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## The effects of corticosterone and social isolation on song stereotypy and neural plasticity in zebra finches

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Psychology

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## Abstract

Birdsong is important to male songbirds' reproductive success. For male birds it is important to consistently reproduce their song with high stereotypy, and the effects of stress on song consistency are little studied. My current study examined the effects of social isolation and corticosterone administration on song production and migrating and apoptotic neurons in the song-control brain region HVC of zebra finches. All males significantly decreased in song stereotypy over time, independent of treatment or housing. Males who were housed in isolation had a significant decrease in their latency to sing, as well as a decrease in arborization in migratory neurons. There were no significant effects of treatment or housing on activated caspase-3 immunoreactive cells, which were used as a measure of apoptosis. In conclusion, there are different outcomes when comparing the effects of social stressors to corticosterone administration. This should be taken into consideration when designing future studies examining stress.

## Keywords

Song Stereotypy, Corticosterone, Social Isolation, Zebra Finch, HVC.

## Co-Authorship Statement

Myself along with Scott MacDougall-Shackleton are responsible for the conception and design of my study. Dr. MacDougall-Shackleton and I completed all of the implant surgeries, whereas myself and my volunteers oversaw the collection of all data, including recordings, QMR scans, and tissue processing. This data was jointly analyzed and interpreted with Dr. MacDougall-Shackleton.

The beeswax implants used in my study were generously created and supplied by Kendra Sewall and Scott Davies, Department of Biology at Virginia Polytechnic Institute.

This thesis will be submitted for publication with the authors being listed in order as, myself, Kendra Sewall, and Scott MacDougall-Shackleton.

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## Chapter 1

### 1 Introduction

It was once thought that imitative vocal learning (the learned acquisition of conspecific vocalizations through sensorimotor systems) was a uniquely human characteristic.

However, the more that is uncovered about birdsong, the more we see that the production of learned vocalizations, via a tutor and vocal practice, are not solely a human trait. Some of the more relevant shared traits between our species include the need for interactions with a tutor with normal vocalizations, a critical period in which to acquire these vocalizations and how these learned sounds are used to communicate relevant information to our social groups (Doupe & Kuhl, 1999; Kuhl, 2010; Nowicki & Searcy, 2004).

In addition to being a model system to study imitative vocal learning, song production in birds is an excellent model to study the behavioral effects of stress. When selecting a mate, females have demonstrated a preference for specific song characteristics, and these selection pressures have allowed birdsong to become an honest indicator of male quality (Catchpole, 1996; Spencer & MacDougall-Shackleton, 2011). Birdsong communicates both direct and indirect benefits to females such as territory quality, stable food sources, early life experience and other genetic factors for offspring such as size (Geberzahn & Aubin, 2014; Nowicki & Searcy, 2004; Spencer & MacDougall-Shackleton, 2011; Yamada & Soma, 2016). Examining the potential cost of maintaining these desirable song characteristics throughout stressful life stages is what allows us to infer an individual's fitness and how they adapt to stress. The potential

quality of a male can be inferred through specific song characteristics or deficits as a result of developmental stress (MacDougall-Shackleton & Spencer, 2012; Nowicki, Peters, & Podos, 1998; Schmidt, Moore, MacDougall-Shackleton, & MacDougall-Shackleton, 2013). However, few studies have examined the plasticity of adult song and stress' effects on song production and neurodegeneration in adult birds.

Below I review the background literature on song learning, production and the effects of stress on them before introducing the main objectives of my thesis.

## 1.1 Song learning

Song is important to male birds specifically because it signals their quality as a potential mate and/or a territorial rival. Male songbirds typically learn their songs from a tutor during what is termed the critical period (Thorpe, 1958). For closed ended learners, like my study species the zebra finch (*Taeniopygia guttata*), their ability to significantly modify or refine their song after 90 days ends; this represents their critical period. Males typically learn their song from their father and their song structure reflects that syntaxial structure as well as that of their geographic region (Anderson, Peters, & Nowicki, 2014; Hunter & Krebs, 1979; Lachlan, van Heijningen, Ter Haar, & ten Cate, 2016). During their first year, male zebra finches can continue to refine this song by increasing stereotypy through a decrease in neurogenesis and motor practice (McDonald & Kirn, 2012).

## 1.2 Song control nuclei

The song control system in the avian brain consists of several brain nuclei which are responsible for song learning and production. Forward auditory connections from the cochlear nucleus to the auditory midbrain ultimately project to the HVC (proper name) which is commonly referred to as the song control center of the brain (Suthers & Zollinger, 2008). Songs are continuously refined through a series of neural projections between the HVC and two other song nuclei, the robust nucleus of arcopallium (RA) and Area X in the medium striatum, ultimately resulting in a crystallized song structure at sexual maturation.

Song is produced by the coordination of two highly specialized systems, the song control system, which are the series of neural regions that control the learning and production of song (Figure 1.1) and the syrinx, which is the motor organ responsible for sound production. Song production heavily relies on the song-control region HVC. We know this because when HVC is lesioned we see the loss of song production. In addition, the pattern of neuronal firing in HVC mirrors the pattern of song structure (Hahnloser, Kozhevnikov, & Fee, 2002; Kozhevnikov & Fee, 2007). Motor neurons projecting from HVC ultimately project to the tracheobronchial syrinx and stimulate fine muscle contractions. These fine muscle contractions cause cartilage nodes in combination with paired medial and lateral labia to modulate air flow in the bronchus resulting in vibrations which produce sound. These audible vibrations are similar to those produced by human vocal cords. Songbirds are able to independently control each lateral side of the syrinx, this allows individuals to maximize their ability to produce complex sounds, as one side of the syrinx can be closed producing one sound or both sides can be open

simultaneously producing different sounds (Suthers & Goller, 1997; Suthers & Zollinger, 2008). This partly explains the diversity of birdsong that we hear. Individuals need to rapidly develop and maintain precise control of this organ by song crystallization in order to send ecologically relevant signals to conspecifics.

### 1.3 Song characteristics

Songs can convey a variety of information from singers to other individuals that receive information. Female songbirds have been shown to prefer three main features of song, including song complexity (song repertoire size), geographic dialects, and vocal performance. Song complexity, which can be characterized by song repertoire size (the number of different songs a male produces), the number of syllables produced, or both, has been shown to positively correlate to male characteristics such as, adult survivorship, territory quality, offspring survival, and immune function (MacDougall-Shackleton & Spencer, 2012; Nowicki & Searcy, 2004; Searcy, Nowicki, Hughes, & Peters, 2002). In terms of the importance of discerning a potential mate from the same geographic region as yourself, males producing the same song structural elements as native regional populations can have lower parasite loads (MacDougall-Shackleton, Derryberry, & Hahn, 2002; Stewart & MacDougall-Shackleton, 2008). Lastly, vocal performance, such as the number of songs produced in a bout, or stereotypy (consistency in song production over time), also relate to fitness. For example, in male stonechats, singing for longer periods of time has been shown to correlate positively with parental care (Greig-Smith, 1982). These song characteristics are further selected for in males by females demonstrating their preference in behavioral studies for males who have larger repertoire sizes, sing songs from their same geographic region, and have higher song production and

stereotypy (Anderson et al., 2014; Buchanan & Catchpole, 2005; Holveck & Riebel, 2007).

The song feature that is the focus of my research is song stereotypy, one element of song production. Stereotypy should be very important to males because they should be highly motivated to consistently communicate information to potential mates or territorial rivals. Song stereotypy is defined as consistency between and within songs on structural components like pitch, frequency, sequence of notes and speed (Schmidt et al., 2013; Smith, Brenowitz, Wingfield, & Baptista, 1994). Having high stereotypy serves two purposes for males, it allows them to communicate their quality consistently and second, it allows listeners to recognize the singer. Females prefer to hear males that they have heard sing before and/or are from the same geographic region as them; this should add selective pressure on males to maintain a stereotyped song (Anderson et al., 2014). High song stereotypy should also allow males to recognize each other as neighbors, facilitating male-to-male communication. Previous studies have found that male songbirds are able to recognize other individuals based on their song and distinguish between territories, familiar, and unfamiliar males (Lein & Lovell, 2005; Stoddard et al., 2017). Further evidence that song stereotypy is important in birdsong lies in the fact that seasonally breeding songbirds sing more stereotyped song during the breeding season than during the non-breeding season (Smith, Brenowitz, Beecher, & Wingfield, 1997). Song stereotypy represents an important element of song production, this measure allows males to compete and display their relative fitness to females.

Sexual selection theory posits that in order for song stereotypy to be an honest indicator of male quality there must be an associated cost to producing stereotyped song. One



potential cost for song production is that song learning may depend on coping with developmental stressors (Spencer et al., 2005; MacDougall-Shackleton & Spencer, 2012; Schmidt et al., 2013), thus it is possible that stressors could also pose costs for the motor production of stereotyped song in adulthood. If a male is able to maintain a stereotyped song through an acute or chronic stressful event, he should in theory be able to outcompete males who cannot maintain stereotypy in the face of stress. Maintaining this element of vocal performance (high stereotypy) depends on an individual's ability to compensate and maintain the coordination between the descending motor pathway and the syrinx throughout a stressful event. This investment and ability is what could represent the cost of maintaining a stereotyped song and make stereotypy a sexually selected trait. Theoretically, the coordination of the song control system and the physical syrinx could potentially be disrupted by stress, but this has not been previously examined.

## 1.4 Stress and compensation

Stress is a part of our everyday lives, and although it is initially adaptive to respond to stressors with the release of glucocorticoids, prolonged exposure to these glucocorticoids results in a number of negative outcomes such as decreased neurogenesis (Gould, Cameron, Daniels, Woolley, & McEwen, 1992). Birds, like humans, have evolved to respond to stress in a compensatory way to help them survive the immediate threat, which could be a predator, unstable food source or infection (MacDougall-shackleton, Schmidt, Furlonger, MacDougall-Shackleton, 2013; Owen-Ashley, Turner, Hahn, & Wingfield, 2006; Schmidt, Macdougall-Shackleton, & Macdougall-Shackleton, 2012). This stress response involves the activation of the hypothalamic-pituitary-adrenal axis (HPA-axis), which begins with the brain recognizing a stressful event, sending signals to

the anterior pituitary, then the adrenal cortex ultimately releasing glucocorticoids which mediate our stress response. It is the release of these glucocorticoids that allow us to respond to stress effectively. However prolonged exposure to these glucocorticoids (the primary of which is corticosterone in birds) also has negative consequences.

Glucocorticoids have been shown to negatively impact the brain in ways such as decreasing the number of mature neurons and resulting in a shift away from hippocampal memory to a lower energy strategy, with more temporary memory formation, the result of which is fewer long term memories are formed (Newman, MacDougall-Shackleton, An, Kriengwatana, & Soma, 2010; Schwabe, 2013).

In birds, corticosterone has been shown to have detrimental effects on both the physiology as well as behavioral characteristics important to avian survival. For example, corticosterone (CORT) has been shown to have a series of negative effects on HVC. Treatment with CORT, resulting in high-induced stress in song sparrows decreases not only overall neuron density but specifically the neuron density, and size of HVC (Newman et al., 2010). However, CORT was not seen to reduce the number of migrating neurons (Wada et al., 2013), thus the neurodegenerative effects of CORT on HVC can most likely be attributed to migrating neurons not turning into mature neurons. This could occur through apoptosis, programmed cell death. CORT implants also resulted in a decrease of newly incorporated neurons into HVC and RA in song sparrows (Newman et al., 2010). Most probable, is the implication that this reduced cell count is a result of neurons undergoing apoptosis to reduce the brains overall energy demands. Apoptosis naturally occurs in the avian brain when individuals are transitioning from the breeding

season, to the non-breeding season. However, the body can also use it as a mechanism to lighten resource loads when experiencing stress.

Additionally, exogenous oral CORT administration affects the body composition of birds, resulting in decreased growth for zebra finch nestlings (Spencer & Verhulst, 2007). A series of negative behavioral effects have also been observed with CORT administration. Postnatal exposure to CORT in zebra finches resulted in delayed behavioral outcomes such as reduced neophobia (in males only) and reduced success when competing for non-food resources such as perches (Spencer & Verhulst, 2007). In song sparrows transdermal CORT treatment has resulted in delayed onset of molt and decreased food consumption, as well as a series of physiological symptoms such as reduced body mass and flight muscle (Busch et al., 2008). Lastly, CORT administration has been seen to decrease higher order branching in migratory neurons; there was a decrease in the number 4<sup>th</sup> and 5<sup>th</sup> order connections of axons and dendrites to each other, while having no effect on migratory cell bodies (McDonald & Kirn, 2012). This exemplifies the subtle yet significant effects that CORT can have on the avian brain. These studies demonstrate that exposure to glucocorticoids, such as CORT, whether or not in response to a stressor, can have significant negative consequences on the brain and behavior.

CORT, as well as actual stressors such as social isolation and food restriction, also negatively affect song development and production. Socially isolated and CORT-treated zebra finches showed a significant decrease in calling activity when compared to controls, however only those individuals treated with CORT showed a dramatic increase in plasma CORT concentrations (Perez et al., 2012). This suggests that stressors can affect vocalizations independent of CORT. CORT-treated zebra finch nestlings have also

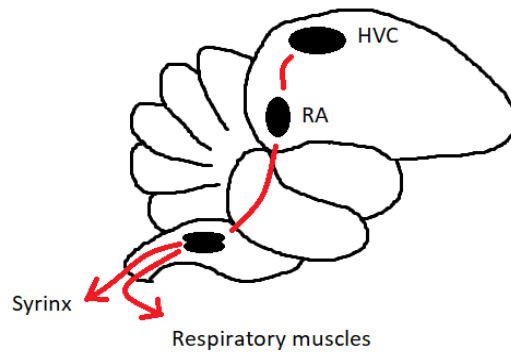
been shown to modify their calling behavior to communicate an increase need to parents, thereby increasing parental care through modified CORT treatment (Perez et al., 2016). Nutritional stress experienced by zebra finches during their first 30 days resulted in decreased accuracy when copying their tutor's song (Brumm, Zollinger, & Slater, 2009). However, few studies have examined the effects of CORT compared to actual stressors such as social isolation in adult males and how this affects their song performance.

## 1.5 Effects of stress on song in adulthood

Currently, we know that developmental stress can be inferred through deficits in the adult song of males, as well as negatively impact song control nuclei (Buchanan, Leitner, Spencer, Goldsmith, & Catchpole, 2004; Spencer, Buchanan, Goldsmith, & Catchpole, 2003; Woodgate, Bennett, Leitner, Catchpole, & Buchanan, 2010), however little is known about how adult song plasticity is affected by stress. The current body of research tells us that the response to an actual stressor compared to CORT treatment is varied, but the specific behavioral and physiological differences have not been examined within the same experimental study. It is important to determine if experimentally it is realistic to simulate a stressful response by administering a single glucocorticoid like CORT, or if a more complete set of glucocorticoids released, like when experiencing an actual stressor, result in a more complete behavioral and physiological result. Additionally, we need to more concretely establish a link between any deficits in behavior and how those deficits may be reflected by neurodegeneration in the brain.

## 1.6 Objectives and hypotheses

My study seeks to examine the effects of social isolation and CORT administration on neural plasticity and song stereotypy in male zebra finches. Specifically, I examine the effects of a stressor as well as a simulated stress response on migrating and apoptotic neurons, as well as specific deficits in song performance. I have two predictions, the primary of which is that CORT administration and social isolation will significantly decrease song stereotypy. More extreme behavioral deficits are predicted for individuals housed in isolation with the vehicle treatment as supplemental CORT may allow those in the isolate condition to compensate more effectively. Second, I predict that CORT administration and social isolation will result in an increase in apoptosis (measured as an increase in activated caspase-3 proteins). Based on prior research (Wada et al., 2013) I predict that CORT and stressors would not decrease migratory neurons in HVC. I also predicted that individuals treated with CORT and socially housed, should not have as dramatic an increase in apoptosis as this positive housing condition could potentially buffer against some of the negative effects of CORT on the brain.



**Figure 1.1 Schematic of a sagittal view of the avian brain, the primary nuclei responsible for song production are in back. Neural projections from the HVC ultimately project to the syrinx, this allows for the precise motor control of the vocal organ to produce highly specific conspecific sounds. RA = robust nucleus of the arcopallium**

## Chapter 2

### 2 Methods

All procedures and animal care was approved by the Animal Care Committee at the University of Western Ontario, AUP# 2016-043 (Appendix 1). All birds used in this study were sourced from a breeding aviary at the Advanced Facility for Avian Research at Western University.

#### 2.1 Pilot study: Corticosterone administration

Although silastic implants have been widely used as a method to chronically dose birds with steroid hormones, their use for delivering corticosterone is problematic (Newman et al. 2010). Newman et al. (2010) found that silastic CORT implants deliver large pharmacological doses that disrupt negative feedback of the HPA axis. I thus conducted four pilot studies to determine the most effective method of corticosterone administration.

##### 2.1.1 Direct oral dosing of CORT dissolved in oil.

My original method consisted of directly pipetting a CORT solution into the mouth of birds. This would allow for consistent dosing between groups and individuals. I made a 0.2 mg/mL solution of corticosterone (CORT) by adding 12.9 mg of corticosterone to 64.5 mL of peanut oil. A small amount of acetone was initially added to the CORT to help the CORT dissolve and was then evaporated during stirring (approx. 10 mins).

Individuals (15) were randomly assigned to one of four time groups, 3 mins, 30 mins, 60 mins or 90 mins and assigned to either CORT treated peanut oil or plain peanut oil conditions. Males were broken up into groups of five and were tested over the course of three days. Each morning I entered the room and manually caught individuals from their home cage and directly pipetted 20  $\mu$ L of the solution into their mouths. I then waited

either 3, 30, 60 or 90 minutes before taking a blood sample of approximately 30  $\mu\text{L}$  from the brachial vein in less than three minutes from re-entering the room. The samples were then spun down using a centrifuge to separate blood cells from plasma. I quantified corticosterone (CORT) levels by using extracted plasma and the EIA Corticosterone Protocol Kit from Enzo Life Sciences (ADI-900-097), adapted by Wada, Hahn, and Breuner (2007).

### 2.1.2 Oral dosing of CORT dissolved in oil in eggfood.

I used the same CORT solution that was used in the direct oil dosing study for each subsequent oral dosing study. Each bird (8) was housed individually with *ad libitum* access to seed and water in addition to being given a quarter teaspoon of eggfood (blended white bread, cornmeal, and hard boiled eggs) with 20  $\mu\text{L}$  of the CORT solution pipetted on top twice a day. Eggfood was placed in the cages between the same hours each day, 09:00h-11:00h and 16:00h-18:00h. Following this, birds were left to consume the eggfood. I then re-entered the room and took a blood sample of approximately 30  $\mu\text{L}$  from the brachial vein in less than three minutes from the time of entry.

### 2.1.3 Oral dosing of CORT dissolved in ethanol in cucumber.

As an alternative to the oil dosing (above) I also examined if an ethanol solution could be used to increase the consistency in timing with CORT treatment with the ingestion of a single piece of cucumber, that birds might eat more quickly than the dosed eggfood (above). An ethanol/CORT solution was mixed by adding 63.1 mg of CORT to 6.31 mL of 95% ethanol, to give an over-all concentration of 20 mg/mL. I pipetted a dose of 2  $\mu\text{L}$  of this solution onto a piece of cucumber, roughly 3  $\text{mm}^3$ , at least 30 mins prior to it being placed in the cage so the ethanol could evaporate leaving only the CORT to be



ingested. A control condition consisted of 4  $\mu\text{L}$  of 95% ethanol pipetted onto a piece of cucumber and administered identically. Eight birds were housed in pairs and *ad libitum* food was removed and a cage divider was inserted to separate individuals one hour prior to placing the treated piece of cucumber in the cage. Birds were treated twice a day during the same hours as in the direct oil study. One hour after treatment I entered the room and within three minutes took a blood sample of approximately 30  $\mu\text{L}$  from the brachial vein. A CORT assay (as above) was conducted to determine if there were differences in plasma CORT concentration between individuals in the control vs experimental groups.

#### 2.1.4 Beeswax implants

Recently, beeswax implants have been successfully used to dose zebra finches with CORT (Beck et al., 2016). Beeswax implants were kindly made by Scott Davies and Kendra Sewall at Virginia Polytechnic Institute following procedures described in Beck et al. (2016). Ten mm long, 0.5 mg CORT beeswax implants were made by dissolving 32.7 mg of CORT in ethanol and adding it to a melted mixture of 1.35 g of beeswax and 0.15 g of peanut oil. Using a 3 mL syringe the slightly cooled mixture was extruded into a long cylinder and cut into 10 mm sections and kept at  $-30\text{ }^{\circ}\text{C}$  for storage until use. I used six individuals to pilot this method, three birds received experimental implants and three control implants. For surgical details see *Surgical Implants* below. Blood samples were taken once a week (procedure described in direct oil dosing) for three weeks to determine the diffusion of CORT into the blood stream compared to controls.

## 2.2 Main study: Effects of CORT and social housing on song and HVC

My study used 28 (n = 7 per group before drop outs) adult male zebra finches (*Taeniopygia guttata*), most which I bred at the Advanced Facility of Avian Research at Western University, with the remaining purchased from a breeder. Subjects were randomly assigned to one of four conditions: social housing with vehicle implant, social housing with CORT implant, isolate housing with vehicle implant and isolate housing with CORT implant (Table 2.1).

For the three-week duration of the treatment period (Figure 2.1) I housed all birds in the same room with *ad libitum* access to food and water, with lights on at 07:00 and off at 21:00 with the temperature between 22-24°C. Birds were either housed three or four to a cage for the social housing conditions or housed individually for the isolate conditions. I only used birds that achieved a minimum score of 75% song stereotypy in their baseline recordings. All males received either a CORT treated beeswax implant or a vehicle beeswax implant and were returned to their home cage for a period of three weeks. A waterfall schedule was used in order to ensure that each bird was implanted, scanned and recording at a similar time and manner as other individuals.

### 2.2.1 Song recording

All individuals received a baseline recording before the start of the experiment. I placed one male per chamber in an isolation chamber over night with *ad libitum* access to food and water. The next day between 08:00 and 11:00 I placed a female in the chamber with the male (in a separate cage) to stimulate singing. Each male was recorded for 10 minutes using a Marantz Recorder PMD671 with a Sennheiser microphone and then placed back

in his home cage once he had completed his recording session. If a male failed to produce a minimum of five songs during his recording, he was stimulated with a different female immediately after the first failed recording session or isolated for another night and stimulated again the following morning. All males were recorded a total of two times, once before the start of the experiment to determine eligibility and a second time at the end of the three-week treatment period (Figure 2.1). The final recording procedure was identical to the initial recording session. Songs from these recordings were used to determine changes in stereotypy.

### 2.2.2 Surgical implants

For implantation, all birds received an intramuscular injection of 0.01 mL of 0.5% Metacam immediately before being anesthetized using 2% isoflurane. The beeswax implants were inserted subcutaneously on the back through a small incision on the left side of the spine. The incision was closed with a skin adhesive, afterward the animal recovered in a private cage in the surgery room under a heat lamp until they were perching normally again. The procedure took no more than 15 minutes per individual and all individuals recovered as expected with no complications. If an implant broke during insertion both pieces of the implant were still inserted subcutaneously. Four individuals lost their implants during the course of the study, through either rejection or infection and these were not replaced once the initial implant fell out, males 801 (social, CORT), 777 (social, vehicle), and 3685 (isolate, CORT) lost their implants 11 days after surgery, while male 708 (social, vehicle) lost his 10 days after surgery. These birds were excluded from the final statistical analysis.

### 2.2.3 Body composition and body mass

Prior to the start of the experiment I recorded all bird's weight using a spring scale, and conducted two Quantitative Magnetic Resonance (QMR) scans to determine fat and lean mass content, at 10 days post-op and 21 days post-op, with a final weight recording as well on day 21. I conducted the QMR scan using a QMR unit (Echo-MRI-B, Echo Medical Systems, Houston, TX, USA) designed to specifically measure the body composition of small birds and bats (Guglielmo, McGuire, Gerson, & Seewagen, 2011; Kriengwatana & MacDougall-Shackleton, 2015). The QMR scanner was calibrated daily using a 5 g vial of canola oil. When conducting a scan, I placed birds (awake) inside the plastic holding tube and inserted it into the instrument. Scans were conducted using the 'small bird' and 'three accumulation' settings where lean and fat mass were scanned three times to create an average per scan by the Echo MRI software. All measurements were recorded to the nearest 0.001 g.

### 2.2.4 Tissue collection

After the final recording, each individual was immediately taken to a post-mortem room for perfusion and brain collection. I deeply anesthetized birds using isoflurane and perfused transcardially first with saline, then with 4% paraformaldehyde. I removed the brain from the skull and stored in 4% paraformaldehyde for 24 h before transferring it to a 30% sucrose solution for a minimum of 24 h for cryoprotection. Brains were then frozen using crushed dry ice and were stored at -80 °C until processed for immunohistochemistry.

## 2.2.5 Immunohistochemistry

I used two immunohistochemistry protocols in this study, Doublecortin (DCX) and Activated Caspase-3 (Csp-3). Doublecortin is an endogenous protein expressed only in immature neurons that are migrating or undergoing differentiation (Wada et al., 2013). Activated Caspase-3 is an effector enzyme expressed by mammalian cells undergoing apoptosis, including neurons (Shalini, Dorstyn, Dawar, & Kumar, 2014).

Brains were bisected at the midline and one hemisphere (randomly selected) sliced sagittally at 40  $\mu\text{m}$  using a cryostat and sections were placed in 0.1% PBS (phosphate buffered saline). Collected sections were alternated between free floating wells for immunohistochemical detection of doublecortin (DCX) and activated caspase-3 (Csp-3).

### 2.2.5.1 Doublecortin

To detect DCX free floating sections were washed twice with 0.1 M PBS for at least five minutes each before being incubated in 0.5%  $\text{H}_2\text{O}_2$  in PBS for 15 mins at room temperature with agitation. After sections were washed three times in 0.1 M PBS and incubated in 10% Normal Horse Serum (in 0.3% PBS/Triton) for 60 mins. To complete the first day of processing sections were incubated in a primary antibody, DCX (C-18; sc-8066 Santa Cruz Biotechnology) diluted at 1:250 in 0.3% PBS/T over night. The following day sections were washed twice in 0.1% PBS/T, then incubated in a horse-anti-goat biotinylated secondary antibody, (1:400 in 0.3% PBS/T) for one hour. Sections were washed again three times in 0.1% PBS/T before another one hour incubation in avidin-biotin horseradish-peroxidase complex (Vectastain ABC, Elite Kit) at 1:200 in 0.3% PBS/T. After a final three washes sections were visualized using diaminobenzadine (SigmaFAST DAB tablet) for approximately 50 seconds.

### 2.2.5.2 Activated caspase-3

To detect Csp-3 free floating sections were washed twice with 0.1 M PBS for at least five minutes each before being incubated in 0.5% H<sub>2</sub>O<sub>2</sub> in PBS for 15 mins at room temperature with agitation. After sections were washed three times in 0.1 M PBS an antigen retrieval step was performed by incubating sections in 10 mM sodium citrate at 80 °C for 30 mins. After sections returned to room temperature and were washed three times with 0.1 M PBS they were incubated in 10% Normal Horse Serum (in 0.3% PBS/Triton) for 60 mins. To complete the first day of processing sections were incubated in a primary antibody, anti activated-caspase 3 (G7481, Promega Corporation) diluted at 1:1000 in 0.3% PBS/T over night. The following day sections were washed twice in 0.1% PBS/T, then incubated in a horse-anti-rabbit biotinylated secondary antibody (1:400 in 0.3% PBS/T) for one hour. Sections were washed again three times in 0.1% PBS/T before another one hour incubation in avidin-biotin horseradish-peroxidase complex (Vectastain ABC, Elite Kit) at 1:200 in 0.3% PBS/T. After a final three washes sections were visualized using SigmaFAST DAB for approximately 90 seconds.

All sections were float mounted onto Histobond microscope slides and coverslipped using a serial dehydration process and permount mounting medium after drying for 12 hours.

### 2.2.6 Microscopy

For each protocol, I visualized HVC using a Leica DM5500B microscope. I selected five sagittal sections where HVC had the largest cross-sectional area and photographed them at 40X, an area of approximately 251  $\mu\text{m}^2$ . For each brain section sampled, one image was taken at the center of the visible HVC and one immediately outside the HVC (Figure

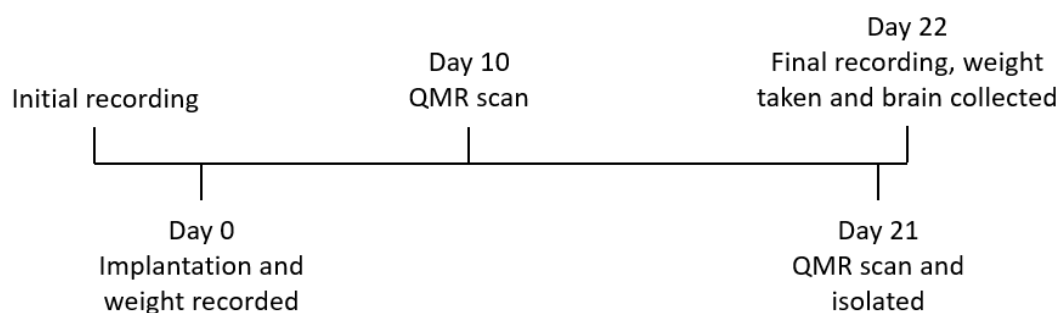
2.2). At 40X the image inside HVC took up almost the entire visible area of HVC, and so care was taken not to include any borders of HVC in the image. Regarding the external HVC image, each image was taken directly ventral to HVC. This resulted in 5 images within HVC and 5 images outside of HVC for each bird.

I manually processed each image blind using ImageJ to count cell bodies as well as examine the overall percent of immunohistochemistry staining in each image. Cell bodies were manually counted for each image. To assess percent immunoreactivity the percent of pixels stained in an image was calculated by manually setting a color threshold to only highlight stained cells and fibers and dividing that value by the total number of pixels in the image. This allowed for both cell bodies as well as dendritic staining to be incorporated into our statistical analysis. Cell counts and percent immunoreactivity in HVC were compared across treatments using a two-way ANOVA (housing x hormone treatment), using immunoreactivity from the images outside of HVC as a covariate.

### 2.2.7 Song stereotypy analysis

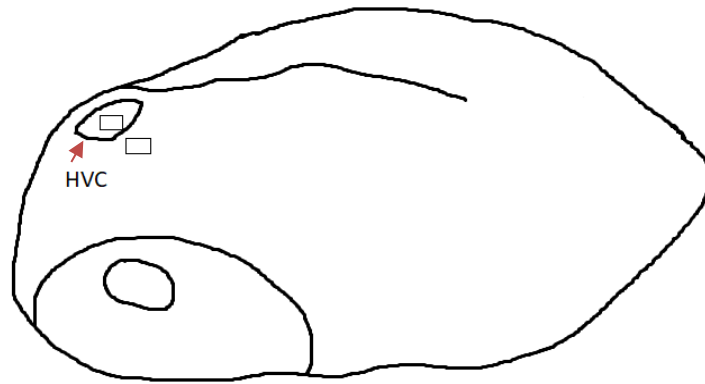
I defined a song as a repeated phrase of syllables or notes, introductory notes were not considered part of the song phrase unless present in the majority of songs (Airey & DeVoogd, 2000). Each recording was visualized as a spectrogram using Raven Pro 1.5 and seven clear song phrases were cut out and saved as separate .wav files. Using Sound Analysis Pro five of the seven selected song samples were compared to each other in all possible permutations to determine the overall percent similarity of songs within a specific recording. Sound Analysis Pro creates similarity measurements by placing two songs on axis and comparing them across sound features present in the spectrogram (Figure 2.3). Using an algorithm to determine the similarity of a model song to a

subsequent song the sound analysis window shows the % Similarity of different components of the song to create an overall % Similarity score for the two songs, higher similarity is denoted by warmer colors (red = 95-100%), while cooler colors represent lower similarity overlap (blue = 35-49%). If one of the .wav files was unable to be processed or read by the software one of the two extra song samples were used in its place as part of the similarity matrix. In the percent similarity analysis songs were measured on % Similarity, % Accuracy, pitch, frequency, entropy and sequence of notes. Two recordings were lost from our database and therefore those individuals were excluded from the song analysis component of this study. The averaged % Similarity for all song combinations was used as the pre/post measure to examine song stereotypy between conditions over time. A repeated measures three-way ANOVA was used to examine the behavioral effects of treatment on song stereotypy, latency to sing and the total number of songs per recording.

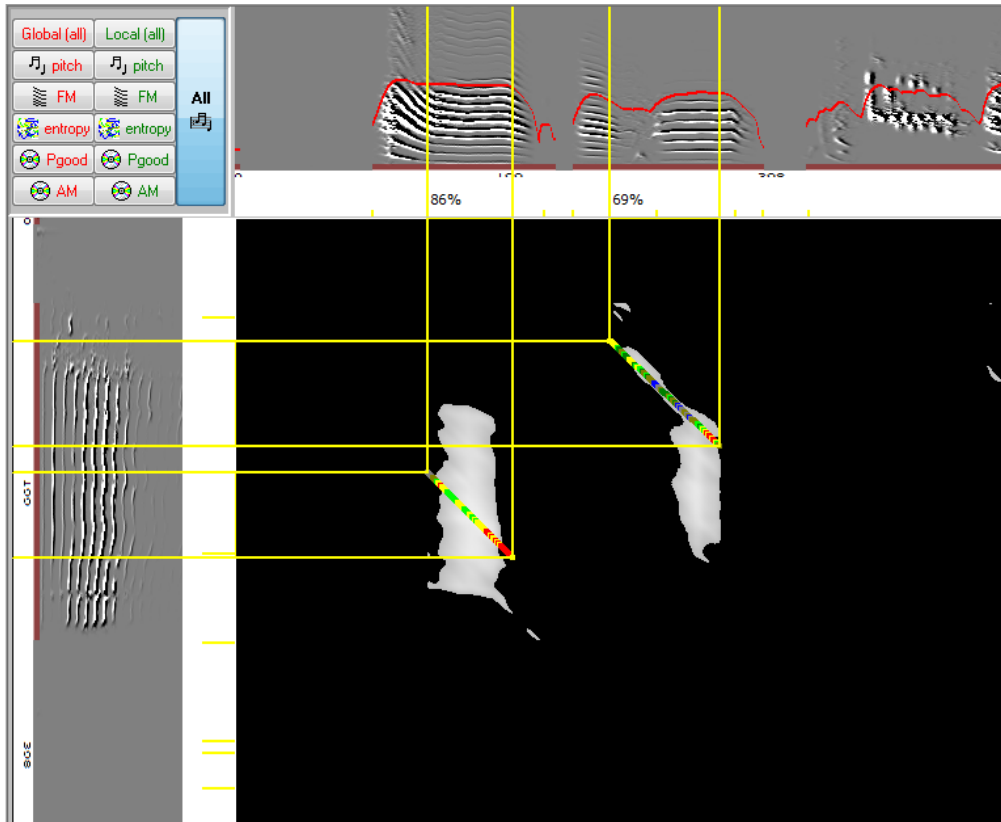


**Figure 2.1 Experimental timeline. After an initial recording session all birds were implanted, received two QMR scans, and their brains were collected the following day after their final recording session.**





**Figure 2.2 Schematic drawing of a sagittal section of a zebra finch telencephalon with two sample squares, one inside HVC, and one outside of HVC representing where images were taken for analysis.**



**Figure 2.3** Sound Analysis Pro window showing the comparison of two songs along the axes of the similarity window. Higher regions of similarity are in red, while lower regions are in blue.

**Table 2.1** Experimental design, with final group numbers. Individuals whose implants fell out during the study were removed from all statistical analyses.

	CORT	Vehicle
Social Housing	5	5
Isolate Housing	6	7

## Chapter 3

### 3 Results

#### 3.1 Pilot study: CORT treatment

Samples sizes for these pilot studies were small and precluded statistical tests. Therefore, the results of the pilot studies are described qualitatively below.

##### 3.1.1 Direct oral dosing of CORT dissolved in oil.

Birds in the vehicle treatment did not show an increase plasma CORT concentration resulting from being handled, but those fed CORT did have a transient increase in plasma CORT comparable to birds experiencing a restraint stressor (Figure 3.1). Based on these results from an acute oral dose with CORT dissolved in oil I proceeded with this method chronically dosing birds twice per day. However, after ten days of treatment birds in both vehicle and CORT conditions became ill and experienced weight loss. These birds were humanely euthanized. I hypothesize that repeated peanut oil treatment was intolerable to the bird's digestion and resulted in chronic wasting sickness. As a result, this method of CORT administration was rejected.

##### 3.1.2 Oral dosing of CORT dissolved in oil in eggfood.

Although this method of topically treating eggfood with CORT dissolved in peanut has been used in previous studies, I found that it did not allow individuals to be housed in groups, or control for the time of ingestion and therefore was also rejected. Birds varied widely in how long they took to consume the eggfood dosed with oil. As a result, this method of CORT administration was rejected.

### 3.1.3 Oral dosing of CORT dissolved in ethanol in cucumber

After one hour of placing the treated piece of cucumber in the cage CORT treated birds had an increase in plasma CORT concentrations (mean = 25 ng/ml), while vehicle bird's plasma concentration remained at an expected baseline (Figure 3.2). Unfortunately, removing *ad libitum* access to food did not result in individuals becoming more synchronized in consuming the treatment, nor did individuals consume the cucumber regularly. Thus, similar to the eggfood treatment above the timing of the dose could not be precisely controlled. Additionally, entering the room multiple times a day and separating the birds so that each bird received an individual dose also added extra unnecessary stress to the overall experimental design, therefore this treatment method was also rejected.

### 3.1.4 Beeswax implant

No individuals rejected their beeswax implant or suffered an infection as result of surgery. Blood samples taken at baseline, week one, and week two showed that CORT treated birds generally had a higher plasma CORT concentration at week one and maintained a higher average until week two (Figure 3.3). One bird had elevated levels of CORT at the baseline bleed, but by week one CORT implanted birds appear to have higher CORT than control birds. These pilot data are consistent with a much larger prior study validating beeswax CORT implants (Beck et al., 2016). This treatment method allowed for individuals to be housed socially, has a low rejection rate and allows CORT to diffuse into the blood stream consistently between individuals for three weeks. Based on these pilot data and a prior validation study (Beck et al., 2016) this method was chosen to treat subjects during the main experiment.

## 3.2 Main study: Effects of CORT and social housing on song and HVC

Following the pilot studies above, birds were treated with beeswax implants, and housed socially or individually as described in the methods, for three weeks. All individuals who lost their implants were removed prior to all statistical analyses (Table 2.1).

### 3.2.1 Body composition data

There were no significant main effects of hormone treatment,  $F(1, 23) = 0.44, p = 0.512$ , or housing,  $F(1, 23) = 0.80, p = 0.378$ , on fat mass. As well there was no significant interaction effect,  $F(1, 23) = 0.26, p = 0.617$ . There was a significant effect of time,  $F(1, 23) = 9.64, p = 0.005$ , and a significant interaction of time and treatment,  $F(1, 23) = 16.17, p = 0.001$ . There was also a significant interaction between time and housing,  $F(1, 23) = 0.709, p = 0.044$ . Bird's fat mass decreased over time, and this relationship was influenced by the implant received as well as housing condition (Figure 3.4 A).

There were no significant main effects of treatment,  $F(1, 23) = 2.21, p = 0.151$ , or housing,  $F(1, 23) = 0.00, p = 0.962$  on lean mass. Additionally, there was no significant interaction effect,  $F(1, 23) = 1.60, p = 0.218$ . There was a significant effect of time,  $F(1, 23) = 25.96, p < 0.001$ , as well as a significant interaction between time and treatment,  $F(1, 23) = 6.71, p = 0.016$ . However, there was no significant interaction of time and housing,  $F(1, 23) = 1.47, p = 0.238$ . Over all, a bird's lean mass decreased over time, and this temporal decrease was affected by implant type (Figure 3.4 B).

There were no main effects of treatment,  $F(1, 23) = 0.75, p = 0.395$ , or housing,  $F(1, 23) = 3.95, p = 0.059$  on total body mass. There also was no significant interaction effect,

$F(1, 23) = 0.01, p = 0.926$ . There was a significant effect of time,  $F(1, 23) = 7.54, p = 0.011$ , however, there were no other significant interactions with time for treatment,  $F(1, 23) = 0.00, p = 0.959$ , or housing,  $F(1, 23) = 0.33, p = 0.574$ . All individuals saw a decrease in total weight over the course of the study, but this was not impacted by either treatment or housing condition (Figure 3.4 C).

### 3.2.2 Song stereotypy and behavioral measures

Contrary to my prediction, hormone treatment and social housing did not affect song stereotypy. A three-way repeated measures ANOVA revealed that there was no significant effect of implant,  $F(1, 18) = 1.12, p = 0.304$ , or housing,  $F(1, 18) = 1.66, p = 0.214$  on song stereotypy. Additionally there was no significant interaction effect between housing and treatment,  $F(1, 18) = 0.70, p = 0.415$ . However, a significant effect of time was found,  $F(1, 18) = 5.12, p = 0.036$ , where birds exhibited a significant decrease in song stereotypy from the initial recording to the final recording (Figure 3.5). There were no significant interactions between time and housing  $F(1, 18) = 1.63, p = 0.218$ , or time and implant,  $F(1, 18) = 1.99, p = 0.175$ .

Using a three-way repeated measures ANOVA I measured latency to first song and number of songs as an index of an individual's motivation to sing. There was no main effect of implant,  $F(1, 18) = 0.06, p = 0.814$  on latency to sing. However, there was a significant main effect of housing on latency to sing,  $F(1, 18) = 5.496, p = 0.031$ , where birds housed in isolation started singing one minute sooner on average compared to birds housed socially (Figure 3.6 A). There was no significant interaction between housing and hormone treatment observed for latency,  $F(1, 18) = 0.26, p = 0.619$ . Lastly, there were no significant interactions between time and housing  $F(1, 18) = 0.44, p = 0.514$ , and time

and implant,  $F(1, 18) = 0.08$ ,  $p = 0.781$ , on latency to sing. Groups did not differ in their latency to sing based on the implant they were given or as a result of time. Housing condition was seen to significantly delay the onset of singing.

With regards to number of song per recording, there were no significant main effects of implant  $F(1, 18) = 0.15$ ,  $p = 0.698$ , or housing,  $F(1, 18) = 2.28$ ,  $p = 0.148$ , on the number songs uttered during the recording session. No significant interaction was seen either,  $F(1, 18) = 0.29$ ,  $p = 0.595$ . There were no significant interactions of time and housing  $F(1, 18) = 2.25$ ,  $p = 0.151$ , or time and implant,  $F(1, 18) = 0.05$ ,  $p = 0.813$ . Groups did not demonstrate a difference in the number of songs produced for either recording based on their implant or housing condition (Figure 3.6 B).

### 3.2.3 Neural effects

#### 3.2.3.1 Doublecortin

The pattern of doublecortin immunoreactivity was consistent with other studies (e.g., Alward et al., 2014; Wada et al., 2013). Both round and fusiform cells were clearly labelled and stained (Figure 3.7 and Figure 3.8). To determine if hormone treatment or housing affected the number of doublecortin immunoreactive (DCX-ir) cells I conducted a two-way ANOVA, with a covariate of the number of cells outside and ventral to HVC. The number of DCX-ir cells outside of HVC was a significant covariate,  $F(1, 18) = 5.34$ ,  $p = 0.033$ . However, there was no significant effect of hormone implant,  $F(1, 18) = 0.522$ ,  $p = 0.479$ , or housing,  $F(1, 18) = 3.16$ ,  $p = 0.092$  on the number of DCX-ir cells (Figure 3.9). Furthermore, there was no significant interaction effect,  $F(1, 18) = 0.16$ ,  $p = 0.690$ , groups did not differ in the number of immunoreactive cells as a result of implant or housing condition.

For the percent DCX immunoreactivity the amount of immunoreactivity outside of HVC was not a significant covariate,  $F(1, 18) = 1.68, p = 0.210$ , and was therefore removed as a covariate from further analyses. Difference in immunoreactivity inside HVC compared to outside HVC can be seen in Figure 3.10 and is similar to prior studies (e.g. Wada et al. 2013)). There was no significant main effect of implant,  $F(1, 18) = 0.39, p = 0.539$ , birds did not differ in immunoreactivity based on whether they received the CORT or vehicle implant. However, there was a significant main effect of housing on the percent immunoreactivity,  $F(1, 18) = 5.19, p = 0.034$ , and a significant interaction effect,  $F(1, 18) = 5.69, p = 0.028$ . Individuals housed in isolation had a lower amount of immunoreactivity (Figure 3.11). Of those in isolate housing, males treated with CORT had higher percent coverage than those treated with vehicle, and this relationship was absent for males housed socially.

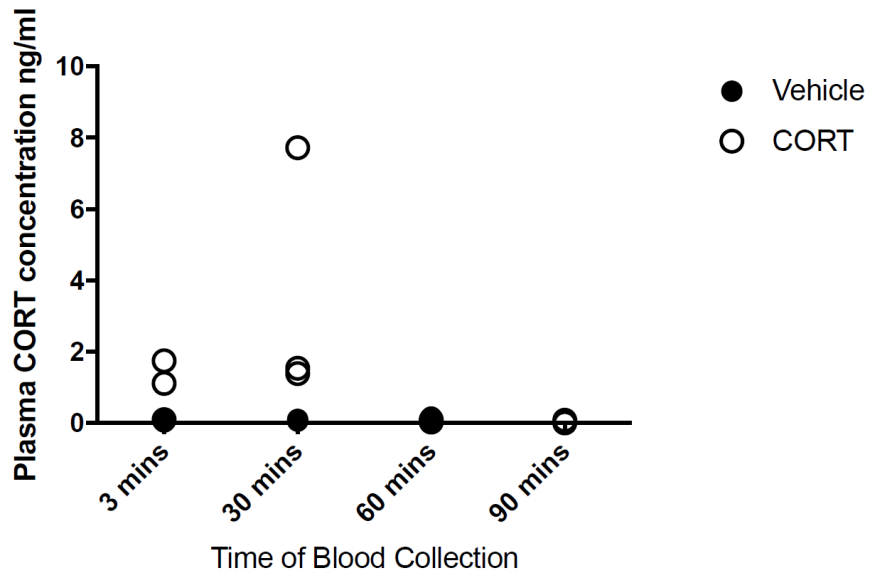
### 3.2.3.2 Activated caspase-3

Activated Caspase-3 immunoreactivity appeared relatively evenly within the central region of the brain with stronger staining occurring near the ventricles and perimeter of the brain. HVC was clearly visible as a lighter stained region (Figure 3.12). There were a class of smaller cells with clear margins and no projections staining that were also observed (Figure 3.13), however as these cells have not been qualified before in previous literature these cells were not included in the final cell body counts for Activated Caspase-3 immunoreactivity. Further work is required to determine if these cells (Figure 3.13) are a result of non-specific binding or represent a previously unreported cell type. For the purpose of this study I counted only cells that were large and sharply defined, had clear projections and dark round or fusiform cell bodies (illustrated in Figure 3.14). The

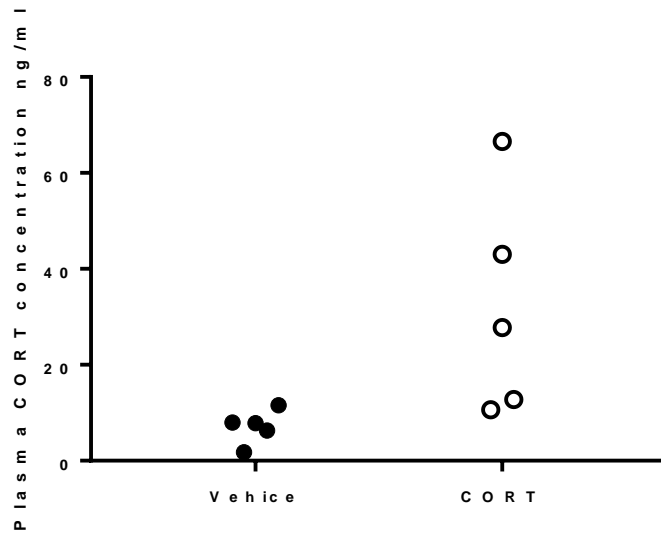


pattern of staining and immunoreactive cells are consistent with a previous study examining caspase-3 immunoreactive cells in HVC (Chen, et al., 2015).

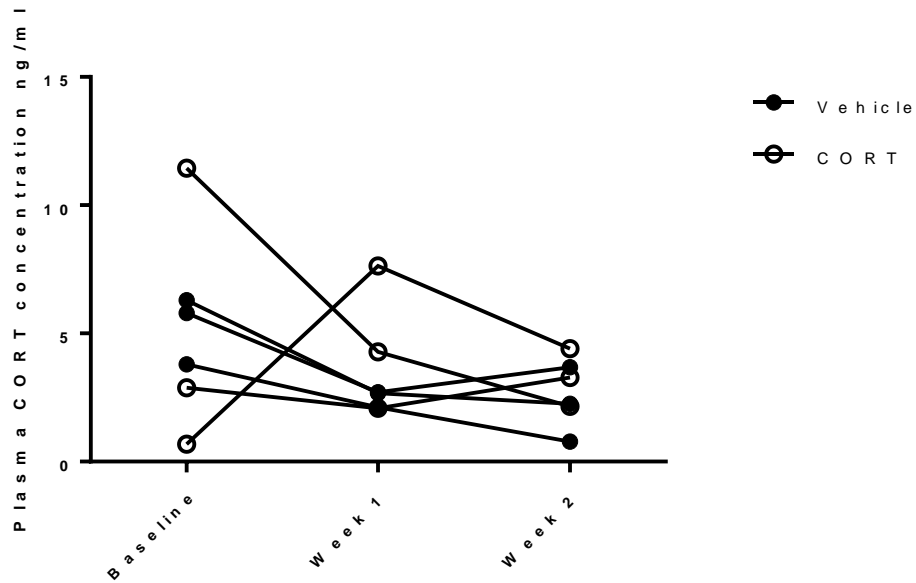
To determine if hormone treatment or housing affected the number of activated caspase-3 immunoreactive cells I conducted a two-way ANOVA, with a covariate of outside-HVC Csp-3 cells. The number of Csp-3-ir cell bodies outside HVC was a significant covariate,  $F(1, 18) = 10.23, p = 0.005$ . Over all there were no significant main effects of implant,  $F(1, 18) = .01, p = 0.925$ , or housing,  $F(1, 18) = 0.375, p = 0.548$  on the number of caspase-3 cell bodies present inside HVC. Thus, birds did not differ in the number of immunoreactive cells based on implant type or housing condition. There was a non-significant trend towards an interaction effect,  $F(1, 18) = 4.29, p = 0.053$ , with males in the isolate/CORT condition having a higher number of caspase-3 cells than those in the isolate/vehicle condition (Figure 3.15), however ultimately not significant.



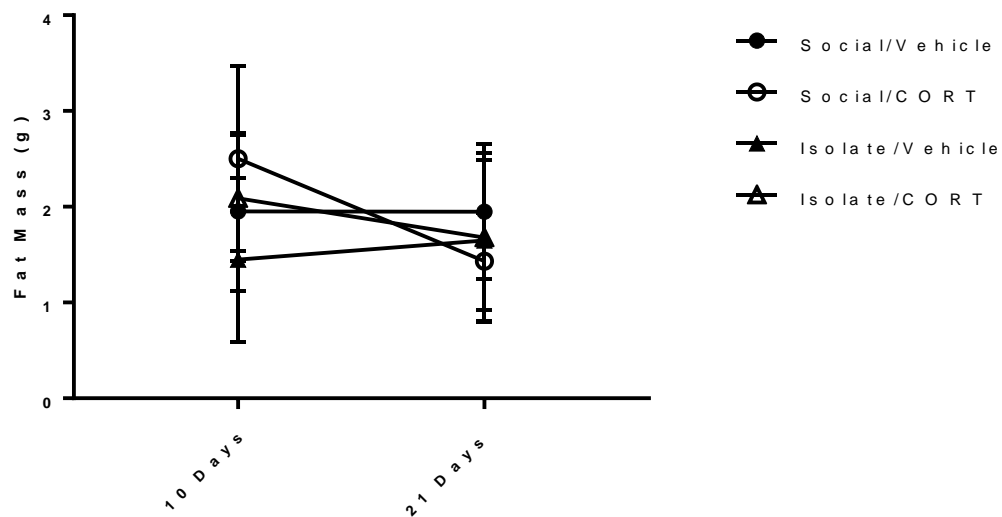
**Figure 3.1. Corticosterone assay results for direct oral treatment of CORT for individual birds. Each line represents an individual bird. Birds in the vehicle condition did not show an increase in plasma CORT due to handling or treatment stress.**

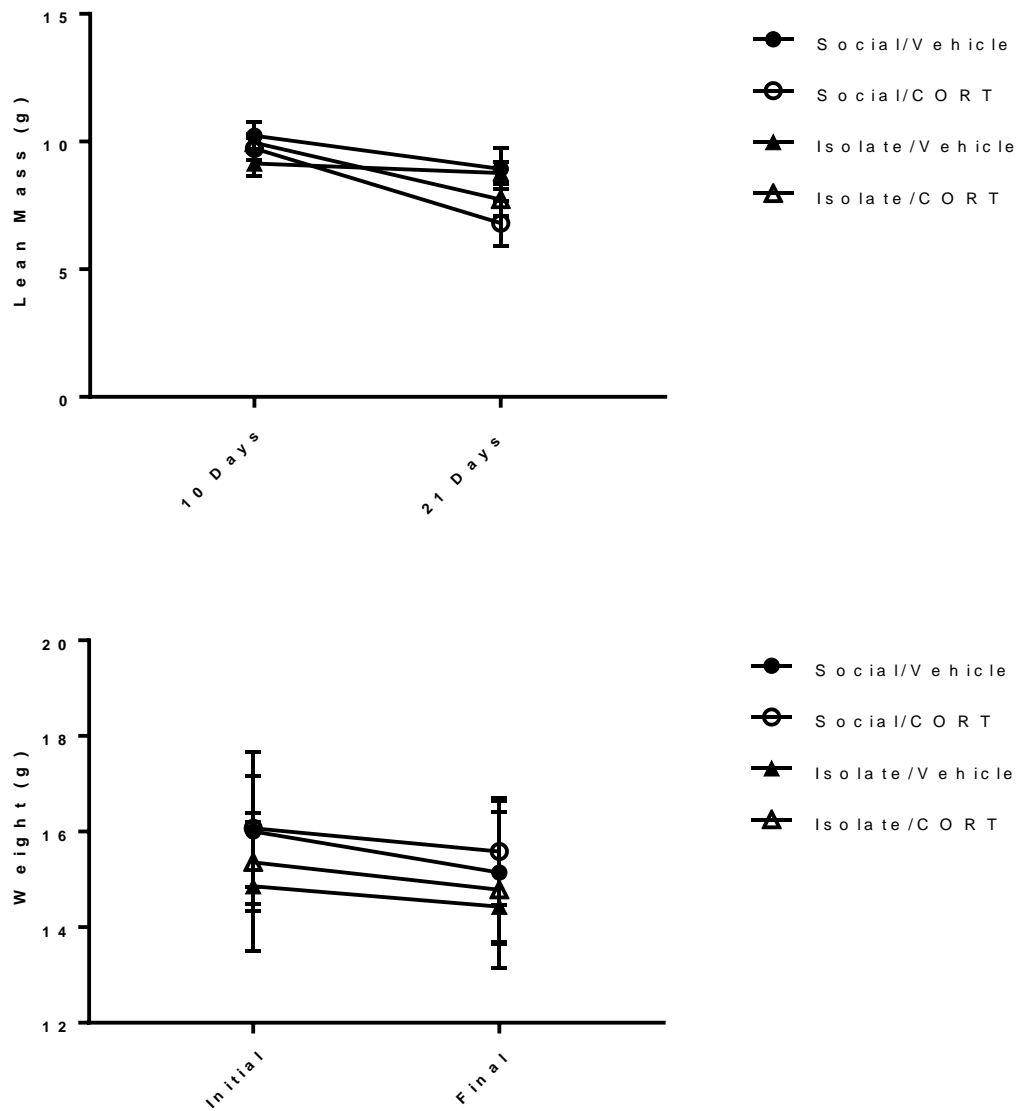


**Figure 3.2. Corticosterone assay results for individual birds in the ethanol oral dosing study. CORT/vehicle was administered through cucumber ingestion and results show after one hour CORT treated birds had an increase in plasma CORT concentrations.**

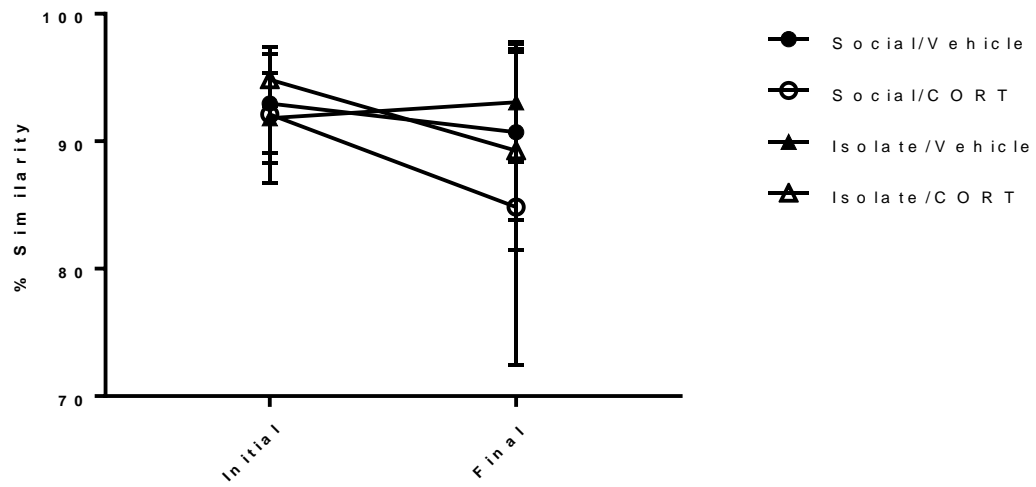


**Figure 3.3. Plasma CORT concentrations of individual birds implanted CORT beeswax pellets or control (vehicle) pellets from baseline to week two. Birds in the vehicle condition saw a decrease in CORT over time, while on average birds in the CORT condition saw an increase in week one and maintained a higher concentration of plasma CORT until week two.**

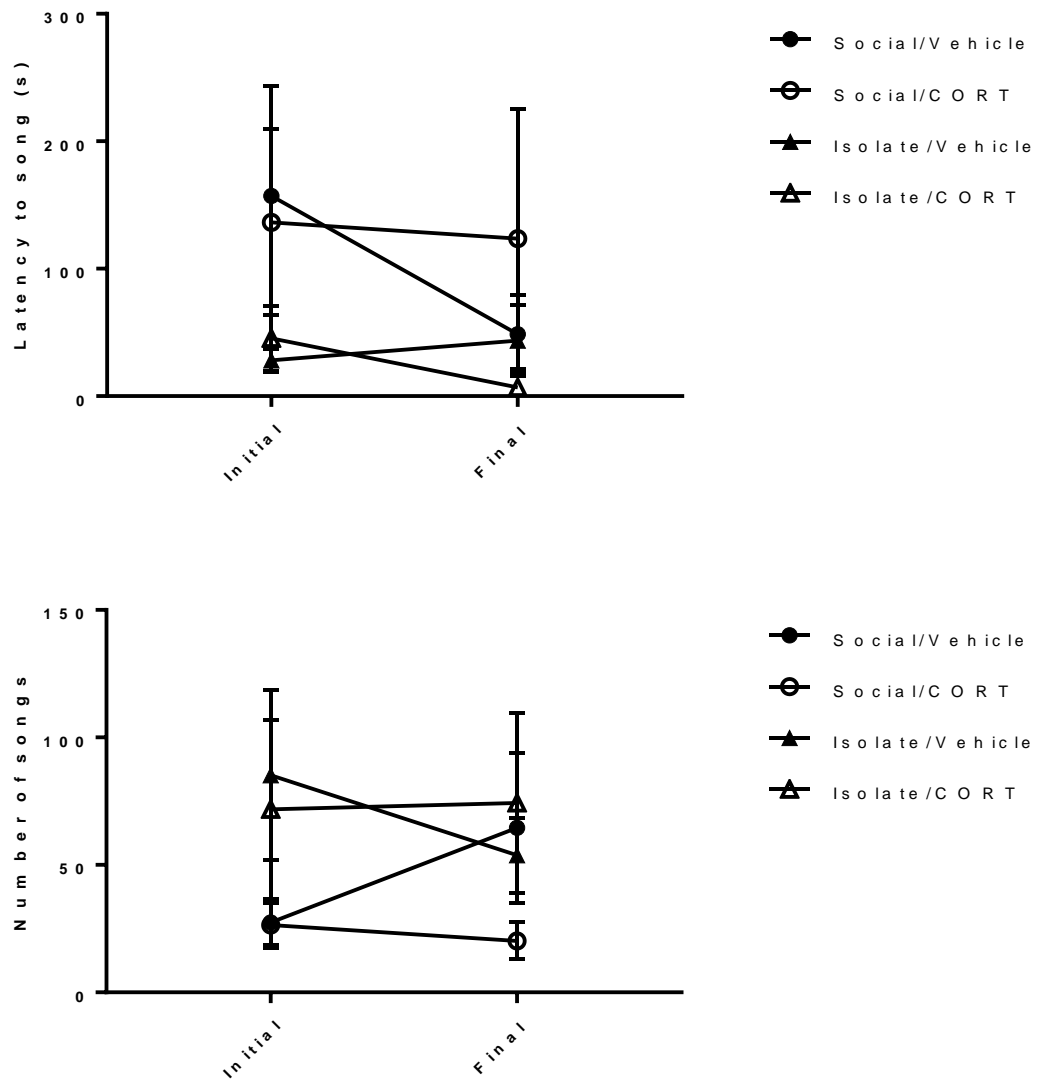




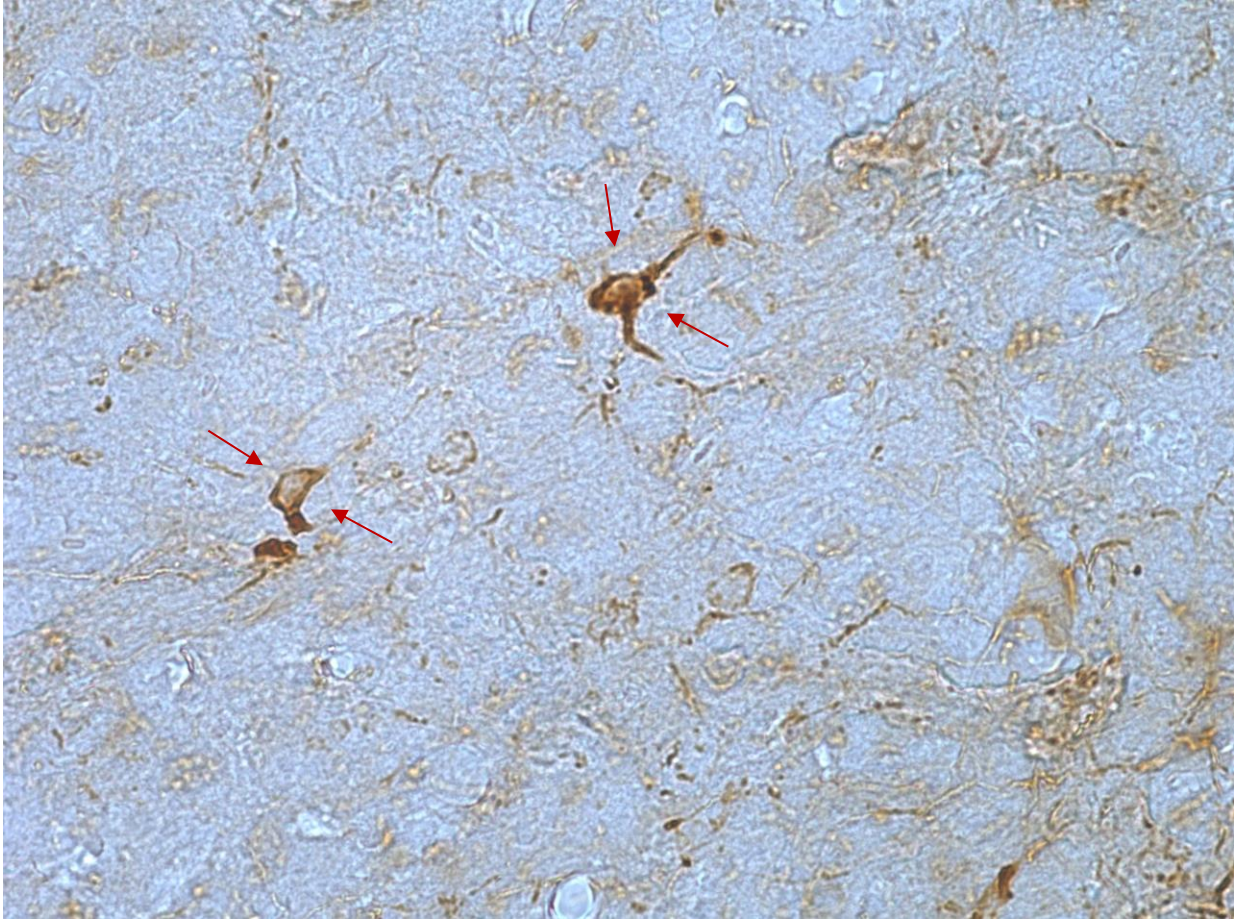
**Figure 3.4. Fat mass (A), lean mass (B) and total body mass (C) of zebra finches housed socially or individually (isolate) and implanted with CORT beeswax pellets or control (vehicle) pellets. Fat and lean mass was measured with QMR 10 and 21 days after implantation. Total body mass was measured with a spring scale on day 1 and 21. Error bars represent SEM. All three measures had a significant effect of time, where fat, lean and total weight decreased from the beginning to the end of the study.**



**Figure 3.5. Overall all groups demonstrated a decrease in song stereotypy from initial baseline recording ( $M = 93.64$ ,  $SEM \pm .718$ ) to the final recording ( $M = 88.63$ ,  $SEM \pm 1.83$ ) regardless of treatment or housing condition.**



**Figure 3.6.** Birds housed in isolation ( $M = 30.94$ ,  $SEM \pm 24.57$ ) decreased the amount of time it took them to initiate singing (A) compared to their baseline recording as well as individuals housed socially ( $M = 116.37$ ,  $SEM \pm 26.91$ ). The number of songs that males sang in their initial recording (B) ( $M = 55.05$ ,  $SD \pm 64.74$ ) did not differ as a function of housing condition or implant when compared to the number of songs produced in their final recordings ( $M = 54.23$ ,  $SD \pm 58.07$ ).

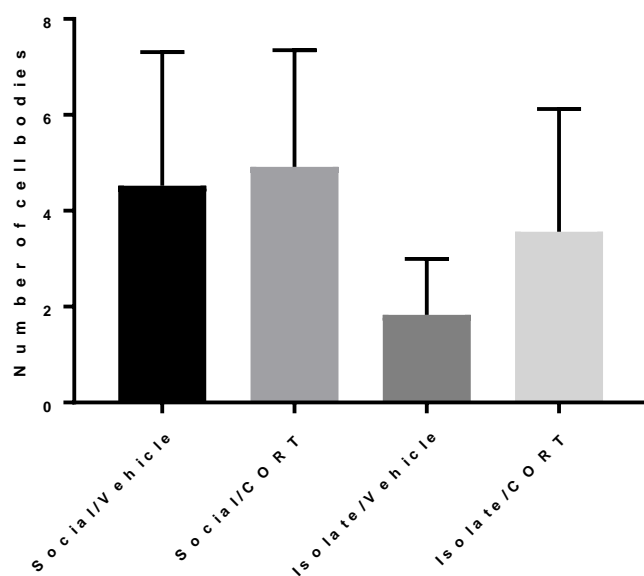


**Figure 3.7. Immunoreactive Doublecortin neurons. Red arrows indicate two round cells found inside the HVC of zebra finch 3682 (40X).**

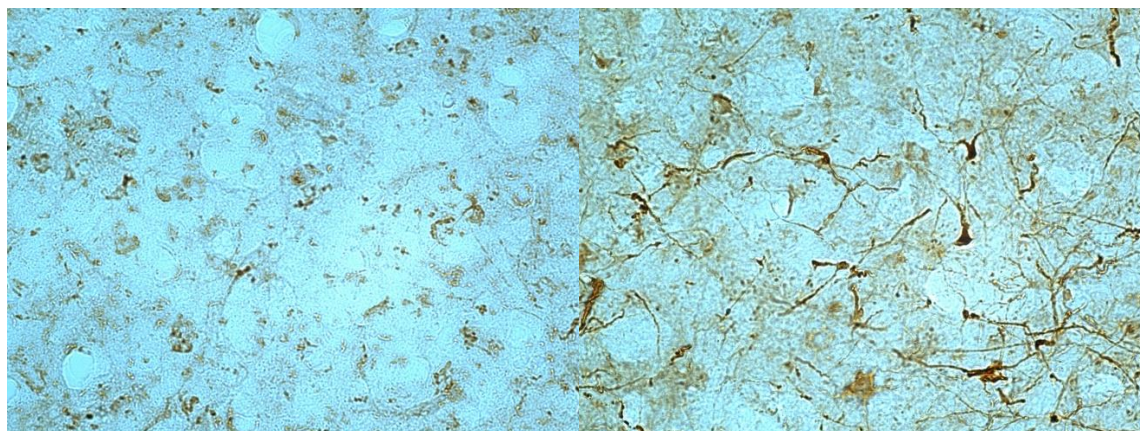




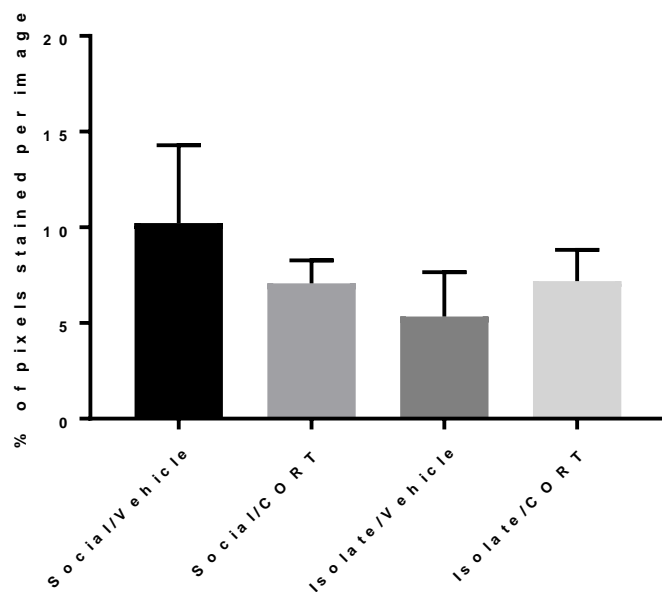
**Figure 3.8. Immunoreactive DCX cells. Red arrows indicate fusiform neurons inside the HVC of zebra finch 3623 (40X).**



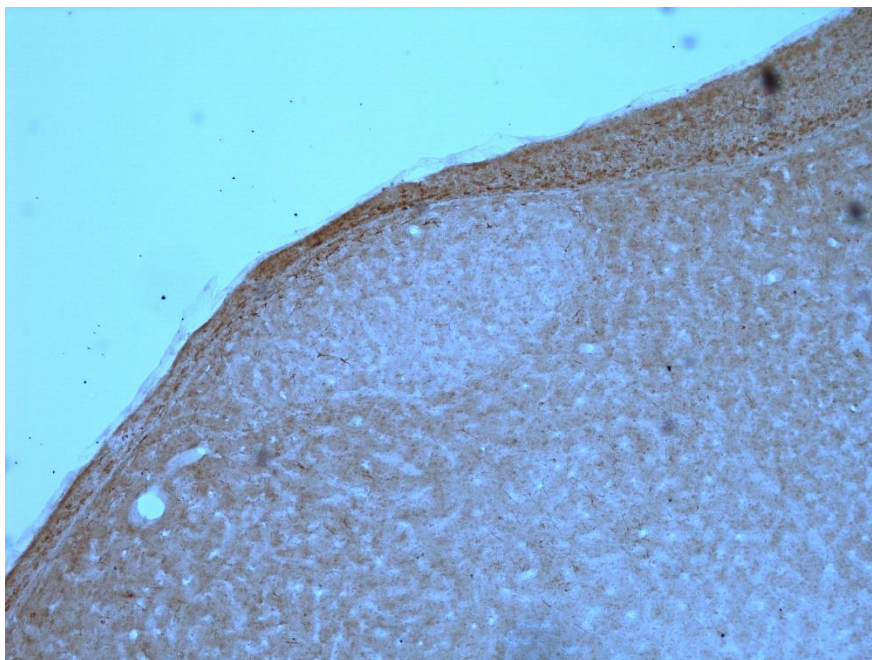
**Figure 3.9. Number of DCX cells inside HVC between groups. There were no significant differences in the number of DCX cell bodies inside HVC between groups based on treatment or housing. Error bars  $\pm$  SEM.**



**Figure 3.10. Difference in percent coverage of DCX staining between the inside of HVC and outside of HVC (40X). Birds in isolate conditions showed less staining overall compared to other groups as well as less staining compared to within group HVC staining.**

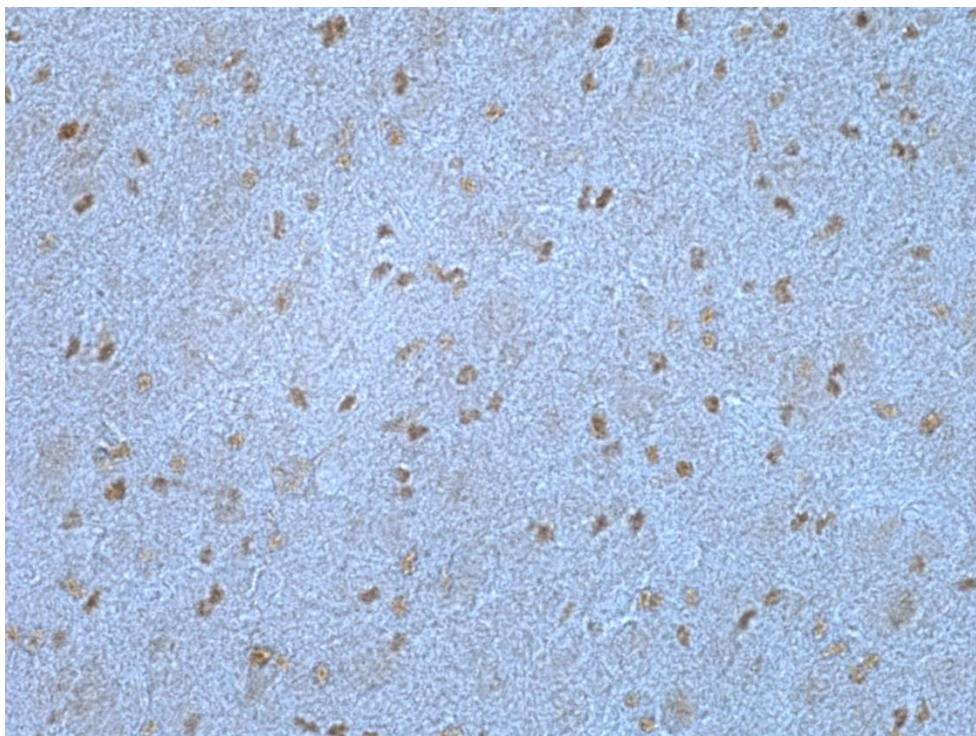


**Figure 3.11. Difference in percent of pixels stained in each inside and outside image of HVC. Males in isolate conditions showed less dendritic staining than those in social conditions. Of those isolate males, those in the vehicle condition showed the least amount of staining, with this relationship being reversed in socially housed males. Error bars represent SEM.**

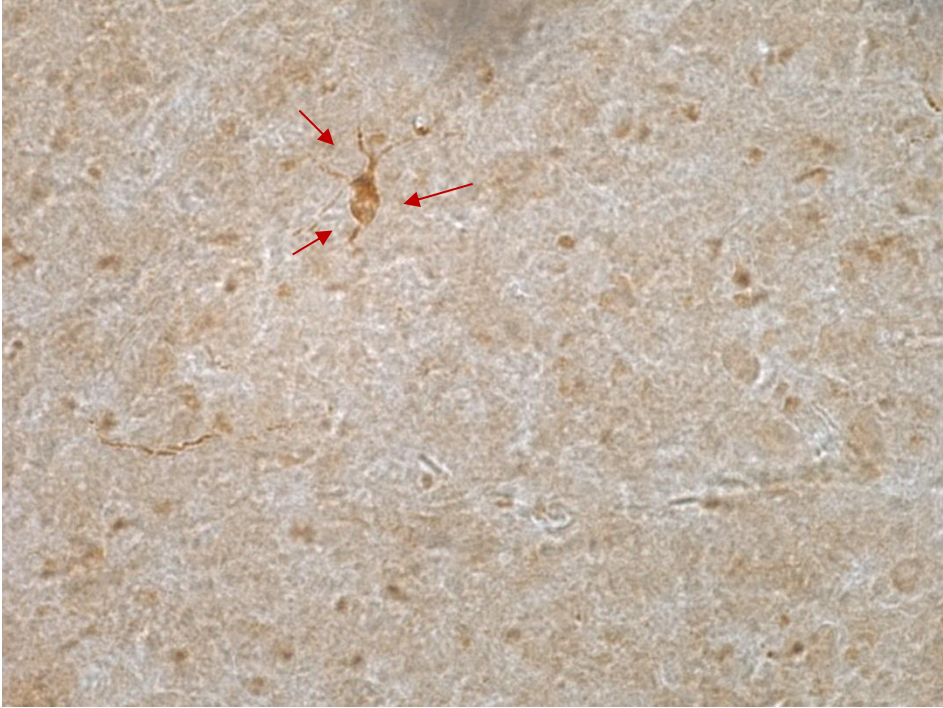


**Figure 3.12. HVC of zebra finch 750, immunoreactivity shows activated caspase-3 like staining within and outside of HVC. HVC is clearly visible as a lighter stained region of the brain.**

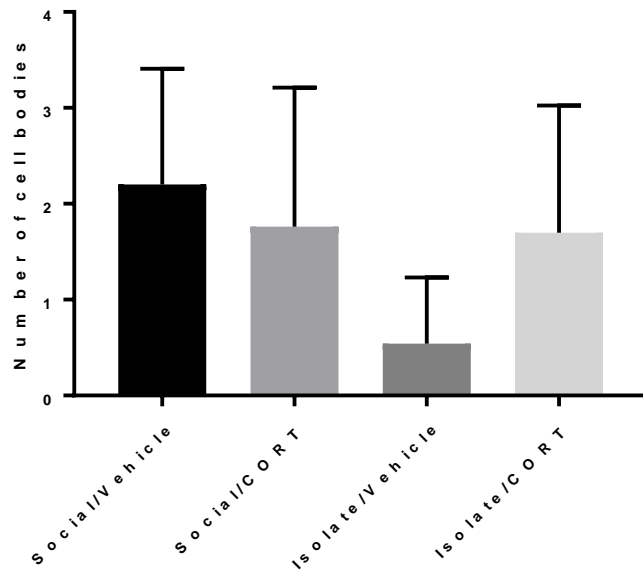




**Figure 3.13. Unknown cells inside HVC of zebra finch 708, with Activated Caspase-3 like immunoreactivity. Cells were at about 1/3 the size of other fusiform and round cells inside HVC.**



**Figure 3.14. Example of immunoreactive activated caspase-3 cell, cells demonstrating the same characteristics were counted as part of the statistical analysis for activated caspase-3. Red arrows indicate a stained fusiform cell inside of HVC.**



**Figure 3.15.** There were no significant differences found in the number of caspase-3 stained cell bodies inside HVC between groups. Stained cell bodies outside of HVC was a significant covariate.

## Chapter 4

### 4 Discussion

The goal of the current study was to examine the effects of two categories of stressors on neuroplasticity and song stereotypy in zebra finches. Overall, there were no significant main effects of social housing or isolation on song stereotypy. However, social housing did affect motivation to sing, with males housed in isolation singing significantly sooner, and an overall larger number of songs, albeit not a significant amount. Although there was no detectable effect of hormone treatment or housing on the number of DCX-ir cells, housing did affect the overall percentage of DCX-ir in HVC. This suggests that housing may have affected the arborization or extension of processes from DCX-ir cells in HVC. Last, a strong trend was observed for an interaction effect with regards to activated caspase-3 stained cells, socially housed birds did not see a change in the number of cells undergoing apoptosis, however in those housed in isolation, CORT treated birds had more Csp-3-ir cells. Below I review the results of my study and place them in the context of the main objectives of this study.

#### 4.1 Body composition data

Based on the body composition data collected during the course of the experiment, there were no significant effects of treatment on body composition, however there was a significant effect of time. With regards to treatment and housing condition, this result is unexpected as other studies have shown that CORT treatment does significantly impact body condition of both nestlings and adults (Busch et al., 2008; Schmidt et al., 2012), however in my study it is possible that the treatment dose was too low to see similar



results. Similarly, it could be that being housed in isolation was not a strong enough stressor to result in a decline in overall body condition. However, the lack of effect on body condition supports the conclusion that any resultant effects on song stereotypy or HVC are a result of treatment and not an indirect effect of decline in body condition.

## 4.2 Song stereotypy and song production

Although there were not significant main or interaction effects of treatment on song stereotypy, song stereotypy still significantly decreased as a function of time. This could be due to the general stress of the experiment experienced by all individuals, such as being weighed, handled every other day post-op to check the implant site as well as conducting QMR scans. This effect should have been buffered by the lack of females housed in the room, as male song sparrows who are housed without the presence of a female are more prolific singers and maintain high stereotypy compared to male song sparrows housed with female partners (Boseret, Carere, Ball, & Balthazart, 2006; Konkle, Hurley, Ball, & Balthazart, 2008). However, general stress can limit the refinement of song and is most likely the reason for the general decrease in song stereotypy over time (Spencer et al., 2003).

When examining other behavioral song measures, such as latency to sing and the number of songs per recording, I saw that males housed in isolation sang significantly faster than those housed in social settings, approximately a minute and a half faster. This is consistent with previous literature, as males housed in isolation, compared to their counterparts housed with females, exhibit higher song rates and higher mean song duration (Alward et al., 2014). This was also seen in my study, as males housed in isolation sang on average 40 more songs per recording, however this difference was not

statistically significant. These males may be more strongly motivated to re-establish lost social connections compared to males who have maintained them. However, one study has found that social isolation, while not resulting in an increase in plasma CORT concentrations, does result in a decrease in calling activity, it is important to note that song production was not examined in that study (Perez et al., 2012). This provides evidence that songs and calls may differentially be effected by stressors as they serve different ecological purposes. While calls may be subject to decline, the maintenance of song production may be more strongly reinforced as this is how males communicate and find a mate to reproduce. It's been seen previously that male zebra finches as well as other species who sing for longer periods of time, as well as sing more gain more female attention (Catchpole, 1996; Holveck & Riebel, 2007; Nowicki & Searcy, 2004). Lastly, it is important to note that there was a significant effect of housing on a behavioural measure, but no effect of hormone treatment, this adds to the body of research demonstrating that CORT administration and the experience of an actual stressful event cannot always be equated and can have differential effects (Alward et al., 2014; Konkle et al., 2008; Perez et al., 2012). In summary, it can be interpreted that social isolation results in an increase in song-related behavioral measures such as an increase in song production.

## 4.3 Neural effects

### 4.3.1 Doublecortin

There were no significant differences in the number of DCX labelled cells in the HVC between groups. This is consistent with previous research, supporting the hypothesis that changes in neuron density in HVC and its size is not a result of a decreased number of

migratory neurons (Newman et al., 2010; Wada et al., 2013). Most likely, this is due to another process, such as apoptosis where the migratory cells are not maturing in HVC and instead are culled.

Although there were no significant effects on DCX cell bodies, there was a significant effect of housing condition on DCX % immunoreactivity. This % measure incorporates all immunoreactivity in an image, not just cell bodies, but dendrites and axons as well. Additionally, there was no significant correlation between staining inside and outside of the HVC, with all birds showing less % staining inside HVC. Males housed in isolation had on average 3% less staining than those housed in social conditions. This mirrors previous research which has shown that isolate housing can maintain cell body number but decrease the number of higher order dendrite and axonal connections in migratory neurons in the HVC (Alward et al., 2014). Here, I saw that birds in the CORT conditions had the same amount of staining, while males in the isolate/vehicle group had the least. This supports my previous hypothesis that those individuals in the isolate/vehicle group may have stronger negative effects as CORT has evolved to help organisms respond to stressors and the addition of supplemental CORT may serve this exact role. Additionally, this, in conjunction with the behavioural data, adds further support to my other hypothesis that CORT administration does not affect the body in the same way as experiencing an actual stressor, as there was no effect of hormone treatment on DCX neurons or staining. As well, CORT treatment significantly interacted with housing to affect the percent immunoreactivity of DCX. CORT-treated individuals had the same amount of staining occur regardless of housing (7.18% and 7.07%), while vehicle groups were nearly 5% apart with the isolate group being the lower of the two. CORT may be

potentially performing its adaptive role and acting as a buffer against stressful conditions, however it would be interesting to see at exactly what levels of CORT administration do we see the same effects as we do under housing conditions.

#### 4.3.2 Activated caspase-3

Overall, no significant results were observed for activated caspase-3 like immunoreactivity. Males in the isolate/vehicle condition showed the lowest amount of caspase-3 stained cells compared to those in the isolate/CORT group which showed the highest, while individuals in both social conditions maintained approximately the same number of caspase-3 stained cells. Percent staining was not examined for activated caspase-3 as caspase-3 has been implicated in not only neural pruning but also dendritic repair with no way to separate the activity of the two. Therefore % staining was not examined to exclude any confounding reactivity as a result of dendritic or axonal repair mediated by activated caspase-3 (Kuo, Zhu, Younger, Jan, & Jan, 2006; Li & Sheng, 2012).

When examining the data on activated caspase-3 reactivity it's important to note that both social groups exhibited similar rates of immunoreactivity to those in the isolate/CORT condition. This could be due to the fact that one group of individuals is undergoing a high rate of cell death in HVC (isolate/CORT) as CORT has been shown to decrease HVC size and neuron number (Wada et al., 2013), while the other group is undergoing high rates of apoptosis as well but as a plastic mechanism and simultaneously undergoing equal rates of neurogenesis. This results in both groups having similar rates of apoptosis but one group replacing the lost cells with the incorporation of new ones as a mechanism of neural plasticity. Activated caspase-3 has been implicated in natural seasonal changes

in neurogenesis and cell death associated with breeding, providing support that in some individuals caspase-3 reactivity could be due to healthy levels of coordination between neurogenesis and apoptosis (Larson, Thatra, Lee, & Brenowitz, 2014; Thompson & Brenowitz, 2008). So ultimately not having a significant main effect of housing or implant does not mean that a detrimental amount of apoptosis is not occurring in one or more groups. Examining neurogenesis would provide the necessary evidence to support this conclusion. Over all this result of activated caspase-3 immunoreactivity is not what I predicted. Previous research such as Newman et al. (2010) and Wada et al. (2013), lend support to the theory that CORT should greatly increase apoptosis, but that is not what was observed. This could be due to species differences or differences in CORT dose as my study had relatively low levels of exogenous CORT. To fully resolve the issue of neurodegeneration and any complimentary neurogenesis Newman et al. should be replicated and examined for activated caspase-3, BrdU and, NeuN labelling of mature neurons.

#### 4.4 Future directions and limitations

One of the limitations of this study was that the exposure period to housing and CORT conditions was only three weeks long. The style of implant that I used has only been validated to release CORT for a three-week treatment period and therefore was the determinant in the methodological design. In my previous undergraduate work where I did see a significant effect of treatment length on song stereotypy, birds were housed individually for 2-3 months while experiencing back-to-back repeated conditions (Faltynek, 2014). Examining specific song production characteristics like song stereotypy may be too fine a measure to see large results in this treatment time.

Second, when examining the immunoreactivity for activated caspase-3, there seemed to be two different patterns of staining occurring. First, fusiform and round cells were clearly identifiable with clear margins and dendrites and axons visible (Figure 3.7 and Figure 3.8), while a second kind of cell was also stained (Figure 3.13). The second much smaller cell bodies had no dendritic or axonal staining. More research should be conducted to specifically examine what kinds of cells these are and why they would bind to activated caspase-3 antibodies.

Lastly, future research should incorporate measures of neurogenesis, such as Brdu, as well as markers of mature neurons (e.g., NeuN) when examining neurodegeneration. Incorporating measurements of neurogenesis will allow future researchers to distinguish between individuals undergoing both neurogenesis and apoptosis simultaneously and those undergoing apoptosis. Over all, I was able to determine that adult song is plastic and can significantly decrease in stereotypy over time. Additionally, I was able to demonstrate that stressors can result in both behavioral (song latency) and histological (doublecortin labelled cell arborisation) changes which are quantitatively different from those observed when just administering corticosterone. This adds to the growing body of work supporting the theory that corticosterone administration is not equivalent to experiencing ecological stress in avian models.

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## Appendices



AUP Number: 2015-043  
PI Name: Macdougallshackleton, Scott A  
AUP Title: Neurobiology of songbird vocal learning

**Official Notification of AUS Approval:** A MODIFICATION to Animal Use Protocol 2015-043 has been approved.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D  
on behalf of the Animal Use Subcommittee

### **Appendix 1. Animal Use Protocol for Effects of Corticosterone and Social Isolation on Song Stereotypy and Neural Plasticity in Zebra Finches**

# Curriculum Vitae

**Name:** Pavlina Faltynek

## ACADEMIC INFORMATION

**Master of Science, Behavioural and Cognitive Neuroscience**      **Expected completion August 2017**

*The University of Western Ontario, Advanced Facility for Avian Research, London, Ontario*

Master's Thesis:

- The Effects of Corticosterone and Social Isolation on Song Stereotypy and Neural Plasticity in Zebra Finches, with Dr. Scott MacDougall-Shackleton

**Honors Specialization in Psychology**  
**2014**

King's University College at *the University of Western Ontario*, London, Ontario

Undergraduate Thesis:

- The Effects of Corticosterone on Song Stereotypy in the Zebra Finch, with Dr. Scott MacDougall-Shackleton
- Dean's Honour Role, 2014

**Ontario Secondary School Diploma**  
**2009**

Medway High School, London, Ontario

- Honour Role

## RESEARCH EXPERIENCE

**Master's Thesis**

August 2015 – Current

- Examined the impact of glucocorticoids and isolation on male zebra finches
- Surgically implanted corticosterone beeswax implants
- Conducted song recordings and analyzed for similarity
- Collected and processed neurological tissue using immunohistochemistry to examine treatment effects on migrating neurons, apoptosis and neuron density

**King's Academic Mentoring Program (KAMP)**

September 2013 - September 2014

Head Researcher

- Designed a peer mentoring program at King's University College to increase student retention and academic performance
- Created a best practices document to facilitate program potentiation
- Designed a 7-month group meeting schedule to assist with common topics
- Developed Mentor Screening Package, Resources for Emergencies Document, Mentor Resources Document and executed training of Academic Mentors

**Honors Thesis**

September 2013 - April 2014

- Examined the impact that corticosterone administration has on song stereotypy in zebra finches
- Administered corticosterone and performed all recording sessions
- Analyzed data collected from recordings to determine song consistency between multiple conditions

### **Independent Study - Religiosity and its Relationship to Organ Donation Acceptability**

January 2013- April 2013

Head Researcher

- Designed a correlational study to examine the relationship between organ donation and religiosity
- Designed and adapted questionnaires
- Collected and analyzed data

### **PUBLICATIONS AND PRESENTATIONS**

Faltynek, P. (2013) Religiosity and its Relationship to Organ Donation Acceptability. Western Undergraduate Psychology Journal 1: 114-122.

Potvin, DA., Faltynek, P & MacDougall-Shackleton, SA. (2014). "Cultural evolution or learning deficiencies? An investigation into the effects of noise on song learning in juvenile zebra finches." Animal Behavior Society Meeting, Princeton, NJ

Faltynek, P., Potvin, DA. & MacDougall-Shackleton, SA. (2014). "The effects of corticosterone on song stereotypy in the adult zebra finch." Ontario Ecology, Ethology & Evolution Colloquium, Guelph, ON.

Faltynek, P., Potvin, DA. & MacDougall-Shackleton, SA. (2014). "The effects of corticosterone on song stereotypy in the adult zebra finch." Western Affiliates Thesis Conference, London, ON.

Faltynek, P., Buckland, C. (2015) "The King's Academic Mentoring Program". King's University College, Teaching and Pedagogical Practices Conference, London, ON.

Faltynek, P., Sweall, K., & MacDougall-Shackleton, S. A. (2017). "The effects of corticosterone and social isolation on song stereotypy and neural plasticity in zebra finches". Ontario Ecology, Ethology & Evolution Colloquium, Kingston, ON.

Faltynek, P., Sweall, K., & MacDougall-Shackleton, S. A. (2017). "The effects of corticosterone and social isolation on song stereotypy and neural plasticity in zebra finches". Animal Behavior Society, Toronto, ON.

### **TEACHING EXPERIENCE**

#### **Psychology 3258 (F/G), (2016-2017)**

- Semester essay course on techniques and methods in behavioural neuroscience using animal models. Partnered with a professor to teach practical neuroanatomy, helped students design behavioural studies using rats, graded lab reports and presentations on methods in behavioural neuroscience.



**Psychology 2800E (2015-2016)**

- Full year essay course on research methodology. Gave lectures in a tutorial style setting to a class size of 20. Content included helping students design, execute, analyze and interpret two correlational studies. Additionally, conducted all of the grading for the course including, four written assignments and a presentation.

**EMPLOYMENT*****The University of Western Ontario***

Teaching Assistant

September 2016 – April 2017

- Psychology 3258 (F/G), Research Methods in Behavioural Neuroscience

***The University of Western Ontario***

Teaching Assistant

September 2015 – April 2016

- Psychology 2800E, Research Methods

**King's University College**

Proctor

September 2015 – Present

- Administered exams

**King's University College**

Faculty Secretary

October 2014- September 2015

- Administrator for the Economics, Business and Math Department, the Sociology Department and the Philosophy and Religious Studies Department
- Managed budgets, organized and edited course syllabi and teaching assistant contracts
- Attended Department Meetings, took minutes and motions
- Secretary for the Research Ethics Review Committee

**King's University College**

King's Academic Mentoring Program, Head Coordinator

May 2014-September 2014

- Developed legal procedures, recruited and interviewed mentors, created manual and organized training
- Organized kick-off event and information sign ups for incoming students and their parents

**Advanced Facility for Avian Research**

Bird care

November 2013- August 2014

- Changed food, water, grit cups and assisted in daily and weekend care

**VOLUNTEER EXPERIENCE****Canadian Association of Girls in the Sciences**

September 2015 – Present

- Organize monthly events based around STEM fields for elementary school aged girls

**Graduate Editor, Western Undergraduate Psychology Journal**

September 2016-August 2017

- Evaluate and determine if submissions should be accepted or rejected
- Revise and edit accepted submissions

**Program Reviewer – Graduate Rep**

September 2016

- Graduate representative for the review of the proposed Master of Data Analytics Program at *The University of Western Ontario*

**Thames River Clean-Up**

May 2015 and October 2015

- Collected and documented waste along Thames River shoreline

**Bylaws Committee Member (Public Service Alliance of Canada)**

September 2015-June 2016

- Reviewed/edited/updated the Teaching Assistant's Bylaws Policy with four other union members

**Member of Student Retention Working Group, King's University College (formerly Student Engagement Committee)**

September 2014- September 2015

- Sat on academic committee to evaluate and develop retention practices
- Specific contributions include; Faculty Handbook, King's One Pager, online resource page

**Member of Student Engagement Committee, King's University College**

September 2013- September 2014

- Member of academic committee to evaluate and develop opportunities for student engagement
- Specific contributions include; proposal for King's Academic Mentoring Program to be adopted by the college and part of the annual budget

**Canadian African Community Healthcare Alliance**

Fall Caravan to Tanzania 2012

- Logistics team
- Assistant to medical staff and surgeons

**Collingwood General and Marine Hospital**

June 2012- January 2013

- Volunteer
- Clerical duties
- Porter
- Determined dietary needs and allergies of incoming patients

**SCHOLARSHIPS AND ACADEMIC HONORS**

- Dean's Honor Role, 2014
- Entrance Scholarship to *the University of Western Ontario*, \$2500