 18.000, Z = -0.775, P = 0.440; U = 13.000, Z = -1.436, P = 0.152; U = 22.000, Z = -0.258, P = 0.796, respectively).

There were no significant differences in the body weight, tarsus length, wing length and body condition between empty implanted birds from FCs (n = 3) and empty implanted birds from IBCs (n = 3), therefore, they were combined as one empty implanted group (n = 6; U = 3.000, Z = -0.655, P = 0.514; U = 2.000, Z = -1.091, P = 0.276; U = 3.500, Z = -0.449, P = 0.654; U = 4.000, Z = -0.218, P = 0.828; respectively). Because there were only two no-

implanted birds from FCs, they were combined with no-implanted birds from IBCs as one no-implanted group (n = 8).

There were no significant differences in body weight, tarsus length, wing length, and body condition between empty (n = 6) and no-implanted (n = 8) adult male birds (U = 21.000, Z = -0.387, P = 0.700; U = 18.000, Z = -0.775, P = 0.440; U = 13.000, Z = -1.436, P = 0.152; U = 22.000, Z = -0.258, P = 0.796; respectively), therefore, they were combined as one control group (n = 14).

There were no significant differences in body weight, tarsus length, wing length, and body condition between Cort treated birds from FCs (n = 7) and Cort treated birds from IBCs (n = 9), therefore, they were combined as one Cort treated group (n = 16; U = 24.000, Z = -0.794, P = 0.428; U = 16.000, Z = -1.641, P = 0.102; U = 25.000, Z = -0.694, P = 0.488; U = 29.000, Z = -0.265, P = 0.792; respectively).

3.4. Discussion

Our study showed that Cort-filled silastic implants continued to release hormone over the course of 21 days *in vitro.* Cort concentration in saline was very high at the end of three weeks, and further dilution was needed to measure hormone levels. Further, all Cort implants still contained Cort at the end of three weeks in saline. Similar *in vitro* validation of Cort implants has been demonstrated previously as well (Newman et al., 2010).

Our results, from *in vivo* experiments with adult birds, showed that the baseline plasma Cort level were significantly higher in the Cort group than in the control group on day 1 (one day after implantion). Baseline Cort levels were not significantly different on subsequent days (on day 3 plasma Cort concentration start declining and by day7, plasma Cort concentration was as

low as controls. This phenomenon was unexpected but it has been observed with previous studies (Newman et al., 2010; Angelier et al., 2007; Herrmann et al., 2009; Meyer et al., 1979), indicating increased plasma Cort concentration only on days 1, 2, and 3 after implantation. Plasma Cort concentration started declining after day 3 and still remained low on day 21 and day 28 after Cort implantation (Newman et al., 2010).

In order to study the effects of early Cort treatment on the development of the avian song control system, male zebra finches were implanted at P4±1 and their plasma Cort concentration was measured at 4 weeks later (as juveniles around P30; 30 days after hatch). Plasma Cort concentration was suppressed in Cort-treated birds. Our previous findings in adult birds and the findings of others (Newman et al., 2010) suggest that Cort-filled silastic implants inserted in zebra finches on P4 (4 days after hatch) release Cort for one day (day 5 after hatch) at least if not more during the critical period of song nuclei formation (which is around P1-P10). Furthermore, we observed a main effect of Cort exposure on adult behavior (song similarity score). It seems that Cort exposure (at least for one day) during the critical period is sufficient to significantly reduce song similarity in Cort-treated birds compared with control birds. Likewise, other studies with Cort-filled silastic implants have shown that Cort increases neurodegeneration of song nuclei in song sparrows (Newman et al., 2010), reduces osteocalcin, and induces bone loss in mice (Herrmann et al., 2009). In these reports, baseline plasma Cort level was significantly higher in the Cort group on day 1 and 2 (only for few days) after implant insertions compared with the control group.

Our findings and others do not explain the temporal dynamic of Cort release (or exposure) from Cort-filled silastic implants at this point and these concerns have yet to be resolved. We are not sure if Cort exposure occurs for only one day, two days, one week or more. It is pos-

sible that Cort-implants did not stop releasing Cort, but that Cort was metabolized and excreted (increased clearance) through the kidney or accumulated in other tissues. Furthermore, maybe endogenous Cort secretion was suppressed by the Cort implant. It is possible that *in vivo*, the Cort implants stopped releasing Cort as a result of encapsulation and isolation from blood vessels. This is unlikely, however, because when song sparrows received Cort, DHEA or both implants adjacent to each other plasma DHEA levels were elevated throughout the treatment period (28 days) whereas Cort levels peaked early and then dropped. The DHEA implants were not encapsulated in any of the birds based on high plasma DHEA level throughout the treatment. Therefore, increased Cort level may not be a central index for chronic stress and other phenomena such as context - related behavior, metabolites excretion rate, gonadal steroid levels may be better indexes for chronic stress and should be considered in future studies.

Early Cort treatment did not affect stress-related biological markers such as body weight, tarsus length, and body condition in juvenile male zebra finches. We did not assess body weight and tarsus length earlier and therefore it is possible that early Cort affected body weight and tarsus length at week 1 or 2 and the birds then recovered. Nevertheless, we found that in juvenile birds regardless of treatment, plasma Cort concentration was negatively correlated with body condition. Early Cort treatment had no significant effects on body weight, tarsus length, wing length, and body condition in adult male birds at age 210 days. Our result that Cort did not affect stress-related biological markers in adult birds is consistent with reports that food restriction has no physical effects by day 200 (Brumm et al., 2009). Although, these authors found a decrease in wing length at age 100 days, however these birds appeared to catch up with the control birds by day 200. The effects of early Cort on stress-related biological markers in birds at age 100 days were not demonstrated in our study.

The effects of early development stress including food restriction (Brumm et al. 2009; Spencer et al., 2003; Zann and Cash, 2008) and manipulating brood size (Gil et al., 2006; Holveck et al., 2008) on song quality in male zebra finches have been investigated and there is inconsistent evidence on which aspect of song parameters are affected by early developmental stress. We found that early Cort treatment contributed to lower similarity and total score between tutor (father) and tutee (son) in adult male zebra finches from FCs and IBC #2. This is consistent with previous studies (Brumm et al., 2009) indicating lower song similarity scores in early nutritional stressed birds. Early Cort treatment on males from IBC #2 resulted in lower accuracy score in addition to lower song similarity score between tutor and tutee. This finding is consistent with previous studies demonstrating the effects of early food restriction or brood size manipulation on song features in zebra finches (Brumm et al., 2009 and Holveck et al., 2008).

Early Cort treatment did not alter song similarity and total score between tutor and tutee in adult male birds from all the IBCs. This different result may be explained by the fact that other variables such as the tutor song rate, brood size and density of the housing conditions may play a role in song learning of the fledgling birds resulting in individual differences in song learn and/or even motivation to learn. Each FC contained about 40-50 mixed-sex and mix-aged birds and IBC #2 contained a pair-bond with seven offspring, whereas the other IBCs contained a pair-bond with an average of 3 offspring. We did not control for the tutor song rate.

Furthermore, we found that early Cort treatment resulted in higher average pitch in adult male birds from all IBCs including IBC #2. Birds from IBCs (not IBC #2) also had higher mean frequency. Nevertheless, early Cort treatment did not alter average pitch and mean frequency in adult male birds from FCs. High average pitch of birds in IBCs may indicate more noisy song which may be a sign of poorer copies of tutor song or less structured song (Feher et al., 2009).

There were no effects of early Cort treatment on duration of song in our birds. This is consistent with other studies reporting on no effects of early food-restriction or brood size manipulation on song duration in zebra finches (Brumm et al., 2009; Holveck et al., 2008; Gill et al., 2006). Though, there are some inconsistent findings reporting either decreased or increased song duration in food restricted birds (Spencer et al., 2003; Zann and Cash, 2008; respectively).

There was no evidence of Cort effect on song amplitude (in terms of song performance) similar to previous study in zebra finches (Brumm et al., 2009). Nevertheless, several other studies showed that the stressed birds had different song amplitude (Brumm and Hultsch 2001; Brumm and Todt 2002; Brumm and Slater 2006; Brumm 2009). Based on our results, mean amplitude may not be an indicator of early Cort treatment in adult male zebra finches.

In addition, we found that early Cort treatment resulted in an increase in the total number of syllables in adult bird song in all IBCs. These results are not consistent with other studies which report no effect of food restriction (Zann and Cash, 2008; Brumm et al., 2009) or brood size manipulation (Gill et al., 2006; Holveck et al., 2008) on total number of song syllables. Though, early Cort treatment did not affect the total number of syllables or number of syllable types in adult birds from FCs and this finding is consistent with the studies mentioned above. On the other hand, only one study reported that early food restriction in zebra finches decreased total number of syllables in adult males (Spencer et al., 2003). The different results from IBCs in our study may be explained by the differences in the experimental conditions including food restriction and manipulation of brood size as well individual differences.

Research indicates that early developmental stress alters song, however the song parameters affected by developmental stress vary from study to study. Consistent with the majority of studies on early developmental stress in zebra finches, song complexity measured as song dura-

tion and / or number of syllable types was not altered by early Cort treatment. These findings confirm that Cort may mediate the adverse effects of developmental stress (such as food restriction and brood size manipulation) on song learning accuracy (or song similarity) but not song complexity. We found that early Cort treatment affects song similarity and results in poorer copies of the father's (tutor) song. An increased average pitch and total number of syllables in early Cort treated birds from all IBCs may contribute to low quality song compared with the tutor song. Early Cort treatment did not alter mean amplitude or song duration. How female zebra finches detect these changes in quality and accuracy of learned song features and whether they use this information to evaluate the early condition of a male remains to be understood.

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3.6. References

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Table 3.1. Validation of corticosterone (Cort) implants *in vitro*
To investigate the release of Cort from silastic implants over a three-week period, the three different sized implants were incubated in 2ml saline (0.75%) over three weeks. The implants included 5mm empty (control), 5 mm Cort, 10 mm Cort, and 15 mm Cort in four different testing tubes. At the end of week one, week two, and week three, an aliquot of 200µl sample from each test tube was transferred into a fresh tube for Cort measurements. Cort was extracted from saline and measured. Cort was released in physiological saline from 5mm, 10mm and 15 mm silastic implants during two week validation period. The saline Cort concentration was very high at the end of three weeks and further dilution was needed for measurement. The data from week 3 was not included in the table.

Silastic implants	Saline Cort concentration (ng / ml) after one week	Saline Cort concentration (ng / ml) after two weeks
5mm (empty)	113	154
5 mm (Cort)	74	148
10 mm (Cort)	83.9	300
15 mm (Cort)	199	289

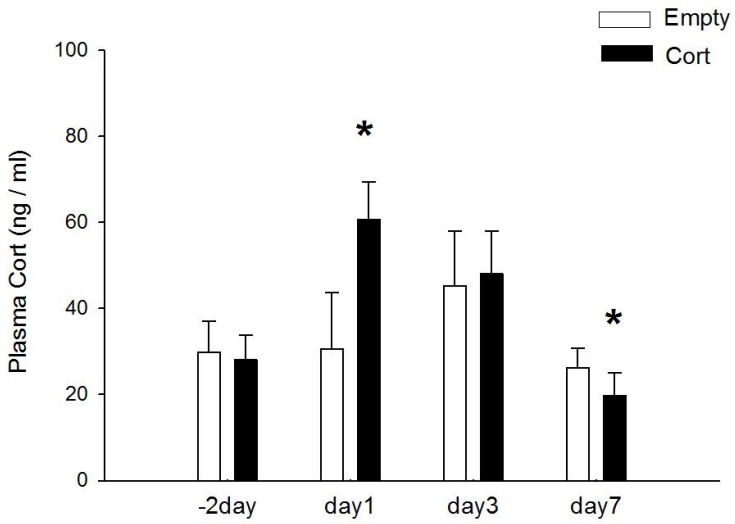

Figure 3.1. Validation of Cort implants *in vivo.* Adult male zebra finches were implanted with either 10 mm Cort (n = 5) or 10 mm empty (n = 4) implants. Two days before implantation (day -2), baseline blood samples were collected. Blood samples were also collected on day 1, 3, and 7 after implantation. In Cort implanted birds (black bars), plasma Cort concentration was significantly higher on day 1 than day -2 and 7 (*P = 0.032, *P = 0.011, respectively). Plasma Cort concentrations in adult birds that received empty implants (white bars) did not differ at any time point. Data represent mean values ± SEM. Asterisks highlight significant differences between groups (*P < 0.05).

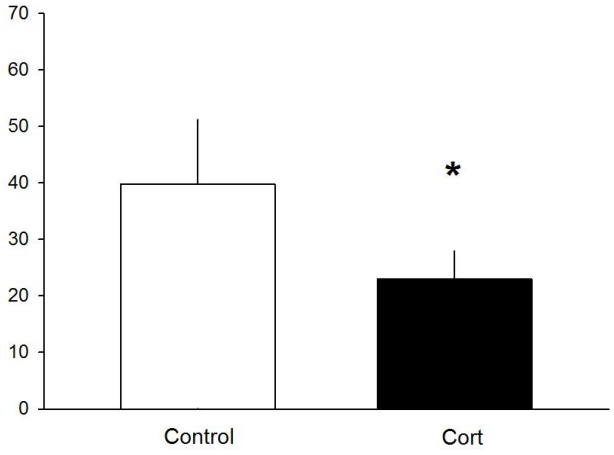

Figure 3.2. Plasma Cort concentration in early Cort treated juvenile birds. Male zebra finches in the Cort group were received 5mm Cort implants at age P4±1 (n = 6). Birds in the control group received either empty implants (n = 3) or no implants (n=3). After an overdose of Isoflurane, the birds were decapitated as juveniles (at age P30±1) and their trunk blood was collected for measuring plasma Cort concentration (see Methods). Cort treated (black bar) juvenile male zebra finches had significantly lower plasma Cort concentration than control birds (white bar; U = 3.000, Z = -2.191, *P = 0.028). Data represent median, 25-75% confidence interval. Asterisks highlight significant differences between groups (*P < 0.05).

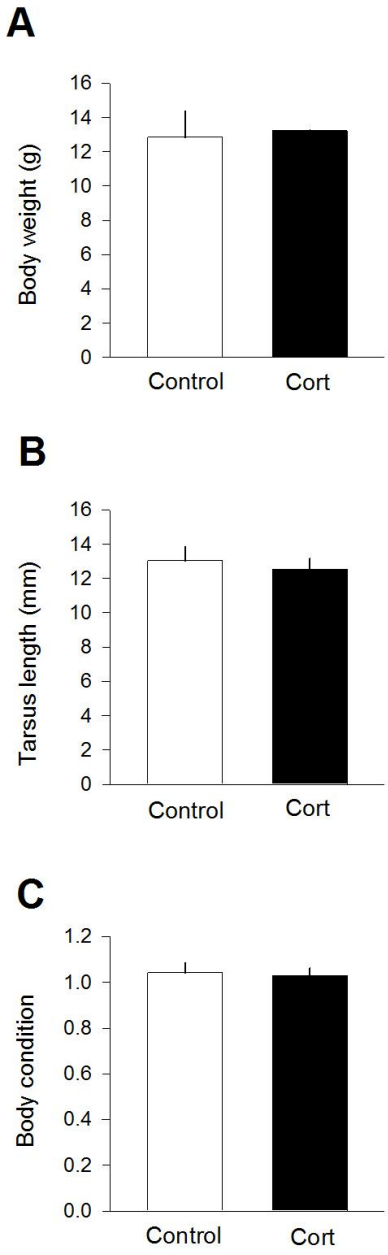

Figure 3.3. The effects of early Cort treatment on stress-related biological markers in juvenile male zebra finches. Male birds in the Cort group were received Cort implants at age P4±1. The control (No-Cort treated) group included male birds that received either empty implants or no implants. All juvenile birds (n = 5 from each Cort treated and control groups) were weighed at day 30 ± 1 and the length of their tarsi were measured. The average of left and right tarsus was used in the statistical analysis. To obtain body condition (relative weight at a given size) for juvenile, body weight was divided by tarsus length. There were no significant differences in the body weight (**A**), tarsus length (**B**), and body condition (**C**) between Cort treated (black bars) and control (white bars) juvenile birds. Data represent median, 25-75% confidence interval.

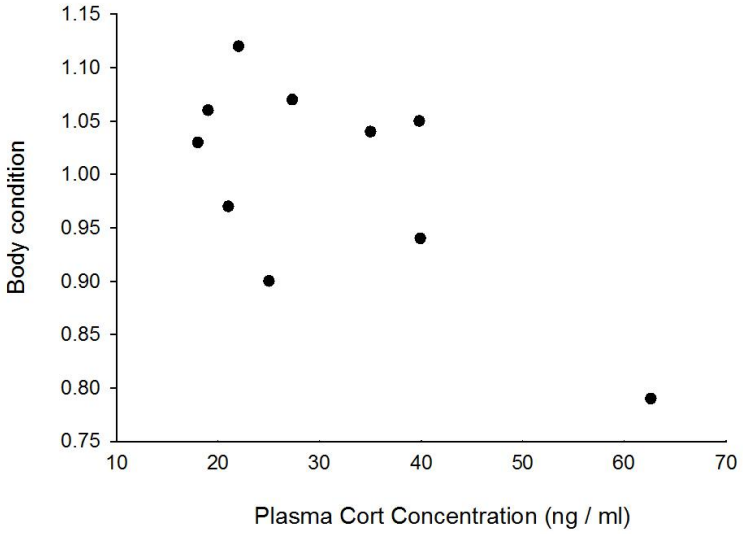

Figure 3.4. Correlation between plasma Cort concentration and body condition in juvenile (P30) male zebra finches. Pearson correlation test was used to obtain the correlation between plasma Cort concentration and body condition in all juvenile male zebra finches without considering their treatment. There was a significant negative correlation between plasma Cort concentration and body condition in juvenile male zebra finches regardless of treatment (Pearson correlation: r = - 0.660, P = 0.038). Juvenile male birds with higher plasma Cort concentration had lower body condition (n =10).

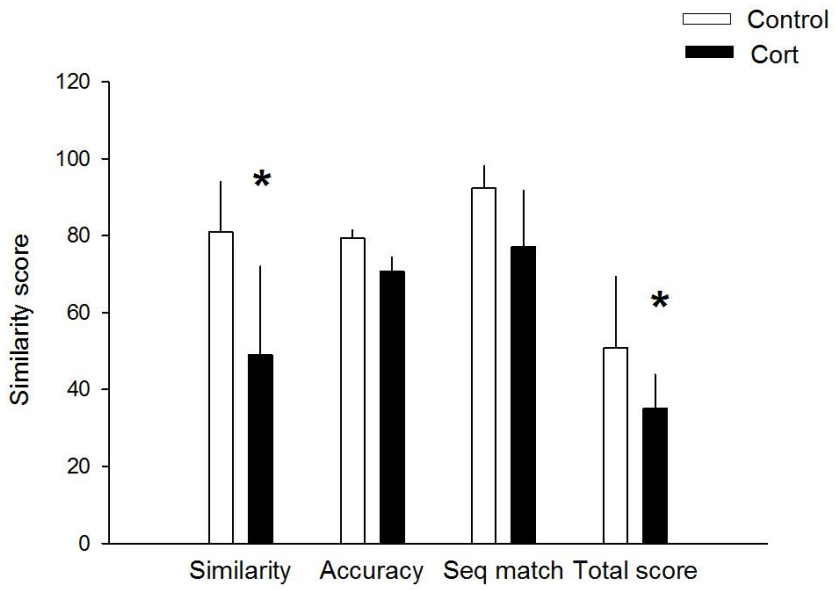

Figure 3.5. The effects of early Cort treatment on song parameters in adult male zebra finches from flight cages (FCs). Male zebra finches in the Cort group received Cort implants at age P4±1. The control group included male zebra finches that received either empty implants or no implants. Songs of early Cort treated and control male birds were recorded and analyzed in adulthood between 150 and 210 days of age**.** Cort treated (black bars) adult male birds from FCs (n = 7) had significantly lower song similarity and total score in comparison with control (combined empty and no implants; n = 14; white bars) adult birds (U = 18.500, Z = -2.283, *P = 0.022; U = 13.000, Z = -2.686, *P = 0.008; respectively). There were no significant differences in song accuracy and song sequential match between Cort treated (black bar) and control birds from FCs. Data represent median, 25-75% confidence interval. Asterisks highlight significant differences between groups (*P < 0.05).

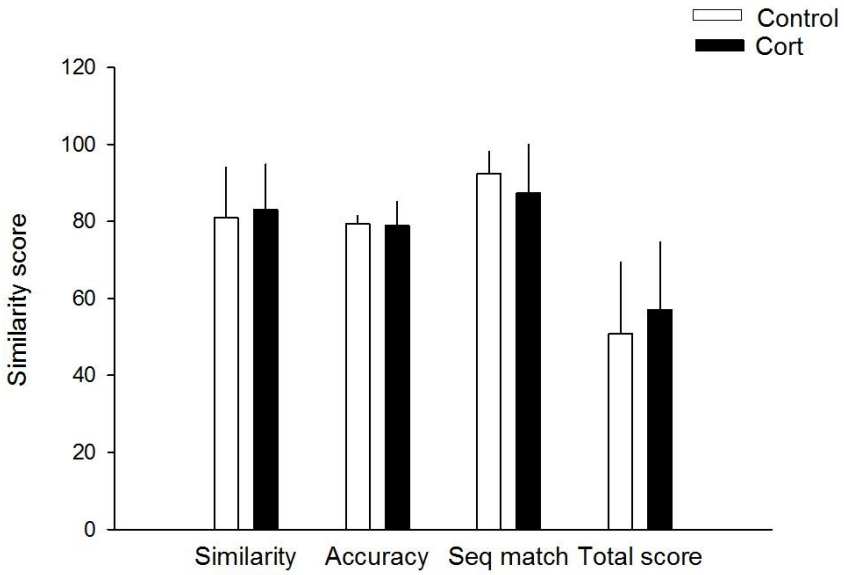

Figure 3.6. The effects of early Cort treatment on song parameters in adult male zebra finches from individual breeding cages (IBCs). Male zebra finches in the Cort group received Cort implants at age P4±1. The control (No-Cort treated) group included male zebra finches that received either empty implants or no implants. Songs of early Cort treated and control male birds were recorded and analyzed in adulthood between 150 and 210 days of age**.** There were no significant differences in song similarity, accuracy, sequential match, and total score between Cort treated (black bars; n = 9) and control (white bars; n = 14) birds from IBCs. Data represent median, 25-75% confidence interval.

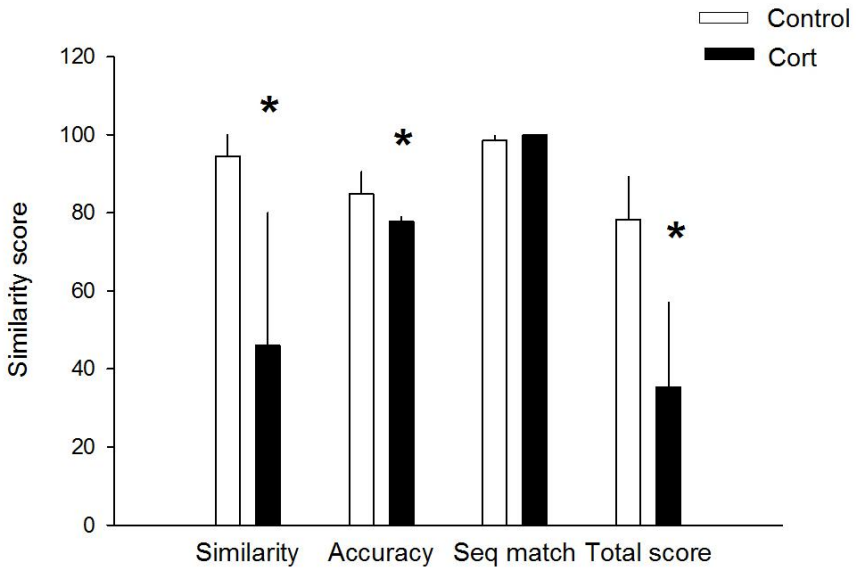

Figure 3.7. The effects of early Cort treatment on song parameters in adult male zebra finches from individual breeding cage #2 (IBC #2). Male zebra finches in the Cort group received Cort implants at age P4±1. The control (No-Cort treated) group included male zebra finches that received either empty implants or no implants. Songs of early Cort treated and control male birds were recorded and analyzed in adulthood between 150 and 210 days of age**.** Cort treated birds (black bars; n = 3) in IBC #2 had lower similarity, accuracy and total score than control (siblings; n = 4; white bars) birds (U = 0.000, Z = -2.141, *P = 0.032; U = 0.000, Z = -2.121, *P = 0.034; U = 0.000, Z = -2.121, *P = 0.034, respectively). Song sequential match was not different between Cort treated and control birds from IBC #2. Data represent median, 25-75% confidence interval. Asterisks highlight significant differences between groups (*****P < 0.05).

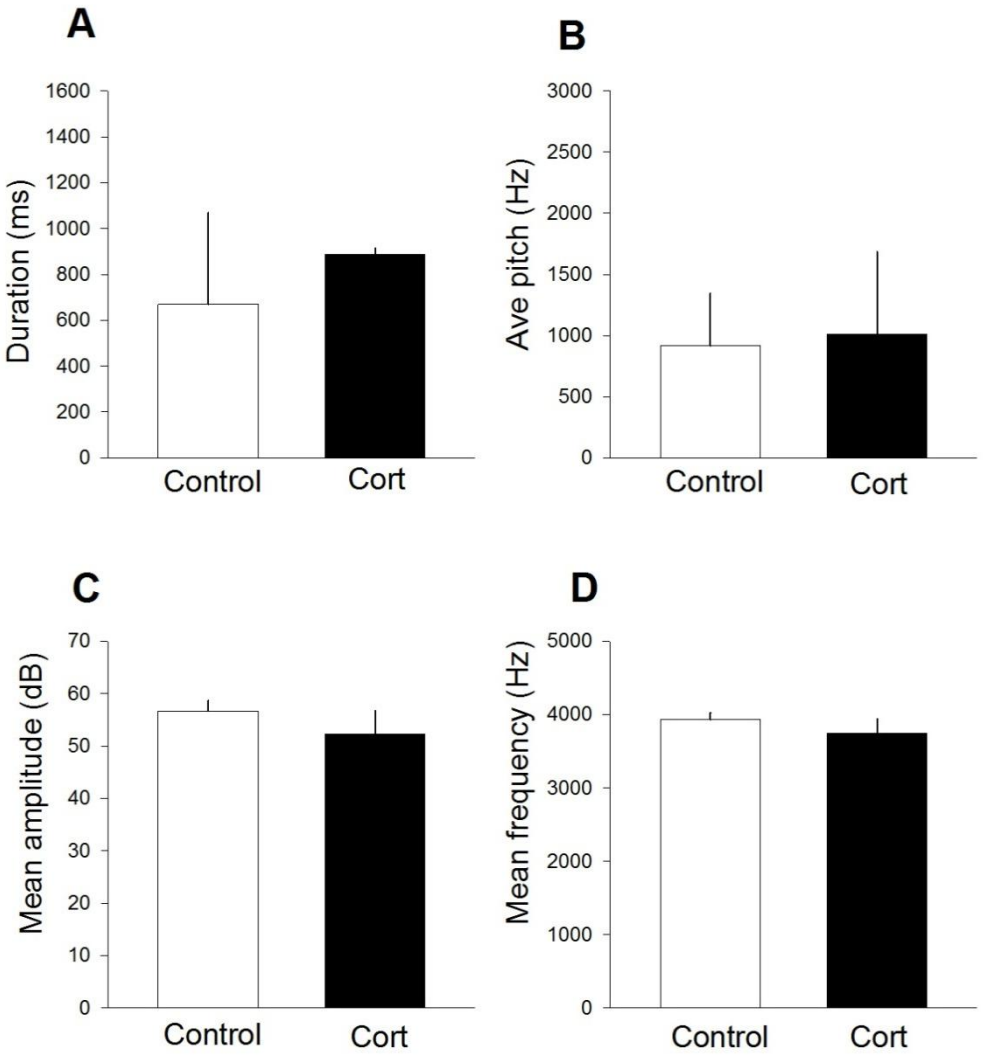

Figure 3.8. The effects of early Cort treatment on song duration, average pitch, mean amplitude, and mean frequency in adult male zebra finches from flight cages (FCs). Male zebra finches in the Cort group received Cort implants at age P4±1. The control group included male zebra finches that received either empty implants or no implants. Songs of early Cort treated and control male birds were recorded and analyzed in adulthood between 150 and 210 days of age**.** There were no significant differences in duration (**A**), average pitch (**B**), mean amplitude (**C**), and mean frequency (**D)** between Cort (black bar) and control (white bar) birds from FCs. Data represent median, 25-75% confidence interval.

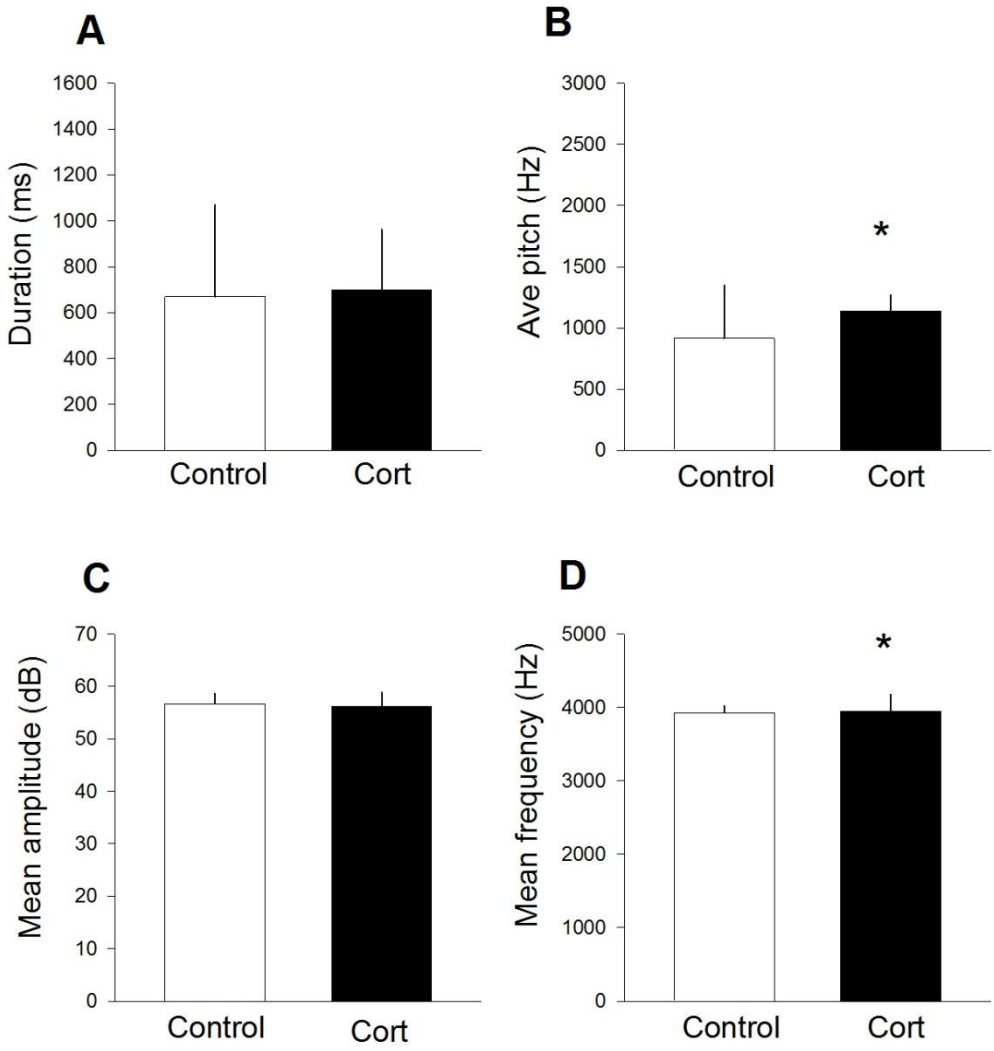

Figure 3.9. The effects of early Cort treatment on song duration, average pitch, mean amplitude, and mean frequency in adult male zebra finches from individual breeding cages (IBCs). Male zebra finches in the Cort group received Cort implants at age P4±1. The control group included male zebra finches that received either empty implants or no implants. Songs of early Cort treated and control male birds were recorded and analyzed in adulthood between 150 and 210 days of age**.** Cort treated (black bars) birds in IBCs had higher average pitch (**B**) and mean frequency (**D**) in comparison with control birds (white bars; U = 34.000, Z = -2.738, *P = 0.006; U = 45.00, Z = -2.241, *P = 0.026, respectively). There were no significant differences in duration (**A**) and mean amplitude (**C**) between Cort (black bars) and control birds (white bars) from IBCs. Data represent median, 25-75% confidence interval. Asterisks highlight significant differences between groups (*****P < 0.05).

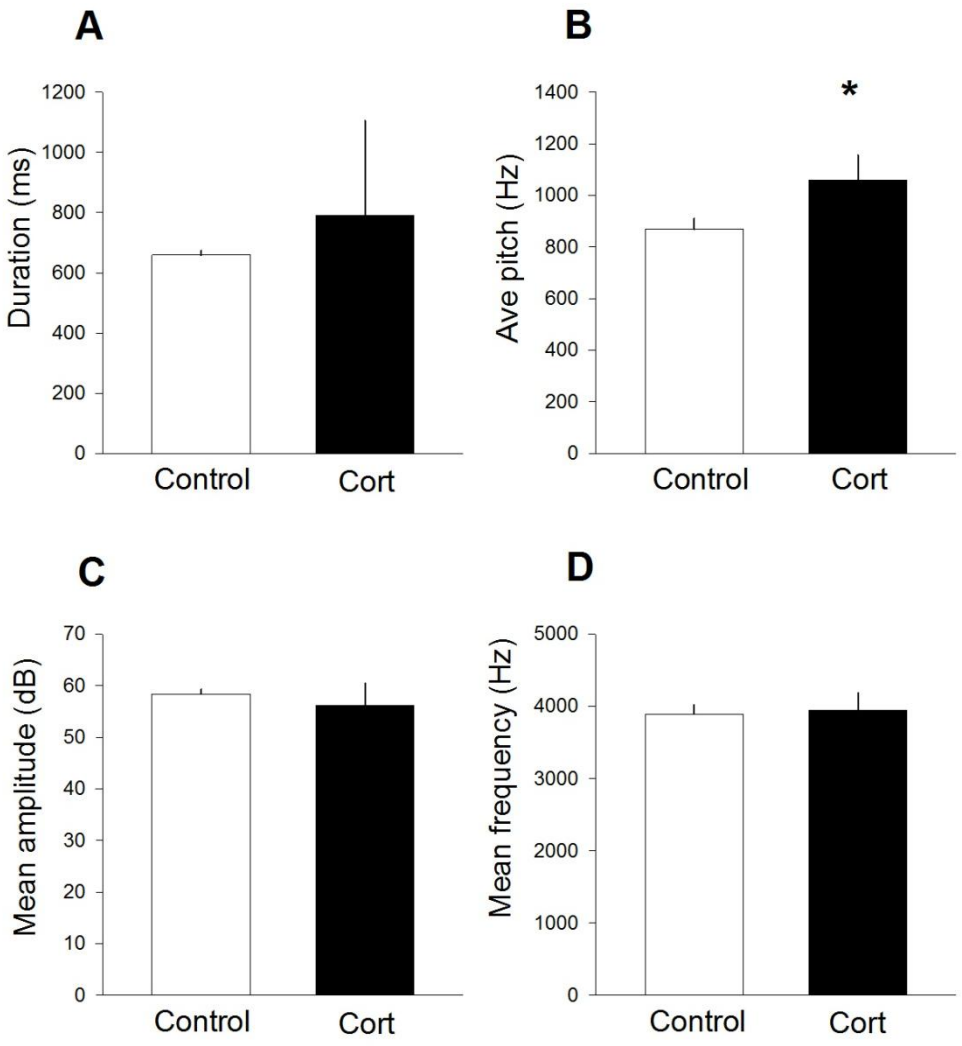

Figure 3.10. The effects of early Cort treatment on song duration, average pitch, mean amplitude, and mean frequency in adult male zebra finches from individual breeding cage #2 (IBC #2). Male zebra finches in the Cort group received Cort implants at age P4±1. The control group included male zebra finches that received either empty implants or no implants. Songs of early Cort treated and control male birds were recorded and analyzed in adulthood between 150 and 210 days of age**.** Cort treated (black bar; n=3) birds in IBC #2 had higher average pitch (**B**) compared with control (white bar) siblings (U = 0.000, Z = -2.121, *P = 0.034; n=4). There were no significant differences in duration (**A**), mean amplitude (**C**), and mean frequency (**D**) between Cort (black bars) treated and control (white bars) birds from IBC #2. Data represent median, 25-75% confidence interval. Asterisks highlight significant differences between groups (*****P < 0.05).

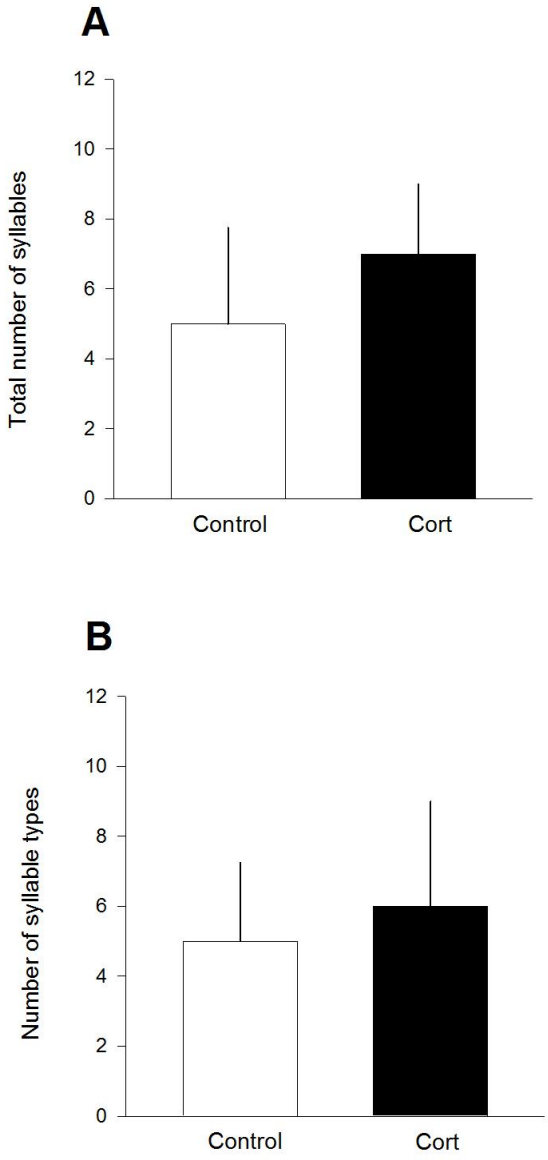

Figure 3.11. The effects of early Cort treatment on the total number of syllables and number of syllable types in adult male zebra finches from flight cages (FCs). Male zebra finches in Cort group received Cort implants at age P4±1. The control group included male zebra finches that received either empty implants or no implants. Songs of early Cort treated and control male birds were recorded and analyzed in adulthood between 150 and 210 days of age**.** There were no significant differences in the total number of syllables (**A**) and number of syllable types (**B**) between Cort (black bars) and control (white bars) birds from FCs. Data represent median, 25-75% confidence interval.

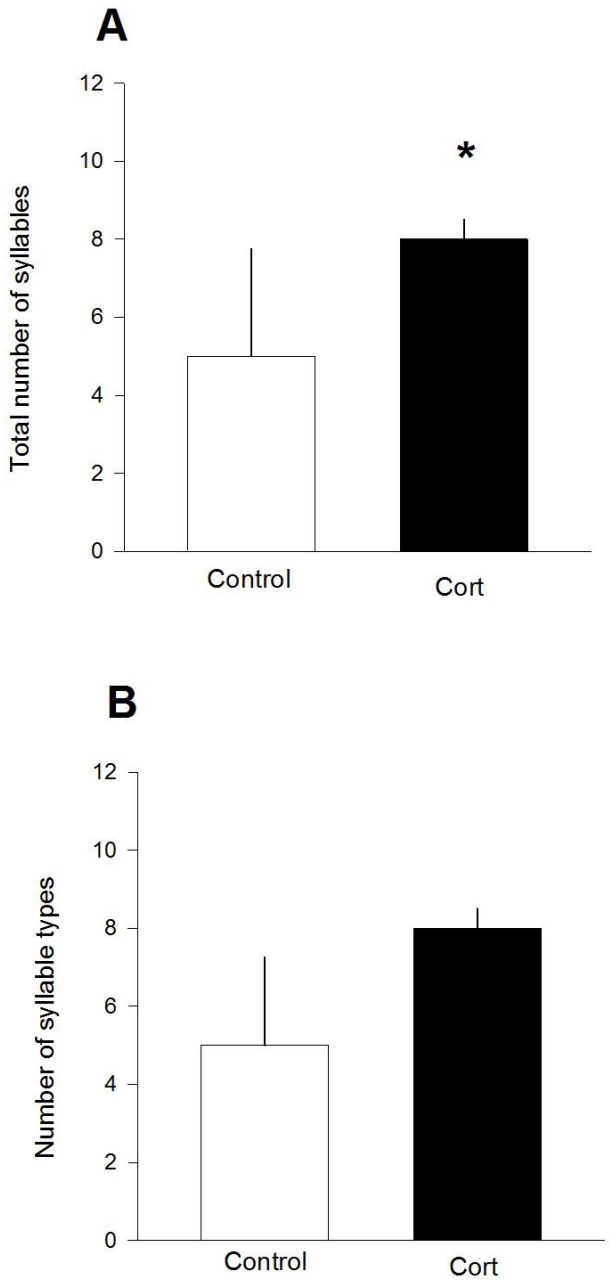

Figure 3.12. The effects of early Cort treatment on the total number of syllables and number of syllable types in adult male zebra finches from individual breeding cages (IBCs). Male zebra finches in the Cort group received Cort implants at age P4±1. The control (No-Cort treated) group included male zebra finches that received either empty implants or no implants. Songs of early Cort treated and control male birds were recorded and analyzed in adulthood between 150 and 210 days of age**.** The total number of syllables (**A**) was higher in Cort (black bars) treated birds from IBCs Compared with control birds (white bars): U = 44.000, Z = -2.308, *P = 0.022.The number of syllable types (**B**) was not significantly different between Cort (black bar) treated and control birds. Data represent median, 25-75% confidence interval. Asterisks highlight significant differences between groups (*****P < 0.05).

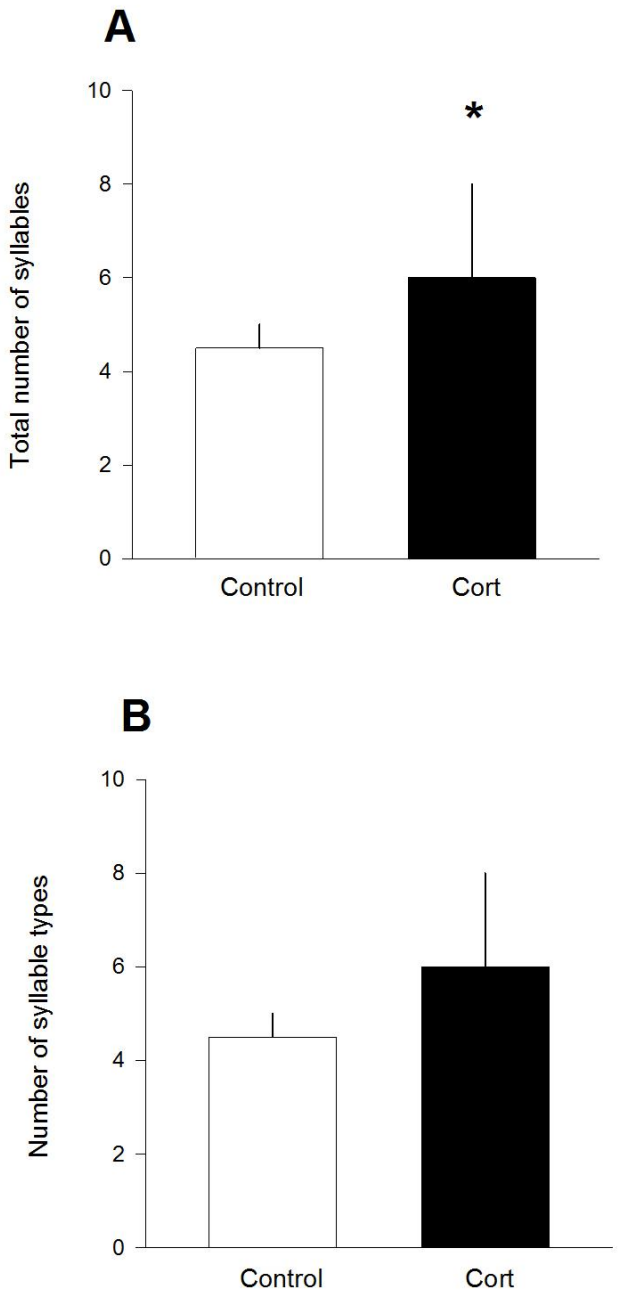

Figure 3.13. The effects of early Cort treatment on the total number of syllables and number of syllable types in adult male zebra finches from individual breeding cage #2 (IBC #2). Male zebra finches in the Cort group received Cort implants at age P4±1. The control (No-Cort treated) group included male birds that received either empty implants or no implants. Songs of early Cort treated and control male birds were recorded and analyzed in adulthood between 150 and 210 days of age. The total number of syllables (**A**) was higher in Cort (black bars) treated birds from IBC #2 compared with control birds (white bars): U = 0.000, Z = -2.181, *P = 0.030.The number of syllable types (**B**) was not significantly different between Cort (black bar) treated and control birds (white bar). Data represent median, 25-75% confidence interval. Asterisks highlight significant differences between groups (*****P < 0.05).

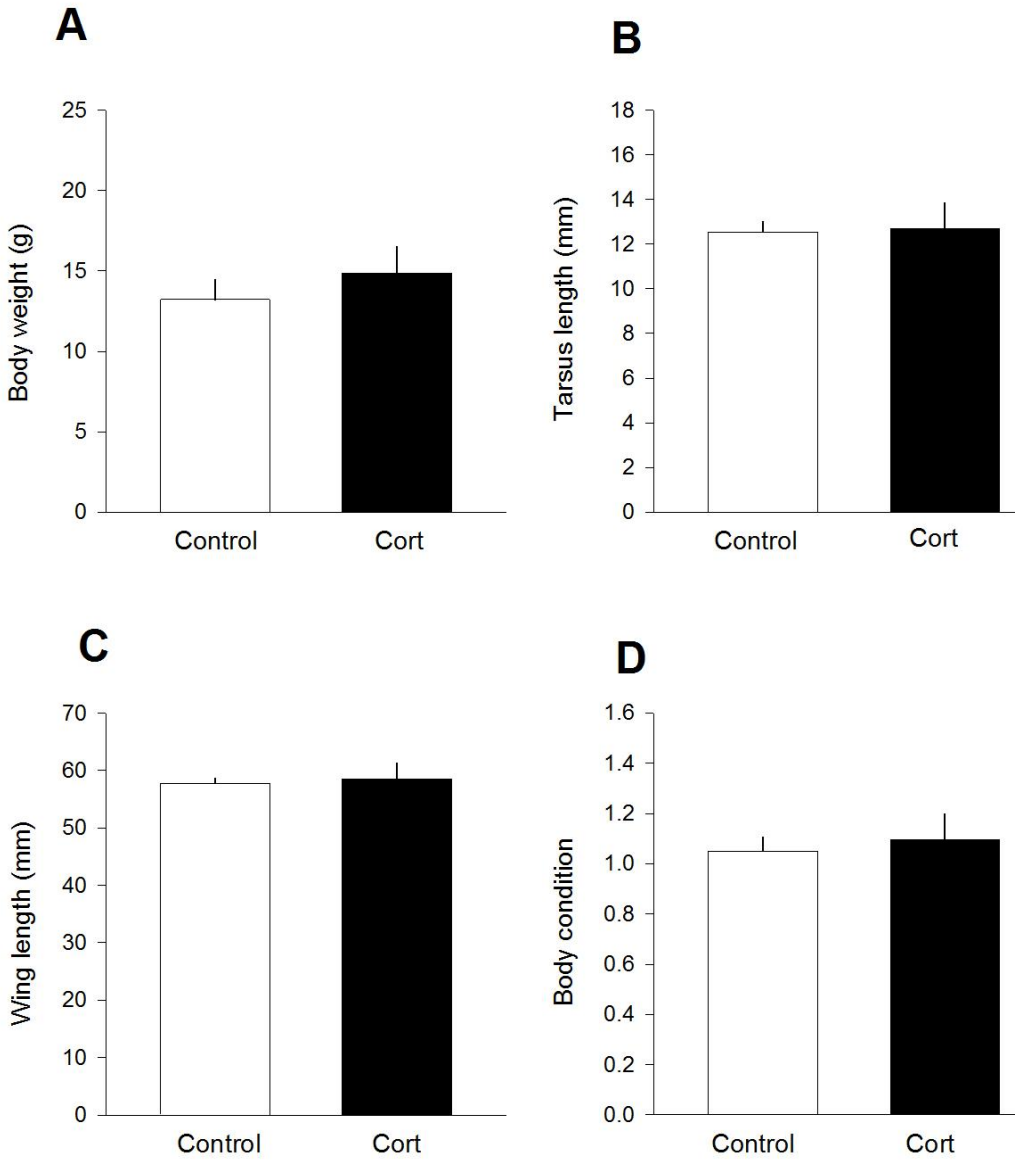

Figure 3.14. The effects of early Cort treatment on stress-related biological markers in adult male zebra finches. Male birds in the Cort group received Cort implants at age P4±1. The control (No-Cort treated) group included male birds that received either empty implants or no implants. All adult male birds from different treatment groups (n=16 Cort and n=14 control group) were weighted at day 210 (after recording their song) and the length of their tarsi and wings were measured and body condition (body weight / tarsus length) was calculated. The average of left and right values for both tarsi and wing was used in the statistical analysis. There were no significant differences in body weight (**A**), tarsus length (**B**), wing length (**C**), and body condition (**D**) between Cort (black bars) and control (white bars) adult birds. Data represent median, 25-75% confidence interval.

Table 3.2. Results of the effects of early corticosterone (Cort) treatment on body weight, tarsus length, wing length and body condition in juvenile (P30) and adult male zebra finches.
Birds in the Cort group (n = 5 for P30; n = 16 for adults) received Cort implants at day P4 until day 30 (for juvenile) and until adulthood (for adults). Birds in the control group (No-Cort treated; n = 6 for P30; n = 14 for adults) received either empty implants or no implants
(See "Methods" for details).

	Control vs. Cort treated birds
Juvenile (P30)	
Body weight (g)	U = 12.000, Z = - 0.104, P = 0.918
Tarsus length (mm)	U = 7.000, Z = - 1.149, P = 0.252
Body condition (Bodyweight/Tarsus)	U = 12.000, Z = - 0.104, P = 0.918
Adult	
Body weight (g)	U = 67.000, Z = -1.871, P = 0.062
Tarsus length (mm)	U = 75.000, Z = -1.538, P = 0.124
Wing length (mm)	U = 70.000, Z = -1.755, P = 0.080
Body condition (Body weight/Tarsus)	U = 87.000, Z = -1.039, P = 0.300

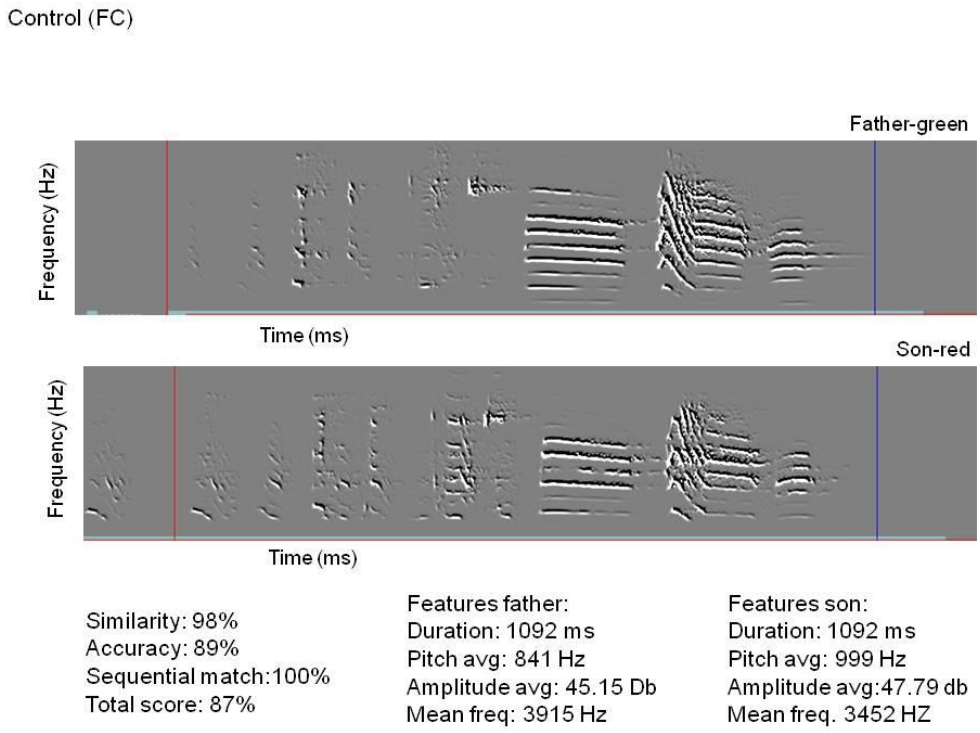

Figure 3.15A Example sonograms of control (No-Cort treated) male zebra finches from a flight cage (FC). The control birds received either empty silastic implants at age P4±1 or no implants and their song were recorded and analyzed in adulthood between 150-210 days of age. The upper and lower sonograms are from a father and his control - reared son, respectively.

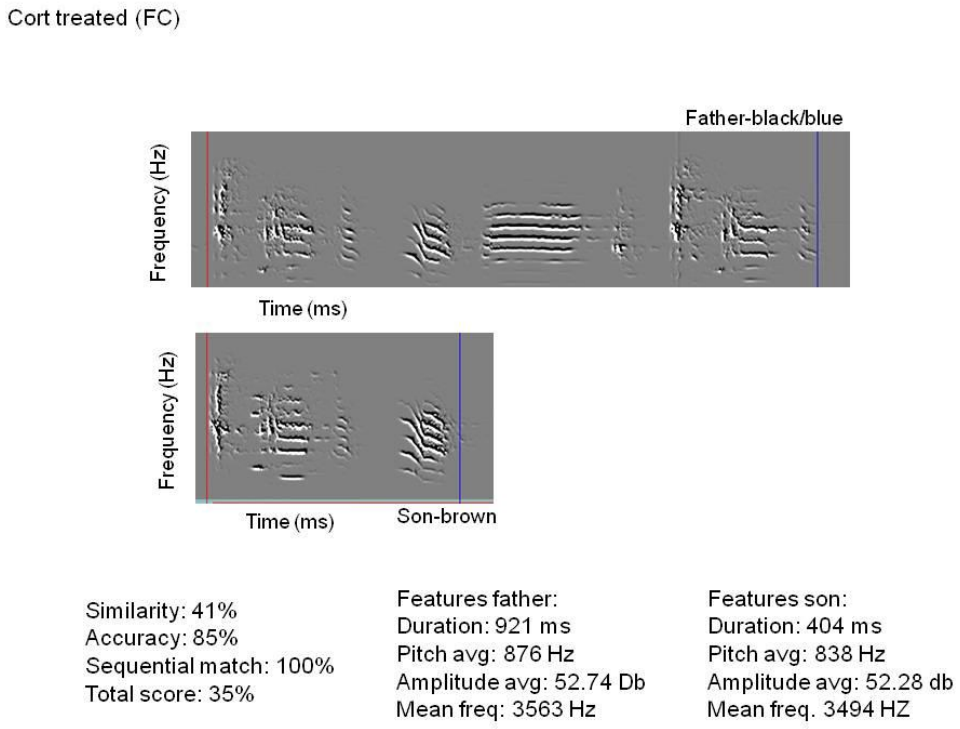

Figure 3.15B Example sonograms of Cort treated male zebra finches from a FC. Cort treated birds received Cort implants at age P4±1 and their song were recorded and analyzed in adulthood between 150-210 days of age. The upper and lower sonograms are from a father and his Cort treated son, respectively.

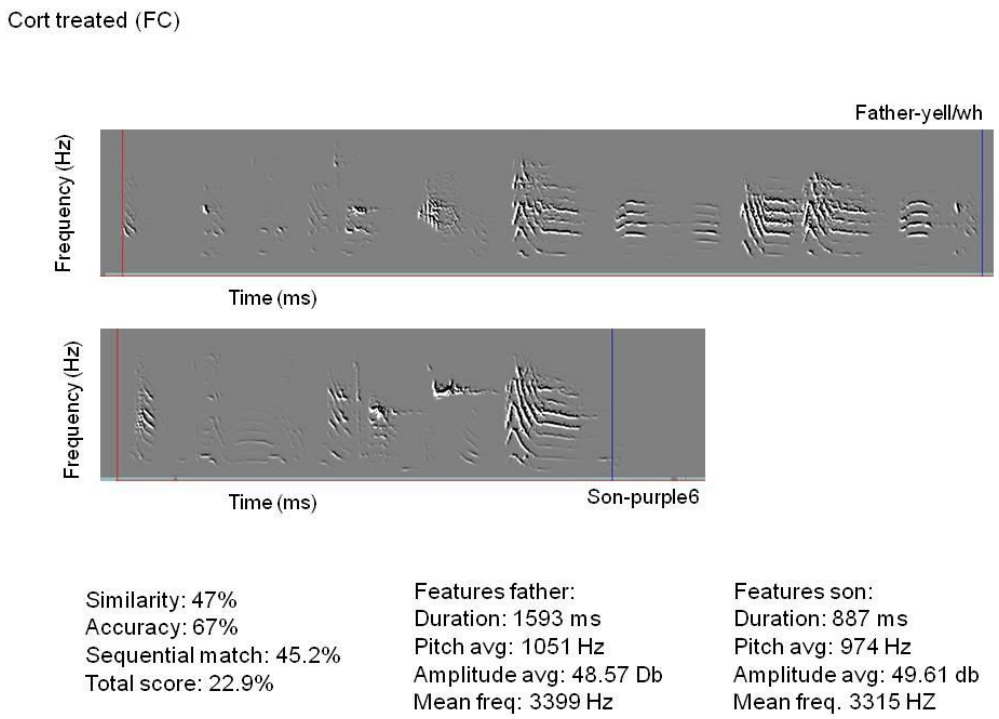

Figure 3.15C Example sonograms of Cort treated male zebra finches from a FC. Cort treated birds received Cort implants at age P4±1 and their song were recorded and analyzed in adulthood between 150-210 days of age. The upper and lower sonograms are from a father and his Cort treated son, respectively.

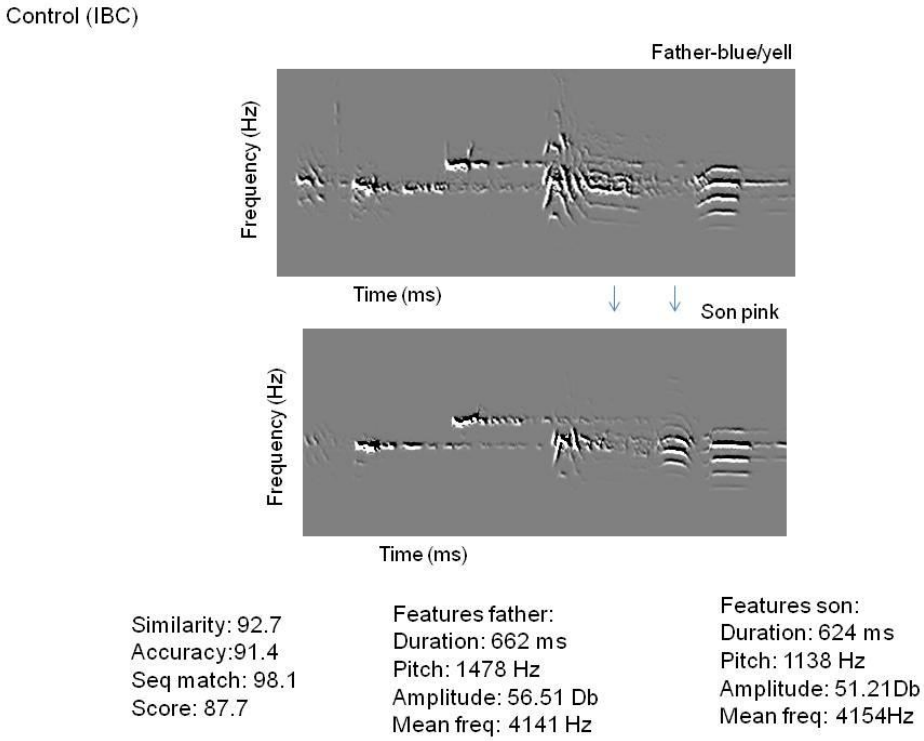

Figure 3.16A Example sonograms of control (No-Cort treated) male zebra finches from an individual breeding cage (IBC). Control birds received either empty silastic implants at age P4±1 or no implants and their song were recorded and analyzed in adulthood between 150-210 days of age. The upper and lower sonograms are from a father and his control - reared son, respectively.

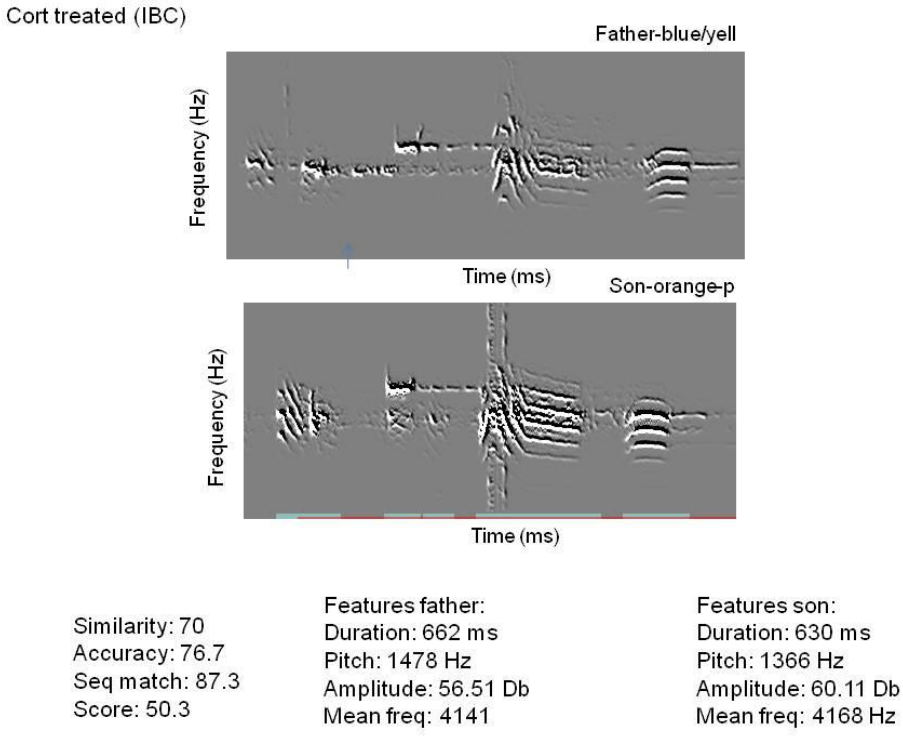

Figure 3.16B Example sonograms of Cort treated male zebra finches from an IBC. Cort treated birds received Cort implants at age P4±1 and their song were recorded and analyzed in adulthood between 150-210 days of age. The upper and lower sonograms are from a father and his Cort treated son, respectively.

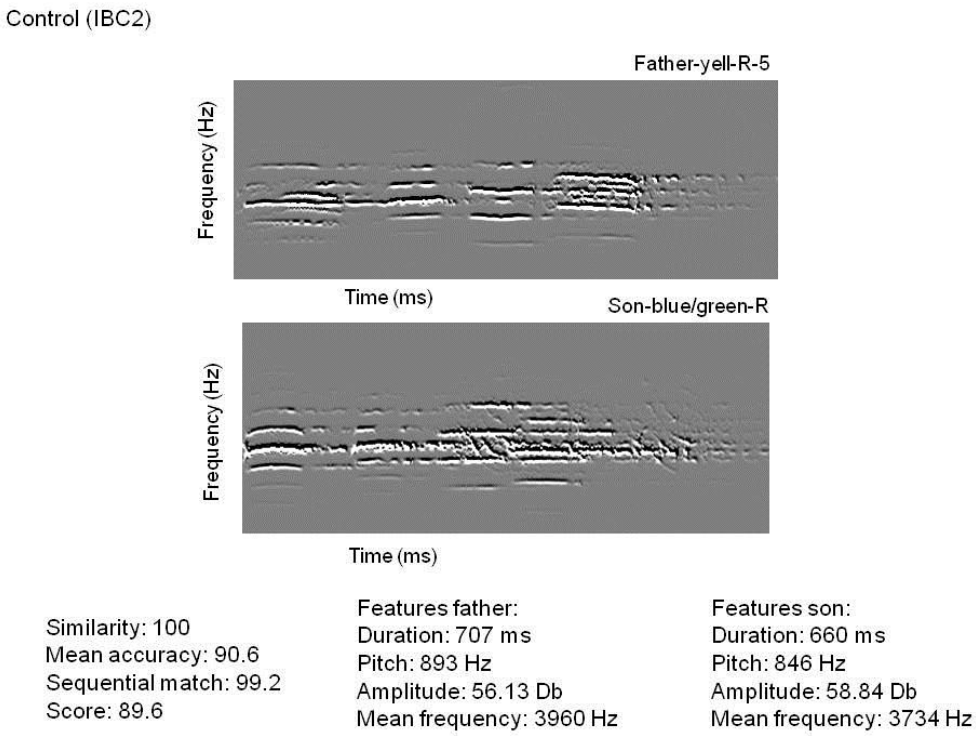

Figure 3.17A Example sonograms of control (No-Cort treated) male zebra finches from individual breeding cage 2 (IBC2). Control birds received either empty silastic implants at age P4±1 or no implants and their song were recorded and analyzed in adulthood between 150-210 days of age. The upper and lower sonograms are from a father and his control - reared son, respectively.

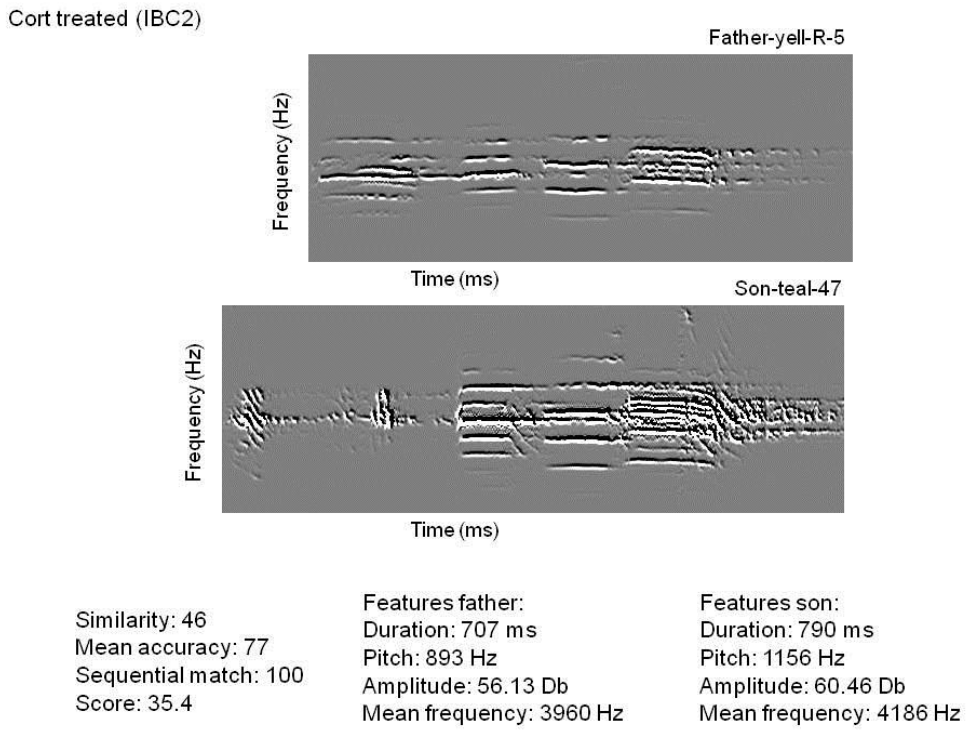

Figure 3.17B Example sonograms of Cort treated male zebra finches from IBC2. Cort treated birds received Cort implants at age P4±1 and their song were recorded and analyzed in adulthood between 150-210 days of age. The upper and lower sonograms are from a father and his Cort treated son, respectively.

CHAPTER 4: EARLY CORTICOSTERONE TREATMENT DIFFERENTIALLY AFFECTS THE SIZE OF THE SONG CONTROL NUCLEUS HVC IN THE JUVENILE AND ADULT MALE ZEBRA FINCH BRAIN

Abstract

Early developmental stress affects the size of song control nuclei and song complexity in many songbirds, suggesting a direct link between brain and behavior. HVC (acronym is a proper name) is required for learning and production of song and song complexity is an indicator of male quality and is important for mate choice in many songbird species. The mechanisms underlying these effects are unknown. We tested the hypothesis that early corticosterone (Cort) treatment selectively reduces the size of song control nucleus HVC in juvenile (post-hatch day 30) and adult male zebra finches (*Taeniopygia guttata*). Male zebra finches received Cort implants at P4 (post-hatch day 4). We measured the volume of HVC, robust nucleus of the arcopallium (RA) and the telencephalon (Tel) containing HVC and RA using Nissl stain at two age points. We found that early Cort treatment significantly reduces the size of HVC in juvenile and adult male zebra finches. Early Cort treatment did not significantly affect the size of RA or Tel. Therefore, the HVC is more susceptible to the effect of early Cort treatment than RA and Tel. This result suggests that the effect of developmental stress (or nutritional stress as previously reported) on the HVC size may be mediated through Cort. Activation of glucocorticoid receptors (GR) within HVC via Cort might be a specific mechanism by which the size of HVC and song complexity decrease in developmentally stressed birds. Our results support the nutritional-stress hypothesis and explain the learned features of song as an indicator of male quality. Furthermore, our findings demonstrated that in addition to genetic factors, environmental factors affect the develop-

ment of song control nuclei (particularly HVC) and highlight the developmental plasticity of songbird brain.

4.1. Introduction

Song complexity (number of song types and / or larger repertoire of syllables; previously described in chapter 1) has evolved through sexual selection (Airey et al., 2000; Buchanan et al., 2004) and is important in mate choice for many songbird species (Anderson 1994, Searcy and Yasukawa 1996). The 'developmental stress hypothesis' suggests that complex songs evolved as indicators of male quality (Nowicki et al., 1998). Song is controlled by a series of interconnected song nuclei. The volume of specific song nuclei in particular HVC (acronym is proper name, previously described as the caudal nucleus of hyperstriatum ventrale or the high vocal center; Reiner et al., 2004) correlates positively with song complexity (Airey et al., 2000; Garameszegi and Eens 2004; DeVoogd et al., 1993). HVC is required for learning and production of song and males with more elaborate songs have a larger HVC (Airey et al., 2000). It is suggested that the development of this song control nucleus may be a mechanism controlling song complexity which indicating male quality (Buchanan et al., 2004).

Early developmental stress can affect the physiology, behavior, reproductive success, and survival (Lindstrom, 1999; Naguib et al., 2006; Naguib and Nemitz, 2007). The mechanisms underlying the effect of early stress on neuronal development are not well understood. It is important to understand these mechanisms in order to understand the impact of stress on human brain and its function.

Zebra finches are powerful models for studying stress-induced brain plasticity because reduction of HVC size following lesion or early chronic stress in zebra finches results in reduc-

tion in song complexity (Spencer et al., 2003; Buchanan et al., 2004), suggesting a direct link between brain plasticity and behavior in songbirds (Pfaff et al., 2007). The mechanisms underlying this effect are unknown.

Early chronic stress or elevated levels of glucocorticoids (GC; such as cortisol and corticosterone) result in brain abnormalities in stress-related mental disorders (Sapolsky, 2000 and 2001) and glucocorticoid receptors (GR) mediate the adverse effects of GC on brain and its function (Sapolsky, 2000 and 2001; Krugers et al., 2006). Thus, GR play a role in stress, and are found in the song nuclei and telencephalon (Shahbazi et al., 2012). Activation of GR via corticosterone (Cort) within HVC may therefore be a mechanism by which HVC size and song complexity decrease in developmentally stressed birds. Others have shown that food-restricted song sparrows have elevated baseline Cort (MacDonald et al., 2006; Kempster et al., 2007). These findings suggest that Cort may mediate the effects of early stress, although Cort is only one of several hormones released during stress response.

Decreasing HVC size, reducing song complexity and changes in blood Cort concentration in developmentally stressed birds have not been examined in a single study. In addition, reduction of HVC size has not been shown in stressed juvenile birds. We tested the effects of Cort on song complexity (Chapter 3) of adults and song nuclei size in juvenile and adult birds (this chapter). To test the effect of Cort on HVC size and song complexity, we used Cort implants that mimic the response to a stressor. Nissl histology was then used to measure the effect of exogenous Cort on song nuclei size at two age points. We hypothesized that early Cort treatment would decrease the size of HVC but not another song nucleus, RA (robust nucleus of the arcopallium) and the telencephalon, which contains many of the song nuclei. This result would sug-

gest that HVC may be more sensitive to the effects of early Cort treatment than other regions of the song control system.

4.2. Methods

4.2.1. Animals

We used male zebra finches from our breeding colony at Georgia State University. The Georgia State University Institutional Use and Animal Care Committee granted approval for all animal procedures. Male birds age P4±1 (4 days post-hatch; then allowed to grow to juveniles or adults) were used. Sex of juvenile birds was determined via PCR (Soderstrom et al., 2007) and by visual examination of the testes at the time of tissue collection.

4.2.2. Experimental manipulation

Male finches at P4±1 were divided into 6 groups. Group 1 (n = 5) were implanted with crystalline Cort subcutaneously above flight muscle for 26 days. Group 2 (n = 3) served as the control for Group 1 and received empty implants. Group 3 (n = 3) served as the no-implant controls for groups 1 and 2. Groups 4 (n = 6), 5 (n = 3), and 6 (n = 6) were duplicates of groups 1, 2 and 3. The first 3 groups were sacrificed as juveniles at age P30±1. The last 3 groups (groups 4, 5, and 6) were sacrificed around age 200 days after their courtship songs were recorded. After song recording was complete, the brains of all animals were processed for Nissl staining (described below) to measure the effect of exogenous Cort on the size of HVC, RA and the telencephalon. We compared the volume of HVC and RA to the volume of telencephalon area to demonstrate that Cort-induced reduction of HVC size is a neuroanatomically specific effect. Blood Cort concentrations were measured via RIA at P30 to validate the implants. Because only male finches (P4±1) were included in the experiments, and we were not able to visually deter-

mine their sex until P30, a sexing PCR (Soderstrom et al., 2007; Agate et al., 2003) protocol was followed to determine the sex of birds at implant insertion or as juveniles. Only two main age groups (juvenile and adult) are included in this experiment.

4.2.3. Measurements of brain song nuclei

To determine the effects of exogenous Cort on the size of HVC, RA and the telencephalon, Nissl stain was performed on the brains of juvenile (P30; n = 11) and adult male (n = 15) birds. After an overdose of Isoflurane, juvenile and adult birds were decapitated as previously described (Duncan et al., 2011). For juvenile birds, the entire skull was immersed in Bouin's fixative for 24 h after removing the skin and feathers. Adult male birds were decapitated after an overdose of Isoflurane and the brains of these animals were removed from the skull and placed in Bouin's fixative for 24 hr. followed by three 12 h rinses in 70% ethanol. The brains of juvenile and adult birds were then ethanol dehydrated, and cleared in CitriSolv Hybrid (Fisher Scientific, King of Prussia, PA). Brains were then embedded in Paraplast (Oxford Labware, St. Louis, MO), sectioned at 10 µm with alternating coronal sections placed on gelatin-subbed microscope slides, and stored at room temperature until use.

Standard Nissl protocols were followed for juvenile and adult tissue. Prior to beginning the Nissl stain, slides with juvenile and adult tissue were cleared with xylene (Fisher Scientific, Fair Lawn, NJ), rehydrated, and then rinsed in distilled water. Slides were incubated in Cresyl violet acetate solution (Nissl; Sigma-Aldrich, St. Louis, MO; at 2g/1L dH_2O) for 20min at room temperature. Slides were then rinsed quickly in distilled water and dehydrated in serial dilutions of ethanol and xylene. Both juvenile and adult tissue was then coverslipped with mounting medium.

One series of sections of both the left and right hemisphere were stained. Volume data were averaged across left and right hemispheres for each bird. Because there were no significant left (L) - right (R) differences in the volume of HVC, RA, and telencephalon, birds with only a left or right HVC or RA were included in the analysis. In addition, there were no significant differences between Cort-treated L+R vs. Cort-treated L or R and there were no significant differences between No-Cort-treated L+R vs. No-Cort treated L or R (statistical analysis discussed below).

Images were captured on a Zeiss light microscope using Axiovision software and measurements were conducted using an image analysis system on a video screen. For volume measurements, the perimeter of the region of interest in each section was traced on digitized images and the area was calculated by a built-in function of the software. Regions of interest were measured in every third section in each bird. The volume of both song nuclei was calculated by summing the area measurements and multiplying by the number of series, number of samples across the series, and section thickness. There were four series of slides (one series was used for the Nissl stain). The number of samples across series is the total number of sections containing the region of interest divided by 3. Neuroanatomical measurements were performed while blind to treatments. The Nissl stain was used for all the samples for economic reasons. Though, the morphometric results depend on the delineation method (Gahr, 1997).

4.2.4. Statistical analysis

All data were analyzed using IBM SPSS Statistics for Windows, version 19.0 (SPSS Inc, Chicago, IL). First, data were examined for assumptions of parametric statistical tests. When assumptions were violated, a non-parametric alternative was used. Alpha was set at $P < 0.05$. One-

Way ANOVA was used to compare volumes of HVC, RA, and Tel. of Cort-treated birds to those in control (No-Cort treated) birds. One-Way ANOVA was also used to compare the ratios of HVC/Tel and RA/Tel of Cort-treated birds to control birds. Mann-Whitney U test was used to compare the mean volume of left HVC, RA, and Tel to right HVC, RA, and Tel. One-Way ANOVA or independent-samples t- tests were used to compare volumes of HVC, RA, and Tel of No-implanted with those of Empty-implanted birds. Non-parametric Spearman correlation tests (Spearman's rho) were used to compare the correlations between the HVC volume (and HVC/Tel ratio) and song similarity, song accuracy, sequential match, total score, and total number of syllables in adult male zebra finches regardless of treatment group. This test was used (instead of parametric-Pearson correlation test) because we had a potential outlier and based on the traditional criteria for outlier (mean ± 2 standard deviation), it was not an outlier. Therefore, this non-parametric correlation test was appropriate.

4.3. Results

4.3.1. Volume asymmetry of HVC, RA, and Tel in the brain of juvenile and adult male zebra finches

Nissl stain was used to determine the volume of HVC, robust nucleus of the arcopallium (RA) and the telencephalon (Tel; containing HVC and RA). Figures 4.1A and 4.1B demonstrate HVC and RA of a juvenile male zebra finch, respectively. Figures 4.1C and 4.1D demonstrate area measurements of HVC and RA, accordingly. Volume data for juvenile and adult were averaged across right (R) left (L) hemispheres for each individual in the statistical analysis. There were some individuals that had only right or left hemispheres due to damage to one hemisphere or other technical issues. For HVC measurements from juveniles, one individual from the Cort and one from the control (No-Cort treated) group had only the left hemisphere intact (total n =

2). For RA measurements of juveniles, three individuals from the control group had only the left hemisphere intact (total n = 3) and one individual from Cort group had only left hemisphere intact. For HVC measurements of adults, one individual from Cort group had only right hemisphere (n = 1) and three individuals from control group had only one intact hemisphere (n = 3; 2 with only left and one with only R hemisphere). For RA measurements of adult, there were two individuals from control group with only one hemisphere intact (n = 2; one with right and one with left hemisphere).

In order to use all the individuals with one intact hemisphere in the analysis, for both age groups, volumes of right HVC, RA, and Tel (containing HVC and RA) were compared to left HVC, RA, and Tel. We found that there were no significant differences in the volume of HVC, RA or Tel containing HVC and RA between right and left hemispheres. Our finding is consistent with other reports of no significant right-left differences in the volume of HVC and RA (Gil et al., 2006).

There were no significant differences in the volume of HVC, RA, and Tel between right and left hemispheres in any juvenile birds (Table 4.1A). U = 29.000, Z = -0.265, P = 0.792; U = 18.500, Z = -0.534, P = 0.594; U = 23.000, Z = -0.900, P = 0.368; U = 20.500, Z = -0.267, P = 0.790 for HVC, RA, Tel (for HVC measurements), and Tel (for RA measurements), accordingly.

There were no significant differences in the volume of HVC, RA, and Tel between right and left hemispheres of all adults used in the study (Table 4.1A). U = 60.500, Z = -0.664, P = 0.508; U = 86.500, Z = -0.529, P = 0.598; U = 64.000, Z = -0.462, P = 0.644; U = 79.000, Z = -0.873, P = 0.384 for HVC, RA, Tel (for HVC measurements), and Tel (for RA measurements), respectively.

There was no significant asymmetry in the ratios of HVC/Tel and RA/Tel from juvenile or adult males (U = 31.000, Z = -0.053, P = 0.958; U = 18.000, Z = -0.600, P = 0.550; U = 66.000, Z = -0.346, P = 0.730; U = 91.000, Z = -0.322, P = 0.748, respectively; Table 4.1A).

There was no significant asymmetry of HVC, RA, Tel (containing HVC and RA), and their ratios in control (No-Cort treated; Table 4.1B) and Cort (Table 4.1C) treated juvenile and adult male zebra finches. In addition to no significant R-L differences, for the individuals with only right or left hemispheres, a statistical analysis was performed to make sure that there were no significant differences between the individual with only R (or L) hemisphere and others with R (or L) hemisphere in control or Cort groups. Furthermore, statistical analysis were performed to make sure that there were no significant differences in the volume of HVC or RA or Tel between an individual with only one hemisphere and the individuals with both hemispheres (individuals with having average of R and L hemispheres).

There were no significant differences in the volume of HVC, Tel, and the HVC/Tel ratio between control (only L hemisphere; L) and control (R + L hemispheres; R + L) juvenile birds (t(2) = 1.415, P = 0.294; t(2) = 0.637, P = 0.590; t(2) = -0.498, P = 0.668, accordingly). There were also no significant differences in the volume of HVC, Tel and the HVC/Tel ratio between Cort (with L) and Cort (with R + L) juvenile birds (t(3) = 0.076, P = 0.944; t(3) = -0.036, P = 0.974; t(3) = 0.290, P = 0.792, respectively). There were no significant differences in the volume of RA, Tel, and the RA/Tel ratio between control (with L) and control (with R + L) juvenile birds (t(3) = -0.312, P = 0.776; t(3) = -0.293, P = 0.790; t(3) = -0.254, P = 0.816, respectively). There were no significant differences in the volume of RA, Tel, and the RA/Tel ratio between Cort (with L) and Cort (with R+L) P30 birds (t (2) = -0.885, P = 0.470; t (2) = -0.513, P = 0.640; t (2) = -1.089, P = 0.396, accordingly).

Furthermore, there were no significant differences in the volume of HVC, Tel, and the HVC/Tel ratio between control (with L) and control (with R + L) adult birds ($t(4) = -0.400$, $P = 0.710$; $t(4) = 0.615$, $P = 0.572$; $t(4) = 0.407$, $P = 0.706$, respectively). There were no significant differences in the volume of HVC, Tel, and the HVC/Tel ratio between control (with R) and control (with R + L) adult birds ($t (3) = -0.805$, $P = 0.480$; $t (3) = 2.176$, $P = 0.118$; $t (3) = -1.335$, $P = 0.274$, accordingly). One adult bird with just right HVC that was excluded because there was a significant difference in the volume of HVC between Cort (with R, $n = 1$) and Cort (with R + L, $n=6$) adult birds ($t (5) = 2.080$, $P = 0.092$). There were no significant differences in the volume of Tel and the HVC/Tel ratio between Cort (with R) and Cort (with R + L) adult birds ($t (5) = 0.333$, $P = 0.754$; $t (5) = 0.788$, $P = 0.466$, respectively). There were no significant differences in the volume of RA, Tel, and the RA/Tel ratio between control (with L) and control (with R + L) adult birds ($t(6) = 1.169$, $P = 0.144$, $t(6) = 1.869$, $P = 0.112$; $t(6) = -1.044$, $P = 0.338$, respectively). There were also no significant differences in the volume of RA, Tel, and the RA/Tel ratio between control (with R) and control (with R + L) adult birds ($t(6) = 0.390$, $P = 0.710$; $t(6) = 0.987$, $P = 0.362$; $t(6) = -1.044$, $P = 0.338$, accordingly).

4.3.2. Effects of early Cort treatment on size of HVC, RA, and Tel in juvenile and adult male zebra finches

We found that juvenile and adult male zebra finches treated with Cort early during development had significantly smaller HVC volume when compared with control (No-Cort) birds. This finding is consistent with previous reports (Buchanan et al., 2004; MacDonald et al., 2006; Nowicki et al., 2002) indicating that developmental stress (such as food restriction) affects size of HVC in adult zebra finches, song sparrows, and swamp sparrows.

Volumes were averaged across left and right hemispheres for each individual in the statistical analysis. Cort-treated juveniles had significantly smaller HVC volume than control juveniles ($F(1, 7) = 12.619$, $P = 0.01$; Table 4.2; Figures 4.2A and 4.2C; Figure 4.3A). Because there were no significant differences in the volume of HVC, Tel, and the HVC/Tel ratio between No-Implant ($n = 2$) and Empty-implant ($n = 2$) birds, both groups were combined as one control (No-Cort treated) group for HVC measurements ($t(2) = 0.061$, $P = 0.958$; $t(2) = 0.468$, $P = 0.686$; $t(2) = -0.350$, $P = 0.760$, respectively).

There were no significant effects of Cort on the volume of RA in juvenile birds ($F(1, 7) = 0.309$, $P = 0.596$; Table 4.2; Figures 4.2B and 4.2D; Figure 4.3B). Because there were no significant differences in the volume of RA, Tel and the RA/Tel ratio between No-implant ($n = 3$) and Empty-implant ($n = 2$) birds, both groups were combined as one control (No-Cort treated) group for RA measurements ($t(3) = -1.040$, $P = 0.376$; $t(3) = -1.039$, $P = 0.376$; $t(3) = -0.953$, $P = 0.412$, accordingly).

There were no significant differences in the volume of Tel (containing HVC and RA) between Cort and control (No-Cort treated) juvenile birds for HVC and RA measurements ($F(1, 7) = 2.062$, $P = 0.194$ and $F(1, 7) = 0.154$, $P = 0.706$, respectively; Table 4.2; Figure 4.3C). Figure 4.3C only shows the Tel containing HVC and RA from HVC measurements (for simplicity, the graph of the Tel containing HVC and RA from RA measurements were not shown). Different values of Tel for HVC and RA measurements were because of different number of birds in each group. Juvenile birds used for HVC measurements had samples of $n = 5$ for Cort and $n = 4$ for control, and the juvenile birds used for RA measurements had $n = 4$ for Cort and $n=5$ for control. The sample sizes for adult birds used for HVC measurements were $n = 7$ for both Cort treated

and control groups and for RA measurements were n = 6 for Cort treated and n = 9 for control groups.

In order to get the volume of Tel containing HVC and RA, the volume of Tel Containing HVC and the volume of Tel containing RA in each hemisphere were average to get the volume of Tel containing HVC and RA for each hemisphere. Then, the volume of Tel (containing HVC and RA) were averaged across right and left hemispheres for each individual in the statistical analysis. Birds with only one intact hemisphere were included in the analysis if they were not significantly different than their group and average group. For example: control with R or L vs. control with R + L and Cort with R or L vs. Cort with R + L.

The HVC/Tel ratio tended to be significant ($F(1, 7) = 5.424$, $P = 0.054$; Table 4.2) between Cort and control juvenile birds. This finding may suggest that HVC is more susceptible to Cort effect during development than RA and Tel and the effect of Cort is specific to HVC. RA/Tel ratio was not significantly different between Cort treated and control juvenile birds ($F(1, 7) = 0.157$, $P = 0.704$; Table 4.2).

Cort treated adult male zebra finches had significantly smaller HVC volume than control birds ($F(1, 11) = 5.841$, $P = 0.034$; Table 4.2; Figures 4.4A and 4.4C; Figure 4.5A). Because there were no significant differences in the volume of HVC, Tel, and HVC/Tel ratio between No-Implant (n = 5) and Empty-implant birds (n = 2), both groups were combined as one control (No-Cort) group ($F(1, 5) = 1.801$, $P = 0.238$; $F(1, 5) = 1.236$, $P = 0.318$; $F(1, 5) = 0.003$, $P = 0.958$, respectively).

There were no significant differences in the volume of RA between Cort treated and control birds ($F(1, 12) = 0.042$, $P = 0.842$; Table 4.2; Figures 4.4B and 4.4D; Figure 4.5B). Because there were no significant differences in the volume of RA, Tel, and RA/Tel ratio between No-

Implanted (n = 6) and Empty-implanted birds (n = 3), both groups were combined as one control (No-Cort treated) group (F (1, 7) = 0.001, P = 0.972; F (1, 7) =1.309, P = 0.290; F (1, 7) = 0.812, P = 0.398, accordingly).

There were no significant differences in the volume of Tel containing HVC and RA between Cort and control adult birds (F (1, 11) = 0.061, P = 0.810 and F (1, 12) = 0.480, P = 0.502 for HVC and RA measurements, respectively; Table 4.2; Figure 4.5C). Figure 4.5C only shows the Tel containing HVC and RA from HVC measurements (for simplicity, the graph of the Tel containing HVC and RA from RA measurement are not shown).

The HVC/Tel ratio tended to be significant smaller compared with control adult birds (F (1, 11) = 4.483, P = 0.058; Table 4.2) between Cort treated and control adult birds. This finding may suggest that HVC is more susceptible to Cort effect during development than RA and Tel and the effect of Cort is specific to HVC. The RA/Tel ratio was not significantly different between Cort treated and control adult birds (F (1, 12) = 0.634, P = 0.442, Table 4.2).

4.3.3. Correlations between HVC volume (and HVC / Tel ratio) and song parameters in adult male zebra finches

There was a significant positive correlation between the HVC volume and song similarity in adult male zebra finches regardless of treatment (n = 10; r = 0.711, P = 0.021; Figure 4.6A). Moreover, the HVC /Tel ratio was significantly correlated to song similarity in adult male birds regardless of treatment (r = 0.832, P = 0.003; Figure 4.6B). Though, there were no significant correlations between HVC volume (and HVC /Tel ratio) and other song parameters.

4.4. Discussion

No significant asymmetry (or lateralization) was observed in the volume of HVC, RA, and Tel (containing HVC and RA) in juvenile and adult male zebra finches. Our finding is consistent with other reports of no significant right-left differences in the volume of HVC and RA (Gil et al., 2006). We tested for asymmetry of the HVC, RA and Tel volumes in order to use all the birds in our study because some of the brain tissue had damage to one hemisphere or damage to a specific area (HVC or RA).

We found that early Cort treatment reduced the size of HVC at both age groups. Furthermore, early Cort treatment tended to reduce the HVC / Tel ratio at both age groups. Early Cort treatment did not affect significantly the size of RA and Tel (containing HVC and RA). This result suggests that HVC is more susceptible to early Cort treatment than RA and Tel. Our results are consistent with previous reports that developmental (or nutritional) stress affects the size of HVC in zebra finches, song sparrows, and swamp sparrows (Buchanan et al., 2004; MacDonald et al., 2006; Nowicki et al., 2002). Furthermore, we demonstrated that early Cort treatment reduces the size of HVC in juvenile zebra finches and this reduction in the HVC volume appears to persist into adulthood. Previous studies on developmental stress demonstrated the effect of early stress on the size of song nuclei in adult birds (Buchanan et al., 2004; MacDonald et al., 2006; Nowicki et al., 2002).

The volumes of HVC and RA are genetically controlled across individual males within a species whereas size of other song nuclei has less control by genetic suggesting that they are more susceptible to developmental and environmental conditions (Airey et al., 2000). In addition, evolvability index, a statistical measure that predicts response to selection, is higher for HVC and its target RA in comparison with all other brain volume measured. This suggests that selection based on functions of these two nuclei structure give rise to changes in their anatomy

(Airey et al., 2000). Our study and previous developmental stress studies indicate that not only genetic but also environmental control are important in the development of the song control nuclei particularly HVC.

The developmental (or nutritional) stress hypothesis (Nowicki et al., 1998 and 2002; Buchanan et al., 2003) has been proposed to explain the evolution of song complexity (as learned features of song) as an indicator of male quality during mate choice by females. Song and male quality are linked because the brain structures involved in song learning develop mainly during the first few months post-hatching. Songbirds are prone to nutritional and other stressors during early life. Exposure to stress such as food-restriction during early life can compromise the development of the song control nuclei (Nowicki et al., 1998; Nowicki et al., 2002; Buchanan et al., 2003). Only individuals that can resist well during exposure to stress are able to devote adequate resources to the brain development necessary for song learning. Therefore, song complexity and song quality become reliable indicators of male quality and are maintained as such by the developmental costs of song (Nowicki et al., 1998; Nowicki et al., 2002; Buchanan et al., 2003). Previous studies suggested that developmental stress reduces the song complexity in swamp sparrows and European Starling (Nowicki et al., 2002; Buchanan et al., 2003). In addition, the effect of early food restriction on song complexity is associated with changes in the volume of the song nuclei in zebra finches, swamp sparrows, and song sparrows (Buchanan et al., 2004; Nowicki et al., 2002; MacDonald et al., 2006). Our study similarly demonstrated that early Cort treatment decreases song learning accuracy and selectively reduces HVC size. All these studies may explain the evolution of accuracy of learned song features as indicator of male quality.

Studies of developmental stress have not investigated the underlying mechanism of reduction of HVC size. In our study, we tested whether HVC volume is susceptible to early Cort

treatment in juvenile and adult male zebra finches. Cort may mediate the effects of early nutritional stress on song complexity and HVC size, however, previous reports failed to show increased levels of plasma Cort in food-restricted birds (Spencer et al., 2003). Our findings demonstrate that early Cort treatment differentially affects the HVC size.

We demonstrated (in Chapter 3) that Cort implanted adult birds had significantly higher baseline plasma Cort level than the control birds on days 1 and 2 and tended to have higher Cort on day 3. Baseline Cort levels were not significantly different on subsequent days. Therefore, Cort-treated birds in our study had higher plasma Cort concentration for at least few days during development. These results were consistent with those of previous studies using silastic Cort implants in adult song sparrows and Black-legged kittiwakes reporting that plasma Cort levels were higher on day1 and day 2 after implantation and plasma Cort levels were not significantly higher on subsequent days (Newman et al., 2010; Angelier et al., 2007). We found that early Cort treatment during development affects the size of HVC but not RA or Tel in both juvenile and adult zebra finches. This finding is consistent with the studies of the effect of developmental stress on the song control nuclei. Therefore, our result suggests that Cort may mediate the effects of developmental stress on the brain song nuclei.

Our previous work (Shahbazi et al., 2011; Chapter 2) demonstrated that glucocorticoid receptors (GR) are present in the song nuclei and telencephalon (other regions of Tel in addition to the song nuclei) of P10 and adult birds. Other studies reported that Cort mediate the adverse effect of stress through GR (Sapolsky, 2000 and 2001; Krugers et al., 2006). Thus, our results suggest that early Cort treatment in juvenile and adult birds may reduce the size of HVC through activation of GR. The reports of seasonal regulation of GR in the brain of house sparrows in ad-

dition to seasonal regulation of the adrenocorticoid response to stress may support this suggestion (Breuner and Orchinik, 2001), though zebra finches are not seasonal.

Estrogen receptors (ER) and possibly androgen receptors may be involved in the effect of early Cort treatment on the size of song nucleus HVC for several reasons. First, ER play an important role in masculinization of the brain. Second, ER are expressed in HVC neurons. Finally, number of the ER neurons changes during ontogeny and decreases after day 40 (Gahr and Konishi 1988; Gahr 1996).

Oxidative stress process may play a role in the reduction of HVC size observed in Cort-treated birds in our study. Oxidative stress is production of free radicals (atoms or molecules) that can damage critical molecules and physiological processes including DNA, proteins, lipids, and cell membranes. Steroid hormones suppress the immune response and result in oxidative stress. Glucocorticoids (such as Cort and cortisol), testosterone, and progesterone are known to impair enzymatic antioxidant defenses and to directly induce oxidative stress in tissues (von Schantz et al., 1999). Therefore, Cort may affect the size of HVC by reducing the number of cells within the HVC through the process of oxidative stress.

We can speculate that the activation of GR in the song nuclei may activate gene transcription which may result in the induction of oxidative stress processes and decrease the number of cells within HVC. In our study, however, it is unknown whether the reduction of HVC volume in Cort treated birds is due to a reduction in the number of neurons (or glial cells) or in the size of the cells within HVC. Testing whether GR (or MR) are necessary for Cort-induced reduction of HVC may help us to understand the mechanism underlying the effect of Cort on the HVC size.

We demonstrated that the song control nucleus HVC is more vulnerable to the effect of early Cort treatment than are RA and Tel in both juvenile and adult male zebra finches. Our study provides important information to support the developmental stress hypothesis and emphasizes the developmental plasticity of the zebra finch brain. Furthermore, our findings highlight that environmental factors in addition to genetic factors can affect the development of the song nuclei, primarily HVC.

Moreover, we found that there is a significant correlation between the volume of HVC and song similarity in adult male zebra finches regardless of treatment. This finding indicates that, even without considering treatment, adult male zebra finches with a larger HVC volume have higher song similarity. This effect is not because of larger Tel but because of a larger HVC, given that the HVC / Tel ratio is correlated with song similarity as well. Furthermore, no correlations were observed between HVC volume and song accuracy, sequential match, or total score, suggesting that the effect is specific to song similarity.

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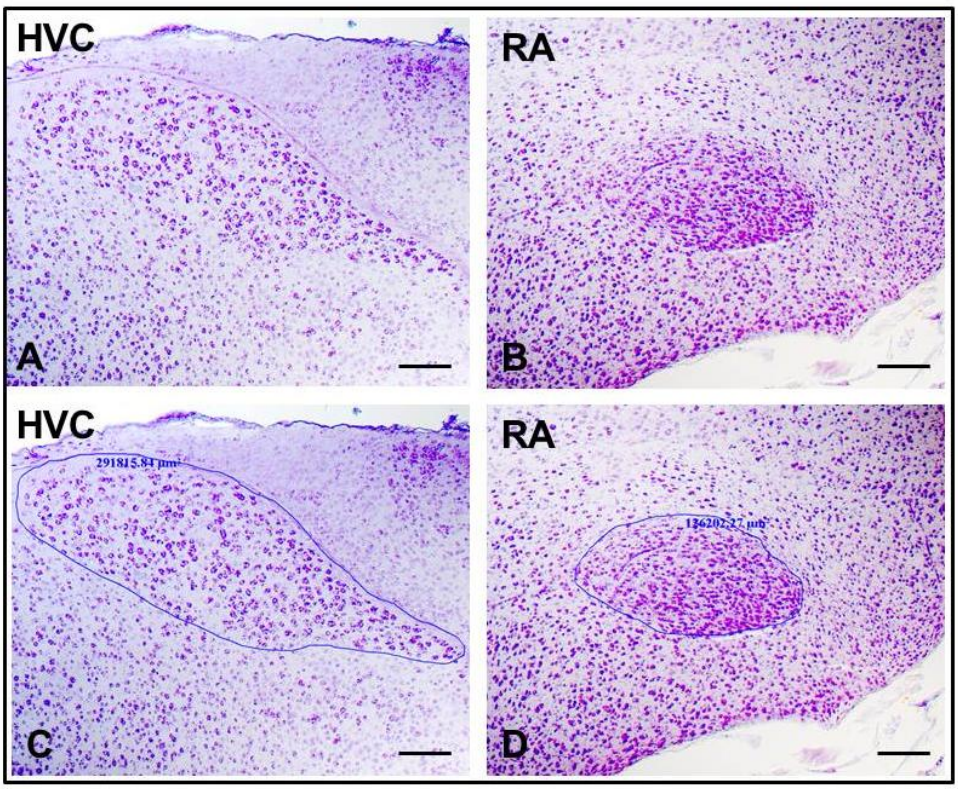

Figure 4.1. Photomicrographs of HVC (a proper name) and robust nucleus of the arcopallium (RA) in the brain of juvenile (P30) male zebra finch using Nissl stain. **A** and **B** show HVC and RA of the juvenile male brain, respectively. **C** and **D** show how the area of HVC and RA were measured, respectively. Scale bar = 100 µm in **A**, **B**, **C** and **D**.

Table 4.1.A Lack of lateralization of HVC (a proper name), robust nucleus of the arcopallium (RA), and telencephalon (Tel) of juvenile and adult male zebra finch brain. Lack of size asymmetry for HVC, RA and Tel in the brain of all juvenile (P30) and adult male zebra finches (P30: n = 7 for right HVC, n = 9 for left HVC and n = 5 for right RA, n = 9 for left RA; adult: n = 12 for right and left HVC, n = 14 for right and left RA). There were no significant differences between right and left HVC, RA and Tel (containing song nuclei) of juvenile and adult male zebra finches.

Brain region	All birds (control and Cort treated birds)
Juvenile (P30)	
HVC volume	U = 29.000, Z = -0.265, P = 0.792
Tel volume	U = 23.000, Z = -0.900, P = 0.368
HVC/Tel ratio	U = 31.000, Z = -0.053, P = 0.958
RA volume	U = 18.500, Z = -0.534, P = 0.594
Tel volume	U = 20.500, Z = -0.267, P = 0.790
RA/Tel ratio	U = 18.000, Z = -0.600, P = 0.550
Adult	
HVC volume	U = 60.500, Z = -0.664, P = 0.508
Tel volume	U = 64.000, Z = -0.462, P = 0.644
HVC/Tel ratio	U = 66.000, Z = -0.346, P = 0.730
RA volume	U = 86.500, Z = -0.529, P = 0.598
Tel volume	U = 79.000, Z = -0.873, P = 0.384
RA/Tel ratio	U = 91.000, Z = -0.322, P = 0.748

Table 4.1.B Lack of lateralization of HVC (a proper name), robust nucleus of the arcopallium (RA), and telencephalon (Tel) of control (No-Cort treated) juvenile and adult male zebra finch brain. Lack of size asymmetry for HVC, RA, and Tel in the brain of control juvenile (P30) and adult male zebra finches (P30: n=3 for right HVC, n = 4 for left HVC and n = 2 for right RA and n = 5 for left RA; adult: n = 5 for right HVC, n = 6 for left HVC and n = 8 for right and left RA). There were no significant differences between right and left HVC, RA and Tel (containing song nuclei) of juvenile and adult male zebra finches.

Brain region	Control (No-Cort treated) birds
Juvenile (P30)	
HVC volume	U = 5.000, Z = -0.357, P = 0.722
Tel volume	U = 3.000, Z = -1.061, P = 0.290
HVC/Tel ratio	U = 4.000, Z = -0.707, P = 0.480
RA volume	U = 4.500, Z = -0.195, P = 0.846
Tel volume	U = 4.000, Z = -0.387, P = 0.700
RA/Tel ratio	U = 4.000, Z = -0.387, P = 0.700
Adult	
HVC volume	U = 14.000, Z = -0.183, P = 0.856
Tel volume	U = 14.000, Z = -0.183, P = 0.860
HVC/Tel ratio	U = 11.000, Z = -0.730, P = 0.466
RA volume	U = 28.000, Z = -0.421, P = 0.674
Tel volume	U = 28.000, Z = -0.420, P = 0.674
RA/Tel ratio	U = 30.000, Z = -0.210, P = 0.834

Table 4.1.C Lack of lateralization of HVC (a proper name), robust nucleus of the arcopallium (RA), and telencephalon (Tel) of juvenile and adult Cort-treated male zebra finch brain. Lack of size asymmetry for HVC, RA, and Tel in the brain of Cort treated juvenile (P30) and adult male zebra finches (juvenile: n = 4 for right HVC, n = 5 for left HVC and n = 3 for right RA, n = 4 for left RA; adult: n = 7 for right HVC, n = 6 for left HVC and n = 6 for right and left RA). There were no significant differences between right and left HVC, RA and Tel (containing song nuclei) of juvenile and adult male zebra finches.

Brain region	Cort treated birds
Juvenile (P30)	
HVC volume	U = 7.000, Z = -0.735, P = 0.462
Tel volume	U = 7.000, Z = -0.735, P = 0.462
HVC/Tel ratio	U = 9.000, Z = -0.245, P = 0.806
RA volume	U = 4.000, Z = -0.707, P = 0.480
Tel volume	U = 6.000, Z = 0.000, P = 1.000
RA/Tel ratio	U = 4.000, Z = -0.707, P = 0.480
Adult	
HVC volume	U = 14.000, Z = -1.000, P = 0.318
Tel volume	U = 16.000, Z = -0.714, P = 0.476
HVC/Tel ratio	U = 18.000, Z = -0.429, P = 0.668
RA volume	U = 16.000, Z = -0.320, P = 0.750
Tel volume	U = 10.000, Z = -1.281, P = 0.200
RA/Tel ratio	U = 16.000, Z = -0.320, P = 0.750

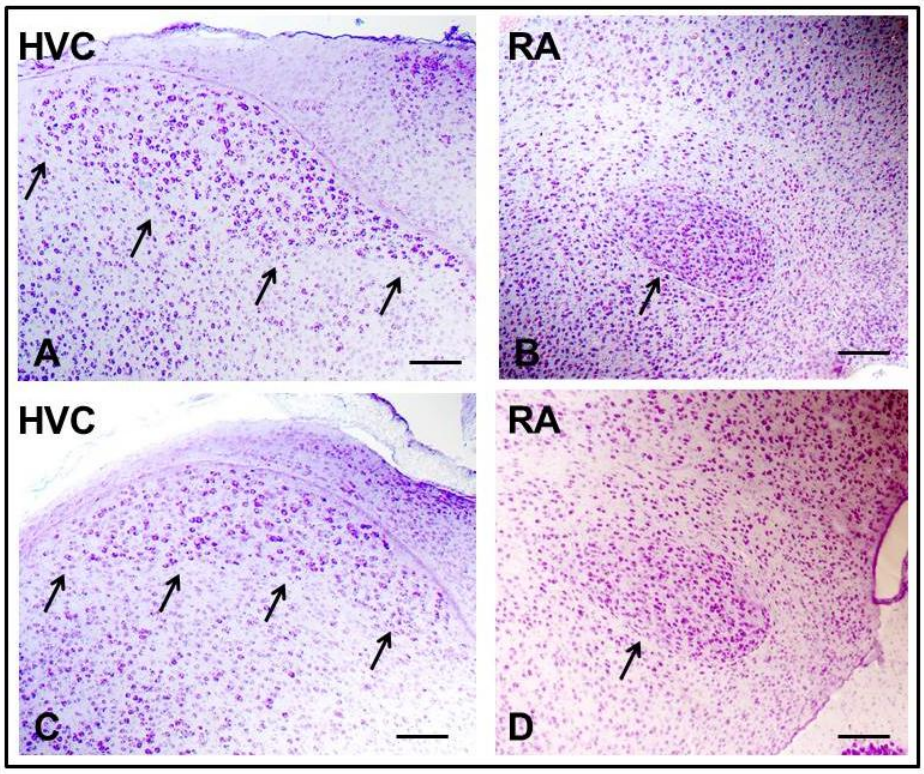

Figure 4.2. Photomicrographs of HVC (a proper name) and robust nucleus of the arcopallium (RA) in the brain of Cort treated and control juvenile (P30) male zebra finch using Nissl stain. **A** and **B** show HVC and RA, respectively, of a control (No-Cort treated) juvenile male. **C** and **D** show HVC and RA, respectively, of a Cort treated juvenile male. **A** and **C**: The dorsal edge of HVC is well-defined by the lateral ventricle and the ventral edge is shown by arrows. **B** and **D**: The arrow points to RA. Scale bar = 100 µm in **A**, **B**, **C** and **D**.

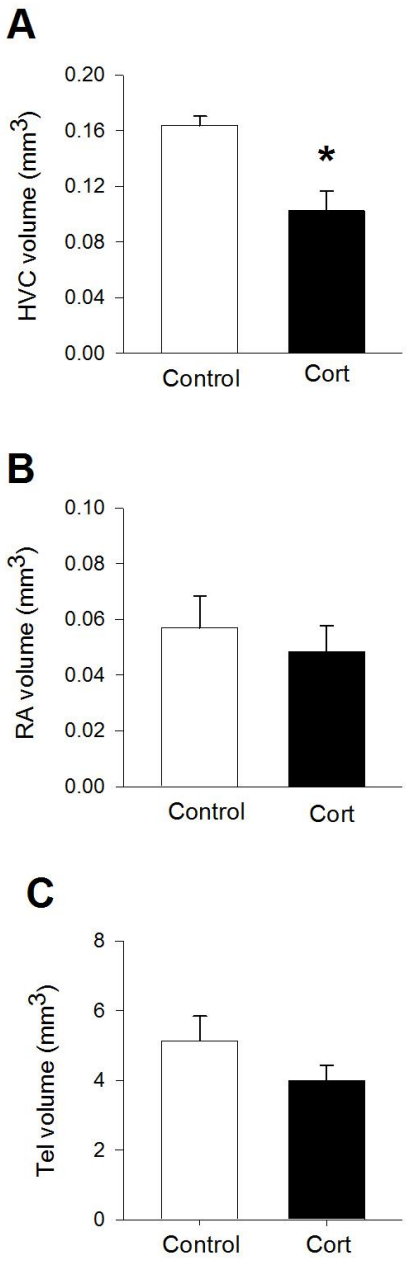

Figure 4.3. Effects of early corticosterone (Cort) treatment on volume of brain areas in juvenile (P30) male zebra finches. **A**. HVC (a proper name): one-way ANOVA $F(1, 7) = 12.619$, $^*P = 0.01$; **B**. Robust nucleus of the arcopallium (RA): one-way ANOVA $F(1, 7) = 0.309$, $P = 0.596$; **C.** Telencephalon (Tel): one-way ANOVA $F(1, 7) = 2.062$, $P = 0.194$. n = 5 Cort- treated and n = 4 control (No-Cort treated) birds. Volume data were averaged across left and right hemispheres for each individual in the statistical analysis. Data represent mean values ± SEM. Asterisks highlight significant differences between groups ($^*P < 0.05$).

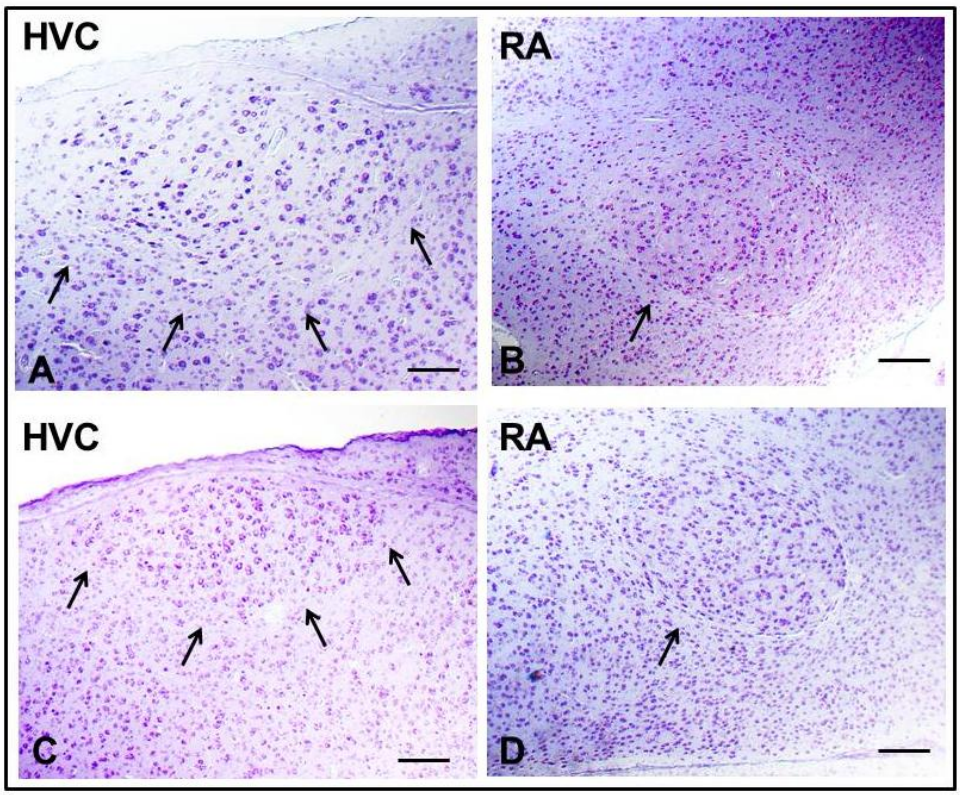

Figure 4.4. Photomicrographs of HVC (a proper name) and robust nucleus of the arcopallium (RA) in the brain of Cort treated and control adult male zebra finch using Nissl stain. **A** and **B** show HVC and RA, respectively, of a control (No-Cort treated) adult male. **C** and **D** show HVC and RA, respectively, of a Cort treated adult male. **A** and **C**: The dorsal edge of HVC is well-defined by the lateral ventricle and the ventral edge is shown by arrows. **B** and **D**: The arrow points to RA. Scale bar = 100 µm in **A**, **B**, **C** and **D**.

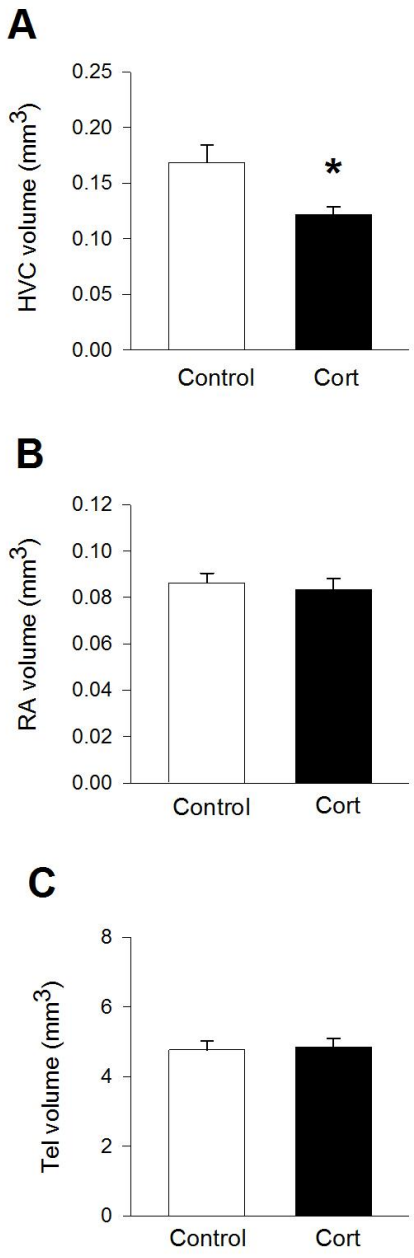

Figure 4.5. Effects of early corticosterone (Cort) treatment on volume of brain areas in adult male zebra finches. **A.** HVC (a proper name): one-way ANOVA $F(1, 11) = 5.841$, $P = 0.034$; **B.** Robust nucleus of the arcopallium (RA): one-way ANOVA $F(1, 12) = 0.042$, $P = 0.842$; **C.** Telencephalon (Tel): one-way ANOVA $F(1, 11) = 0.061$, $P = 0.810$. n = 6 Cort-treated birds for HVC, RA and Tel. n = 7 control (No-Cort-treated) birds for HVC and Tel. n = 8 control birds for RA. Volume data were averaged across left and right hemispheres for each individual in the statistical analysis. Data represent mean values ± SEM. Asterisks highlight significant differences between groups (*$P < 0.05$).

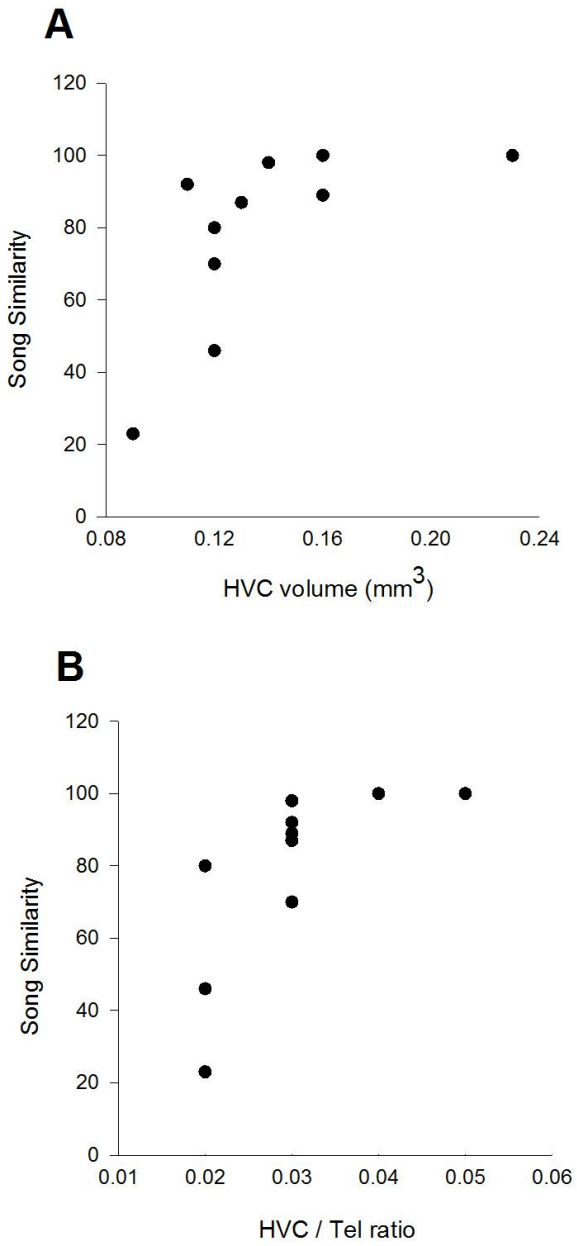

Figure 4.6. Correlation between HVC size, HVC / Tel ratio, and song similarity in adult male zebra finches regardless of treatment group. **A.** The volume of HVC (a proper name) was significantly correlated to song similarity: Spearman rho, r = 0.711, P = 0.021. **B.** HVC / Telencephalon (HVC / Tel) ratio was also significantly correlated to song similarity: Spearman rho, r = 0.832, P = 0.003. Volume data were averaged across left and right hemispheres for each individual in the statistical analysis. Tel is containing the song nuclei. n =10 for the analysis.

Table 4.2. Effects of the early corticosterone (Cort) treatment on HVC (a proper name), Robust nucleus of the arcopallium (RA) and telencephalon (Tel) volumes of juvenile and adult male zebra finch brain. Birds of the Cort group were treated with corticosterone (Cort) through silastic implants around day 4 (P4±1). The control (No-Cort treated) group received either empty implants at the same time or no implants. HVC (a proper name), Robust nucleus of the arcopallium (RA), and telencephalon (Tel; contain song nuclei) volumes were measured in the brain of juvenile (P30) and adult male zebra finches. n = 5 for juvenile and n = 6 for adults for Cort-treated birds. n = 4 for juvenile and n = 8 for adults for control birds (see methods for details). Data represent mean values ± SEM. Asterisks highlight significant differences between groups (*P < 0.05).

Brain region	Control (No-Cort treated) vs. Cort treated birds
Juvenile (P30)	
HVC volume	F(1, 7) = 12.619, P = 0.01*
Tel volume	F(1, 7) = 2.062, P = 0.194
HVC/Tel ratio	F(1, 7) = 5.424, P = 0.054
RA volume	F(1, 7) = 0.309, P = 0.596
Tel volume	F(1, 7) = 0.154, P = 0.706
RA/Tel ratio	F(1, 7) = 0.157, P = 0.704
Adult	
HVC volume	F(1, 11) = 5.841, P = 0.034*
Tel volume	F(1, 11) = 0.061, P = 0.810
HVC/Tel ratio	F(1, 11) = 4.483, P = 0.058
RA volume	F(1, 12) = 0.042, P = 0.842
Tel volume	F(1, 12) = 0.480, P = 0.502
RA/Tel ratio	F(1, 12) = 0.634, P = 0.442

CHAPTER 5: GENERAL DISCUSSION

This series of studies addressed the effects of early corticosterone (Cort) treatment on several levels of analysis, from circulating hormone levels (plasma Cort concentration), behavioral analysis (song learning accuracy) to neuroanatomical analysis (neuronal glucocorticoid receptor distribution and size of song control nuclei).

5.1. Glucocorticoid Receptor distributions and subcellular localizations

Exposure to chronic stress or elevated Cort in animals results in hippocampal atrophy and cognitive dysfunction and glucocorticoid receptors (GR) mediate the adverse effects of glucocorticoids (such as Cort and cortisol) on hippocampal functioning during prolonged stress (Ferguson and Sapolsky, 2007; Krugers et al., 2006). Early developmental stress in male zebra finches (*Taeniopygia guttata*) decreases HVC size and song complexity, suggesting a direct link between brain and behavior. HVC is required for learning and production of song and song complexity is important for mate choice. Although the mechanisms behind the effect of stress on brain and behavior are unknown, we have demonstrated that the elevated levels of Cort (at least for few days) and/or changes in the sensitivity of GR may play a significant role.

Chapter 2 addressed the question whether GR are distributed across the development of the zebra finch song control nuclei. The presence of these receptors in the regions of the brain where the song control nuclei develop or in the song nuclei themselves indicates that these brain regions are likely to be stress (and/or Cort) sensitive. The results of the Chapter 2 demonstrated that GR immunoreactive (GR-ir) neurons were present in zebra finch brain in regions homologous to regions of the mammalian, quail, frog, and fish brain where GR-ir neurons are distributed

(Ahima and Harlan, 1990; Carruth et al., 2000; Kovacs et al., 1989; van Eekelen, 1987; Yao et al., 2008). Furthermore, GR-ir neurons were present in two song control nuclei, HVC and RA (robust nucleus of the arcopallium).

Distribution of GR-ir neurons did not vary at either age point, P10 or adult, however there were differences in labeling intensity. Furthermore, there were significant differences in total GR-ir neuronal numbers and number of neurons with both nuclear and cytoplasmic GR immunoreactivity between the HVC representative section of P10 and adult. These findings are supported by the reports of HVC size reduction and subsequent decrease in song quality in developmentally stressed zebra finches (Buchanan et al., 2004; MacDonald et al., 2006; Nowicki et al., 2002) and the fact that HVC is required for song learning and production. Though, there were no significant differences in total number of GR-ir neurons and number of neurons with both nuclear and cytoplasmic GR immunoreactivity in the RA and TeO representative sections of P10 and adult. These findings suggest GR within HVC may play a role in mediating the effects of developmental stress (via Cort) on HVC size and song quality in male zebra finches.

A main concern in GR distribution study was antibody specificity. In our study, in most areas of the zebra finch brain, there were mainly two classifications of staining reactivity. One group of neurons had both cytoplasmic and nuclear GR immunoreactivity and the other group of neurons had only cytoplasmic GR immunoreactivity (i.e., HVC of P10 and adult had about 50% of each). GR-ir neurons widely distributed in rat brain with mostly nuclear or both nuclear and cytoplasmic staining (Ahima and Harlan, 1990). Most quail brain areas showed weak to moderate GR immunoreactivity in nuclei of neurons using a mouse monoclonal antibody (Mab49/1; Kovacs et al., 1989). The labeling intensity of GR-ir neurons reported in these studies is not consistent with what we report in our study. Inconsistency in the protein expression patterns in pub-

lished results may be explained by a combination of the type of antibody, epitope accessibility, type of fixative, fixative delivery procedures, and local cellular context may explain inconsistency in the protein expression patterns in published results (Sarabdjitsingh, et al., 2010). Two polyclonal primary antibodies (PA1-510A and PA1-511A-both peptides from human GR) were used in our study. Both antibodies gave the same pattern of GR immunoreactivity in Bouin's-fixed tissue in DAB-immunohistochemisty (IHC, which was used for GR distribution, labeling intensity and neuronal cell count in zebra finch brain). Despite the low percentage identity between the human GR and zebra finch, there is some evidence to suggest that the immunoreactive protein being visualized here is, in fact, GR. First, the predicted GR for zebra finch is the only protein sequence available for BLAST, and it is a "predicted" sequence based on zebra finch genome and not based on GR protein itself. Second, there is higher percentage identity between the region of the human GR (a region including 22aa which PA1-511A was raised against) and the same region of the predicted zebra finch GR (82% identity), and because these two antibodies gave the same pattern of immunoreactivity in Bouin's-fixed tissue in DAB-IHC, therefore they both likely recognize the same immunoreactive protein. Third, the human GR sequences that PA1-510A and PA1-511A antibodies were raised against are present in rat GR with high identity (88% and 95%, respectively), and rat hippocampus was used as a positive control in the current study. Fourth, Western blotting with PA1-510A determined that a protein with a molecular weight similar to the mammalian GR (≈97 KDa) is present in the zebra finch brain. Finally, control experiments including the omission of primary antibody PA1-510A and preabsorption of PA1-511A with GR antigens resulted in a loss of GR immunoreactivity.

One of the future directions that we can follow based on our findings is whether mineralocorticoid receptors (MR) are distributed across the development of the zebra finch song nuclei.

MR regulate the basal circadian rhythm of Cort level. In contrast to GR, MR have high affinity for Cort and are heavily occupied and activated at basal conditions (Breen et al., 2004). The function of MR is important for the sensitivity and feedback of neuroendocrine responses. According to the MR / GR balance hypothesis (de Kloet, 1991), an increase in the amount of MR relative to GR will result in a reduced responsiveness of the HPA axis to stress (Oitzl et al., 1995). The increased activation of the HPA axis in many stress-related disorders is due to a MR / GR imbalance (Bao et al., 2007). Another direction to follow is whether early Cort treatment affects GR and MR distribution in brain song nuclei of male zebra finches.

5.2. Circulating hormone level and hormone delivery method

In order to study the effect of early Cort treatment on the development of the avian song system, male zebra finches were implanted at P4 (4 days after hatch) and their plasma Cort concentration was measured as juveniles 4 weeks after implantation (around P30; 30 days after hatch). Plasma Cort concentration was suppressed in Cort-treated birds. Our previous findings with adult birds and others (Newman et al., 2010; Angelier et al., 2007) suggest that Cort-filled silastic implants inserted in zebra finches on P4 release Cort for one day (day 5 after hatch) at least, if not for longer during the critical period of song nuclei formation (which is around P1-P10). Moreover, we observed a main effect of Cort exposure during the development on adult behavior (song quality) and brain (HVC size). It seems that Cort exposure (at least for one day) during the critical period is sufficient to significantly reduce song similarity and HVC size. Likewise, other studies have shown that treatment with Cort-filled silastic implants reduces HVC volume and neuron numbers in HVC of adult male song sparrows (Newman et al., 2010), decreases osteocalcin and induces bone loss in mice (Herrmann et al., 2009). In these reports,

baseline plasma Cort level was significantly higher in the Cort group than in control group on day 1 and 2 after implant insertions.

This transient increase in plasma Cort concentration in response to Cort treatment (Cort implants) in our study, and some avian studies, is similar to that reported in rodents (Herrmann et al., 2009; Meyer et al., 1979). One of the challenges in this area is to maintain consistent elevated level of Cort for at least 3-4 weeks to mimic chronic stress (Herrmann et al., 2009). Three drug delivery methods for consistent delivery of Cort including subcutaneous injection of Cort (which increased Cort level only for a few hours depending on dose), implantation of micro-osmotic slow release pump (which did not result in elevated level of plasma Cort), and implantation of slow-release pellets (which increased Cort level only within 7 days) were compared and none of these delivery methods provided consistent elevated plasma Cort level (Herrmann et al., 2009). Using Cort pellets in rats (Meyer et al., 1979) resulted in an increased plasma Cort concentration for about one week with the highest plasma Cort level in the first 3 days.

Our findings and those of others do not explain the temporal dynamic of Cort release (or exposure) from Cort-filled silastic implants and it remains to be addressed. At this point, we are not sure whether our implants provided Cort exposure for only one day, two days, one week or more. It is possible that Cort-implants did not stop releasing Cort, but Cort was metabolized and excreted (increased clearance) through the kidney or accumulated in other tissues. It is likely that endogenous Cort secretion was also suppressed. Chronic stress in free-living European starlings reduces plasma Cort concentration and reproductive success (Cyr and Romero, 2007). Although it is possible that *in vivo*, the Cort implants stopped releasing Cort due to encapsulation and isolation from blood vessels. This is unlikely because when 3 groups of adult song sparrows were implanted, Cort-implanted, DHEA-implanted and birds with both Cort and DHEA implants (ad-

jacent to each other), plasma DHEA levels were high throughout the treatment period (28 days) whereas plasma Cort levels were higher only for the first three days (Newman et al., 2010). These findings suggest that encapsulation of DHEA-implants is unlikely. Therefore, increased Cort level may not be a reliable marker of chronic stress and other phenomena such as context - related behavior, metabolites excretion rate, and levels of steroids in gonadal tissue (or other tissues) may be better markers of chronic stress and it needs to be revisited.

5.3. Early Cort treatment and song quality

Behavioral analysis using zebra finches as a model addressed whether early Cort treatment reduces song quality and song learning accuracy in adult male zebra finches (Chapter 3). Studies of early developmental stress (including food restriction and manipulation of brood size) are reflected in adult song and the song parameters affected by developmental stress vary from study to study (Spencer et al. 2003; Zann and Cash, 2008; Brumm et al., 2009; Gill et al., 2006; Holveck et al., 2008). Like the majority of studies of early developmental stress in zebra finches, we showed that song complexity measured as song duration or repertoire size (or number of syllables in zebra finch song) is not altered by early Cort treatment. Nevertheless, only one study showed differing results in song complexity indicating that early food restricted zebra finches had song with lower number of syllables (Spencer et al., 2003).

We found that early Cort treatment lead to lower similarity and total score between tutor (father) and tutee (son) in adult male zebra finches from FCs and IBC2. This result is consistent with previous studies (Brumm et al., 2009) indicating lower song similarity scores in early nutritional stressed birds. Though, early Cort treatment did not alter song similarity, accuracy, and total score between tutor and tutee in adult male birds from other IBCs. The FC housing condi-

tions differed from the IBC housing condition. Each FC contained approximately 40-50 mixed-sex and mix-aged birds (each pair with an average of 3-4 nestlings), whereas IBC #2 contained a single pair-bond with seven nestlings and the other IBCs contained a single pair-bond with an average of 3 nestlings. Developmental stress studies using brood size manipulation approach in zebra finches indicated that birds from larger broods had lower syntax learning accuracy and consistency than birds from smaller broods (Holveck et al., 2008). Although the Cort treated birds with the largest brood size (IBC #2) in our study had lower similarity and accuracy, the order of the syllables (sequential match) was not affected by either treatment or housing condition. By contrast, another study of brood size manipulation in zebra finches showed that early nestling condition does not affect song learning scores and song characteristics (Gill et al., 2006).

Thus, the different results between FCs (small-medium brood size), IBC #2 (large brood size) and IBCs (small-medium brood size) regarding song quality may be explained by the fact that early condition in particular social-context can affect song development. Early condition may affect male's social status and therefore may in turn affect his exposure to singers (Holveck et al., 2008). In our aviary, especially for birds from FCs, it is possible that dominance relationships differed among the experimental birds. Moreover, the tutor's song rate, song consistency, level of song difficulty (easier song vs. more difficult song) and the number of males in tutoring groups may affect song quality. For example, it is possible that the learned features of the model song (tutor song) available in the IBC #2 or FCs were more difficult to learn or produce than the tutor song in the other IBCs or that birds from IBC #2 had a choice of the model songs to copy. We did not control for social status, tutor's song rate, tutor's song consistency, difficulty level of tutor's song, and number of males in the tutoring group. Therefore, all these factors during early

development (nestling and fledgling) may alter the effects of early Cort treatment on song quality of male zebra finches.

In conclusion, our findings confirm that Cort may mediate the adverse effects of developmental stress (such as food restriction and brood size manipulation) on song learning accuracy (or song similarity between tutor and tutee) but not song complexity. We found that early Cort treatment affects song similarity and total score and results in poorer copies of father (tutor) song. Although song similarity, song accuracy, and total score were not affected in the Cort treated birds from IBCs, other aspects of song features were affected such as average pitch and total number of syllables. Early Cort treatment affected these parameters in all birds from IBCs (including IBC #2) the same way. Cort treatment may contribute to lower quality song. Early Cort treatment did not alter mean amplitude, song duration or repertoire size. Our study supports developmental stress hypothesis and emphasizing that song learning accuracy but not song complexity can be an indicator of male quality and past condition. How female zebra finches detect these changes in quality and accuracy of learned song features and whether they use this information to evaluate the early condition of a male remains to be understood.

5.4. Early Cort treatment and HVC size

We found that early Cort treatment reduces the size of HVC in both juveniles and adults. Early Cort treatment did not significantly affect the size of RA and Tel (telencephalon containing HVC and RA). This suggests that HVC is more susceptible to early Cort treatment compared with RA and Tel. Our results are consistent with reports that developmental (or nutritional) stress affects the size of HVC in zebra finch, song sparrows, and swamp sparrows (Buchanan et al., 2004; MacDonald et al., 2006; Nowicki et al., 2002). Furthermore, we demonstrated for the first

time, to our knowledge, that early Cort treatment reduces the size of HVC in juvenile zebra finches and this reduction in the HVC volume appears to persist to adulthood. Previous studies of developmental stress demonstrated the effect of early stress on the size of song nuclei in only adult birds (Buchanan et al., 2004; MacDonald et al., 2006; Nowicki et al., 2002).

Avian studies of the effect of developmental stress on HVC size and song quality did not investigate the underlying mechanism of reduction of HVC size. In our study, we investigated whether HVC volume is susceptible to early Cort treatment in juvenile and adult male zebra finches. Cort may mediate the effects of early nutritional stress on song quality and HVC size. Previous reports, however, failed to show increased level of plasma Cort in food restricted zebra finches (Spencer et al., 2003).

In addition to these findings, we found a positive correlation between the volume of HVC and song similarity in adult male zebra finches regardless of treatment. This result indicates that even without considering treatment, adult male zebra finches with larger HVC volume had higher song similarity and because the HVC / Tel ratio is also correlated positively with song similarity, this effect is not because of larger Tel. Furthermore, no correlation was observed between HVC volume and song accuracy, sequential match, and total score suggesting that this effect is specific to song similarity. Our finding is consistent with previous studies indicating a positive correlation between HVC volume and song complexity in songbirds (Airey et al., 2000; Garameszegi and Eens 2004; DeVoogd et al., 1993), however, in our study we observed a positive correlation between HVC volume and song similarity only, not song complexity. Together, these findings suggest that HVC volume plays a crucial role in song quality and learned featured of song in adult male zebra finches.

5.5. Conclusions

In summary, Chapter 2 demonstrated that GR are present in the song nuclei and telencephalon (Tel) of P10 and adult birds. The total number of GR-ir neurons (per representative section) was higher in adult birds than in P10 birds in HVC but not RA and TeO suggesting a potential role for GR within HVC in stress-induced reduction of HVC size and song quality. Chapter 3 showed that by using Cort implants (that mimics the response to a stressor) plasma Cort concentration can be elevated for at least one day during development in Cort treated birds. Plasma Cort concentration then declined as in previous report (Newman et al., 2010; Angelier et al., 2007). Furthermore, Chapter 3 demonstrated that early Cort treatment reduced song similarity and total score which is consistent with previous studies of developmental stress on song quality (Brumm et al., 2009; Holveck et al., 2008). Chapter 4 showed that early Cort reduced the size of HVC, indicating that the effect of early Cort treatment is specific to HVC and not RA or Tel. This finding is consistent with reports of the effect of developmental stress on HVC size (Buchanan et al., 2004; MacDonal et al., 2006; Nowicki et al., 2002). Moreover, because HVC is necessary for learning and production of song and Cort mediate the adverse effect of stress through glucocorticoid receptors (Sapolsky, 2000 and 2001; Krugers et al., 2006). Thus, this result suggests that early Cort treatment in juvenile and adult birds may drive the effect of early developmental stress on the size of HVC and song learning accuracy through activation of GR within HVC despite the fact that plasma Cort concentration was not elevated after first few days.

We can speculate that the activation of GR in the song nuclei may activate gene transcription which may induce oxidative stress processes, decreasing the number of cells within the HVC and in turn causing poorer copying of the tutor song. finally the smaller volume of HVC and poorer song copies of the tutor song in Cort treated birds. Although in our study, it is un-

known that the reduction of HVC volume in Cort treated birds is due to reduction in the number of cells or reduction in the size of the cells within the HVC.

In seasonally breeding songbirds such as canaries and song sparrows HVC volume and number of neurons fluctuates seasonally. Canaries learn new songs in adulthood (Nottebohm et al., 1986) and song sparrows do not learn new songs in adulthood but their songs become variable (Smith et al., 1997). In zebra finches, new neurons are added to HVC of adult birds (Walton et al., 2012), contrary to seasonal songbirds, these neurons are not part of neuronal replacement process. Zebra finches are different from canaries and song sparrows and they learn their song once during development (Immelmann, 1969) and their song remains the same. Neuron additions in zebra finches result in increased number of neurons that project from HVC to RA, in this process HVC volume remains the same and the packing density of neurons increases. These HVC-RA projecting neurons are important for the pattern of learned song features. In addition, increased number of these projecting neurons is dependent on their social context (this effect is more acute in socially housed zebra finches; Walton et al., 2012). Therefore, early Cort treatment in our study may affect number of neurons projecting from HVC-RA and resulting in lower song learning accuracy (in FCs and IBC#2 but not in IBCs) and reduction of HVC volume in adult male zebra finches.

We demonstrated decreasing HVC size, reducing song learning accuracy (song similarity and total score) and altered blood Cort concentration in early Cort treated birds in a single study. Taken together, the results from chapters 2, 3, and 4 suggest a potential role of Cort in mediating adverse effects of developmental stress on HVC size and song quality in adult male zebra finches. Our results clearly support the developmental stress hypothesis and emphasize the developmental plasticity of the zebra finch brain.

There are several directions that we can follow based on our findings. The next step is to investigate whether GR are necessary for Cort-induced reduction of HVC size and song learning accuracy and whether this effect is specific to cytosolic GR and independent of MR. If GR are necessary and specific for Cort-induced reduction of HVC size and song learning accuracy, then we will investigate the potential roles of corticotropin-releasing hormone (CRH), vasopressin (VP), adrenocorticotropin (ACTH) and other hormones in stress-induced reduction of HVC size and song learning accuracy to test sufficiency of GR in early stress-induced reduction of HVC size and song learning accuracy. Furthermore, we will investigate potential cellular mechanisms involved in Cort-mediated regulation of HVC size and song learning accuracy.

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APPENDICES

Appendix A: Validation of glucocorticoid receptor antibodies

Table A.1. Validation of glucocorticoid receptor (GR) antibodies. The table demonstrates the results of validation of glucocorticoid receptor (GR) antibodies. Two primary polyclonal antibodies (1°Ab); PA1-510A and PA1-511A) and one primary monoclonal antibody were used (with different dilutions) and different antigen retrieval solutions tested. Rat and zebra finch brain tissue were used in two different fixatives including paraformaldehyde (PFA) fixed, gelatin embedded tissue and Bouin's fixed, paraffin embedded tissue. DAB-immunohistochemistry (DAB-IHC) and fluorescence immunohistochemistry (F-IHC), and western blot were performed. Abbreviations for the table are: C = cytoplasmic GR, N = nuclear GR, C&R = cytoplasmic and nuclear GR, 1°Ab = primary antibody, 2°Ab = secondary antibody, BSA = bovine serum albumin and NGS = normal goat serum.

	PA1-510A	**PA1-511A**	**Monoclonal**
F-IHC, 4% PFA (Adult-Male-ZF)	Mostly C (some w both N&C)	NO	N (mostly)
F-IHC, Bouin (Adult- Male-F)	C	C (not much staining)	(not staining)
F-IHC, 4% PFA (Adult-Male-at)	C	C	N
Embedding	Gelatin	Gelatin	Gelatin
Vibratome sections	50µm	50µm	50µm
Dilution (1° Ab)	1:2000	1:2000	1:1000
2° Ab	CY3-goat anti-rabbit IgG	CY3-goat anti-rabbit IgG	CY3-goat anti-rabbit IgG
Dilution (2° Ab)	1:400	1:400	1:400
Blocking agent	3% BSA+5% NGS	3% BSA+5% NGS	3% BSA+5% NGS
Antigen retrieval	Hot citrate buffer and/or Trypsin	Hot citrate buffer and/or Trypsin	Hot citrate buffer and/or Trypsin
DAB-ICC (PFA-rat)	C	No staining	N
DAB-ICC (PFA-ZF)	C (C&N)	No staining	N (some C)
DAB-ICC (Bouin-ZF)	C	C	C
Western blot	Positive, band of appropriate size	No band	Positive (2 close bands-around 93-95)- High back-ground

Appendix B: Curriculum Vitae

4/10/2012

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EDUCATION

POSITION: Doctoral Graduate Student (PhD Candidate; Oct, 2007)

MAJOR and ADVISORS: Neuroscience, Laura L. Carruth

INSTITUTION: Georgia State University, Atlanta, GA (2005-present)

Expected Graduation: August 2012

TOPIC: The Effects of Early Corticosterone Treatment on the Development of the Avian Song Control System

DEGREE: Master of Science

MAJOR and ADVISORS: Neurobiology and Behavior, Kyle J. Frantz

INSTITUTION: Georgia State University, Atlanta, GA (2003-2005)

TOPIC: Age and Sex Differences in the Acquisition and Maintenance of Intravenous Amphetamine Self- Administration in Rats

DEGREE: American Society of Clinical Pathologists Certificate

MAJOR and ADVISORS: Clinical Laboratory, Mary Lee

INSTITUTION: Georgia State University, Emory University Hospital, Atlanta, GA (1993-1996)

DEGREE: Master of Science

MAJOR and ADVISORS: Biochemistry, Manouchehr Messripor

INSTITUTION: Esfahan University, Esfahan, Iran (1986-1990)

TOPIC: Alterations of Striatal Acetylocholinesterase Activity by Catecholamines

DEGREE: Bachelor of Science

MAJOR and ADVISORS: Medical Technology, Tahmorece Jalayer

INSTIRUTION: Esfahan University, Esfahan, Iran (1979-1985)

EXPERIENCE

POSITION: Medical Technologist

INSTITUTION: Grady Memorial Hospital, Atlanta, GA (1997-2005)

AWARDS & HONORS

- Brains and Behavior Fellowship, Georgia State University, Atlanta, GA, 2009 - 2012.
- Center for Behavioral Neuroscience Travel Award to attend the annual CISAB (Center for the Integrative Study of Animal Behavior) at Indiana University, Bloomington, IN on April 7 and 8, 2011.
- Center for Behavioral Neuroscience Fellowship, Georgia State University, Atlanta, GA, 2007- 2009.
- Steven Kudravi Memorial Award for an outstanding teaching assistant, Georgia State University, Atlanta, GA, Spring 2008.

- Best poster presenter award in the poster preview for Society for Neuroscience, Emory University, Atlanta, GA, 2005.

PEER-REVIEWED PUBLICATIONS

Shahbazi, M., Schmidt, M., and Carruth, L.L. 2011. Distribution and subcellular localization of glucocorticoid receptor-immunoreactive neurons in the developing and adult male zebra finch brain. *Gen Comp Endocrinol*, 174(3): 354-61.

Shahbazi, M., Moffett, A.M., Williams, B.F., and Frantz, K.J. 2008. Age- and Sex-Dependent Amphetamine Self-administration in Rats. *Psychopharmacology* (Berl), 196(1):71-81.

INVITED PRESENTATIONS

- **Shahbazi, M.** and Carruth, L.L. The effects of chronic corticosterone treatment on the development of the avian song system. Center for the integrative study of animal behavior (CISAB). Indiana University, Bloomington, IN, April 8, 2011. (Presented data as an invited speaker at the Animal Behavior Conference at Indiana University)
- **Shahbazi**, M. The effects of chronic corticosterone treatment on the development of the avian song system. NIBL, GSU, April26, 2011. (Presented data as an invited speaker at the Neuroscience Institute Breakfast & Lecture at Georgia State University.)

POSTERS PRESENTED

1. **Shahbazi, M.** and Carruth, L.L. The effects of chronic corticosterone treatment on song nuclei size and song production in the male zebra finch. Brains and Behavior

Spring Symposium, Atlanta, GA, April, 2011.

2. **Shahbazi, M.** and Carruth, L.L. The effects of chronic corticosterone treatment on song nuclei size and song production in the male zebra finch. Society for Neuroscience. San Diego, CA, Nov. 2010.

3. **Shahbazi, M.** and Carruth, L.L. Glucocorticoid receptor distribution across the development of the avian song system. South East Nerve Net, Atlanta, GA, March, 2010.

4. **Shahbazi, M.** and Carruth, L.L. Glucocorticoid receptor distribution across the development of the avian song system. Brains and Behavior Spring Symposium, Atlanta, GA, April, 2010.

5. **Shahbazi, M.** and Carruth, L.L. Glucocorticoid receptor distribution across the development of the avian song system. Society for Integrative and Comparative Biology. Seattle, WA, Jan, 2010.

6. **Shahbazi, M.** and Carruth, L.L. The role of glucocorticoid receptors and stress on the development of the avian song system. Society for Neuroscience. Chicago, IL, Oct, 2009.

7. **Shahbazi, M**., Moffett, A.E., Williams, B.F. and Frantz, K.J. Acquisition of Amphetamine Self-administration in Periadolescent and Adult Rats. Society for Neuroscience. Washington, DC, Nov. 2005.

8. **Shahbazi, M.** Moffett, A.E., Williams, B.F. and Frantz, K.J. Acquisition of Amphetamine Self-administration in Periadolescent and Adult Rats. Poster pre-view for Society for Neuroscience, Emory University, Atlanta, GA, 2005.

9. **Shahbazi**, M., McQueen, A. and Frantz, K.J. Acquisition of Amphetamine

Self- administration in Periadolescent and Adult Rats. College on Problems of Drug Dependence. Orlando, Florida, June, 2005.

10. Messripor, M. and **Shahbazi, M.** 1991. Alteration of Striatal Acetylcholinesterase Activity by Catecholamines. *Journal of the Neurological Science*. 98,332. Presented in 7th International Congress of Neuromuscular Diseases.

PROFESSIONAL ORGANIZATIONS

Society for Neuroscience

The Society for Integrative & Comparative Biology

Sigma Xi (The Scientific Research Society)

Center for Behavioral Neuroscience

The Atlanta Chapter of the Society for Neuroscience

RESEARCH METHODS

- Radioimmunoassay to measure the concentration of plasma steroids
- Western Blotting
- Immunohistochemistry & Immunofluorescence
- Histological techniques (cryostat, microtome, Vibratome and nissl stain)
- Capturing images using Axiovision software (Zeiss) & confocal microscopy
- Bird general dissections
- Making silastic implants
- Song recording and analyzing in songbirds
- Intravenous drug self-administration paradigms in rodents

- Making intravenous catheters for self-administration
- Surgical procedures in rats including catheter implant in jugular vein
- Cell biology techniques such as neurite out growth
- Laser Capture microscopy

TEACHING EXPERIENCE

- Guest Lecturer, Hormones & Behavior, April 5, 2010, "Stress, Behavior and the Brain", Georgia State University, Atlanta, GA.
- Guest Lecturer, Hormones & Behavior, April 8, 2009, "Homeostasis and Behavior", Georgia State University, Atlanta, GA.
- Guest Lecturer, Hormones & Behavior, March 25, 2009, "Stress, Behavior and the Brain", Georgia State University, Atlanta, GA.
- Teaching Assistant for Animal Biology Laboratory, Georgia State University, Atlanta, GA (Spring 2008 – Spring 2010).
- Guest Lecturer, Animal Biology, Nov. 17, 2008, "Arthropod Diversity", Georgia State University, Atlanta, GA.
- Teaching Assistant for Foundations in Biology II laboratory, Georgia State University, Atlanta, GA (Fall 2006 - Fall 2007).
- Teaching Assistant for Introductory for Biology I laboratory, Georgia StateUniversity, Atlanta, GA (Spring 2003- Fall 2005).