

Cognitive and Brain Consequences of Early Life Immune System Challenge in a
Songbird

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ABSTRACT

Cognition, defined as the mechanism by which an animal acquires, processes, stores, and uses information present in the environment, is a trait that is sensitive to developmental conditions. Existing research supports the idea that the ability to develop and maintain cognitive abilities depends on the physiological condition of the individual, which can be influenced by the early environment. Alterations in maternal care, social stress, and malnutrition are some examples of environmental conditions that impact development and resulting cognitive abilities across taxa. The primary goal of this research was to determine whether immune system challenge during the critical song learning period in zebra finches (*Taeniopygia guttata*) would lead to long term negative impacts on song quality and learning, spatial learning, and neophobia. Immune challenge during this period of development did not produce long term impacts on learning or memory, nor did it lead to any changes in neophobic responses. However, birds that were hatched later in a clutch performed better on the motoric and spatial tasks, and were less neophobic. Future research in zebra finches that can describe the variation in song attributes as a function of hatching order would be a useful first step in determining a mechanistic link between hatch order and song learning outcomes.

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1. Introduction

Early environment impacts phenotype

Animals can exhibit phenotypic plasticity, in that they are able to adjust their phenotype in response to environmental variation. This plasticity can occur during critical periods of development, where a given genotype can result in a range of phenotypes depending on the environmental context in which the organism develops (Gilbert, 2001). In many instances, an animal's fitness is maintained, because the resultant phenotype is suited to the demands of the adult environment (Monaghan, 2008). In other instances, when an environment is constraining, an animal may have to adopt a less adaptive strategy in order to survive (McEwen & Wingfield, 2003). Such adjustments may promote survival in the short-term, yet impose negative consequences on the animal later in life (Metcalf & Monaghan, 2001; Monaghan, 2008). Phenotypic change resulting from environmental influences during development may impose costs on the adult animal such as reduced lifespan, or reproductive output (Metcalf & Monaghan, 2001). Thus, under certain circumstances, characteristics of the early environment can act as developmental constraints that can negatively impact fitness in adulthood (Stamps, 2003).

Cognition is influenced by developmental condition

Cognition, which has been defined as the mechanisms by which an animal acquires, processes, stores, and uses information present in the environment (Shettleworth, 2010), is a trait that is sensitive to developmental conditions. Existing research supports the idea that the ability to develop and maintain cognitive abilities depends on the physiological condition of the individual, which can be influenced by the

early environment (Buchanan, Grindstaff, & Pravosudov, 2013). Alterations in maternal care, social stress, and malnutrition are some examples of environmental conditions that impact development and resulting cognitive abilities across taxa. For example, rat mothers subjected to stress during gestation exhibit reduced maternal care behavior and have offspring that express greater behavioral anxiety, potentially facilitating attentiveness to important environmental stimuli (Champagne & Meaney, 2006). Wistar rats reared in social isolation demonstrate deficits in filtering out unimportant or irrelevant environmental stimuli, also known as sensorimotor gating, as adults (Ellenbroek & Cools, 2002). Humans that were undernourished as children show a higher frequency of mental disorders such as attention-deficit disorder (Layer & Gómez-pinilla, 2008). Birds that experience malnutrition during early development demonstrate impaired spatial learning (Pravosudov, Lavenex, & Omanska, 2005).

The developmental stress hypothesis

Birds are a particularly well-established model for examining the effects of the early post-natal environment on cognition, as nestlings are not buffered from the effects of environmental change by a lactating mother, and could face a range of potential early-life stressors in the wild. The developmental stress hypothesis, studied in songbirds, posits that a variety of early-life stressors can negatively impact brain growth and subsequent song learning and memory, posing

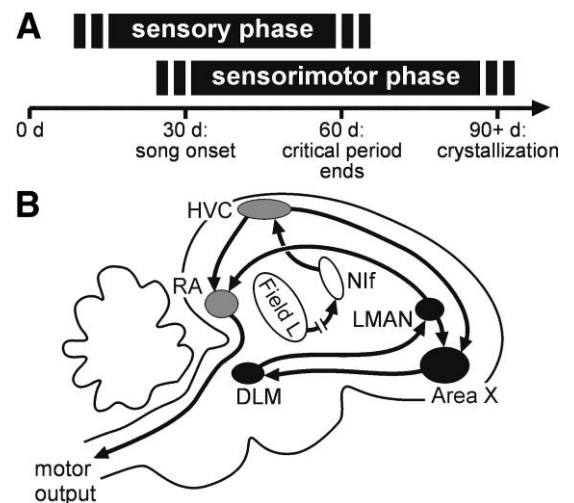


Figure 1.1. Timeline of song learning and the avian song system. Song learning (A) and the song system (B). (Kojima and Doupe, 2007).

consequences for an animal's later-life fitness (Stephen Nowicki, Peters, & Podos, 1998). Female songbirds demonstrate preferences for particular attributes of song that can be impacted by stressors that impair brain development, implicating song as an honest signal of male quality (Stephen Nowicki & Searcy, 2004). In most species, juvenile males learn songs from tutors during an early-life critical period, which is arguably considered a cognitive trait (Marler, 1990; Stephen Nowicki et al., 1998). The process of song learning has been well studied in male zebra finches (*Taeniopygia gutatta*), which learn their songs from adult tutors, generally their father, in three phases within the first 90 days of life. The sensory phase, during which a juvenile acquires memory of tutor song begins at about 20 days post hatch and ends around day 60 post hatch. The sensorimotor phase, during which juveniles practice vocalizing and replicating memorized song, begins around day 30. Crystallization of mature, stereotyped song occurs when sexual maturity is reached around day 90 (Immelmann, 1969). During this learning period, areas in the brain that make up a neural circuit, called the song control system, grow in size and connectivity (Fig. 1) (Brainard & Doupe, 2002). Specifically, in the sensory and sensorimotor periods of song learning, young males are sensitive to factors such as limited food resources and parasitic infection (Stephen Nowicki & Searcy, 2004).

Developmental stress particularly impacts the song control nucleus HVC, the volume of which is positively correlated with song complexity and accuracy (DeVoogd, 2004; S Nowicki, Searcy, & Peters, 2002). For example, juvenile male swamp sparrows (*Melospiza georgiana*) that were fed 70% of the average volume of food consumed by controls produced poorer quality songs as adults, which were less attractive to females (S Nowicki et al., 2002). Additionally, the volume of the song control nucleus HVC is

smaller in the brains of these males, suggesting that food restriction impacted song learning through effects on brain development (S Nowicki et al., 2002). Subsequent studies have shown that males that experience early-life stressors ranging from parasitic infection to sibling competition produce poorer quality songs, likely as a result of compromised brain development (Peters, Searcy, & Nowicki, 2014). Studies comparing males that were food restricted as nestlings to males that were treated with corticosterone as nestlings, found reductions in song quality relative to controls, suggesting that corticosterone may mediate the impact of these early-life stressors (Schmidt, McCallum, MacDougall-Shackleton, & MacDougall-Shackleton, 2013; K. a. Spencer, Buchanan, Goldsmith, & Catchpole, 2003).

The developmental stress hypothesis was originally applied only to song learning and the underlying song control system, but has been expanded to address a wide variety of phenotypic traits including spatial cognition and hippocampal volume (Farrell, Weaver, An, & MacDougall-Shackleton, 2011; Pravosudov et al., 2005). For instance, hippocampal volume and spatial retrieval ability is reduced in food-caching scrub jays that experience early-life nutritional stress (Pravosudov et al., 2005). This effect was corroborated in a study in zebra finches from the Sewall lab (unpub data). Thus, developmental stress has the potential to influence the trajectory of brain development, which consequently impacts learning and cognition.

The immune system, early immune challenge, and learning

Developmental stress in songbirds has been studied extensively in the context of nutritional and glucocorticoid manipulations (Peters et al., 2014). Additionally, early-life parasitic infection has been demonstrated to lead to a reduction in song complexity and

the growth of HVC (Karen a Spencer, Buchanan, Leitner, Goldsmith, & Catchpole, 2005). However, the effects of immune system challenge in birds are relatively unresolved, despite some evidence that there may be programming effects of immune system activation on behavior on birds (Grindstaff, 2015). Grindstaff et al., (2012) examined the interaction between maternal and developmental immune challenges in zebra finches. They determined that male birds that were challenged with a different antigen than their mother had reduced performance in a novel foraging task, and exhibited less neophobic behavior, suggesting that early-life immune system activation has potential programming effects on cognition.

Evidence from rodent studies suggests that early-life immune challenge directly impairs cognition and brain architecture through programming microglia, the primary immunocompetent cells of the brain. Specifically, rats infected with *E. coli* show evidence of neuroinflammation during early infection as well as an elevated pro-inflammatory cytokine IL-1 β secretion from microglia when subsequently exposed to the same antigen (S. Bilbo & Schwarz, 2009; Bland et al., 2010). Within the brain, cytokines are constitutively expressed at low levels and are regulators of sleep, metabolism, and memory (Yirmiya & Goshen, 2011). Pro-inflammatory cytokine levels are elevated in the brain when peripheral cytokine levels are elevated, indicating an interaction between immune activity in the periphery and the central nervous system, likely through permeable areas of the blood-brain barrier (Verma, Nakaoka, Dohgu, & Banks, 2006). This interaction in the brain induces sickness behavior, which includes reduced activity and increased sleep (Dantzer & Kelley, 2007). In the rodent brain, IL-1 β receptors are highly expressed in the hippocampus, and seem to be necessary for the regulation of

hippocampus-dependent memory (Goshen et al., 2007). Mice without receptors for IL-1 β have memory impairments, and administration of a range of doses of IL-1 β into the lateral ventricles of wild-type mice following fear conditioning demonstrated that IL-1 β impacts learning in an inverted U shaped fashion (Goshen et al., 2007). Rodents that are infected with *E. coli* in early life show memory deficits as well as exaggerated IL-1 β levels upon a secondary immune challenge, compared to controls, suggesting that early-life immune system challenge sensitizes the system, leading to exaggerated IL-1 β expression (S. D. Bilbo et al., 2005; Bland et al., 2010). Specifically, infected animals show poor learning in fear conditioning tasks after initial infection and subsequent immune challenge. Additionally, early-life infected rodents show reduced neurogenesis and greater numbers of microglia in the hippocampus in adulthood after a secondary challenge, suggesting that early-life immune activation leads to changes in brain architecture in areas that underlie cognition.

In the songbird brain, the same microglial cytokines localize around the site of a penetrating injury, suggesting that similar mechanisms underpin neuroinflammation in rodents and birds (Duncan & Saldanha, 2011). However, the songbird brain has at least two specializations that may protect against neuroinflammation. First, inflammation in the avian brain promotes increased expression of aromatase from glia, which has a neuroprotective role and may potentially mitigate the negative effects of prolonged cytokine expression in the brain (Duncan & Saldanha, 2011; Saldanha, Duncan, & Walters, 2009). Second, the songbird brain shows neurogenesis across a wider range of brain regions than does the mammalian brain (Doetsch & Scharff, 2001), and may therefore be more resilient to any damage induced by the inflammatory response than has

been reported in rodents. Thus, it is unclear if immune challenge could be an early life stressor that would impact the songbird brain and subsequent cognition through similar mechanisms as have been described in rodents.

If immune challenge does impact brain and cognitive development, it may be through other downstream effects of an immune response, such as reduced food intake, or elevated glucocorticoid levels, which can act as indirect developmental stressors that negatively impact the brain and cognition. In particular, immune system activation may trigger the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the production of glucocorticoids in the brain (Karrow, 2006). Glucocorticoid receptors are present within the HVC and the hippocampus of songbirds (Hodgson et al., 2007; Suzuki, Matsunaga, Kobayashi, & Okanoya, 2011), and exposure in excess or during a sensitive period of development may be detrimental.

If early-life immune system challenge can shape brain development in a way that limits learning, either directly or indirectly, then immune-challenged birds should perform songs of lower quality than control birds, as well as show poorer performance on a motoric and spatial task. If the effects of early-life immune challenge are achieved through neuroinflammation, we expected fewer neurons in the song control nucleus HVC as a result of reduced neurogenesis (S. Bilbo & Schwarz, 2009). However, regardless of whether immune challenge acts through microglial-mediated or indirect mechanisms on the brain, such as increased HPA axis activity, there may be a reduced number of new cells in these respective areas, and these brain regions may be smaller, overall, in immune-challenged birds. Finally, if learning deficits are not observed, it may be indicative of a protective mechanism in the songbird brain, possibly mediated through the

induction of aromatase (Duncan & Saldanha, 2011; Saldanha et al., 2009), or higher resiliency as a result of active neurogenesis (Doetsch & Scharff, 2001).

2. Impacts of early-immune system challenge on cognition and song learning

Introduction

The goal of this study is to determine how an early-life immune system challenge impacts cognitive performance and underlying brain architecture in zebra finches. Early immune challenge could impact songbirds through either immune system-mediated mechanisms, or through non-immune mediated processes such as by negatively impacting appetite (and thus nutrition), elevating glucocorticoid levels, or reducing attention to song tutors. The first step in resolving how immune challenge could result in variation in cognition requires evaluating behavior, and brain architecture.

To determine how early-life immune challenge impacts the brain, cognition and behavior, we treated juvenile male zebra finches with lipopolysacharride (LPS), which produces a well characterized inflammatory response (Wang, Rousset, Hagberg, & Mallard, 2006), twice during the first 90 days of life (see figure 2), and compared them to males that received either no injection, or a vehicle injection of phosphate buffered saline (PBS). We then evaluated their spatial cognition, neophobia, and accuracy of song learning before collecting their brains. We then correlated attributes of song to the volume and density of HVC and the telencephalon.

Methods

Experimental Overview

After receiving an initial injection at D30 post hatch, birds were group housed with an adult male tutor on D35. At D40, birds were trained on a motoric learning task, and subsequently were given a second injection at D50. Ten days later at D60, birds were administered the spatial learning task. At adulthood (D100), birds were assayed for neophobic responses and their final songs were recorded before their brains were collected on D115. A depiction of the experimental timeline can be viewed in Figure 2.

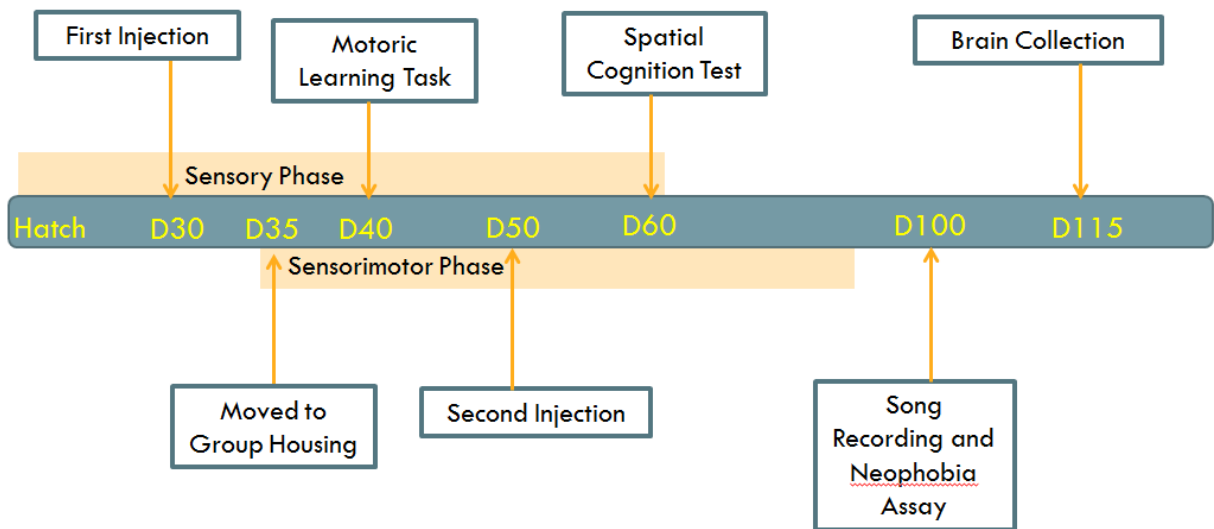


Figure 2.1. Experimental timeline.

Breeding

All methods were approved by the Virginia Tech Institutional Animal Care and Use Committee (15-015). The animal room was maintained on a 14:10 light-dark cycle. Male and female adult domesticated zebra finches were obtained from two separate

breeding colonies (The College of William and Mary, Williamsburg, VA and Duke University, Durham NC). Birds were housed in 11 breeding pairs in a divided flight cage (48 x 47 x 52 cm, Prevue Pet Products, Chicago, IL). Pairs were randomly assigned, and any birds that failed to show any breeding activity were re-assigned a new partner after approximately two weeks. Pairs were supplied with a nest box and ample nesting material (shredded twine and strands of string) for building. Birds were supplied with *ad libitum* access to finch seed mix (Kaytee Supreme Bird Food) supplemented with egg food (Quiko Classic Eggfoods), water supplemented with vitamins (Vita-Sol 8-in1), grit, and cuttlebone.

Hatching and sexing

Nest boxes were checked daily for eggs, and the date each new egg was found was recorded. Upon hatching, young were uniquely color marked with a non-toxic marker (Crayola Easton, PA), in order to keep track of individuals and nestling hatch order. Because the experimental timeline required the identification of males before molt into sex-specific adult plumage, approximately 5 uL of blood was collected from the brachial vein and used for genotyping, once nestlings had fledged (D20-D22). DNA was extracted using a Qiagen DNEasy kit (Qiagen INC, Valencia, CA). Sex determination was made using standard polymerase chain reaction (PCR) procedures. The microsatellite primers P2 and P8 were used to amplify sex-linked CHD genes (CHD-W for females, CHD-Z for both sexes) (Griffiths, Double, Orr, & Dawson, 1998). Once hatched, young were weighed to the nearest 0.01g on D5, 10, 20, 30, 35, 50, and 115.

Juvenile Treatment

Once males were identified, they were assigned to one of three treatment groups: LPS derived from *Escherichia coli* (n = 11), sterilized phosphate buffered saline (PBS) (n = 10), or no injection (n = 10). Treatments were distributed such that no siblings from a given clutch received the same treatment. On day 30, during the sensory learning phase (S Nowicki et al., 2002), juvenile males received either an initial injection of 1 mg/kg of LPS (Sigma Aldrich, L3012) in 40 uL of a 1:1 solution of sterile PBS and pre-sterilized Freund's Incomplete Adjuvant (n=11) (Owen-Ashley et. Al., 2006, Grindstaff et.al 2012), 40uL filter-sterilized PBS (n=10), or received no injection (n=10). All injections were done subcutaneously near the keel, after cleansing the skin with ethanol. A secondary injection was administered on day 50, during the sensorimotor phase of song learning.

Group Housing and Tutors

On day 35 post hatch, juvenile males were separated from their family and moved to group housing with a genetically un-related tutor male. Two males per treatment group were assigned to a tutor, for a total of six unrelated juveniles per tutor (i.e, siblings were not assigned to the same tutor).

Motoric and Spatial Learning

Motoric and spatial learning tasks were adapted after (Boogert, Giraldeau, & Lefebvre, 2008). Twenty-four hours before behavioral testing, birds were moved from group housing and were individually housed in a wire-mesh cage placed inside a soundproof chamber. Two 2.5 cm x 2.5 cm x 2 cm blocks with a center hole of 1.2 cm diameter and 1cm depth were also placed in the cage to allow the bird to habituate to them. The holes were to be covered with lids consisting of a blue plastic disc (diameter 1.5cm) with a rubber bumper affixed to one side to weigh the lid down. Six hours before

trials, birds were deprived of food and the blocks and lids were removed from the cage. The motoric learning task consisted of a shaping procedure in which birds were trained to pull the lids off of the wells contained in two blocks placed at the center of the cage, in order to retrieve a food reward. This was done at three levels: level 1, with the lid next to the well; level two, with the lid half-covering the well; and level three, with the lid covering the well. Birds advanced to the next level when they successfully completed three consecutive trials at each level. The motoric learning task was completed when a bird could complete level three in three consecutive trials, with a maximum of 12 two-minute trials per day, with six minutes between consecutive trials. The number of trials required for each bird to successfully complete the motoric learning task was summed across days. One bird was removed from the study after failing to learn the motoric task after five days (60 trials). Next, birds were trained to recognize that food could be found in a well located in each of four corners of their cage. Just before the start of a trial, all four blocks were given four minutes to visit and feed from the wells located in the corners of the cage. The first corner the bird visited during training trials was recorded to determine corner preference. The corners that were visited first the most often and the least often were not chosen to be baited during the spatial learning task. On day 60 post hatch, birds started a four-corner spatial learning task. One corner of the cage was selected to be baited. This remained constant, requiring the bird to remember where to locate the food reward. A bird passed a trial if the first well it attempted to eat from is the well containing the food reward. If the bird failed the trial, the bird was given until the end of the two-minute trial period to visit other corners in order to find the baited corner. A bird passed the spatial learning task when it visited the baited corner first in 6 out of 7

consecutive trials, with a maximum of 12 trials per day. The number of trials required to finish the spatial learning task was summed across days.

Neophobia

After birds reached adulthood (100-105 days), subjects were tested on their response to a novel food source. Twenty-four hours before the neophobia assay began, birds were removed from group housing and individually housed in a cage located in a soundproof chamber. The test cage had a circle with a diameter of 25 cm drawn on the cage floor, and a white food cup (familiar cup) was placed in the center of the circle. On the morning of the neophobia assay, the food cup was removed from the cage for a period of 6 h before trials began. One bird was tested at a time. A webcam was placed outside the chamber so the bird could be observed during the trials. At the start of the assay, motivation to eat was established by replacing the familiar food cup in the center of the cage and timing the latency to approach the food cup (entering the black circle), perch, and eat from the food cup. As soon as the bird began to feed, the cup was removed from the cage and a five-minute waiting period was started. Next, a completely novel food cup (yellow, with red plastic discs glued to the exterior) was placed in the center of the cage and the bird was timed on the latency to approach, perch, and eat from the food cup. The food cup was removed immediately after the bird started to feed. Birds were timed (in seconds) for a period of up to 90 minutes. After another 5 minute waiting period, a second motivation check was done by replacing the familiar food cup back in the cage. Neophobia was measured as the difference in the latency to eat from the familiar cup during the motivation check and the latency to eat from the novel food cup (Roth et. al., 2010).

Song recordings and scoring

The songs of each male tutor were recorded and scored for attributes of song (Airey & DeVoogd, 2000). Recordings of male subjects were made after mature song was produced around day 100. All recordings were made in sound-proof chambers, with a Shure S57 Instrument Microphone, and a Prosonus AudioBox 1818VSL mixer. Twenty-four hours before recording, birds were moved from group housing to individual cages located in the sound-proof chambers. Songs were recorded and stored using Sound Analysis Pro software on a Dell Latitude 3440 laptop computer. Using Syrinx software, songs were visualized into a spectrogram (figure 3), and one motif was selected and scored on several measures: motif duration, total number of syllables per motif, and total number of unique syllables per motif.

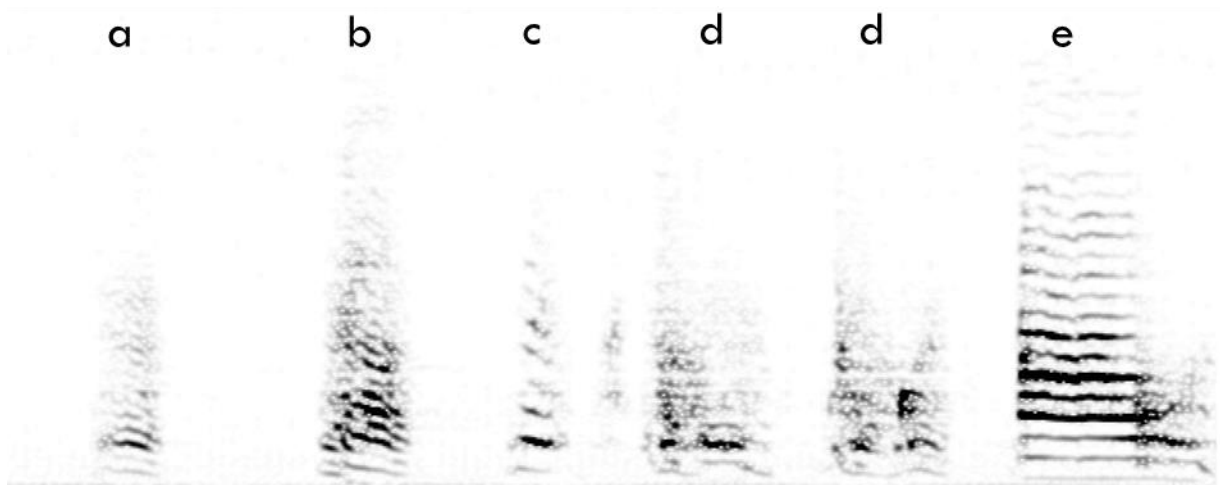


Figure 2.2. Example song motif visualized using Syrinx software. Unique syllables are labeled with a single letter. In this motif there are five unique syllables, with six syllables in total.

Song similarity

The motif of each male subject was compared to its tutor to assess similarity. Before analysis, all motifs were amplitude normalized using Audacity software. Next, using the

batch similarity process with asymmetric comparison in Sound Analysis Pro (Tchernichovski, Nottebohm, Ho, Pesaran, & Mitra, 2000), tutor and tutee motifs were compared. The amplitude was set at 49dB, and all other settings were kept at default. The percent similarity of each subject's motif to its tutor was used in analysis.

Histology

On day 115, birds were deeply anesthetized with isoflurane, and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were collected, and immersion fixed overnight in 4% paraformaldehyde, then transferred to 30% sucrose until saturated a day later. Brains were flash frozen in powdered dry ice, and stored at -80°C until processing. Brains were sectioned in the coronal plane at 40µm on a cryostat. Using standard techniques tissue was mounted on slides and Nissl stained using cresyl violet (Sigma C5402, St. Louis, MO). Volume measurements of the telencephalon and HVC were quantified using the optical fractionator method as described in Ladage et al., 2009 and Pravosudov and Omanska, 2005. Measurements were taken using Stereologer 2000 (Stereology Research Center, Inc.) and a Leica 1-4016 microscope using the 5X objective. HVC volume measurements were taken within a sampling area of 1000 µm² and every third section was measured. Telencephalon volume measurements were taken within a sampling area of 250000 µm² and every twelfth section was measured.

Verification of LPS injections

To verify whether the LPS injections were causing sickness behavior, 6 adult male zebra finches were assigned to be injected with LPS (n=3) or PBS (n=3). Birds were housed individually in a wire mesh cage located in a sound-proof chamber described previously.

Birds had *ad libitum* access to food and water. At 09:30 am the following day, birds were injected with either 1 μ g/ μ L LPS or an equivalent volume of PBS. Behavior was recorded for 30 min, 8 hours after injection and again 24 hours after injection using a Logitech webcam (Logitech, Newark, CA, USA). The camera was turned on 10 minutes before recording in order to minimize disturbance, and no one was in the room during recording. Scoring procedures were adapted after Moyers et. al. (2015) and Burness et. al. (2010). Briefly, videos were scored manually by an observer blind to the treatment group. Each bird was assigned one of five behaviors every 30s during the 30 minute period: immobile, hopping, eating, preening, and drinking. If a bird performed more than one behavior in the 30s period, the period would be coded as the behavior the bird spent a greater amount of time doing.

Statistics

All response variables were checked for normality before analyses using Kolmogorov-Smirnov tests and normal probability plots, and log transformed when appropriate. Treatment effects on song performance, and performance on the motoric and spatial tasks, were tested with generalized linear models with a Poisson distribution and a log link function using hatch order, and treatment nested in parent as factors. Treatment effects on mass were tested with a linear model, using hatch order, and treatment nested in parent as factors. For hatch order, nestlings were sorted in three categories: 1st hatched, 2nd hatched, and 3rd or later hatched. All nestlings hatched 3rd or later were combined into one group due to smaller sample number at later hatch orders. A second simpler model analysis was done examining main effects of hatch order. Treatment effects on latency to eat during the neophobia assay were tested using a general linear model with log₁₀

transformed values. Data from the sickness behavior observations were analyzed using an independent samples t-test with the percentage of time spent on a given behavior as the dependent variable. Pearson's correlations were used to determine relationships between attributes of song and performance on the motoric and spatial tasks.

Results

Mass

There was no effect of treatment on mass ($F(2,18) = 0.0025$, $p = 0.99$) (Figure 6).

Motoric Task

There were main effects of both hatch order ($\chi^2 = 10.392$, $p = 0.016$), and treatment ($\chi^2 = 79.81$, $p < 0.001$) on performance in the motoric task, (Figure 7), as well as an interaction between hatch order and treatment ($\chi^2 = 20.22$, $p < 0.001$). First hatched LPS treated birds performed worse than 1st and 2nd hatched controls and 1st and 3rd vehicle birds (all $p < 0.05$).

Spatial Learning Task

Spatial task performance was influenced by treatment ($\chi^2 = 46.44$, $p < 0.001$), (Figure 8), with PBS treated birds performing better than no injection controls and LPS treated birds. Time to success in the spatial task was not impacted by hatch order or the interaction between treatment and hatch order (all $\chi^2 \leq 6.437$, $p \geq 0.092$).

Neophobia

There was no effect of hatch order ($F(2,4) = 0.480$, $p = 0.096$), treatment ($F(2,4) = 40.171$, $p = 0.174$), or the interaction between treatment and hatch order on neophobia responses ($F(2,4) = 0.058$, $p = 0.945$) (Figure 9).

Song

There was no effect of treatment, hatch order, or the interaction between treatment and hatch order on the total number of unique syllables in a motif (all $\chi^2 \leq 8.403$, $p \geq 0.753$), (Figure 10). There was an effect of treatment on the total number of syllables per motif ($\chi^2 = 41.77$, $p < 0.001$), with vehicle treated birds having fewer total syllables than LPS or no injection controls, but neither hatch order alone nor the interaction were statistically significant (all $\chi^2 \leq 4.837$, $p \geq 0.211$), (Figure 11). There was a significant effect of hatch order ($\chi^2 = 93.51$, $p < 0.001$) treatment ($\chi^2 = 9.704$, $p = 0.008$) and the interaction between treatment and hatch order ($\chi^2 = 15.73$, $p = 0.003$) on motif duration, with first hatched PBS birds having the shortest motifs (Figure 12). There was no effect of treatment or hatch order alone on percent similarity of song (all $F(2,6) \leq 1.655$, $p \leq 0.596$), but there was an effect of the interaction between the two ($F(3,848) = 6.014$, $p = 0.31$), with LPS treated birds that were first to hatch producing songs that were significantly more similar to those of their tutors having significantly higher similarity than no injection birds that were first to hatch ($p = 0.043$), (Figure 13).

Relationships among cognitive and brain measures

There was a significant positive correlation between the number of trials to complete the motoric task and total number of syllables per motif (Pearson's $R = 0.724$, $p < 0.001$), such that birds that took longer to learn the task produced more syllables in a motif. Similarly, there was a significant positive correlation between the trials to complete the motoric task and motif duration such that birds that took longer to learn the task had longer motifs (Pearson's $R = 0.747$, $p < 0.001$) (Figure 14). No other measures of song learning and cognition were correlated (Table 1). There were no correlations

between song characteristics and HVC and telencephalon volume measurements (Table 2).

LPS treatment validation

Birds treated with LPS spent significantly more time immobile than did PBS treated birds ($t(3) = 6.225$, $p = 0.006$), but this depended on the time period examined. At the 24 hour time point, there was no difference between LPS and PBS birds in time spent immobile. ($t(3) = 0.004$, $p = 0.288$). LPS injected birds spent less time feeding 8 hours after injection ($t(3) = 3.687$, $p = 0.032$), but there was no difference between PBS and LPS treated groups 24 hours after injection ($t(3) = 0.383$, $p = 0.369$) (Figure 15).

Simplified models: effect of hatch order alone

When we examined the simplest models, which omitted treatment and included only hatch order as an explanatory factor, four main effects were found. First, there was a significant effect of hatch order on motoric task performance when parental pair was accounted for ($\chi^2 = 134.355$, $p < 0.001$), with third or later hatched birds performing better than first or second hatched subjects (Figure 16). Second, performance on the spatial task was influenced by hatch order ($\chi^2 = 10.71$, $p = 0.005$), with third or later hatched birds performing better than first hatched birds ($p < 0.001$) (Figure 17). Third, birds hatched third or later were the least neophobic of all ($F(17,6) = 7.724$, $p = 0.009$), (Figure 18). Fourth, birds hatched first had a greater number of syllables in their motif than birds hatched second or third or later ($\chi^2 = 59.697$, $p < 0.001$) (Figure 19). No other models examining a main effect of hatch order alone yielded statistically significant results (Table 3).

Table 2.1. Relationships Between song and cognitive measures

	Mean	SD	Motoric	Spatial	Unique	Total Syll.	Duration	Similarity
Motoric	18.3	10.9	-					
Spatial	21.3	9.40	0.21	-				
Unique	5.62	2.01	0.09	0.13	-			
Total Syll.	8.68	5.42	0.72*	0.22	0.33	-		
Duration	1.04	0.73	0.74*	0.21	0.21	0.96*	-	
Similarity	50.3	17.3	0.07	0.07	0.37	0.32	0.34	-

* denotes significance (all $p \leq 0.001$)

Table 2.2. Relationships between song and brain measures

Measure	Mean	SD	Unique	Total Syll.	Duration	Telen. vol (μ^3)	HVC vol(μ^3)
Unique	5.62	2.01	-				
Total Syll.	8.68	5.42	0.33	-			
Duration	1.04	0.73	0.21	0.96*	-		
Telen. vol (μ^3)	1.44×10^{10}	1.89×10^9	0.18	0.69	0.61	-	
HVC vol(μ^3)	2.22×10^7	7.63×10^6	0.01	0.29	0.42	0.49	-

* denotes significance (all $p \leq 0.001$)

Table 2.3. Summary of hatch order effects, part one

Measure	First Hatched		Second Hatched		Third or Later Hatched		DF	χ^2	<i>p</i>
	Mean	SE	Mean	SE	Mean	SE			
Trials to complete motoric task	17.5	1.45	15.54	1.28	14.44	1.28	22.00	134.30	> 0.001
Trials to complete spatial task	23.0	1.62	19.93	1.70	15.95	1.39	22.00	10.70	0.005
Number of unique syllables	6.08	0.92	4.87	0.83	5.91	0.86	18.00	1.10	0.58
Number of total syllables	9.72	1.18	7.19	1.11	7.35	0.96	18.00	59.64	> 0.001

Table 2.4. Summary of hatch order effects, part two

Measure	First Hatched		Second Hatched		Third or Later Hatched		DF	F	<i>p</i>
	Mean	SE	Mean	SE	Mean	SE			
Neophobia	1448.00	621.00	1077.00	621.00	893.00	753.00	17,6	7.72	0.009
Motif duration	1.35	0.13	1.18	0.13	0.90	0.121	16,7	3.40	0.054
Percent Similarity	51.70	6.31	46.30	6.31	52.30	5.64	15,3	1.24	0.49

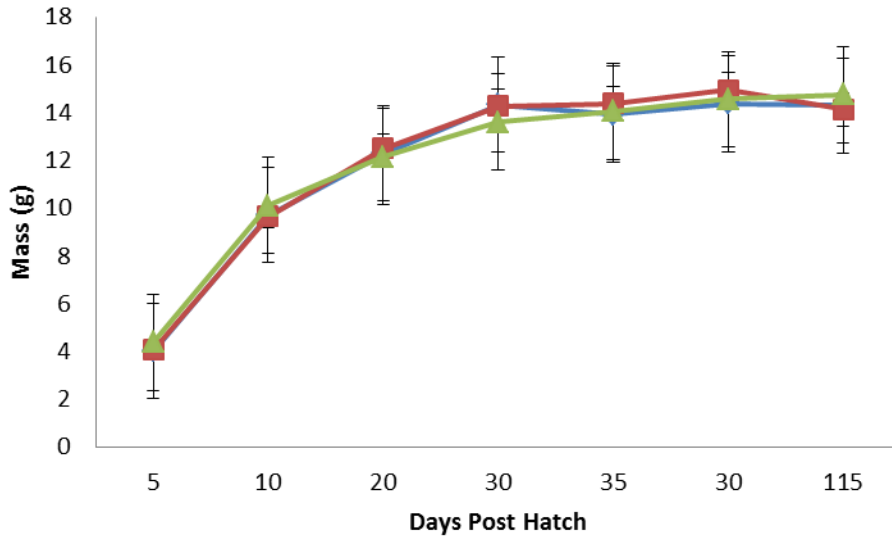


Figure 2.3. Bird mass over time. Birds did not receive experimental treatments until day 30 post hatch. Colors indicate mass of the LPS treated birds (blue), PBS treated birds (red), and untreated birds (green).

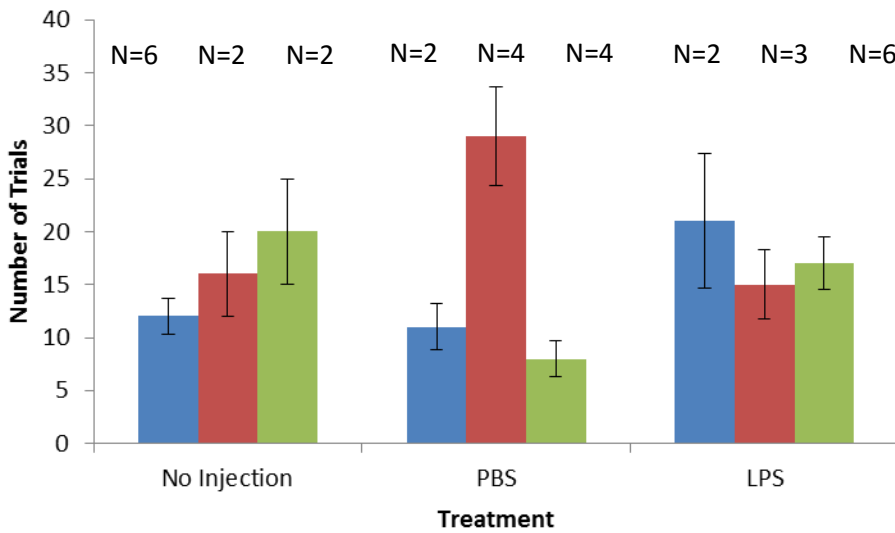


Figure 2.4. Number of trials to complete the motoric task. Number of trials required for subjects treated with LPS, or receiving no treatment to complete the motoric task. Colors indicate the performance of the first (blue) second (red), and third or later (green) hatched subjects with each treatment.

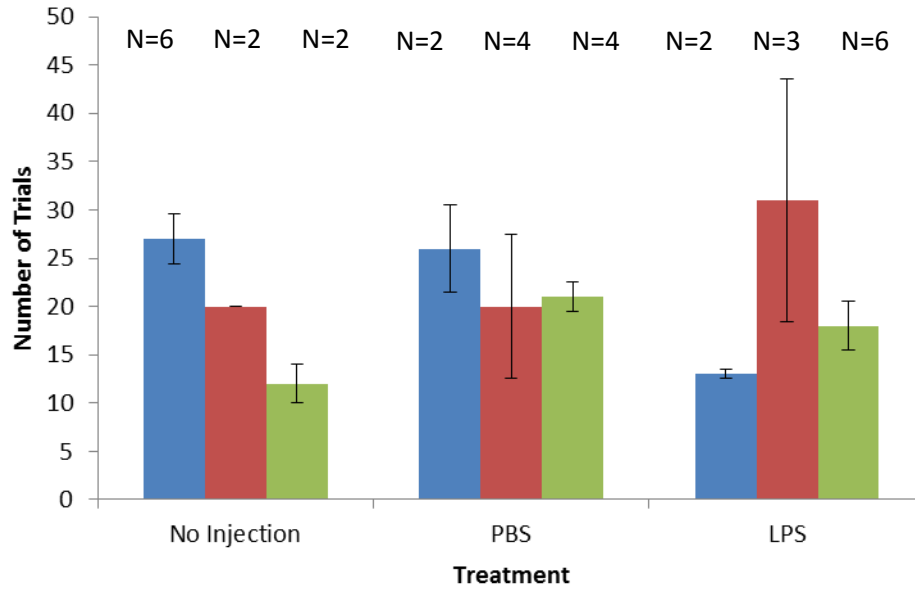


Figure 2.5. Number of trials to complete the spatial learning task.

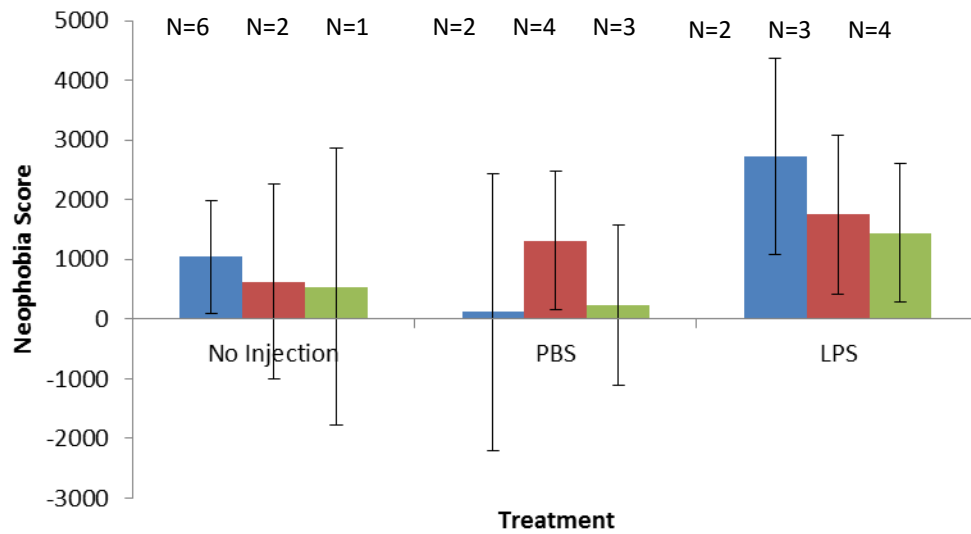


Figure 2.6. Effects of early-life immune challenge on neophobia.

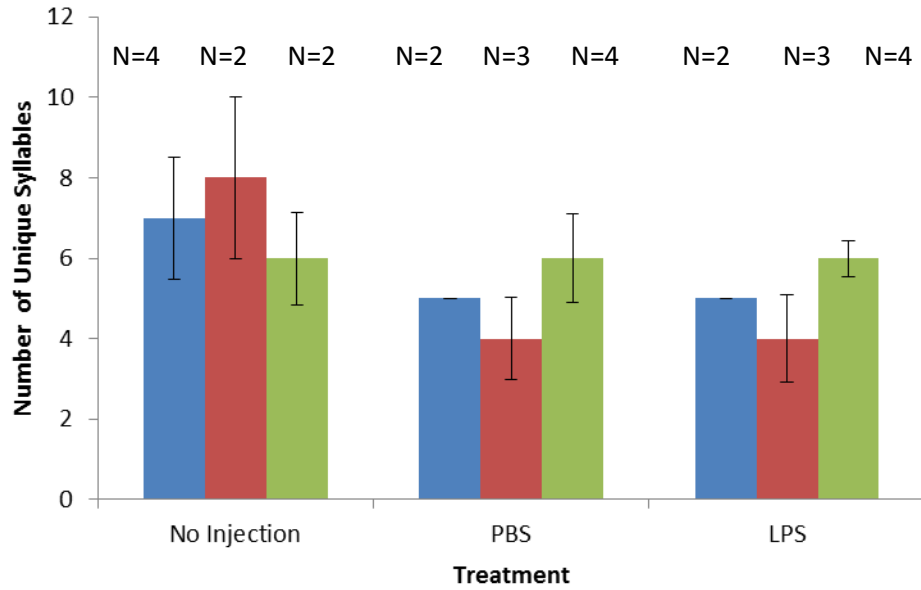


Figure 2.7. Total number of unique syllables per motif.

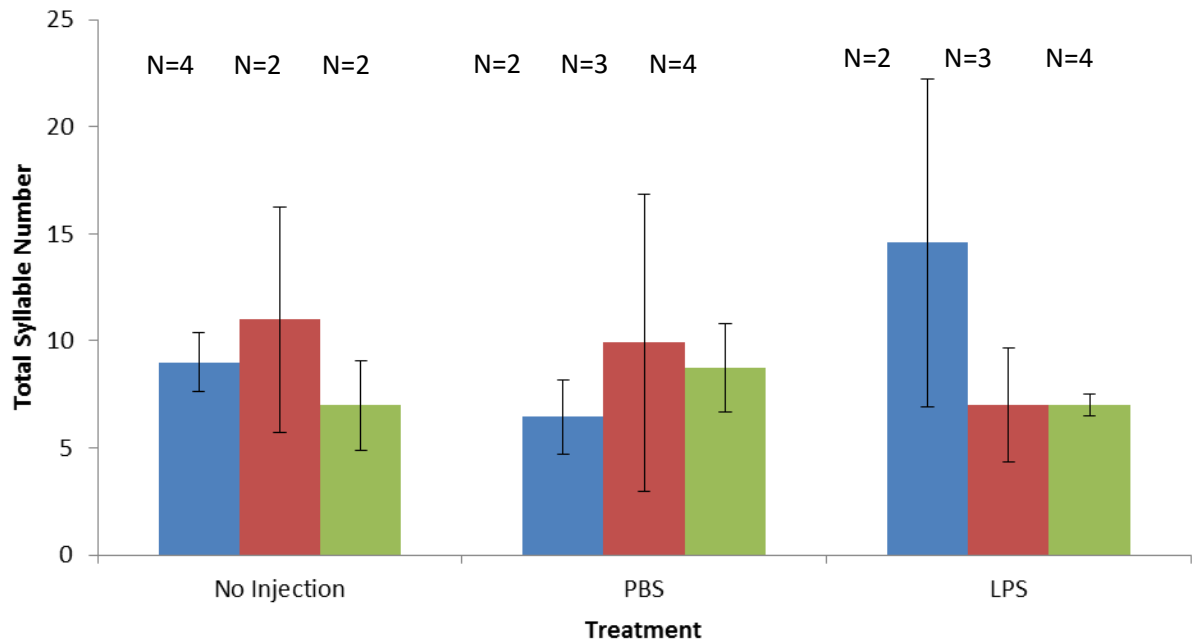


Figure 2.8. Total number syllables per motif.

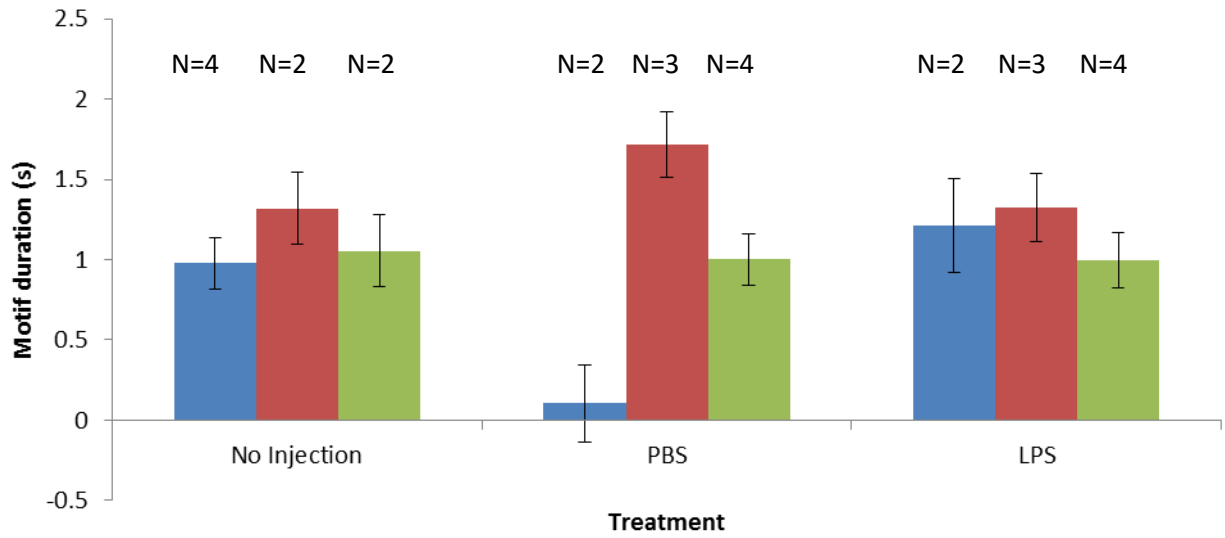


Figure 2.9. Average motif duration

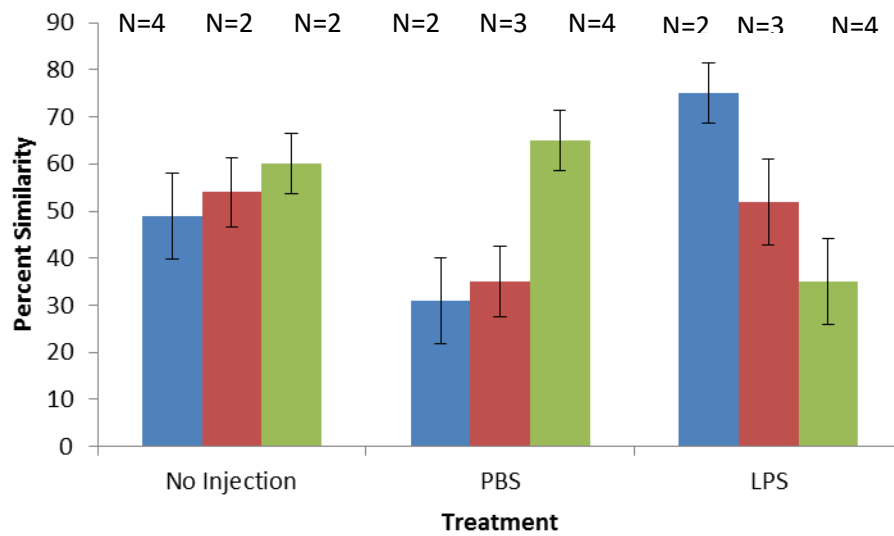


Figure 2.10. Percent similarity of juvenile motif to tutor.

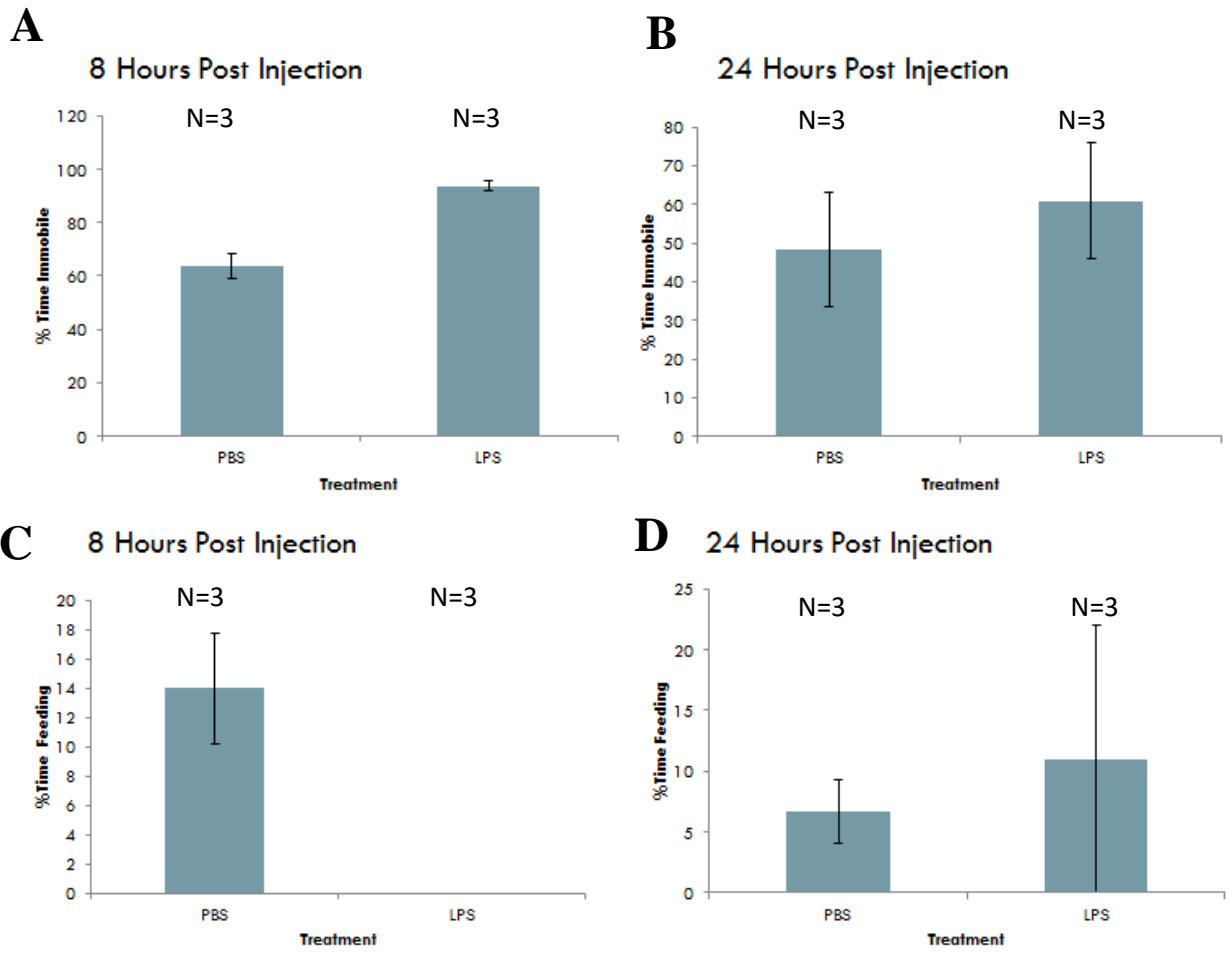


Figure 2.11. Impacts of LPS injection on immobility and feeding behavior 8 and 24 hours post injection.

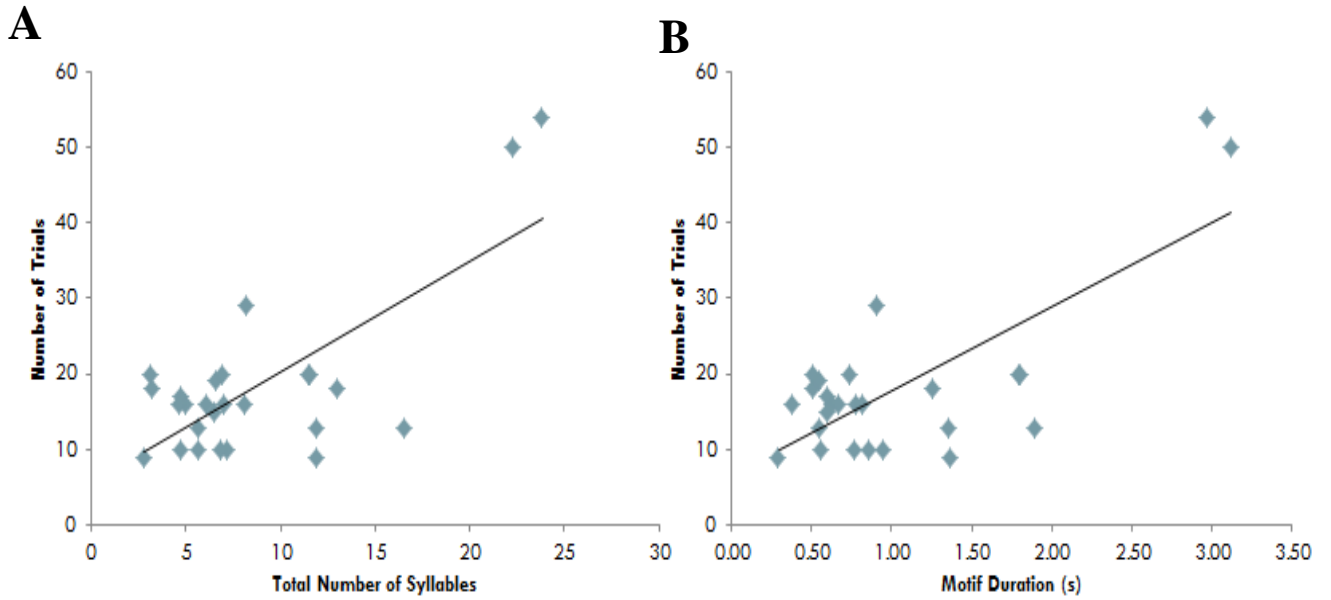


Figure 2.12. Correlation between motoric task data and total number of syllables, and motif duration.

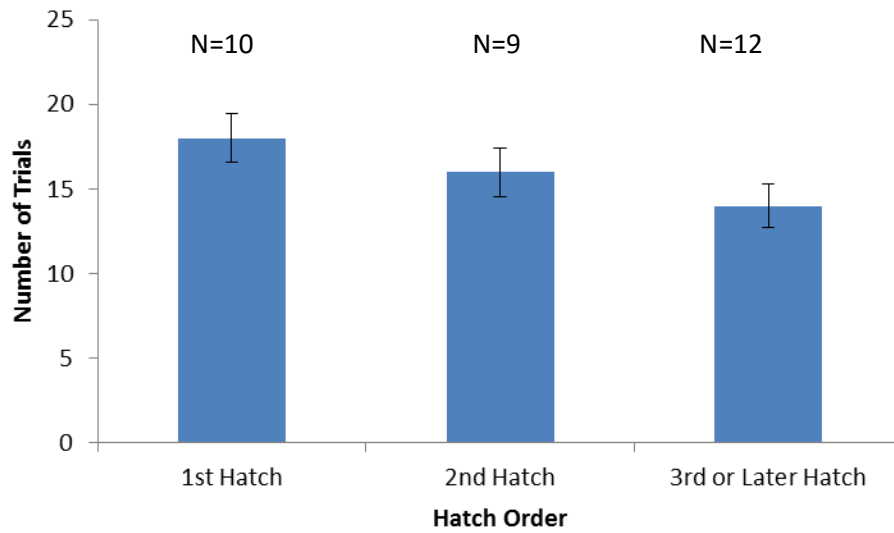


Figure 2.13. Impacts of hatching order on performance on the motoric task.

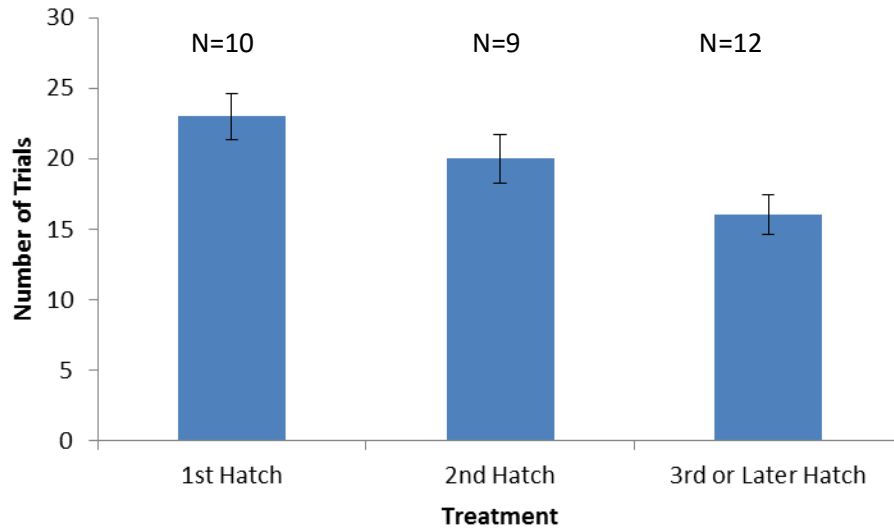


Figure 2.14. Impacts of hatching order on performance on the spatial task.

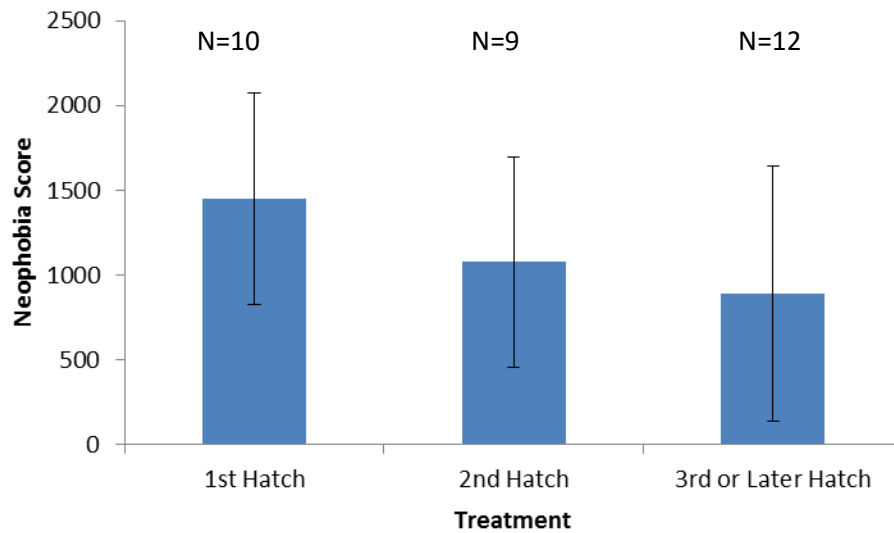


Figure 2.15. Impacts of hatching order on neophobia.

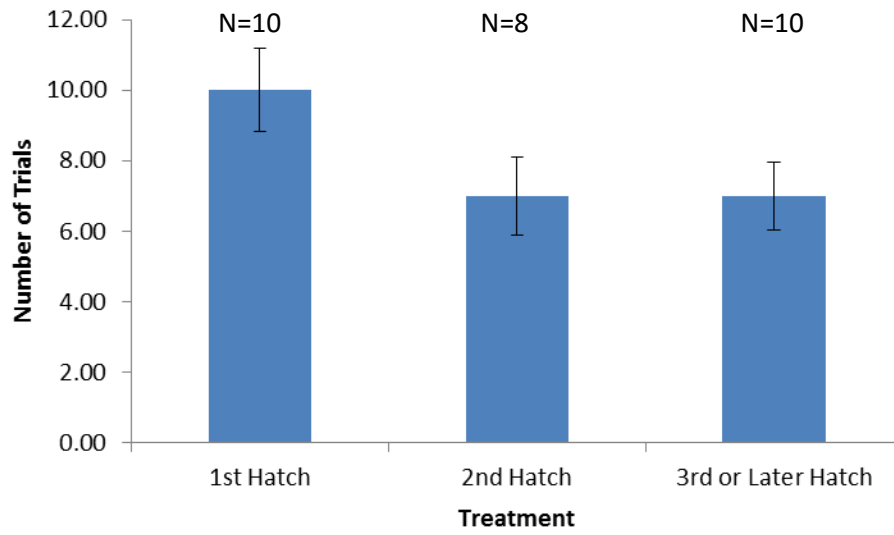


Figure 2.16. Impacts of hatching order on total syllable number per motif.

Discussion

The present study demonstrated that the immune system challenge induced by administration of LPS during the song development period had limited effects on song quality, and no direct impact on cognitive performance or neophobia. We predicted that if early-life immune system challenge acted as a developmental stressor, then male zebra finches should experience deficits in song learning and spatial learning behavior, and show differences in neophobia. Our tests to validate our experimental dose of LPS (also the same as that of Grindstaff et. al. (2012)), demonstrated that the treatment did induce sickness behavior in adults, suggesting that immune challenge during the song development period may not lead to negative impacts on later life learning and behavior. A previous study in zebra finches investigating whether maternal immune challenge had any effect on developmental immune challenge of their offspring, showed no effect of maternal challenge but that male offspring that were challenged with LPS required more trials to complete a spatial foraging task similar to the task in this study (Grindstaff et al., 2012). Grindstaff et. al. (2012) also found that males challenged with LPS early in life were less neophobic than controls, lending support to the hypothesis that developmental stressors can impact animal personality (Kortet, Hedrick, & Vainikka, 2010). However, Grindstaff et. al. (2012) treated nestlings earlier, at day 5 and day 28, which could indicate that the timing of immune challenge could play a role in the potential programming impacts of early-life immune system challenge.

The long-term impact of a developmental stressor on phenotype may be a function of the stressor itself. Previous studies of developmental stress have employed a variety of stressors and found a varying degree of long term impacts. While (K. a.

Spencer et al., 2003) found that food restriction negatively impacted attributes of song, Gil et al., (2006) and Naguib et al., (2008) found that manipulation of brood size did not lead to negative impacts on attributes of song. Similarly, Spencer et al., (2005b) found that early life administration of corticosterone has negative impacts of measures of song quality, suggesting that corticosterone may be mediating the negative impacts of developmental stressors. The degree to which these manipulations are stressful to the animal may vary in severity, leading to these differences in outcome of the quality of song attributes. Lipopolysaccharide stimulates increased levels of glucocorticoids and of pro-inflammatory cytokines such as IL-1 β . At physiological levels both glucocorticoids and IL-1 β are required in normal learning and memory processes, but can be deleterious to these same processes in excess (Diamond, Bennett, Fleshner, & Rose, 1992; Goshen et al., 2007). It may be possible that the dose of LPS used in this study induced an intermediate level of cytokines, glucocorticoids, or both, which in turn did not produce a response that was strong enough to lead to negative cognitive outcomes.

Hatching order significantly impacted the motoric task, spatial task, neophobia and total number of syllables per motif, such that individuals that were hatched later performed better on the motoric and spatial task, and were less neophobic. In species that exhibit hatching asynchrony, first hatched birds are notably larger than later hatched birds. shorter song motif duration. Previous studies have found several phenotypic differences amongst siblings based on hatching order. In a study with barn swallows, later hatched birds begged at a higher rate compared to earlier hatched siblings. These barn swallows were lighter than their siblings, and also had higher circulating concentrations of immunoglobulins in their blood (Saino, Incagli, Martinelli, Ambrosini,

& Møller, 2001). This increase in begging has an energetic cost (Kilner, 2001), and competition for parental care amongst siblings can lead to differences in baseline corticosterone levels. Differences in baseline corticosterone based on hatching order have been observed in collared doves, such that later hatched birds have higher levels than earlier hatched birds (Eraud, Trouve, Dano, Chastel, & Faivre, 2008), suggesting that exposure to elevated levels of corticosterone during development as a function of hatch order may have programming effects on the stress response.

If zebra finches that are later in the hatching order possess higher baseline corticosterone levels than earlier hatched birds, as was found in other species, our results may be consistent with studies that have observed improvements in cognition as a result of higher baseline corticosterone levels. Previous studies have shown that zebra finches that were fed corticosterone as nestlings performed better on a novel foraging task relative to controls (Crino, Driscoll, Ton, & Breuner, 2014), and zebra finches that were fed corticosterone twice a day were less neophobic than control birds (K. A. Spencer & Verhulst, 2007). In our study, we found that in the case of song learning, later hatched birds possessed fewer total syllables than earlier hatched birds. Again, if later hatched birds have higher baseline corticosterone levels, our results would be consistent with studies which found a negative impact of corticosterone on song (Schmidt, Moore, MacDougall-Shackleton, & MacDougall-Shackleton, 2013). Taken together, our finding that later hatched birds performed better on cognitive measures while exhibiting a reduction in song quality suggests that hatch order may impact some aspects of learning differently than others. This idea is further supported by our finding that performance on the motoric learning task was negatively correlated with motif duration and the total

number of syllables per motif. Studies that examine cognition and behavior as a function of hatch order are uncommon in songbirds. It is important to conduct further studies to investigate the impact of hatching order on phenotype.

3. Conclusions

The goal of this research was to determine whether immune system challenge during the critical song learning period in zebra finches would lead to long term negative impacts on song quality and learning, spatial learning, and neophobia. Immune challenge during this period of development did not produce long term impacts on learning or memory, nor did it lead to any changes in neophobic responses. However, birds that were hatched later in a clutch performed better on the motoric and spatial tasks, and were less neophobic. These findings raise three possibilities that deserve future testing. First, it is possible that birds are more resilient against the possible neurotoxic impacts of early immune system challenge. Second, the timing of vulnerability to any stressors, including immune challenge, may be important for understanding phenotypic outcomes and challenge during the song learning period may have less impact than exposure earlier in life. Third, differences in sibling competition amongst hatchlings may influence later-life learning and memory.

Possible mechanism of neuroinflammation in songbirds

The mechanisms of inflammation in the songbird brain in response to peripheral immune challenge are largely unresolved. However, there is some indication that songbird brains possess a protective mechanism against the possible detrimental effects of neuroinflammation. When the inflammogen phytohemagglutinin is directly administered

to the surface of the brain, the subsequent inflammatory response characterized by the release of Il-1 and Il-6, presumably from microglia, induces the production of aromatase (Duncan & Saldanha, 2011). The resulting estrogen production has been found to promote neuroprotection and limit neurodegeneration (Saldanha et al., 2009). It is possible that this response is mitigating the possible negative impacts of early-life immune challenge. To gain a better understanding of the neuroimmune mechanisms of immune system challenge in zebra finches, the expression profiles of inflammatory cytokines such as Il-1 β and Il-6 should be quantified at various life stages (in the periphery and in the brain) after LPS challenge, and compared with controls. If peripheral immune challenge does impact the brain through communication across the blood-brain barrier, then cytokine levels in the brain should be elevated as a result (Goshen et. al., 2009). In addition, if these neuroimmune consequences are mediated through microglial activation, as they are in rodents, then more microglial activation should be observed in the brains of challenged animals (S. Bilbo & Schwarz, 2009). If increases in cytokine levels are observed in the brain, then aromatase induction should also be measured in order to determine whether aromatase plays a role in the brain's response to peripheral immune challenge (Duncan & Saldanha, 2011).

Effects of Timing of Stressor Exposure on Phenotypic Development

Our finding that early-life immune challenge during the song development period did not lead to a clear pattern of deficits on cognition, but Grindstaff et. al. (2012) did find effects when challenging zebra finches during the nestling period, suggests that the timing of immune challenge interacts with the extent of cognitive outcomes. In young birds, different behavioral outcomes have been observed as a result of the timing of

exposure to a stressor. Boogert et. al. (2011) determined that Japanese quail differed in their response to social information, such that birds that were exposed to elevated corticosterone prenatally were more likely to respond to social instruction than birds that were subject to unpredictable food availability. Birds that were exposed to corticosterone before hatching were also found to be more active and exploratory, and had a permanently reduced acute stress response (Zimmer et. al., 2013). Several studies conducted in birds suggest a possibly adaptive value for elevated corticosterone levels. For instance, Japanese quail exposed to unpredictable stressors performed better on a spatial memory task (Calandreau et. al., 2011). Crino et. al. (2014) found that zebra finches fed corticosterone as nestlings performed better on a novel foraging task relative to controls. Stressors can range in severity and impact on a variety of phenotypes. Glucocorticoids have been shown to have a dose-dependent impact on cognitive and behavioral outcomes (Diamond et. al., 1992). These results support the idea that adverse early conditions can program individuals to be better at exploiting novel environments as adults (Love and Williams, 2008). In addition to results shown in developmental stress studies in birds, in rodents, elevated levels of glucocorticoids during development have been demonstrated to lead to deficits in learning and memory

Impact of Sibling Competition on Phenotypic Development

The conditions of the rearing environment have been demonstrated to lead to impacts on development and adult phenotype. For instance, several previous studies of developmental stress, addressing nutritional stress specifically, have used brood size manipulations to simulate a natural range in variation in early developmental conditions. While some have found no effect on song learning or quality (Gil et. al., 2006, Naguib et.

al., 2008), others have found negative effects on a variety of attributes of song (Holveck et. al. 2008, Tscherren et. al., 2009). With larger broods, there is greater constraint on parents because they have more offspring to attend to (Stearns 1992) Additionally, increased competition amongst siblings, and the increased begging that results, can be energetically costly and can influence early condition (Kilner 2001, Neuenschwander 2003). Zebra finch nestlings raised in larger broods have reduced mass prior to independence, and though they are able to compensate after fledging, in adulthood, their body condition returns to being lower than that of birds that were raised in smaller broods (Naguib 2004).

Although many of these studies examine the impact of brood size on developmental condition, few have examined these variations in the context of hatching order. However, hatch order has been observed to lead to differences in phenotype. For example, later hatched ring dove nestlings had higher baseline levels of corticosterone, a primary stress response hormone in birds, than earlier hatched nestlings (Eraud et. al., 2008). In separate studies, differences in corticosterone levels have been found to impact cognition and the brain. Spencer et. al. (2003) found that zebra finch nestlings treated with corticosterone from day 5 to day 30 post hatch had higher baseline corticosterone levels and had a reduced syllable number per motif. In song sparrows, the song control nucleus RA volume is decreased in individuals with elevated corticosterone (Schmidt et. al. 2013), indicating that exposure to elevated corticosterone can produce long term effects on song and its underlying neural correlates.

The present study demonstrated that later hatched birds had fewer total syllables, per motif, indicating that developmental condition as a function of hatching order has

impacts on song. Therefore, it is possible that hatch order may influence song by way of differential levels of corticosterone. Future research in zebra finches that can describe the variation in baseline corticosterone levels and relate these levels to song attributes as a function of hatching order would be a useful first step in determining a mechanistic link between hatch order and song learning outcomes.

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