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Honors Thesis

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Abstract

Stress represents an adaptive response to overcome external threats, yet stress can have adverse effects on the body and the brain. Previous research has shown that stress in juvenile songbirds can detrimentally affect song learning and production. However, little is known about the effects of stress hormones, such as corticosterone on adult birdsong. I used male zebra finches to conduct a within-subjects experiment to examine the effects of corticosterone administration on song stereotypy. I administered corticosterone and peanut oil (vehicle) both for a period of 24 hours and three weeks to each individual before recording and comparing their for similarity. I determined percent similarities between and within conditions. Results show a significant effect of experiment duration regardless of treatment and a significant linear trend in the decrease of percent similarity across treatments. Although stereotypy was seen to decrease as corticosterone and time increased, song latency and the number of songs per recording showed no trends. These results suggest that the primary neural structures responsible for aspects of song development and maintenance (HVC) are vulnerable to the negative effects of stress and corticosterone, while other aspects of song production such as motivation to sing seem to be maintained. This study demonstrates that adult songbirds along with juveniles can be just as susceptible to the negative aspects of stress.

The Effects of Corticosterone on Song Stereotypy in the Adult Zebra Finch

The effects of glucocorticoids and stress on humans have been extensively studied, although many gaps in our knowledge remain regarding the effects of stress on behavioural systems. During a stressful event glucocorticoids are released; a process that has been shown to cause a shift away from hippocampal memory (Schwabe, 2013). The role of hippocampal memory is explicit in that it assists in the development of procedural learning processes (Anderson, 1987). Glucocorticoids modulate a shift in memory consolidation to form more habit-forming memory instead of more intensive cognitive memory, which decreases energy demands in the brain (Schwabe, 2013). Furthermore increases in habitual behaviour, such as those associated with drug use and addiction, have been linked with decreases in activity of neural areas controlling for executive functions (Everitt & Robbins, 2005). The study of glucocorticoids is an important area of research to fully understand the function of stress and its relation to the brain and behaviour.

In humans, the main glucocorticoid released from the adrenal cortex is cortisol, in rodents and birds it is corticosterone (Schwabe, 2013). In the adult rat an increase in adrenal hormones such as corticosterone, have been shown to decrease neurogenesis in the brain (Gould, Cameron, Daniels, Woolley, & McEwen, 1992). The same study also demonstrated that adrenalectomized rats increased neurogenesis in the dentate gyrus, but this effect could be suppressed with corticosterone supplementation. This demonstrates that corticosterone can physiologically affect the adult brain in mammals, although potential behavioural effects of this physiological change were not examined in this study.

An excellent animal model to observe potential behavioural effects of corticosterone are songbirds. Songbirds have complex interacting motor vocal and neural system's which translate into precise musculature movement for the production of song (Brenowitz, Margoliash, & Nordeen, 1997), making them ideal for testing the effects of stress on behaviour. When comparing birdsong and human speech, there are many similarities. Both humans and birds use auditory feedback to practice and learn their tutors vocalizations (Doupe & Kuhl, 1999). Interestingly, both human babies and infant songbirds need to be exposed to the normal vocalizations of their species in order to learn it themselves. In addition, both show the presence of a critical sensitive period in learning vocalizations, such that if vocalizations are not mastered by a certain age a deficiency persists throughout the adult life in the production of speech and song (Doupe & Kuhl, 1999). Compared to other species, both forms of vocalization are shown to be more complex, bird and human vocalizations share the trait that timing beyond that of a single syllable is an important part of communication. Many bird species include a 'sentence' timing structure that is similar to our human syntax (Doupe & Kuhl, 1999). In combination with the similar vocal learning models songbirds' exhibit, their neural structures are also physiologically affected by corticosterone. In songbirds the descending motor pathway related to song production consists of the HVC (proper name) and the RA (Robust nucleus of the Arcopallium), through which the brain stem eventually projects to the birds vocal organ, the syrinx (Wild, Williams, & Suthers, 2000). The HVC's relation to song production has also been seen in the pattern of neuron bursts exhibited complimentary to song syllables and structure, meaning that when a bird sings the pattern of the song profile is reflected in the activation of the HVC (Kozhevnikov & Fee, 2007). The anterior forebrain pathway which is implicated in song learning, also projects from HVC, which suggests the dual role HVC may play in song learning

and song production (Kozhevnikov & Fee, 2007). Corticosterone have been shown to affect many aspects of the song control system, including HVC and RA, which would theoretically also affect song production. Both HVC and RA size significantly decrease with corticosterone administration, as well as decreasing the overall number of neurons in the HVC (Newman, MacDougall-Shackleton, An, Kriengwatana, & Soma, 2010).

As previously mentioned there has not been significant investigation into the behavioural effects of corticosterone, specifically on birdsong in adult birds. However, some work has addressed the developmental effects. The Developmental Stress Hypothesis examines the relationship between early life stressors and potential physiological and behavioural detriments a bird may experience as a result of increased stress (Spencer, Buchanan, Goldsmith & Catchpole, 2003). First termed the Nutritional Stress Hypothesis (Nowicki, Peters, & Podos, 1998), it is based on the theory that song is an honest indicator of male quality. Females prefer males who are better singers, indicating that song may be an evolutionary selected trait and that it should be mediated by cost (Spencer & MacDougall-Shackleton, 2011). The Nutritional Stress Hypothesis states that early life stressors during a fledgling's development can be reflected in phenotypic traits. Growth rates were seen to be negatively affected by nutritional stress in zebra finches, and it was further hypothesized that because the song control system in the brain is one of the last regions to develop that it would be susceptible to negative effects from nutritional stress as well (Nowicki et al., 1998). Since many songbird species show that a female is more likely to choose a male if he has a larger song repertoire and can sing for longer periods of time (Clayton & Prove, 1989), it is important to understand the value of environmental stressors placed on song birds.

Spencer, Buchanan, Goldsmith and Catchpole (2003) separately tested the effects of corticosterone and food restriction stress in relation to the Developmental Stress Hypothesis. Three groups of zebra finches were hatched through random pairing and were randomly assigned to a parent group to be raised. Each parent group raised a brood in an experimental condition and a control condition, there were three parent groups in total. The two experimental groups of zebra finches would either be exposed to nutritional stress or corticosterone administration, while the control group was raised in normal conditions absent of stressors. Birds were raised in this fashion for a period of 30 days and songs were recorded between 100 and 200 days of age. It was found that both food restriction and corticosterone administration significantly affected the total number of syllables, the total length of song and the peak frequency reached by the young songbirds. Although the hatchlings that were in the food restriction experimental condition did not have elevated circulating corticosterone levels, there were still significant negative effects on song production and learning. This demonstrates that both environmental stressors and endogenous hormone levels can independently effect song learning and development. It was also hypothesized that since the food restricted birds did not show elevated levels of corticosterone, that corticosterone may act as a mediator in resource allocation in development, which still led to deficiencies in song learning that lasted until adulthood (Spencer, Buchanan, Goldsmith, & Catchpole, 2003). Spencer et al. (2003) shows that a variety of stressors may affect behavioural systems in developing songbirds, however there is still a lack of investigation into the effects of stress on the adult brain in songbirds.

Songbirds are also shown to have one of the most extreme examples of neural plasticity in adult vertebrates. Through environmental cues such as seasonal changes, birds undergo many parallel physiological changes (G T Smith, Brenowitz, Beecher, & Wingfield, 1997). These

changes can be linked to many circulating hormones such as testosterone and other androgens (G

Troy Smith, Brenowitz, Wingfield, & Baptista, 1994). This implies that during adulthood when

songbirds are undergoing natural changes in neural physiology they may be particularly

susceptible to the negative neurological effects of corticosterone. Based on the significant level

of neural plasticity seen in the avian song control system it implies that song production could be

negatively affected by stress.

Zebra finches make an excellent model to examine the direct effects of corticosterone on

adult songbird neuroplasticity and behaviour. Like the majority of songbirds, sexual selection is

driven by females, therefore song may be an honest indicator of male quality (Nowicki &

Searcy, 2004). Additionally, their HVC size has been seen to positively correlate with song

repertoire size (Airey & DeVoogd, 2000) indicating that HVC size may facilitate fitness through

the capacity of a larger song repertoire. The zebra finches' neural structures and song structures

have already been extensively studied, providing previous relationships between song and neural

structures (Airey & DeVoogd, 2000; MacDougall-Shackleton, Hulse, & Ball, 1998; Spencer et

al., 2003). A final quality that makes them an excellent model is that they are non-seasonal

breeders: they breed all year round, limiting the fluctuation of hormone levels associated with

breeding cycles during different times of the year, such as testosterone (G T Smith et al., 1997).

The importance of using a non-seasonal breeder is based on a number of previous studies.

For example, research examining the seasonal fluctuations of testosterone in male song sparrows

has shown that, it is crucial to limit hormone fluctuation because it can physiologically and

behaviourally affect song (G T Smith et al., 1997). In early to late spring plasma testosterone

levels in song sparrows were seen to rise significantly above the values seen in fall, correlating

with the sparrow's breeding season. Furthermore, a decrease in size in both the HVC and the RA

were seen in this time period (G T Smith et al., 1997). Finally song stereotypy, the variation

between same songs, was found to significantly differ over this duration as well. It was

hypothesized by the authors along with Lieberberg and Nottebohm (1979) that since the vocal

motor tissues of the syrinx contain androgen binding receptors, changes in testosterone levels

may change the direct abilities of the musculature tissue to produce songs consistently (G T

Smith et al., 1997). This potential confound can be eliminated by using non-seasonal breeding

songbirds such as the zebra finches.

This study seeks to examine the relationship between stress and its effects on adult

birdsong. Current gaps exist in the literature on examining the relationship between these two

variables. This study uses quantitative measures of song stereotypy and song production to

examine specific relationships between birdsong and stressors. For the purpose of this study,

song stereotypy is defined as the consistencies in pitch, total length of the song and the number

of syllables between songs. The hypotheses for this study state that corticosterone exposure will

significantly decrease song stereotypy, while chronic exposure will have increased detrimental

effects compared to acute exposure. Ten male zebra finches experienced all four conditions, an

acute exposure period to corticosterone, a chronic exposure period to corticosterone and two

control conditions matching the treatment conditions in duration. The effects on song production

and stereotypy were quantified by analyzing the similarity of specific song elements between and

within conditions.

Materials and Methods

Subjects

In this experiment I used ten male and two female zebra finches. The males were bred at the Advanced Facility for Avian Research Western University in July 2013, and were at least three months old at the time of the experiment. All of the birds were taken from the free flight aviary at the beginning of the study. I housed them in individual cages, they received food, water and grit ad libitum, and were housed on 14:10 h light:dark cycles with an average room temperature of 24ºC.

Recordings

I performed all song recordings in a walk-in isolation chamber equipped with a cage holding platform, using a Marantz Recorder PMD671 with a Sennheiser microphone to record all songs. During each recording the microphone was placed on top of the male's cage while the recorder remained outside of the recording chamber. For a recording session I transferred a male from its home cage into a cage in the isolation chamber, this was done at approximately 10:00 h for each recording. After approximately 24 hours in the isolation chamber I randomly paired the male with a female and moved the female into the isolation chamber in a separate cage. Immediately after the female was introduced I recorded all vocalizations for a period of ten minutes. Two of the birds have failed to sing on their specified recording dates, I gave them an additional rest period of two weeks and then they were re-recorded in addition to their original specified dates.

Corticosterone Administration

At the beginning of the experiment I made approximately 270 mL of 0.2 mg/mL corticosterone (CORT) solution; dissolving 53.2 mg of corticosterone in approximately 5 mL of acetone, then adding it to 266 mL of peanut oil. I stirred the solution for 20 minutes and the

acetone evaporated off. Before each administration of corticosterone I also stirred the solution

for approximately 5 minutes. During the experimental conditions I administered 20 µL of the

prepared CORT solution twice a day to the males, which I pipetted onto their egg food. Through

previous unpublished research from the Advanced Facility for Avian Research, this oral dose has

been shown to mimic endogenous levels of mean corticosterone in zebra finches when

experiencing restraint stress, elevating plasma concentrations to between 40 and 60 ng/mL.

Experimental Design

Each bird experienced all four treatment conditions. The four conditions consisted of a

short control period, a long control period, a short experimental period and a long experimental

period. The order of treatments was assigned using a randomized block design. Short exposure

conditions were executed first to avoid any carry over effects of the long exposure. The duration

of the long period was 21 days and the short period was 24 hours. During experimental

conditions I administered the CORT solution, while pure peanut oil was used as the vehicle in

the control conditions. I isolated the birds for the last 24 hours of each condition, and then

recorded them following the procedure described above. There was a seven day recovery period

after the first and second short conditions and a 31-day recovery period after the first long

condition. During the 31-day recovery period birds were placed back into group housing under

the same conditions experienced in individual housing.

Procedure

I fed the birds a quarter teaspoon of egg food twice a day, every day during each

condition. I made egg food fresh daily by combining one hardboiled egg, ¾ of a slice of white

bread and one tablespoon of cornmeal in a commercial food processor and blending it until

coarsely ground. For birds in experimental conditions I pipetted 20 µL of CORT onto their egg

food and for birds in control conditions 20 µL of pure peanut oil prior to it being placed in their

individual cages. I distributed the egg food at approximately 10:00 h and 18:00 h every day on

plastic dishes. For the last 24 hours of each condition birds were isolated for approximately 24

hours and then randomly assigned to a female and their vocalizations recorded as described

above. The purpose of isolation was to evoke singing when reintroduced to a female. Note that

for both short conditions the birds spent the entirety of that condition in isolation. After the 10

minute recording session both the male and the female were returned to their home cages.

Song Nomenclature and Analysis

In this study I defined the smallest unit of song as a syllable or note, the combination of

these elements in a cluster as a song and multiple song clusters as strophes (MacDougall-

Shackleton, Hulse & Ball, 1998). Using a USB cable I transferred songs from the digital recorder

to a computer and analyzed them using sound analysis software (Sound Analysis Pro 2011),

developed by Ofer Tchernichovski at City University, New York. Sound Analysis Pro 2011 is a

specially designed software program used to analyze birdsong and compare features of song to

one another. This software was used to examine the differences in note and song consistency

within and between each recording from the same bird. Using Raven Pro sound analysis

software, each recording was visualized as a spectrogram and five songs were sectioned out and

saved as individual .wav files representing the average song profile for that recording. Using

Sound Analysis Pro 2011 a similarity batch was run. The similarity batch measure allows the

comparison of two columns of .wav files. Analyses were conducted on an MxN matrix, which

compares all possible combinations of wav. files in the two columns and produces an output for

each specific comparison which includes % similarity, accuracy, % sequence and entropy. The %

similarity scores for all individual comparisons for a particular batch were averaged to come up with the overall % similarity score for that recording. All batch comparisons involved comparing recordings to each other by the same bird.

Song stereotypy was analyzed in two separate ways, consistency within the same recording and consistency between recordings. Song consistency within the same recording was measured by placing the same five song samples in each similarity batch column and determining an overall % similarity for each recording. Using Graph Pad Prism (version 6) the % similarity score for each recording was put into a two-way repeated measures ANOVA with duration and treatment as the variables. Song consistency between recordings was measured by taking five song samples from each eggfood/short condition (baseline) and comparing them to the five song samples from the remaining three conditions. This produced a total of three similarity scores for each bird, eggfood/short compared to eggfood/long, eggfood/short compared to corticosterone/short and eggfood/short compared to corticosterone/long. Data was entered into a repeated measures one-way ANOVA to compare differences between conditions.

Other aspects of song performance such as latency to singing and number of songs per recording were also examined using a repeated measures two-way ANOVA. Song latency was recorded in seconds and represents the amount of time before the first song in each recording. I calculated the number of songs per condition by examining each recording as a spectrogram in Raven Pro and counting the number of songs.

Results

Four major analyses were conducted to determine the effects of corticosterone on birdsong stereotypy. A significant effect of duration was found when examining stereotypy

within the same condition, $F(1, 9)=6.435$, $p=0.03$, birds in longer conditions showed a significant

decrease in song stereotypy within the same recording compared to the short conditions. A

strong negative trend was found for the effects of treatment within the same recording, $F(1,$

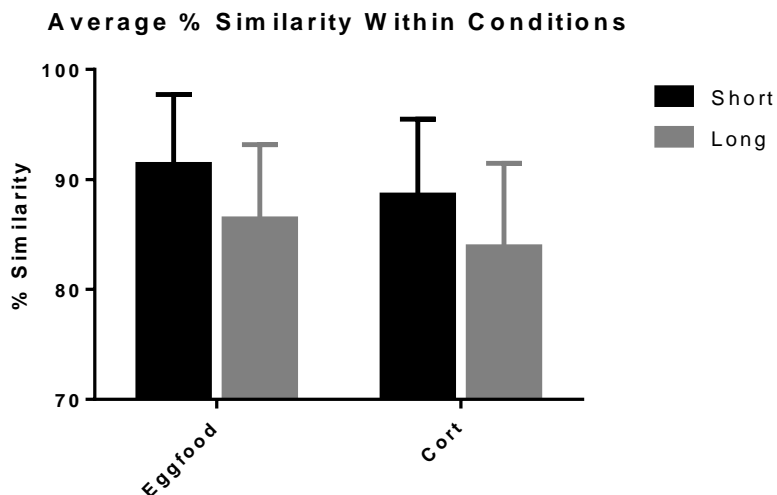$9)=4.054$, $p=0.07$, although not significant (Figure1.). No significant interaction was found

Figure 1. The effects of treatment and control conditions on song stereotypy within the same recording (mean + SE), n=10. Effect of duration, $p=0.03$, effect of treatment, $p=0.07$. Short refers to the short duration conditions and long to the long duration conditions.

between these effects, $F(1, 9)=0.001$, $p=0.97$. Song stereotypy was not found to significantly

decrease between conditions, $F(2, 18)=2.963$, $p=0.07$, although a similar negative trend exists

demonstrating that songs from the eggfood/short condition directly compared to the other

conditions are less stereotyped (Figure 2.). There were no significant effects when examining

other measures of song performance such as song latency and number of songs per condition

(Figure 3.), $F(1, 9)=0.643$, $p=0.44$ and $F(1, 9)=0.000$, $p=0.99$ respectively.

Average % Similarity Between Baseline Recording and Alternate Conditions
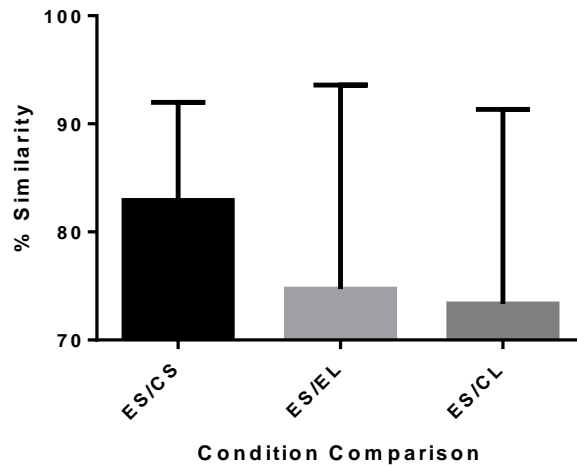

Figure 2. The average % similarity in songs when comparing the eggfood/short condition to all other conditions (mean + SE), n=10. Effect of treatment, *p*=0.07.
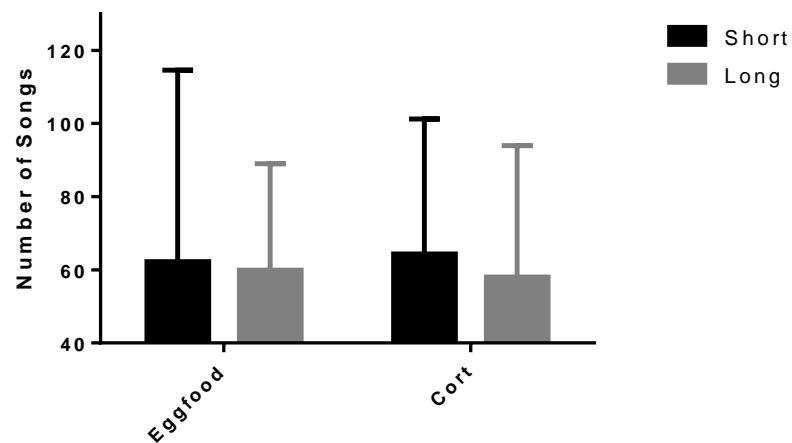
Average Number of Songs Per Condition

Figure 3. The average number of songs per condition sung (mean + SE), n=10. Birds were not seen to significantly decrease in the number of songs sung as corticosterone and duration increased, *p*=0.99. Short refers to the short duration conditions and long to the long duration conditions.

Discussion

Results from this study demonstrate that not only can corticosterone administration decrease song stereotypy but also long periods of disturbance stress, even after song

crystallization has occurred. Based on current literature there is a gap in the effects of stress on

song stereotypy in adult avian models, therefore my study sought to examine the relationship that

exists between stress and consistencies in song production while indirectly examining the effects

on the song control pathway in the avian system. Previous research has shown that females

prefer males who sing for longer periods of time and sing songs that contain a larger number of

syllables (Clayton & Prove, 1998), thus decreases in song stereotypy, such as a reduction in the

number of syllables, can directly influence an individual's fitness through processes of female

choice.

In Brumm et al. (2009) it was shown that developmental stress affected song learning but

not song complexity in zebra finches. Developmental stress was manipulated through food

restriction, and fledglings that were exposed to the experimental treatment did not see a

significant decrease in the total number of song syllables, syllables copied from a tutor or the

syllable type (Brumm, Zollinger, & Slater, 2009). These results contrast with the findings of this

study that stress can in fact decrease song complexity. If song complexity is examined through

the number of syllables and the number of different syllables it can be stated that birds in this

experiment saw an overall decrease in song complexity as well as song stereotypy.

During this experiment, zebra finches experienced two different types of stress,

corticosterone administration and disturbance stress. As the primary stress hormone in avian

models corticosterone mimics the internal chemical state of a bird when experiencing stress. The

disturbance stress resulted from the control conditions where eggfood was placed inside bird's

cages twice a day for the period of each condition. The significant effect of durations speaks to

the vulnerability of the song control pathway to stressors (Figure 1.). These results also provide

evidence as to the multiple ways the avian system defines and perceives stress and adjusts its internal state to compensate for these stressors.

Corticosterone administration has been shown to significantly decrease the size of the HVC in previous studies (Newman et al., 2010), which would theoretically also affect song stereotypy through the song control pathway. Birds in this study did show an overall linear decrease in song stereotypy due to corticosterone administration. The strong negative trend towards decreasing song stereotypy when comparing stereotypy within conditions and between conditions (Figure 2.) suggest that there is some effect on the HVC, and that the HVC may also be responsible for controlling the consistency of songs produced. The results from the primary analyses on song stereotypy support the hypotheses for this study and show that as both corticosterone and duration increase, song stereotypy decreases.

The maintenance of other aspects of song performance, such as the number of songs per condition (Figure 3.), suggest that the effects of corticosterone and stress are isolated to detriments in song stereotypy and not other factors such as motivation to sing or energy allocation. When finches experienced long control or treatment conditions they did not show a significant trend in decreased motivation to sing or an increase in the amount of time to start singing. Since the number of songs and the latency to start singing did not significantly decrease or show any trends it is likely that the effects of corticosterone administration and disturbance stress are effecting neural structures in the brain associated with song production instead of altering more emotional factors such motivation, signifying a link between stress, the song control pathway and behaviour.

In conclusion the results from this study provide the first clear example of the relationship between stress and song stereotypy in adult songbirds. Although treatment effects were not seen to be significant at $p<0.05$, strong negative trends exist implying the independent ways that both corticosterone and stress can affect behaviour. The demonstrated negative effects on song stereotypy have the ability to impact an individual's fitness, directly through sexual selection but also indirectly through methods such as obtaining decreased territory quality, and therefore merit further investigation.

Limitations

In natural environments zebra finches are social animals. Throughout this experiment however, finches were housed individually. This was to ensure that each male received and ate the full dose of eggfood or CORT that they were assigned for that condition. Both of the short conditions were carried out first in order to avoid carry-over effects, however at the end of the first long condition (control or treatment) birds had been housed individually for a period of approximately 70 days. Although all birds were housed in the same room, the isolation of being alone in a cage could have evoked stress in the birds, resulting in additional stress at the end of the first long period which could have contributed to effects on song. After all birds completed the first long period they were placed in group housing for the 31-day recovery period, then individually housed again for the final long condition. Although the results of this study show that % similarity was lower in the long corticosterone administration period than the long control period, birds could experience these conditions in any order, the specific contribution of isolation stress on song stereotypy was not examined in this study.

Future Research

Future research should focus on quantifying the specific contribution of isolation stress on song stereotypy in zebra finches. Furthermore, all birds were presumed equal in resiliency, although song profiles differed between birds. It should be examined if birds with a more attractive song are more resilient to stress and maintain a good song throughout exposure to stressors. While birds with poor songs would have received poor feedback from mates and might theoretically under-value the necessity of maintaining a consistent song throughout stressful periods. Based on the time limitations for the completion of this study, the recovery curve for song stereotypy was not examined. It is strongly recommended that after exposure to a stressor birds are recorded multiple times during recovery to track the relationship between stress, song stereotypy and baseline song recovery. Finally to establish a concrete relationship between stress, the brain and birdsong a similar between subjects design should be conducted with post mortem analysis examining the effects on HVC and other neural structures.

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