June 2017

Effects of an Early Life Immune Challenge on Body Growth, Personality, Mating Behaviors, and Brain Development of Zebra Finches (Taeniopygia guttata)

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Effects of an Early Life Immune Challenge on Body Growth, Personality, Mating Behaviors, and Brain Development of Zebra Finches (*Taeniopygia guttata*)

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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College of Arts and Sciences
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Date of Approval
June 12, 2017

Keywords: stress, poly I:C, activity, birds, zenk, HVC

DEDICATION

This work is dedicated to my parents, brother, and sister-in-law. With their full support, I am now able to finish what I started. They helped me tremendously at the hardest times of this work. I owe you a great debt of gratitude. Thank you. This one is for you all.

ACKNOWLEDGMENTS

I would like to thank the following students for helping me with different aspects of this project: Amanda Oram, Alexandra Krajcevska, Manuela Florez, Samantha Schwartz, Yeisa Torres, and Alexandra Gryzbowski. Their patience and effort to help me in this long-term project will always be greatly appreciated.

I would also like to thank Dr. Leilani Kellog in USF's Psychology department. Dr. Kellog was generous with her time to train me in an experimental procedure, which greatly facilitated the completion of this project.

I would be remiss if I did not acknowledge the very patient and accommodating Dissertation Committee members: Dr. Lynn B. Martin, Dr. Thomas Sanocki, Dr. Cheryl Kirstein, and Dr. Michael Brannick.

Last, but not least, I would like to thank my Major advisor and mentor, Dr. Toru Shimizu. Without his patient guidance, this dissertation would not be possible. The experience I gained in our discussions shaped my approach to science and will continue to guide me in future studies.

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ABSTRACT

The developmental stress hypothesis predicts that an aversive condition, such as decreased food intake, predation, and social isolation, in the early developmental stage could have long term effects on behaviors and brain development of an animal. In nature, bird nestlings are susceptible to various factors, such as malnutrition, infections, and parasites. Effects of early life stress on adulthood have been extensively studied with some stressors including malnutrition. However, immune challenges as an early life stressor and their long-term programming effects on adult behaviors are yet to be studied in detail. The goal of the current study was to investigate changes in growth rate, personality, mate selection behaviors and brain development in zebra finch nestlings after injection with a viral infection mimicking agent, Polyinosinic: polycytidylic acid (Poly I:C). By using Poly I:C, it was possible to isolate long-term effects to the immune response of the bird. After Poly I:C injection on post-hatch day (PD) 14, morphological measures were conducted to detect changes in body growth rate. When birds became sexually mature (> ~PD 200), behaviors of birds were observed in different conditions to detect changes associated with the personality traits of animals. In mate choice trials, both attractiveness of males and mate selection behaviors of males and females were investigated. Finally, the development and neuronal activity of specific brain nuclei involved in courtship (i.e., HVC and RA) and social/sexual behaviors (nucleus taeniae of the amygdala, TnA) were investigated. The results showed that nestlings' growth rate was not affected. However, Poly I:C injection had some effects on certain, but not all, personality traits observed in the study. Such

effects were found only in female zebra finches, suggesting that there was a limited sex-specific influence of an early life immune challenge on personalities of adults. The results also showed that Control females tended to choose untreated males over Poly I:C injected males in mate choice trials. Finally, Poly I:C injection negatively affected the overall development of targeted brain nuclei. In addition, neuronal activity in TnA was higher in Poly I:C injected birds. Results of the present study suggest that one time injection with Poly I:C early in the life causes long term effects on adulthood. These findings are further discussed regarding their relevance to the developmental stress hypothesis.

INTRODUCTION

Experiences in the early developmental stage can have significant effects on behaviors in adults (Grindstaff, 2016; MacDougall-Shackleton & Spencer, 2012; Zimmer, Boogert, & Spencer, 2013). In song birds, Nowicki, Searcy and Peters (2002) proposed the Developmental Stress Hypothesis (DSH), which predicts that a limited period of stress, such as undernutrition in early life of male songbirds, will adversely affect subsequent developments of their brain structures and the quality of courtship songs. In addition to undernutrition, it is known that nestling birds are also susceptible to parasites, bacteria, and viruses that are causing immune challenges. The goal of this study was to test some predictions of DSH with an early life immune challenge (ELIC) as a stressor and to detect how a viral infection would program adult behavior. Specifically, this study examined whether an ELIC as a stressor in young songbirds affects their well-established robust behaviors such as exploratory, social, and mate choice, when they become adults. While there are a few studies on ELIC in birds, little is understood about the mechanism, extent, and degree of its effects. For example, although there are avian studies using ectoparasites such as the hen flea (*Ceratophyllus gallinae*) (Bischoff, Tschirren, & Richner, 2009) and endoparasites such as malaria (*Plasmodium relictum*) (Spencer, Buchanan, Leitner, Goldsmith, & Catchpole, 2005), it is unclear whether the observed effects (i.e. decreased song overlap, and reduced song nuclei volume) was due to either immune system activation or parasites. The current study focused on long-term effects of immune system activation by using a

synthetic double-stranded RNA, Polyinosinic: polycytidylic acid (Poly I:C), which mimics viral infections.

This study had four specific aims:

Aim 1: Does Poly I:C injection affect the body growth rate in nestlings of zebra finch (*Taeniopygia guttata*);

Aim 2: Does ELIC affect personalities of adult zebra finches;

Aim 3: Does ELIC affect behaviors in mate choice trials of adult zebra finches; and

Aim 4: Does ELIC affect the brain development of adult zebra finches.

In order to accomplish these specific aims, the present study used multidisciplinary approaches including physiological and anatomical measurements, behavioral observations, and analysis of neuronal activity using an immediate early gene (IEG). The present study was one of the first systemic studies on ELIC using viral infections on birds. The results provided information about changes in the behavior and brain development of infected birds. Studying behavioral changes can be useful for ecological studies on disease transmission in wild populations, as a permanent change in a birds' behavior may eventually affect transmission rate of a parasite within the social group (Ezenwa et al., 2016).

General Background

Developmental Stress Hypothesis

Nowicki, Peters, and Podos (1998) developed DSH, which was initially named as the nutritional stress hypothesis that suggests song complexity of songbirds may act as an honest signal of bird's developmental history and conditions. Later, they included effects of other possible environmental stressors and renamed it to DSH (Nowicki, Searcy, & Peters, 2002). Consistent with the DSH, subsequent studies showed that malnutritioned male nestlings

developed smaller song brain nuclei associated with courtship song learning and production (Buchanan, Leitner, Spencer, Goldsmith, & Catchpole, 2004; MacDonald, Kempster, Zanette, & MacDougall-Shackleton, 2006; Schmidt, Moore, MacDougall-Shackleton, & MacDougall-Shackleton, 2013). Furthermore, these males expressed less complex songs repertoires and were thus less attractive to females than normally developed males were (Buchanan, Spencer, Goldsmith, & Catchpole, 2003; Nowicki et al., 2002; Spencer, Buchanan, Goldsmith, & Catchpole, 2004; Spencer, Wimpenny, et al., 2005).

The exact mechanism of how malnutrition affects the development of song nuclei and the quality of songs is not yet clear. The predominant explanation is that the hypothalamic-pituitary-adrenal (HPA) axis is involved in the development of the song system (MacDougall-Shackleton & Spencer, 2012). The HPA axis is a set of endocrine glands that becomes activated under stress. As a result, a glucocorticoid hormone, such as corticosterone (CORT), is released from adrenal cortical glands of HPA axis (Brown & Spencer, 2013; Sapolsky, Romero, & Munck, 2000; Siegel, 1980). Supporting involvement of HPA axis in malnutrition, daily calorie restriction was found to increase the CORT level in the plasma of birds (Ottinger et al., 2005). CORT then affects brain structures (including song nuclei) that contain glucocorticoid receptors (GRs) (C. Zimmer & Spencer, 2014). Chronic exposure to stress decreases the number of GRs (Banerjee, Arterbery, Fergus, & Adkins-Regan, 2012). Fewer receptors would delay the termination of CORT release, exposing cells longer to negative effects of stress (Lupien, McEwen, Gunnar, & Heim, 2009). Furthermore, CORT administration *per se* is known to affect song development in a similar manner to malnutrition (Spencer & MacDougall-Shackleton, 2011). Thus, treatment with CORT in early life reduced song nucleus volume and deteriorated song quality (Buchanan et al., 2004; Shahbazi, Jimenez, Martinez, & Carruth, 2014; Spencer, Buchanan, Goldsmith, &

Catchpole, 2003). In addition to similar effects of CORT exposure on song nuclei development, the presence of GR in song nuclei also supports involvement of HPA axis in a malnutrition experienced early in the life (Shahbazi, Schmidt, & Carruth, 2011; Suzuki, Matsunaga, Kobayashi, & Okanoya, 2011).

However, there is a major difference of effects between malnutrition and CORT feeding treatments. Following stressful events, such as physical restraining and heat, CORT-fed birds showed a delayed negative feedback of circulating CORT in adulthood (Marasco, Robinson, Herzyk, & Spencer, 2012; Spencer, Evans, & Monaghan, 2009). Malnutritioned nestlings, on the other hand, did not show a significant change in feedback to such stress stimuli in their adulthood (Schmidt, MacDougall-Shackleton, Soma, & MacDougall-Shackleton, 2014; Soleimani, Zulkifli, Omar, & Raha, 2011; Zimmer et al., 2013). The delay observed in CORT-fed birds suggests that the HPA axis was permanently affected by the early experience of CORT administration while malnutrition did not have such a long-term significant effect. Long-term effects on HPA axis by CORT exposure, but not by malnutrition, has also been supported by artificial adrenocorticotropic hormone administration, which isolates variation in stress response to the sensitivity of adrenal cortical glands of HPA axis (Simpson & Waterman, 1988). When nestlings of song sparrows (*Melospiza melodia*) were fed with CORT, they showed increased CORT release in adulthood; but in nestlings with a restricted diet, CORT release was similar to control birds (Schmidt et al., 2014).

It is also possible that malnutrition might directly affect song system nuclei, rather than indirectly through the HPA. When daily intake is limited, available energy might be devoted more to brain areas essential for immediate survival rather than those for necessary later in life (MacDougall-Shackleton & Spencer, 2012; Nowicki & Searcy, 2005b). For example, under a

similar nutritional stress in early life, western scrub jays (*Aphelocoma californica*) showed decreased hippocampal volume and caching performance (Pravosudov, Lavenex, & Omanska, 2005).

Natural Stressors and Immune Challenges

Feeding CORT to nestlings is an artificial stressor while malnutrition can occur in the natural environment (Nowicki & Searcy, 2005a). It would be difficult to know if the quality and level of stress experienced in CORT-fed animals are comparable to stress experienced in natural settings (Gil, Naguib, Riebel, Rutstein, & Gahr, 2006). It is necessary to study different natural stressors and their effects to fully understand the natural mechanism of early experience influence.

Only a few studies have been conducted to examine other natural stressors. For instance, a study showed that enlarged brood size negatively affected parental care for individual nestlings (Gilby, Mainwaring, Rollins, & Griffith, 2011). However, long term effects of brood size have been controversial. One study found negative effects of increased brood size on song development (e.g., less accurate copy of tutor's song) (Holveck, de Castro, Lachlan, ten Cate, & Riebel, 2008) while there are studies which found either no effect (Gil et al., 2006) or even a positive effect (e.g., increased song rate in the presence of a female) (Tschirren, Rutstein, Postma, Mariette, & Griffith, 2009) of enlarged brood size.

Interaction between song system and immune system makes ELIC a good representation of potential natural stressors in songbirds. Previous studies proposed that the song quality of adult birds is correlated to the strength of their immune system response (Folstad & Karter, 1992). Parasitized Sedge warblers (*Acrocephalus schoenobanenus*) tended to have smaller song repertoires (Buchanan, Catchpole, Lewis, & Lodge, 1999). In European starlings (*Sturnus*

vulgaris), song rate was positively correlated with immune response, which was measured as an accumulation of T-cells to infected areas (Ball, Sockman, Duffy, & Gentner, 2006). Only a few studies have examined the effects of ELIC on behavior and physiology in adults (Grindstaff, 2016). For example, hen flea-infected great tits (*Parus major*) dispersed for a rather short distance and tended to sing less frequently than control birds (Bischoff et al., 2009; Heeb et al., 1999). In this species, adult males are known to disperse quite far from the original nest sites to search for potential mates. Similarly, when male canary nestlings (*Serinus canaria*) were infected with endoparasite malaria they tended to produce simpler song repertoires and to have a smaller song brain nucleus (i.e., HVC) compared to controls (Spencer, Wimpenny, et al., 2005).

In addition to interaction between song system and immune system, immune challenges are also known to activate HPA axis (Buckingham, Loxley, Christian, & Philip, 1996). It was shown that injection of lipopolysaccharide (LPS) (a membrane component of Escherichia coli) in rats and that Poly I:C injection in mice increased plasma level of CORT (Gandhi, Hayley, Gibb, Merali, & Anisman, 2007; Takemura et al., 1997). In birds, repeated injections of LPS also caused higher basal CORT levels in Pekin ducks (Manette Marais, Maloney, & Gray, 2011). Field studies also showed effects of immune challenges on CORT level of birds. In red grouses (*Lagopus lagopus scotica*), with higher parasite loads, CORT level in plasma increased (Mougeot, MartÍNez-Padilla, Bortolotti, Webster, & Piertney, 2010). In tree swallows (*Tachycineta bicolor*), treatment of nests with an insecticide for ectoparasites caused lower basal CORT levels in treated nests (Harriman, Dawson, Clark, Fairhurst, & Bortolotti, 2014). Overall increase in CORT levels after an immune challenge suggests a common pathway for malnutrition and immune challenges to affect development through HPA axis activation.

However, infections with ecto- or endo-parasites may not necessarily be optimal for ELIC studies on the brain and behavior development of birds. For ectoparasites, such as the hen flea, immune reactions to parasites can be quite diverse and complex, as they may carry different types of antigens (Weil, Martin, & Nelson, 2006). Ectoparasites may also be a vector for various pathogens, which makes isolating possible immune responses even more complicated (Wikel, 1999). Endoparasites (e.g., malaria) can affect host metabolism, often resulting in a variety of abnormal conditions, such as anemia and anorexia. This makes it difficult to isolate the exact factor affecting the brain and behavior. Instead of using agents with complex immune reactions, an artificial agent with a simple and a known mechanism of immune reaction would give clearer results. By using an artificial agent, it is possible to restrict the immune activation to a narrow period of time and associate it to a certain stage of the development in a dose dependent manner (Reisinger et al., 2015). However, it needs to be noted that artificial agents such as Poly I:C would elicit only a limited set of immune responses (Fortier et al., 2004)

Several different agents have been used for studies of ELIC. LPS, influenza virus, sheep red blood cells (SRBC), and cytokines are examples of often-used agents (Meyer, Feldon, & Fatemi, 2009). However, the number of studies that looked at long-term effects of ELIC on adult bird behavior is still few, and additional studies are required for the better understanding of its exact effects (Grindstaff, 2016). In this study, since the most common cause of brain inflammation is due to viral infections (Davis, 2000), Poly I:C was used as an immune challenging agent in this study. Poly I:C is a double-stranded RNA analog used for mimicking viral infections (Boksa, 2010; Meyer, 2014). A viral infection mimicking agent like Poly I:C is advantageous for three reasons. First, since it is not a pathogen, but is directly affecting the immune system, it will be possible to isolate effects of the immune responses to viral infections.

The second reason for using Poly I:C is the well-documented mechanisms of immune reactions to Poly I:C, which would help for understanding the physiological changes occur during development. Finally, studying long-term effects of Poly I:C on adult behavior is helpful to understand possible changes in transmission rate of viral infections in animal populations. Incorporating behavioral changes into transmission rates is important for accurate predictions of spread (Ezenwa et al., 2016). Accurate predictions of viral disease spread are ultimately important as it may affect human lives such as avian influenza, and West Nile virus.

Zebra Finches as Subject Animals

In this study, zebra finches were used as subject animals. Zebra finches are Australia originated passerines with a lifespan of two to five years. They are social animals and form strong pair bonds with their mates before reproduction. Zebra finch nestlings have altricial development and need parental care for the first few weeks after they hatch. Adult males and females show sexual dimorphism in which males have bright red bills and orange cheek patches on their head (Koephh, 1985). They also show dimorphism in their vocalizations. Male finches learn species-specific songs from their tutors and later they act as a tutor for babies, while females do not learn how to sing as males do (Jin & Clayton, 1997).

Zebra finches were used as subjects in this study for three major reasons. First, among over 10,000 species of birds, zebra finches were extensively studied as a subject animal for various developmental and cognitive studies (Warren et al., 2010). Physiology and brain mechanisms of their song learning and social behavior are well-known (Bolhuis & Gahr, 2006; Scharff & Nottebohm, 1991; Wild, 1997). Second, there are numerous studies on their natural behaviors, foraging, social behaviors, and mating strategies. Results of the present study could easily be incorporated in other fields of research. And finally, they are highly social and

gregarious animals, which makes housing and breeding easy. In the present study, in order to inject all nestlings at the same age, a breeding colony had to be developed and it was possible to raise the zebra finch nestlings in a captive environment.

Background on Specific Aim 1: Does Poly I:C injection affect the body growth rate in nestlings of zebra finch?

Developmental Stress and Body Growth

During development, many body structures of an animal develop simultaneously, creating competition for resources (Gil, Bulmer, Celis, & López-Rull, 2008). Various stressors during early life can cause abnormality in body growth. When there are not enough nutrients, animals either decrease their growth rate or balance their investment to more vital structures for survival (Schew & Ricklefs, 1998). Male Bengalese finches (L*onchura striata var. domestica*) that hatched in a large brood with a harsh food competition among siblings had lighter body weights and developed shorter tarsus and wings than those from a small brood (Soma et al., 2006). A great tit population living close to a polluted environment (i.e., non-ferrous smelter) had slower growth rates compared to populations living in a cleaner environment (Eeva & Lehikoinen, 1996). Swamp sparrows (*Melospiza georgiana*) tended to have delayed growth during dietary restriction treatment (Nowicki et al., 2002). CORT exposure is also known to have resulted in a slower growth rate in zebra finches (Spencer & Verhulst, 2007), but see (Farrell, Morgan, Sarquis-Adamson, & MacDougall-Shackleton, 2015; Reed et al., 2012).

A few studies showed that ELICs also negatively affect the development of nestlings. In chickens, LPS injections decreased average daily weight gains (Liu et al., 2015). In zebra finches, keyhole limpet hemocyanin (KLH) decreased the growth rate of birds almost immediately although the effects lasted only for five days (Grindstaff, Hunsaker, & Cox, 2012).

Nestlings of mountain bluebird (*Sialia currucoides*) and tawny pipits (*Anthus campestris*) showed decreased body mass gains after they were parasitized by blow flies (*Protocalliphora spp.*) or infected by malaria respectively (Calero-Riestra & García, 2016; O'Brien & Dawson, 2008; Shutler, Ankney, & Mullie, 1999; Verhulst, Riedstra, & Wiersma, 2005).

Acute Phase Responses to Immune Challenge

The Acute phase responses (APRs) are systemic reactions to an inflammatory chemical, mediated mostly by central nervous system. APRs may be both physiological such as HPA axis activation, fever, hypermetabolism, and behavioral such as anorexia, lethargy (Blatteis, 2006). In the present study, APRs after Poly I:C injections were measured to examine whether treatments had immediate effects on animals.

Fever, anorexia, and hypermetabolism as APRs may result in loss of body weight, fat, and muscle loads (LeGrand & Alcock, 2012). Keeping body temperatures high for long periods creates an energetic cost to animals (Martin, Scheuerlein, & Wikelski, 2003). Increased body temperatures accelerate the metabolic processes. To keep up with the energy demand due to increased metabolic process, proteins start breaking down and eventually muscle loads of the animal decrease. Together with anorexia, animals eventually lose body weights during the fight with the pathogen (Hart, 1988; Manette Marais, Maloney, & Gray, 2013).

With the conserved evolution of the immune system, birds are vulnerable to adverse effects of fever and other APRs. Several studies showed increased fever in birds in response to pathogen-associated molecular patterns, such as LPS, muramyl dipeptide (Gray, Marais, & Maloney, 2013). Fever response to Poly I:C has been observed in different species (Cunningham, Campion, Teeling, Felton, & Perry, 2007; Zbikowska, Cichy, & Papierkiewicz, 2013) including birds. In fowls (e.g., chicks and ducks), body temperature significantly increased after an

injection of Poly I:C and stayed elevated for hours (Kent, Dedda, Hale, & Crowe, 2007; M. Marais, Gugushe, Maloney, & Gray, 2011). However, in adult house sparrows (*Passer domesticus*), no fever response or weight changes was detected after Poly I:C injection (Coon, Warne, & Martin, 2011).

Regarding energetic costs of fever, several studies found increased metabolism after experimentally induced fever. For example, after phytohaemagglutinin challenge, the metabolic rate of house sparrows increased by 30% (Martin et al., 2003). In another study, LPS injected zebra finches showed decreased body mass gain over two days (Sköld-Chiriac, Nord, Tobler, Nilsson, & Hasselquist, 2015). However, contradictory to these findings, greenfinches (*Carduelis chloris)* did not show an increase in their metabolic rate after injected with SRBC (Hõrak et al., 2003).

Background on Specific Aim 2: Does ELIC affect personality of adult zebra finches?

Behavioral Effects of Poly I:C in Mammals

In mammals, Poly I:C's effects on behavior were extensively studied (Boksa, 2010). Three common observed effects were on sensory processing, social behavior, and spatial learning and memory. As for effects on sensory processing, infecting pregnant rats and mice with Poly I:C impaired latent inhibition and pre-pulse inhibition in offspring (Zuckerman, Rehavi, Nachman, & Weiner, 2003). However, such impairments disappeared with dopamine antagonistic drugs, suggesting that changes in dopamine metabolism after Poly I:C injection play an important role (Ozawa et al., 2006; Zuckerman et al., 2003).

Neonatally infected mammals also showed abnormal social behavior. In rhesus monkeys (*Macaca mulatta*), offspring of Poly I:C injected animals tended to have a longer latency and a shorter duration of eye fixation to faces of other animals (Machado, Whitaker, Smith, Patterson,

& Bauman, 2015). Adult mice that were infected neonatally spent less time interacting with other mice either compared to the preference for a doll or an empty chamber (Aavani, Rana, Hawkes, & Pittman, 2015; Bitanihirwe, Peleg-Raibstein, Mouttet, Feldon, & Meyer, 2010; Xuan & Hampson, 2014).

Finally, mammalian studies showed that the administration of Poly I:C affected spatial learning and memory. In a spatial recognition task which relies on rodents' preference between a familiar arm and a novel arm, mice injected with Poly I:C tended to spend less time in the novel arm and made fewer alternations between the novel and familiar arms (Giovanoli et al., 2015). In rats, during acquisition trials of a water maze, Poly I:C injected neonates had longer paths to reach the target than control rats (Vorhees et al., 2015). These authors suggest that deficiencies in those two tasks imply poor working memory after Poly I:C injections.

In contrast to mammals, there is only one behavioral study with birds using Poly I:C. Thus, one-day-old Black Australorp, white Leghorn chicks showed deficiencies in passive avoidance trials if they were injected with Poly I:C two hours before acquisition trials (Kent et al., 2007).

Personality Traits

In birds, previous studies showed that early life stress affects not only the song system, but also affects many different behavioral traits including those which can be grouped as personality. Personality, or behavioral syndromes, can be defined as different types of behavior that are consistently observed in individuals across time and context (Sih & Bell, 2008). To consider a set of animal behaviors as personality, both within and between individual consistencies in different contexts are required (Sih, Bell, Johnson, & Ziemba, 2004). For example, if an animal is persistently approaching different novel objects (within individual

consistency) while other members of conspecifics do not (between individual consistency), it is possible to say that the animal is expressing a different personality. While personality can include various traits, such as shyness-boldness and aggressiveness (Reale, Reader, Sol, McDougall, & Dingemanse, 2007), the present study focused on three traits: activity, exploration-avoidance, and sociability. In birds, previous studies showed that early life stress could affect general activity (Pakkala, Norris, Sedinger, & Newman, 2016), exploratory (Brust, Wuerz, & Krueger, 2013) and social behaviors (Farine, Spencer, & Boogert, 2015).

General Activity

In mammals, stress is known to increase animals' general activity, which is defined as all possible behavior in a habituated, non-dangerous environment (Reale et al., 2007). Rats reared under isolation showed increased spontaneous locomotor activity in open-field apparatus with no novel objects (Pryce, Bettschen, Bahr, & Feldon, 2001). Similar to isolation conditions, rats that were raised in standard housing cages were more active in a glass box compared to rats raised in complex housing (Lomanowska, Ammari, & Kraemer, 2010). In guinea pigs, prenatal maternal stress also increased males' movement in open-field apparatus (Emack & Matthews, 2011).

In birds, CORT-treated nestling Savannah sparrows (*Passerculus sandwichensis*) were easily captured with line of mist nests during pre-migratory period, suggesting an increase in their overall movements (Pakkala et al., 2016). So far, there was only one study about ELIC on general activity of birds. SRBC treated mallard ducks (*Anas platyrhynchos*) were more active in a novel environment compared to untreated animals (Butler, Toomey, McGraw, & Rowe, 2012). In their study, general activity was measured in the number of transitions between the different sections in the apparatus and the frequency of ducks recorded as standing or sitting.

Exploratory Behaviors

Exploratory behaviors can be defined as an animal's reactions to a new situation, which can include a new habitat and novel food or objects (Reale et al., 2007). As in mammals (Hohmann, Hodges, Beard, & Aneni, 2013), exploratory behaviors of birds are known to be affected by early life stress. In great tits, those that experienced rationed feeding between post-hatch day (PD)13 and PD25 more frequently visited trees in a novel environment (Carere, Drent, Koolhaas, & Groothuis, 2005). In Japanese quails (*Coturnix japonica*), unpredictable food restriction between PD4 and PD20 increased exploratory behavior as the treatment group spent more time in the novel area of behavioral apparatus (Zimmer et al., 2013).

In zebra finches, depending on type and duration of stress, effects on exploratory behaviors appear to differ. While a short period of food restriction decreased the latency to approach food in a novel environment (Krause, Honarmand, Wetzel, & Naguib, 2009), longer restrictions resulted in no significant differences between groups (Krause & Naguib, 2011). Similar contradictory results were also seen in reactions of zebra finches to novel objects. In one study, CORT administration to female nestlings decreased their latency to approach to a novel object in the cage (Spencer & Verhulst, 2007). However, a low-quality diet caused no difference when compared to a high-quality diet. Both diet groups spent the equal time on perch with a novel object (Kriengwatana, Farrell, Aitken, Garcia, & MacDougall-Shackleton, 2015).

Only a few studies have investigated the effects of ELIC on exploratory behaviors of birds and the results are not clear. In one study (Butler et al., 2012), mallard ducks were divided into three groups and injected with SRBC when they were 3, 8, or 13 weeks old. When ducks became adults, their exploratory behavior was studied in a novel environment. Ducks treated at 8 and 13 weeks old approached novel objects more frequently than those injected at 3 weeks old

(Butler et al., 2012). However, in zebra finches, LPS infection on PD5 only resulted in a non-significant trend of getting closer to a novel object in adulthood (Grindstaff et al., 2012).

Social Behaviors

Effects of early life stress have also been studied in social behaviors, which can be defined as either competitive or affiliative behavior with conspecifics (Reale et al., 2007). As in mammals (Aavani et al., 2015; Machado et al., 2015), social behaviors in birds are known to be affected by early life stress. In male great tits, food rationing between PD13 and PD20 decreased the latency to attack an intruder male (Carere et al., 2005). In zebra finches, CORT administration to nestlings decreased the competitive ability in adulthood for a perch long enough for only one bird (Spencer & Verhulst, 2007). Early life stress also affects affiliative behaviors (i.e., being close to conspecific members). In Japanese quails, both malnutritioned males and control males saw a video in which another quail chose one of the two different food cups (Boogert, Zimmer, & Spencer, 2013). When given options, malnutritioned individuals approached and pecked to food in the food cup which was earlier avoided in the demonstrator video (Boogert, Zimmer, & Spencer, 2013). In zebra finches, CORT administration negatively affected their association with parents and stressed nestlings became less selective about their foraging mates (Boogert et al., 2013; Farine et al., 2015).

Only one study looked at effects of ELIC (using ectoparasite hen fleas) on social behaviors in birds (Bischoff et al., 2009). Great tit nestlings exposed to parasites reduced the degree of song overlap with a playback of a challenging male in their breeding territory, which was positively correlated with the social status of the male.

Background on Specific Aim 3: Does ELIC change behaviors in mate choice trials of adult zebra finches?

Developmental Stress on Male Attractiveness

In songbirds, females have much higher parental investment than males. Females spend more time and energy for reproduction and care of offspring. As a result, females are often the ones to choose potential mates in sexual selection (Alcock, 2013). Females evaluate males by comparing traits that are associated with quality of male's genes, parental care, health, and so on. These traits include beak color, tail length, quality of nest, territory size, and courtship displays such as songs (Alcock, 2013). For example, in house finches (*Carpodacus mexicanus*), females prefer males with bright color plumage as a trait, which has a positive relationship with parental care by males (Hill, 1991).

However, effects of early life stress on male bird attractiveness are still in debate. Early-life stress could negatively affect males' traits, and therefore, females would prefer normally-developed males to stress-experienced ones (Nowicki et al., 2002). In song sparrows, females reacted with courtship-solicitation displays more frequently to songs of normally developed males compared to those of nutritionally stressed males (Schmidt, McCallum, MacDougall-Shackleton, & MacDougall-Shackleton, 2013; Searcy, Peters, Kipper, & Nowicki, 2010). On the other hand, another study showed that female zebra finches did not differentiate between males developed in small or large broods (Naguib, Heim, & Gil, 2008). In canaries too, females showed equal preference to males raised in large broods or malnutritioned (Müller, Vergauwen, & Eens, 2010). In addition, CORT-fed zebra finch males sired more offspring in a common garden breeding experiment compared to control males (Crino, Prather, Driscoll, Good, & Breuner, 2014).

Many studies on developmental stress have focused on the song quality or song rate of the males. In this study, the focus was to explore the roles of behaviors other than songs and vocalizations since little is known about effect of developmental stress on mate selection-related behavioral traits on males.

Developmental Stress on Females' Mate Choice Behavior

Initial studies of developmental stress in birds has mostly focused on effects on males (MacDougall-Shackleton & Spencer, 2012; Nowicki et al., 2002; Spencer et al., 2003). However, DSH proposed that females could also be affected (Nowicki et al., 2002). One of the earliest studies showed that stressed (malnutritioned) female zebra finches were less active than controls and made fewer sampling visits to stimulus males in mate choice trials (Woodgate, Bennett, Leitner, Catchpole, & Buchanan, 2010). In another study, females from large brood sizes showed decreased preference to long over short songs in an operant conditioning test (Riebel, Naguib, & Gil, 2009).

It is not clear whether the change in the females' behavior was due to an impairment in discrimination or a shift in the male preference (MacDougall-Shackleton & Spencer, 2012). Females' discrimination abilities might be affected according to Schmidt et al. (2013), who showed that malnutritioned birds did not show a clear preference between conspecific and heterospecific songs. In addition, malnutritioned birds showed less neuronal activity in auditory forebrain nuclei when they listened to conspecific songs compared to control birds (Farrell, Neuert, Cui, & MacDougall-Shackleton, 2015).

It is also possible that an early life stress could affect females' behavior by changing their male preference. For example, zebra finches from a large brood tended to prefer songs of males from large brood sizes over those from smaller brood sizes (Holveck & Riebel, 2010). In

addition, previous studies showed that females' physical condition might affect its mate choice. When wings of female zebra finches and canaries were experimentally shortened, females decrease their preference toward attractive males (Burley & Foster, 2006; Lerch, Rat-Fischer, & Nagle, 2013).

Background on Specific Aim 4: Does ELIC affect the brain development of zebra finches?

Immune System and Brain Interactions

After an infection with a pathogen-associated molecule, T-cells of the immune system secrete chemical messengers, such as cytokines (Coico & Sunshine, 2015). Cytokines secreted by T-cells can then be transferred to the brain through several routes, such as binding to receptors on vagal afferents and passing through the blood-brain barrier (Bilbo & Schwarz, 2012). In normal conditions cytokines are constitutively expressed in a healthy brain and regulate homeostasis and behaviors (Szelenyi, 2001); however, peripheral cytokines transferred to brain activate microglia in response to immune challenge (Kreutzberg, 1996; Nimmerjahn, Kirchhoff, & Helmchen, 2005). Active microglia then have neurotoxic effects with the release of cytotoxic chemicals (e.g., nitric oxide), which can lead to neuronal loss (Block, Zecca, & Hong, 2007).

For ELIC's to be effective until adulthood, they should cause long-term effects in the brain. There are two proposed mechanisms for long-term effects of an ELIC (Meyer, 2013). The first mechanism is through activation of microglia, which remain active until adulthood. Neonatally immune-challenged rats had more active microglia in their brain than controls when they reached adulthood (Wang et al., 2013). After an immune challenge, active microglia release an exaggerated amount of a cytokine, interlukin-1β (IL-1β). In mammals, it is known that IL-1β is associated with learning. For example, 24 hours after fear conditioning, expression of IL-1β

doubled in normal rats (Goshen et al., 2007). The excess of IL-1β, due to either direct injections into brain or a release from active microglia following an immune challenge, impaired the fear conditioning (Bilbo et al., 2005; Goshen et al., 2007). It was hypothesized that a long-term sensitized state of microglia due to an ELIC might impair learning (Williamson & Bilbo, 2014).

The second proposed mechanism for long-term effect of ELICs are physiological and morphological changes in the brain (Meyer, 2013). For example, in neonatal mice, an exposure to Poly I:C or a cytokine, interferon-β, decreased the number of neuronal progenitor cells (Lathia et al., 2008) and delayed the formation of cortical lamina (Soumiya, Fukumitsu, & Furukawa, 2011). Also, depending on the timing of immune challenge, the numbers of dopamine and glutamate receptors could be affected (Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008). It is suggested that in humans, these changes are associated with psychological problems in adulthood, such as schizophrenia and depression (Knuesel et al., 2014).

Poly I:C can exert long-term effects on the brain in different ways. First, the administration of Poly I:C can trigger neurochemical changes in the releasing rate of certain neurotransmitters or the number of specific receptors (Juckel, 2015). For example, mice born to Poly I:C injected mothers showed an increased level of dopamine in the prefrontal cortex (Winter et al., 2009) and fewer cells expressing glutamate receptor in the hippocampus (Meyer, Nyffeler, Schwendener, et al., 2008). Poly I: C's long-term effects can also be based on anatomical changes during development. These changes may happen through neuronal loss or sensitized microglia after infection. Direct injections of Poly I:C to the hippocampus in adult rats resulted in the activation of microglia and astrocytes 72 hours after injection, followed by neuronal loss after two weeks (Melton et al., 2003). In mice, neonatal injection with Poly I:C decreased occurrence of neurogenesis (Meyer et al., 2006) and reduced the number of reelin-

protein-containing neurons in the hippocampus (Meyer et al., 2006; Ratnayake, Quinn, Castillo-Melendez, Dickinson, & Walker, 2012). Often, these phenomena are coupled with sensitized microglia, an increase observed in adult mice and rats after an immune challenge (Juckel et al., 2011; Ratnayake et al., 2012) but see (Giovanoli et al., 2015). Sensitized microglia then cause neuronal loss by release of pro-inflammatory cytokines (Block et al., 2007). For example, IL-1β inhibits glial uptake of excitatory neurotransmitter, glutamate, which in turn causes excitotoxicity leading to cell death (Melton et al., 2003). Poly I:C is also known to increase mRNA of IL-1β in hypothalamus (Fortier et al., 2004), through binding Toll-like receptor 3 (TLR3) (Maelfait et al., 2008). TLR3 is a member of receptor family found in both mammalian and avian immune cells and detects pathogen-associated molecules, and activates pathways for cytokine secretion (Kawai & Akira, 2007).

Song System Nuclei

One of the predictions based on DSH is the underdevelopment of song brain nuclei and, as a result, the deterioration of song learning and production (Nowicki et al., 2002). Song learning occurs in two critical phases, sensory phase, and motor phase. During the sensory phase, songbird nestlings memorize the tutor songs. To accurately copy tutor's song, the nestling needs to listen to a tutor's song within the first month after hatching. During the motor phase, nestlings start practicing tutor's songs until it crystallizes (Jin & Clayton, 1997). Nestlings deprived of a tutor's song during the sensory phase are known to produce abnormal song types, such as smaller repertoire sizes and decreased notes per song (Marler & Sherman, 1985). These two critical phases are also periods of song nuclei development. In zebra finch males, the number of neurons

in song nuclei increased till 60 days post-hatching. In the female brain, this increase was absent, creating sexually dimorphic brains in songbirds (Kirn & Devoogd, 1989; NixdorfBergweiler, 1996).

The song system includes several distinct brain structures in the forebrain, such as HVC, Robust Nucleus of the Arcopallium, (RA), and Area X. Although these song nuclei are closely connected for song learning and production, lesion effects of individual nuclei can be different. For example, in canaries, lesions in HVC and RA resulted in the elimination of songs while those to Area X did not. These results suggested different roles of these nuclei in the song system (Nottebohm, Stokes, & Leonard, 1976). In general, song nuclei are grouped into the anterior and posterior pathways, which appear to have different functions (Figure 1). Based on physiological and anatomical studies, it was shown that the anterior pathway is generally involved in song acquisition while the posterior pathway is involved in both the acquisition and production of songs (Scharff & Nottebohm, 1991). Both pathways include HVC and RA. In the posterior pathway, HVC projects to RA, which sends a projection to the motor nuclei in trachea to control syrinx, the bird's vocal organ (Wild, 1997). In the anterior pathway, HVC projects to Area X in the basal ganglia, to nucleus dorsolateralis anterior (DLM) in the thalamus, then to the magnocellular nucleus of the nidopallium (lMAN), and ultimately to RA (Bolhuis & Gahr, 2006).

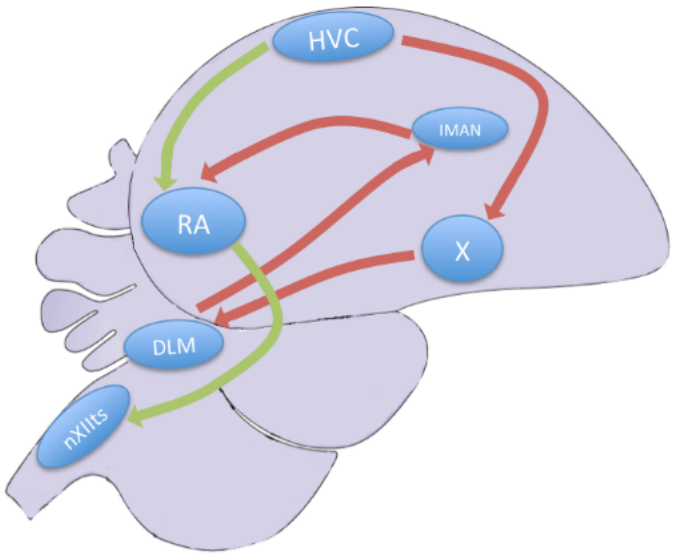

Figure 1. Anterior and posterior pathways in songbird brain for song acquisition and production. Green lines are for posterior and red lines are for anterior pathways. DLM, nucleus dorsolateralis anterior, pars medialis; HVC, a letter based name; lMAN, lateral magnocellular nucleus of the anterior nidopallium; nXIIts, tracheosyringeal portion of the nucleus hypoglossus; RA, robust nucleus of the arcopallium; X, Area X. Adapted from (Bolhuis & Gahr, 2006).

The DSH predicts that limited resources due to stress will negatively affect the development of these song nuclei (Nowicki et al., 2002). Several studies showed changes in the song nuclei volume after an early life stress. In adult swamp sparrows (*Melospiza georgiana*) food restriction resulted in smaller HVC and RA compared to normally fed sparrows (Nowicki et al., 2002). Similar results after food restriction were also observed in both juveniles (PD 35) and adult zebra finches (Buchanan et al., 2004; Honarmand, Thompson, Schatton, Kipper, & Scharff, 2016). However, the effects of developmental stress on HVC and RA are not always the same. For example, in canaries, malaria infected nestlings developed a smaller HVC while the RA volume remained unaffected (Spencer, Buchanan, et al., 2005). In song sparrows, the HVC volume was smaller in nutritionally stressed juveniles while RA volume was not significantly affected (MacDonald et al., 2006). On the other hand, in song sparrows, RA of malnutritioned

birds was smaller compared to that of normally fed birds (Schmidt, Moore, et al., 2013). In contrast to these studies supporting the prediction of DSH on song nuclei, enlarged brood size as another early life stressor did not cause any change in song nuclei volume (Gil et al., 2006).

Even if the size development of nuclei doesn't show clear effects of stress, such effects can be observed by an examination of neuronal activity of these nuclei. Neuronal activities can be visualized by studying the expression of a class of genes called IEGs such as *c-fos and c-jun* (Kaczmarek & Chaudhuri, 1997; Patzke, Manns, & Gunturkun, 2011). IEGs are a set of transcription factors that can be induced by neuronal stimulation (Cole, Abushakra, Saffen, Baraban, & Worley, 1990; Morgan, Cohen, Hempstead, & Curran, 1987). One IEG that is extensively studied in songbirds is *zenk*, which is acronym of four gene names of the avian homologue: *zif268*, *EGR-1*, *NGFI-A* and *krox24* (Mello, Vicario, & Clayton, 1992). It has been shown that singing induces *zenk* expression in HVC and RA of songbirds, positively related with the duration of singing (Jarvis & Nottebohm, 1997). Previous studies showed that ZENK, the protein product of *zenk*, tended to peak about one hour after the stimuli and stays elevated for another 2 hours (Mello & Ribeiro, 1998). This makes ZENK a useful tool for studying brain activity.

Nucleus Taeniae as a Medial Amygdala Homologue in Birds

In addition to song nuclei, one of the targeted brain regions in this study is nucleus taeniae of amygdala (TnA), which is compared to part of the mammalian amygdala based on connection and neurochemical studies (Reiner et al., 2004; Zeier & Karten, 1971). Immunohistochemical studies revealed that TnA expresses limbic-system associated protein, chicken ovalbumin upstream promoter-transcription factor II, similar to medial amygdala of mammals (Yamamoto, Sun, Wang, & Reiner, 2005). With anterograde and retrograde tracers,

input projections from olfactory bulb (Patzke et al., 2011) and output projections to the hippocampus (Cheng, Chaiken, Zuo, & Miller, 1999) were also revealed. These results suggest that the avian TnA is specifically corresponding to the medial amygdala of mammals. As in mammals, lesion studies revealed the role of TnA in social and sexual behaviors. After bilateral lesions to TnA, social facilitation of feeding behavior in starlings had decreased. In addition, lesioned birds joined their cage-mates less often in foraging than controls (Cheng et al., 1999). Lesions to TnA in Japanese quails resulted in decreased responses in males toward females (Thompson, Goodson, Ruscio, & Adkins-Regan, 1998) but see (Absil, Braquenier, Balthazart, & Ball, 2002). In zebra finches, TnA lesioned males were not chosen by females, and lesioned males suppressed their courtship behavior to females if there is a normal male nearby (Ikebuchi, Hasegawa, & Bischof, 2009). In addition, in zebra finches, ZENK expression in TnA of females and the interactions with a pair-bonded male were positively related but this relation was absent in the interactions with a novel male (Svec, Licht, & Wade, 2009). There was an increase in ZENK expression in TnA of male Japanese quails after being exposed to a sexually mature female (Charlier, Ball, & Balthazart, 2005).

There were no avian studies about effects of developmental stress on TnA. In mammals, early life stress was shown to affect the development of amygdala (Teicher, Samson, Anderson, & Ohashi, 2016). Some studies showed that stress could result in an increase in amygdala volume in humans (Pechtel, Lyons-Ruth, Anderson, & Teicher, 2014) whereas other studies showed a decrease in the amygdala volume after a stressful early life environment (Hanson et al., 2015; Lyons-Ruth, Pechtel, Yoon, Anderson, & Teicher, 2016). Later, some researchers suggested that the amygdala initially reacts to stress with an increase in volume and activity. This

increase in the activity, then gets neurotoxic and damages amygdala, resulting in smaller volume in stressed individuals later in life (Teicher et al., 2016).

Rationale

As described above, effects of ELIC on adult brain and behavior had not been investigated sufficiently in birds compared to mammals. Animals are often infected with a parasite in nature and those infections may have long term effects similar to chronic stress or malnutrition. There is only a little information on whether ELIC affects growth of zebra finch nestlings (Specific Aim 1), changes personalities (Specific Aim 2), behavior of adult zebra finches in mate choice (Specific Aim 3), and development and neuronal activity of brain nuclei (Specific Aim 4). The present study capitalized on activating immune system of zebra finches without a parasite using a viral infection mimicking agent, Poly I:C.

For Specific Aim 1, fever and weight loss as APRs to Poly I:C were analyzed to operationalize nestlings' immune response. After Poly I:C injection, body temperatures, weight, fat and muscle loads of birds were measured. When birds were ~PD30, morphological measures, such as wing and tarsus length together with body weights, were measured again to analyze ELICs effect on growth rate. It was hypothesized that Poly I:C injected birds had shorter tarsus lengths and wing lengths due to adverse effects on morphological development. It is also predicted that Poly I:C injection in zebra finch nestlings to cause APRs which would be detected as an increased body temperature, together with loss of body weight, and fat and muscle loads.

For Specific Aim 2, the behavioral patterns of males and females under four different conditions were tested. The four conditions were necessary to understand whether ELICs affect animals' behavior in general or under certain circumstances. Furthermore, whether males and females react differently to ELICs was investigated by injecting both males and females as

subjects. It was hypothesized that being exposed to Poly I:C, early in life stress would affect personality of birds. Specifically, general activity, exploratory, and social behaviors of Poly I:C injected birds would be negatively affected.

For Specific Aim 3, effects of ELIC on male attractiveness and females mate choice trials was examined. Previous studies so far had focused mostly on males' attractiveness after a developmental stress. The present study would provide a more comprehensive picture of how males and females' behaviors can respond to developmental stress. It was hypothesized that ELIC would change males' behavior in mate choice trials and Poly I:C injected males would behave differently from Control males according to the behavioral measurements and/or females' reaction. It was also hypothesized that ELIC would change females' behavior in mate choice trials and Poly I:C injected females would behave differently from Control females according to the behavioral measurements and/or males' reaction.

For Specific Aim 4, the volumes of and ZENK expression patterns in HVC, RA, and TnA were examined to understand effects of ELIC on brain development. It was hypothesized that ELIC would impair brain development and that Poly I:C injected males would have smaller HVC, RA, and TnA. It was also hypothesized that the numbers of ZENK-positive neurons would be different between injection and control groups.

METHODS

Breeding Colony

Thirty-six (36) adult zebra finches (18 females and 18 males) were purchased from local breeders across the Tampa Bay area. They were housed in flight cages, each of which contained three males and three females in the College of Medicine, University of South Florida. Birds were kept on a 12h light: 12h dark cycle and given *ad libitum* food, water, and cuttlebone. Birds were also provided with fresh greens, wicker nest baskets, and nesting material to stimulate breeding. Nests were checked daily for eggs. All procedures were in accordance with the NIH guidelines and approved by the University of South Florida Institutional Animal Care and Use Committee (Appendix C).

Specific Aim 1: Does Poly I:C injection affect the body growth rate in nestlings of zebra finch?

Early-Life-Immune Challenge and Measurements

Forty-nine (49) zebra finches that hatched in the colony were used. On PD 14, they were randomly assigned to either Control or immune-challenged (Poly) group. Birds in the Control group were injected (subcutaneous) with 100 µl of 0.9% saline solution whereas those in the Poly group were injected with Poly I:C (25mg/kg) in 100 µl 0.9% saline solution. Figure 2 illustrates the experimental timeline of the measurements. In order to assess APRs of fever and anorexia to Poly I:C (Coon et al., 2011), body temperature and morphometric measurements

were conducted. In addition, to assess effects of Poly I:C injection on body growth rate, tarsus (part of leg between the heel and the ball) and wing lengths were also measured.

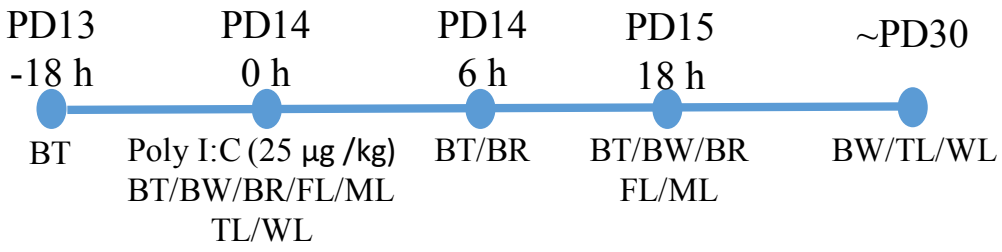

Figure 2. Experimental timeline for measurements during the Poly I:C injection treatment. PD : post-hatch day, h : hours. BT: Body Temperature, BW: Body Weight, BR: Breathing Rate, FL: Fat Load, ML: Muscle Load, TL: Tarsus Length, WL: Wing Length

The body temperatures were measured using a thermistor thermometer (Cole-Parmer Economical Thermistor Thermometers, Cole-Parmer, Illinois, USA). The tip of the thermometer was inserted ~1 cm into the cloaca and the temperature after 15 seconds of insertion was recorded. Temperatures were measured at four time points: the evening before the injection day (-18h), during injection (0 h), the evening of the injection day (6 h), and the morning following the infection day (18 h).

Morphometric measures of body weight, breathing rate, and fat and breast muscle loads were recorded at different time points (Figure 2). The body weights of birds were measured individually using a digital balance (PK-601, Denver Instrument, Bohemia, NY, USA). Breathing rate was measured by holding a bird on its back and counting the number of breast movements for 10 seconds. Fat and breast muscle loads were measured based on the Manual of Field Methods (Bairlein et al., 1995) (Figure 3a). For both fat and breast muscle loads, nestlings were first held back and birds' furculum (wishbone) and sternum (breastbone) were exposed by gently blowing the feathers. Fat and muscle loads were then visually examined and scored

according to Table 1. The lengths of tarsus and wings were measured on the day of injection (0 h) and around PD30. The tarsus and wings were defined as seen in Figure 3b and c.

Table 1. Scoring guideline for fat and muscle loads.

	0	**1**	**2**	**3**
Fat	No fat in the furculum	The furcular hollow is half full of fat.	The furcular hollow is full of fat.	Fat is bulging above the furculum.
Muscle	Sharp sternum, depressed muscles	Not sharp sternum, not depressed nor round muscles	Sternum yet distinct, slightly rounded muscles	Sternum difficult to distinguish, rounded (full) muscles

**Based on (Bairlein et al., 1995)*

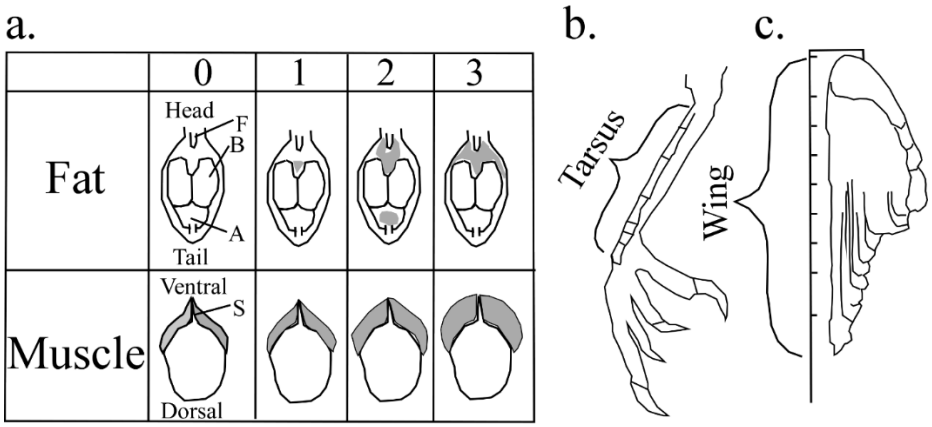

Figure 3. Morphological measures for APR and growth rate. a. Visual guide for measuring fat and muscle load of birds. Muscle load drawings are transverse sections of bird body with sternum on top and back on bottom. Shaded areas are showing fat and muscle load; b. Tarsus of birds measured between toes and intertarsal joints; c. Wing length of birds is measured between bend of wings to the tip of primary feathers. F= Furcular (wishbone) depression, B = Breast muscle, A=Abdomen, S=Sternum (breastbone).

Immediately after the measurements on ~PD30, fledged nestlings were transferred to another flight cage in the Department of Psychology. In each flight cage, 10 birds were placed (5 males and 5 females; 5 Control birds and 5 Poly birds in each cage).

Statistical Analysis

Statistical analyses of body temperatures, breathing rates, body weights, fat loads, muscle loads, tarsus lengths, and wing lengths were conducted with the Statistical Package for the Social Sciences (SPSS) (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.). To detect overnight changes in body temperatures, body weights, and breathing rates, generalized linear mixed models (GLMMs) were used. Treatment (Control or Poly), sex, sex * treatment, and time were fixed effects and bird id was random effect. Repeated covariance type was First-Order Autoregressive. Target distribution was selected as linear. Degrees of freedom was fixed for all tests. For fixed effects and coefficients, robust estimations were used to handle violations of model assumptions. Finally, multiple comparisons were adjusted using sequential Bonferroni.

Differences in changes of fat and muscle loads, and changes in body weights, tarsus lengths, and wing lengths during growth between Control and Poly group were analyzed with Mann-Whitney U-test.

Specific Aim 2: Does ELIC affect personality of adult zebra finches?

Subjects and Treatment

The nestlings injected with the Poly I:C or saline control in Specific Aim 1 were used for Specific Aim 2 when they became over 7-11 months old. Both male and female birds were tested in order to examine whether ELIC affected their personality.

Behavioral Conditions

General arousal level under four different conditions was examined by scoring four types of behaviors (Table 2) in order to examine whether birds with ELIC have different behavioral patterns compared to those in the Control group.

Table 2. Definitions of scored behaviors and conditions in which birds were tested.

Scored Behaviors	
Hopping on one perch	Sliding on the same perch more than one body width or changing directions
Hopping between two perches	Jumping from one perch to another
Visiting Food Cup	Landing on food cup
Pecking at food	Eating food
Conditions	
Familiarity	With familiar birds, visible in different cages.
Solitary	With familiar birds in different cages, but not being visible by separating with a white plastic board.
Exploration	With novel items in the same cage. These items included 2-3 cm sized pink letter, yellow golf ball, and blue meshed soup cup, which were placed in or next to the food cup.
Sociality	With novel birds, visible in different cages. These stimulus birds were four males and four females that subjects had not seen before.

Behavioral Apparatus

Familiarity Condition

Male and female birds were individually placed in their own cages (51wX45hX51d cm) in the housing room. Each cage contained a food cup, water cup, and two perches separated by 15 cm. In this room, birds could see other familiar birds in other cages. Two observation cameras

(Foscam, FI9821W) were mounted to record behavioral reactions of birds in cages. All cameras were connected to a laptop computer (HP G71) outside of the room, through which behaviors of birds were recorded.

Solitary Condition

Male and female birds were tested in their own cages in the observation room. In this room, birds could be visually separated from each other with cardboards between cages. The room of behavioral trials were lit with full-spectrum fluorescent bulbs with UV (Zoo Med, Repti Sun). An observation camera (Foscam, FI9821W) was mounted above the apparatus and connected to a PC (Dell, Optiflex 7010), through which behaviors of birds were recorded.

Exploratory Condition

Male and female birds were tested in their own cages in the observation room. In this room, birds could be visually separated from each other with cardboards between cages. Three novel objects were placed in each cage: a pink Q letter, a yellow golf ball (2wX2hX2d cm), and a blue meshed soup cup, (10wX10hX10d cm). Pink Q letter, and yellow golf ball were placed inside the food cup. Blue meshed soup cup was hanged next to the food cup.

Sociality Condition

Male and female birds were placed and tested in an apparatus consisting of three flight cages (90w X 51h X 51d cm) (Figure 4) in the observation room. Each flight cage had metal wire walls, through which birds could see and interact to each other. An observation camera (Foscam, FI9821W) was mounted above the apparatus and connected to PC (Dell, Optiflex 7010), through which behaviors of subject birds were recorded.

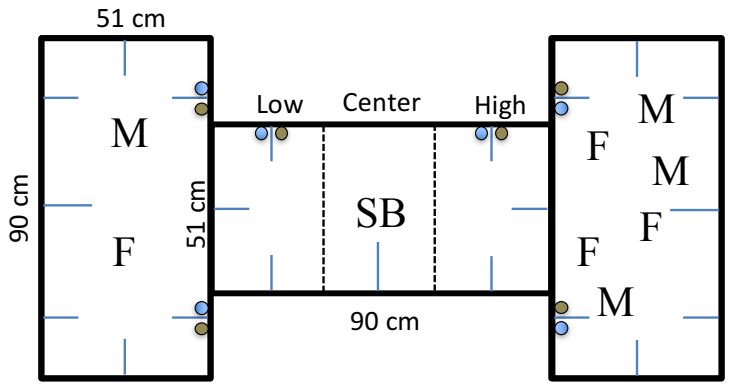

Figure 4. Sociality condition apparatus from a top view. All three cages are identical in size (90 X 51 X 51 cm) Subject bird was in middle cage, and on opposite sides, cages with 2 or 6 finches with same sex ratio were placed. Blue lines are perches, blue circle is water cup and brown circle is food cup. Dashed lines are showing portions of cage for data analysis. M: Male, F: Female, SB: Subject bird.

Behavioral Procedures

When birds were 7-11 months old, behavioral trials started. The presentation order of the four conditions was same for all the birds: Familiarity, Solitary, Exploratory and Sociality. In each condition, a 15-minutes trial per day was conducted on three consecutive days. Between conditions, there were two to three days of intervals. In all four conditions, subject birds could hear other birds in the same room.

Familiarity Condition

Subject birds in their own cage were recorded for 15 minutes twice a day – immediately after the room lights were turned on after 12 hr of a night-darkness period and just before the lights were turned off after 12 hr of a day-light period – for three consecutive days. There was a food cup in each cage for this condition.

Solitary Condition

One day before the experiment started, each subject bird in an individual cage was acclimated for one hour in their own cages in the observation room. On each trial day, the food cup in each cage was removed two hours before trials in order to ensure bird's motivation to

approach food cup. Fifteen (15) minutes before the beginning of each trial, four birds in their own cages were randomly selected and brought to the observation room. Following the 15 minutes of the habituation period, the food cup was placed back to bird's cage to start a 15 minutes trial, during which four behaviors were recorded. After each trial, birds were brought back to the housing room.

Exploratory Condition

All procedures were the same as the Solitary Condition except one novel object was placed in the food cup each 15 minutes trial. After each trial, birds were brought back to the housing room.

Sociality Condition

Each bird was tested once a day for three consecutive days. A subject bird was placed in the center flight cage 15 minutes before each trial. Following the habituation period, two other flight cages were placed adjacent to the center cage. One cage contained two (one male and one female) novel untreated birds (low-density) whereas the other cage contained six (three males and three females) novel untreated birds (high-density) (Figure 4). In each trial, the position of low density and high-density cages were counterbalanced.

Behavior Scoring

In each condition, the following four types of behaviors were scored using JWatcher software (version 1.0, California, 2006) (Blumstein, Evans, & Daniels, 2006). They were: hopping on one perch, hopping between two perches, visiting the food-cup, and pecking at food. In the Sociality Condition, behaviors in the area close to the low-density cage and that close to the high-density cage (30 X 51 cm each) were separately scored.

Statistical Analysis

For all conditions mentioned above, statistical analyses were conducted with the SPSS. To detect effects of Poly I:C injection as ELIC on hopping on one perch, hopping between two perches, visiting the food-cup, and pecking at food, GLMMs were used. Treatment (Control or Poly), cohort, sex, treatment*cohort, treatment*sex, and trials were fixed effects in the model, and bird id was used as a random effect. Repeated covariance type was First-Order Autoregressive. Target behaviors distribution was selected as log-linear. Degrees of freedom was fixed for all tests. For fixed effects and coefficients, robust estimations were used to handle violations of model assumptions. Finally, multiple comparisons were adjusted using sequential Bonferroni.

Specific Aim 3: Does ELIC change behaviors in mate choice trials of adult zebra finches?

Subjects and Treatment

The nestlings injected with the Poly I:C or saline control in Specific Aim 1 were used for Specific Aim 3 following the experiments for Specific Aim 2. Male and female birds were tested for their mate selection in order to examine whether ELIC affected sexual behaviors. In order to prevent sibling effect, birds from different nests were selected for trials.

Behavioral Apparatus

Figure 5 shows the experimental apparatus for Specific Aim 3. The apparatus consisted of one main chamber (40wX40dX45h cm) connecting to two arms (56wX68dX45h cm). The end of each arm had a male subject chamber (56wX56dX45h cm) in which subject males were placed in their own cages. In the center of main chamber, there was a perch (12wX23h cm), located 15 cm behind the partition between the arm and the chamber. The main chamber was separated from arms with a plexiglass (60wX45h cm) placed 10 cm from the partition. When a

female subject entered an arm, only the same-side of main chamber was visible to the female. In each arm, there were two perches placed 33 cm and 63 cm from the partition 23 cm above the ground. Walls of stimulus chambers were covered with soundproof foams (Auralex, 2" Sonomatt). One directional microphone (Sennheiser, ME66/K6) was placed inside each male subject chamber connected to a four-channel field recorder (Roland, R-44) to record male's vocalizations for further analysis for duration of singing. An observation camera (Foscam, FI9821W) was mounted above the apparatus and connected to PC (Dell, Optiflex 7010) and behaviors of birds were recorded. The observation room was lit with full-spectrum fluorescent bulbs with UV (Zoo Med, Repti Sun).

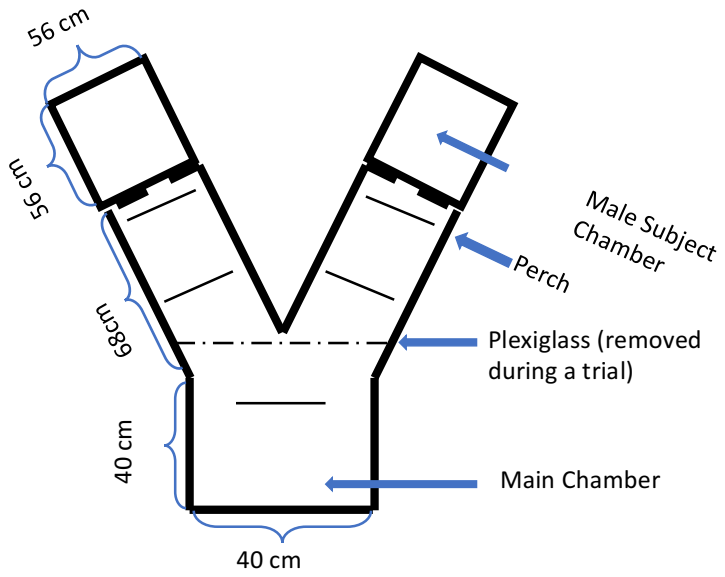

Figure 5. Top view of the behavioral apparatus for mate choice trials and brain development trials.

Behavioral Procedures

About 3-4 weeks after the experiments for Specific Aim 2, behavioral trials started for Specific Aim 3. One day before the experiments started, each subject bird was acclimated for one hour in the Y-maze in the observation room. Fifteen (15) minutes prior to each trial, the

subject female was placed in the main chamber of the apparatus. Following the habituation, lights were turned off and a male was placed in each male subject chamber. For the subsequent three minutes (sampling period), a female could observe both males, but could not enter either arm. Following the sampling period, lights were turned off again to remove the plexiglass between the main chamber and two arms and females had 12 minutes to choose between males by entering one of the arms (choice period). At the end of the 12 minutes, room lights were turned off and a female was guided to the main chamber by holding a flashlight above the chamber.

Males were switched between male subject chambers to avoid possible side biases. The plexiglass was placed back and then lights were turned on. After lights turn on, a trial was repeated. At the end of second 12 minutes, all birds were placed back to their own cages in the housing room. Each bird was used once per day for three consecutive days. A subject female observed different pairs of subject males each day.

Behavior Scoring

Video recorded behaviors were examined with JWatcher software. For males, the number of hopping on one perch, and the number of hopping between two perches were scored. For females, the number of visits to the front perch and the number of hops on the front perch were scored.

Statistical Analysis

Statistical analyses were conducted with the SPSS. To detect effects of Poly I:C injection as ELIC on mating behavior of male and female birds, GLMMs were used. Male treatment (Control or Poly), female treatment (Control or Poly), male treatment* female treatment, and trials were fixed effects in the model, and bird id was used as a random effect. For the model of male

behaviors during 12 minutes of female choice, presence of female was also included as a fixed effect. Repeated covariance type was First-Order Autoregressive. Target behaviors distribution was selected as log-linear. Degrees of freedom was varied across tests because of the small sample size. For fixed effects and coefficients, robust estimations were used to handle violations of model assumptions. Finally, multiple comparisons were adjusted using sequential Bonferroni.

Specific Aim 4: Does ELIC affect the brain development of zebra finches?

Subjects and Treatment

The nestlings injected with the Poly I:C or saline control in Specific Aim 1 were used for Specific Aim 4 following the experiments for Specific Aim 4. The brains of only male birds were histologically examined to study whether ELIC affected the brain development and cellular activity levels. Prior to perfusion, males were exposed to females in order to study the relationship between cellular activity and behavioral reactions to the opposite sex.

Behavioral Procedures

Individual male birds were placed to one of the male subject chambers in their individual cages in the Y-maze used in Specific Aim 3 (Figure 5). After 30 minutes of a habituation period, an untreated novel female was introduced in the main chamber which was separated from the two arms of the Y-maze with a plexiglass. During the subsequent 60 minutes, the following four types of behaviors were recorded and scored using JWatcher software. They were, hopping on one perch, hopping between two perches, visiting the food-cup, and pecking at food. Food and water were not removed from male's cage. After the 1-hour of stimulus exposure, male subjects were sacrificed for brain tissue analysis.

Histology

Tissue Preparation

At the end of behavioral trials, each male bird was deeply anesthetized with an intramuscular injection of Ketamine (50 mg/kg) and Xylazine (10 mg/kg), and then sacrificed by transcardial perfusion with a 0.9% phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PFA) at pH 7.4. Brains were harvested, put in PFA at least for 24 hours followed by a cryoprotectant solution of 30% sucrose in PB for 24 hours. Brains were then frozen with dry ice and left hemispheres were cut sagittally in 40-μm thicknesses using a sliding freezing microtome (Microm HM-400).

Brain Volume Analysis

One set of every fifth section were collected for volume analysis and anatomical reference. Brain sections were mounted on slides with 40% gelatin mounting solution, and air-dried for a day. After a day of air dry, slides were stained with Cresyl Violet (Sigma Chemical, St. Louis, MO), dehydrated with series of alcohol (Ethyl-Alcohol 190 Proof, Pharmco-AAPER, Brookfiedl, CT), cleared with CirtriSolv™ (Fisherbrand™, Pittsburgh, PA) and coverslipped with Permount (Fisherbrand™, Pittsburgh, PA) for storage.

Immediate Early Gene Expression Analysis

A set of adjacent sections of volume analysis were collected for immunohistochemical analysis of IEG expression. Sections were first washed for ten minutes in PBS, and then were incubated in 30 % hydrogen peroxide (H_2O_2) in PBS for 15 minutes at room temperature. After washing three more times in PBS for 10 minutes each, sections were incubated in EGR- antibody solution (1:5000, SANTA CRUZ Biotechnology, C19, made in rabbit) buffered in 0.3% Triton-X/PBS overnight at 4°C. Following day, sections were washed initially three times in PBS for 10

minutes each, before incubating them in secondary antibody (1:5000 biotinylated anti-rabbit IgG, VECTOR Labs, BA-1000) for an hour at room temperature. After a secondary wash in PBS for three times for 10 minutes each, sections were then incubated in avidin-biotin complex (avidin-biotin reagent + PBS + 0.3% Triton X-100 (NaCl) for an hour for signal amplification (Vectastain Elite ABC kit, VECTOR Labs, PK-6100) at room temperature, and then washed another time in PBS for 10 minutes each. Finally, antibody labeled neurons were visualized by immersing them in diaminobenzidine (DAB) solution (0.025% 3,3′ diaminobenzidine + PBS solution) for 10 minutes and adding 8-12 drops of 3% H_2O_2 with additional 10 minutes' incubation. To stop reaction sections were washed for three times in PBS for 10 minutes each. Sections were mounted on slides with mounting solution containing 40% gelatin and air dried for a day. Slides were then coverslipped for long-term storage.

Microscopy Analysis

Volume Analysis

Brain sections were examined under a macroscope (Wild M420 and Nikon SMZ 1500) and a microscope (Nikon Microphot FX). Regions of interest (ROI, including HVC, RA and TnA) were photographed with CCD/digital cameras (Spot Insight QE or Nikon DXM1200) mounted on either macro- or microscopes. Volume measurement was based on a method described by MacDougall-Shackleton, Hulse, and Ball (1998). Digital images were uploaded to graphics software Canvas X (version 16, Texas, 2014). Boundaries of ROI were traced to compute the area. Measured areas were then multiplied by 200 μm, and summed to produce to the volume of the nuclei. For the telencephalon volume, corresponding telencephalon sections for ROIs were measured and volume were calculated multiplying measured areas by 200 μm to obtain the total volume.

Immediate Early Gene Expression Analysis

Quantification of ZENK activity were based on (Castelino & Ball, 2005). Brain sections were examined under a macroscope (Wild M420 and Nikon SMZ 1500) and a microscope (Nikon Microphot FX). The boundaries for ROI were determined from cresyl violet stained adjacent sections that were also used for volume analysis. For each animal, two sections of ROIs for each male bird were photographed with CCD/digital cameras (Spot Insight QE or Nikon DXM1200) mounted on either macro- or microscopes. For HVC, RA, and TnA, sections about 2.3 mm lateral to the midline were used. In order to control for differences in staining densities, the lateral striatum (lSt) was used, as it was known to show a relatively stable level of ZENK expression regardless of different stimulus exposure conditions (Reiner, Medina, & Veenman, 1998). Sections for lSt were about 2.5 mm lateral to the midline. Digital images were uploaded to graphics software NIH ImageJ (version 1.51, Maryland, 2011) (Rasband, ImageJ, & Health) in 8-bit grayscale. In the center of each ROI, a box (0.2X0.3 mm) was drawn. Within the box, ZENK-expressing neurons were counted. Regardless of intensity, a signal darker than the background was considered to be ZENK positive cells.

Statistical Analysis

Statistical analyses were conducted with the SPSS. For effects of Poly I:C injection on brain development, One-way Multivariate Analysis of Covariance (MANCOVA) was conducted on volumes of ROI with Treatment (Control or Poly) as the fixed effect and telencephalon volume as the covariate.

To measure effects of ELIC on brain activity, due to the violations of assumptions required for MANOVA analysis, Mann-Whitney U test was used. The number of ZENK expressing cells were compared between Control group and Poly group for each ROI.

RESULTS

Specific Aim 1: Does Poly I:C injection affect the body growth rate in nestlings of zebra finch?

It was hypothesized that Poly I:C injected birds had shorter tarsus lengths and wing lengths due to adverse effects on morphological development. It was also hypothesized that Poly I:C injection in zebra finch nestlings to cause APRs which would be detected as an increased body temperature, together with loss of body weight, and fat and muscle loads. The results showed that their growth rate of tarsus and wings was not affected by Poly I:C injection. The results also showed that Poly I:C injected birds did not exhibit a significant APR except that Control birds lost more muscle load after saline injection compared to Poly birds.

Body temperatures of Control and Poly birds at all time points were normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$). GLMM analysis revealed that body temperatures of both groups were lower in the evening before injection compared to post-injection measurement points ($\beta = -0.775 \pm 0.245$, $t = -2.121$, $p = 0.034$, Table A1). After the injection, temperatures did not significantly differ between groups (Figure 6a). Breathing rate of both groups were normally distributed as assessed by Shapiro-Wilk's test ($p > 0.05$). Breathing rates remained the same between Control and Poly groups at all post-injection measurement points (Table A2, Figure 6b). Weights of both Control and Poly groups significantly differed from normality as assessed by Shapiro-Wilk's test ($p < 0.05$). Control group showed a strong peak (Kurtosis = 1.9), and Poly group showed a flat distribution (Kurtosis = -0.91). After transforming data by ranking cases,

GLMM analysis revealed no significant weight change between groups after injection (Table A3, Figure 6c). A Mann-Whiney U test was run to determine if there were differences in fat and muscle load score changes overnight between Control and Poly birds. Distributions of the changes in fat and muscle load scores for both groups were similar, as assessed by visual inspection. Changes in fat load scores were not statistically different between Control (Mdn =0.00) and Poly I:C (Mdn =0.25) birds (U=323, z=0.75 p=0.453, Figure 6d) birds. Changes in muscle load scores were statistically different between Control (Mdn=0.0) and Poly (Mdn=0.0) birds (U=423.5, z= 3.011, p = 0.003, Figure 6e).

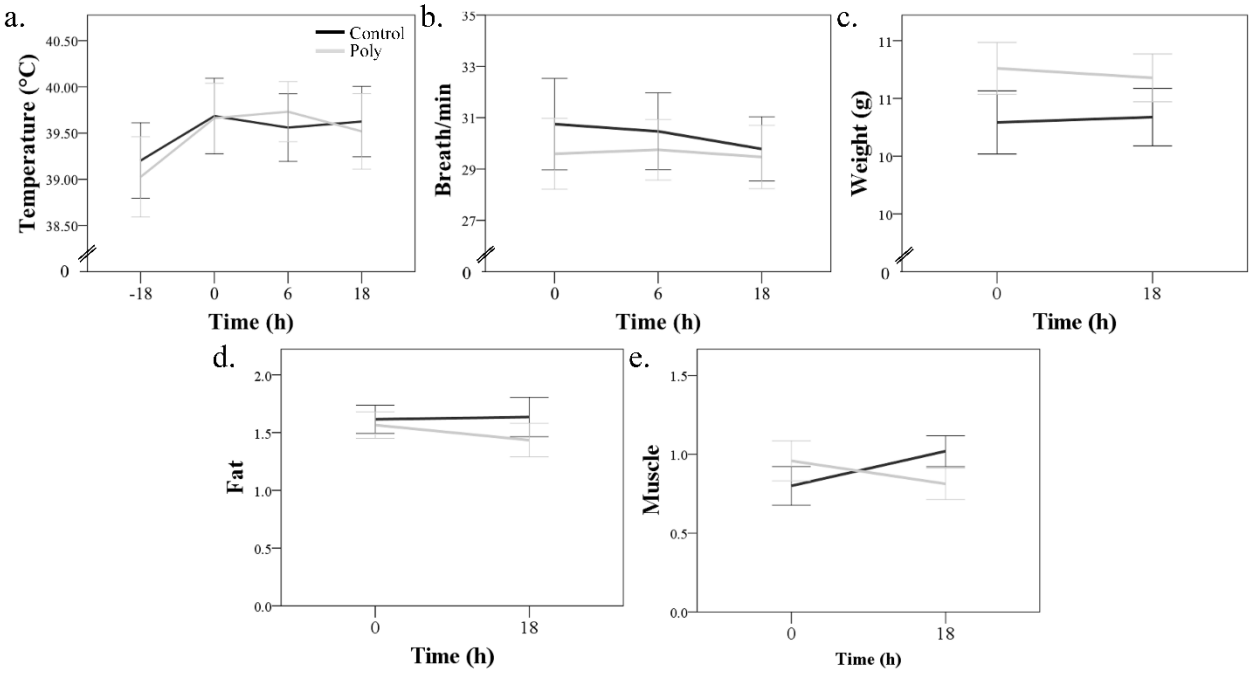

Figure 6. Acute-phase responses of zebra finches to Poly I:C injection. a. The average temperature patterns between the 18 h prior to Poly I:C injection and the 18 h after the injection. b. Breathing rate, c. Body weight, d. Fat load, and e. Muscle load of zebra finches between the Poly I:C injection and 18 h after the injection. Mean values and standard errors are shown.

To detect differences in growth rates (i.e. body weight, tarsus length, wing length) of Control and Poly birds on the day finches were separated from home cage, a Mann-Whitney U test was run (Table A4). Changes in body weight, tarsus and wing length from the Poly I:C

injection day (PD14) to fledging day (~PD30) were not significantly different in terms of these measurements between groups (Figure 7).

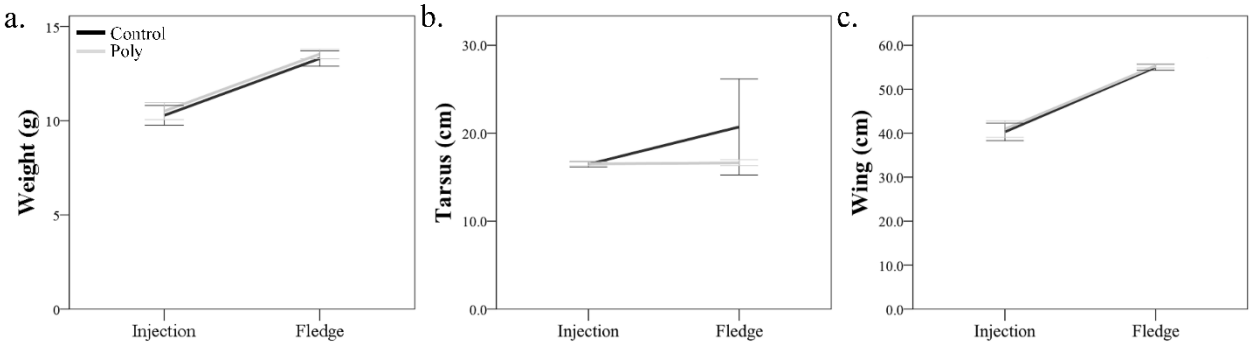

Figure 7. Development of zebra finches from day of injection to the fledging day. a. Body weight, b. Tarsus length, and c. Wing length of the birds. Mean values and standard errors are shown.

Specific Aim 2: Does ELIC affect personality of adult zebra finches?

It was hypothesized that being exposed to Poly I:C, early in life would affect personality of male birds. Specifically, general activity, exploratory, and social behaviors of Poly I:C injected birds would be negatively affected. The overall results showed that there was a negative effect of Poly I:C injection on females on feeding-related behaviors (i.e., visiting food cup and pecking at food) in certain conditions (i.e., familiarity, solitary, and sociality conditions). However, no effects were found in males. Below is the detailed description of analyses for the scored behaviors in four conditions.

For all behaviors, GLMM were used to detect possible effects of Poly I:C injection. Treatment, cohort, sex, treatment*cohort, treatment*sex, and trials were fixed effects in the model, and bird id was used as a random effect. Repeated covariance type was First-Order Autoregressive. Target behaviors distribution was selected as log-linear. Degrees of freedom was fixed for all tests. For fixed effects and coefficients, robust estimations were used to handle

violations of model assumptions. Finally, multiple comparisons were adjusted using sequential Bonferroni.

Familiarity Condition

GLMM analysis revealed no main effects or interactions on hopping on one perch behavior of Control and Poly birds (Table A5, Figure 8a). In hopping between two perches behavior, there was no main effect of treatment, but there was a main effect of cohort on the number of hops (Figure 8b). The first cohort hopped between two perches more often than second and third cohorts ($\beta = 0.657 \pm 0.294$, $t = 2.292$, $p < 0.05$, Table A6).

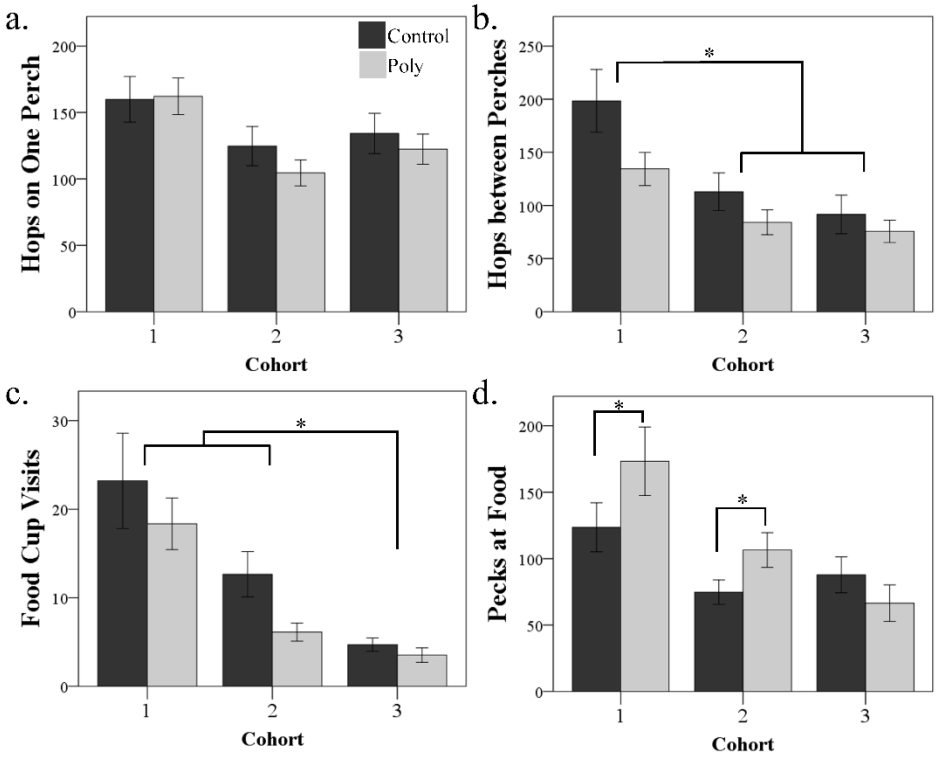

Figure 8. Behaviors of cohorts in familiarity condition. Number of a. Hops on one perch, b. Hops between perches, c. Food cup visits, and d. Pecks at food. * : $p < 0.05$. Mean values and standard errors are shown.

In addition, there was a significant interaction between treatment and sex, as Control females hopped between two perches more often ($\beta = 0.756 \pm 0.362$, $t = 2.086$, $p < 0.05$, Figure 9b). Finally, both Control and Poly birds hopped between two perches more often on the first and

third trials (β = 0.405 ± 0.192, t = 2.106, p < 0.05 / β= 0.325 ± 0.171, t = 1.554, p < 0.05 respectively). In visiting the food cup behavior, treatment was not a significant main effect, but there was a main effect of cohorts. The first and second cohorts visited the food cup more often than the third cohort (β =1.786 ± 0.333, t = 5.37, p < 0.05, β = 0.714 ± 0.317, t = 2.254, p <0.05, respectively (Table A7, Figure 8c)). There was also an interaction between treatment and sex as Control females visited the food cup more often (β =0.789 ± 0.371, t = 2.130, p < 0.05, Figure 9c).

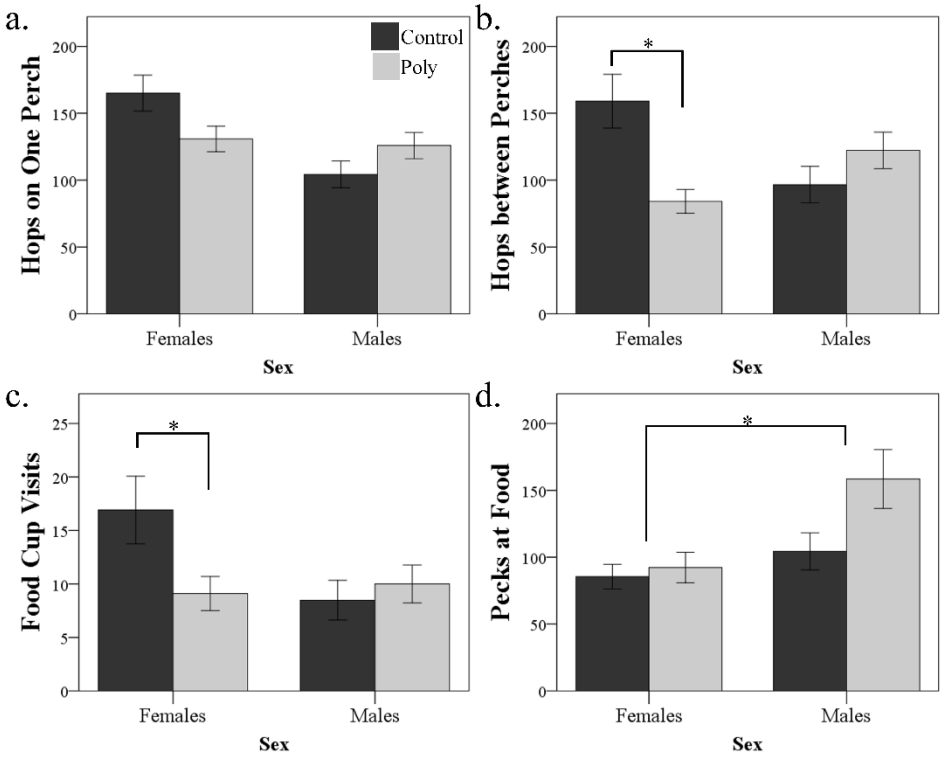

Figure 9. Behaviors of sexes in familiarity conditions. Number of a. Hops on one perch, b. Hops between perches, c. Food cup visits, and d. Pecks at food. * : p < 0.05. Mean values and standard errors are shown.

Finally, there was the main effect of trials on visiting the food cup. On first, third and fifth trials, birds visited the food cup more often (β = 1.841 ± 0.465, t = 3.960, p < 0.05 / β = 1.816 ± 0.468, t = 3.889, p < 0.05 / β = 1.784 ± 0.510, t = 3.502, p < 0.05, respectively). In pecking at food behavior; although there was no main effect of treatment, there was a main effect

of cohort (Figure 8d). The first and second cohort of birds pecked at food significantly more often the third cohort ($\beta = 0.986 \pm 0.203$, $t= 4.855$, $p <0.05$/ $\beta = 0.754 \pm 0.182$, $t= 4.14$, $p <0.05$, respectively, Table A8). There was a significant interaction between treatment and cohort as Control birds of first and second cohorts pecked at food less often ($\beta = -0.573 \pm 0.292$, $t = -1.966$, $p < 0.05$ / $\beta = -0.877 \pm 0.274$, $t = -3.236$, $p < 0.05$, respectively). There was a main effect of sex on pecking at food. Females pecked at food significantly less often than males ($\beta = -0.555 \pm 0.154$, $t= -3.615$, $p <0.05$, Figure 9d). There was also a significant interaction between treatment and sex as Control females pecked at food more often ($\beta = 0.436 \pm 0.214$, $t = 2.401$, $p < 0.05$). Finally, there was a significant main effect of trials on pecking at food, and both Control and Poly groups pecked at food more often in trials 1, 3 and 5 ($\beta = 1.575 \pm 0.266$, $t= 5.917$, $p <0.05$/ $\beta = 1.692 \pm 0.255$, $t= 6.625$, $p <0.05$/ $\beta = 1.651 \pm 0.267$, $t = 6.176$, $p < 0.05$, respectively).

Solitary Condition

GLMM analysis revealed no significant main effect or interaction on the hopping on one perch behavior of Control and Poly birds except trials (Figure 10a). On the first trial, birds hopped on one perch more often than the second and third trials ($\beta =0.366 \pm 0.177$, $t = 2.070$, $p <0.05$, Table A9). On hopping between perches behavior, there was no main effect of treatment between Control and Poly birds (Table A10, Figure 10b). However, there was a trend of Control birds to hop between perches less often than Poly birds ($\beta = -1.37 \pm 0.779$, $t = -1.759$, $p = 0.081$). There was also a main effect of trials, as birds hopped between perches more often on the second trial compared to first and third trials ($\beta = 0.597 \pm 0.248$, $t = 2.406$, $p <0.05$). On visiting the food cup behavior, there was no main effect (Table A11, Figure 10c), but there was again a strong trend of Control birds to visit the food cup less often compared to Poly birds ($\beta = -1.257 \pm 0.724$, $t = -1.735$, $p= 0.085$). There was significant interaction between treatment and sex, in which

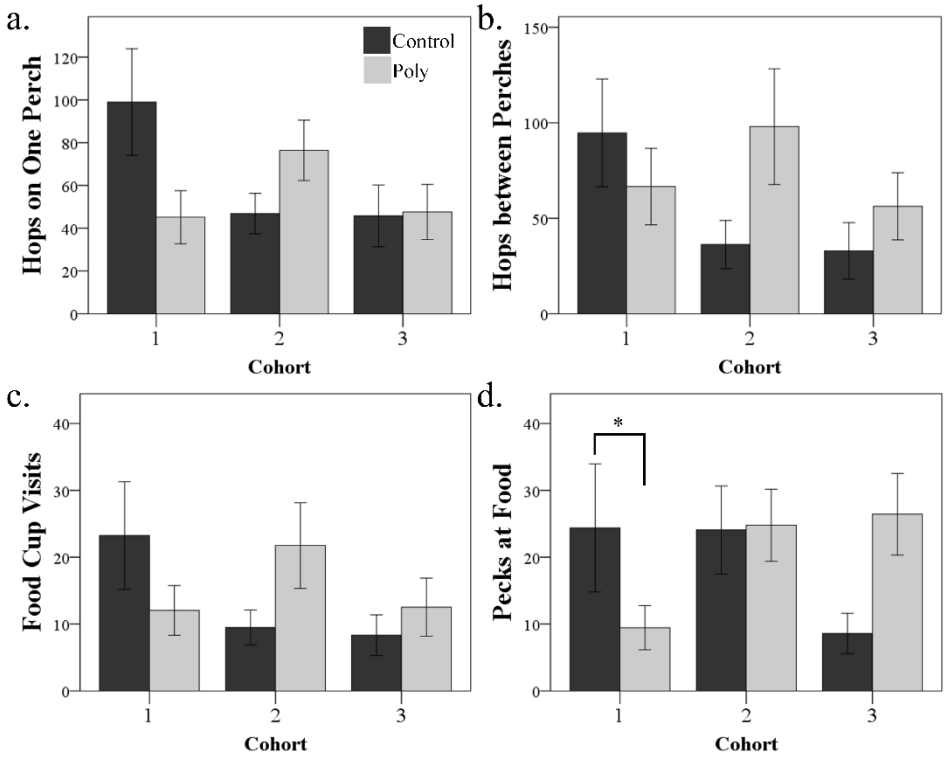

Figure 10. Behaviors of cohorts in solitary condition. Number of a. Hops on one perch, b. Hops between perches, c. Food cup visits, and d. Pecks at food. * : $p < 0.05$. Mean values and standard errors are shown.

female birds of Control group visited the food cup more often ($\beta = 1.347 \pm 0.677$, $t = 1.991$, $p < 0.05$). On pecking at food behavior, there was a significant main effect of treatment (Table A12, Figure 10d). Control birds pecked at food less often compared to Poly birds ($\beta = -1.463 \pm 0.656$, $t= -2.229$, $p < 0.05$). There was also a main effect of cohort as first cohort pecked at food less often ($\beta = - 0.886 \pm 0.455$, $t = -1.992$, $p = 0.048$). Finally, there was a significant interaction between treatment and cohort as Control birds of first cohort pecked at food more often ($\beta = 1.991 \pm 0.735$, $t = 2.708$ $p < 0.05$).

Exploratory Condition

A GLMM analysis revealed no significant main effect of treatment on hopping on one perch, hopping between perches, visiting the food cup, and pecking at food behaviors. However, there was a main effect of cohort on the hopping one perch, hopping between perches, and

visiting the food cup behaviors. The first and second cohorts hopped more often on one perch (β = 1.722 ± 0.328, t = 5.241, p < 0.05 / β = 1.388 ± 0.434, t =3.198, p < 0.05, Table A13, Figure 11a), hopped between two perches more often(β = 2,696 ± 0.369, t = 7.302, p = 0.05 / β = 1.885 ± 0.567, t = 3.327, p < 0.05, Table A14, Figure 11b), and visited the food cup more often (β = 2.558 ± 0.605, t =4.229, p < 0.05 / β = 1.892 ± 0.664, t = 2.851, p < 0.0 / Table A15, Figure 11c) compared to the third cohort. In addition, there was a main effect of trials on visiting the food cup behavior. On trials 1 and 2, birds visited the food cup more often compared to the trial 3 (β = 1.174 ± 0.349, t = 3.359, t = 3.359 p < 0.05, β = 1.197 ± 0.351, t = 3.415, p < 0.05, respectively). There was only a main effect of trials on pecking at food behavior, as on second trial, birds pecked at food more often (β = 0.720 ± 0.313, t = 2.3, p <0.05, Table 16, Figure 11d).

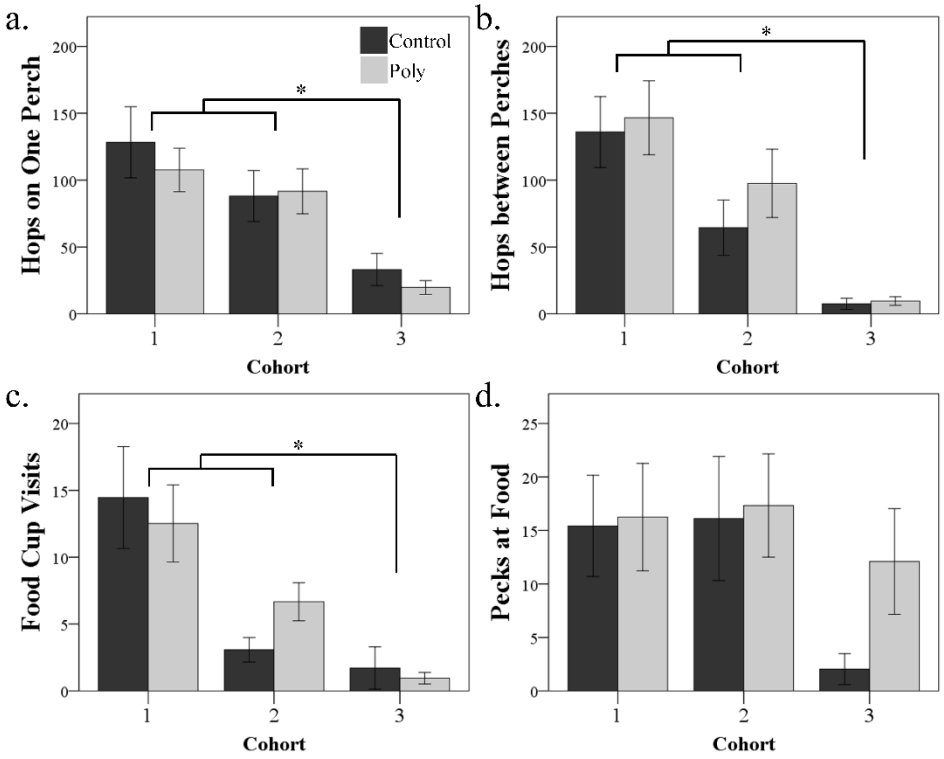

Figure 11. Behaviors of cohorts in exploratory condition. Number of a. Hops on one perch, b. Hops between perches, c. Food cup visits, and d. Pecks at food. * : p < 0.05. Mean values and standard errors are shown.

Sociality Condition

A GLMM analysis revealed no significant main effects or interactions on hopping on one perch, hopping between perches, and visiting the food cup behaviors in the high-density side of the cage (Table A17, Table A18, and Table A19, Figure 12a, b and c). On pecking at food behavior, there was no significant main effect of treatment, but cohort was a significant main effect. Second cohort pecked at food less often than the first and third cohorts on the high-density side of the cage ($\beta = -3.012 \pm 0.807$, $t = -3.735$, $p < 0.05$, Table A20). There was also an interaction between treatment and cohorts as Control birds of Cohort 2 pecked at food more often ($\beta = 2.465 \pm 1.074$, $t = 2.294$, $p < 0.05$, Figure 12d). Sex was another main effect on pecking at food behavior. Female birds pecked at food more often than males ($\beta = 1.816 \pm 0.78$, $t = 2.33$, $p < 0.05$, Table A20).

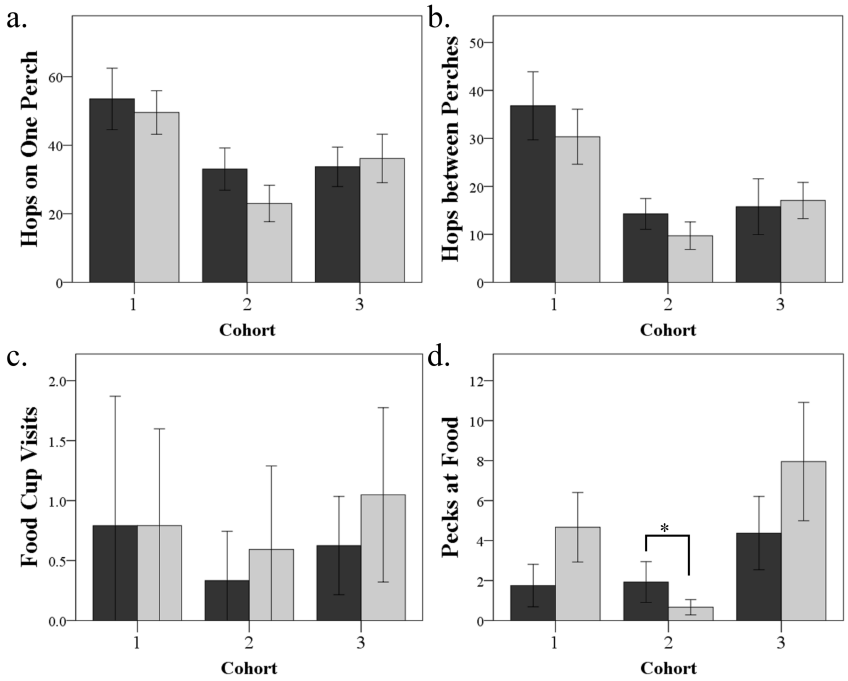

Figure 12. Behaviors of cohorts on the high-density side of the sociality condition flight cage. Number of a. Hopping on one perch, b. Hopping between perches, c. Food cup visits, and d. Pecks at food. * : $p < 0.05$. Mean values and standard errors are shown.

In the low-density side of the cage, there were no main effects or interactions on hopping on one perch and hopping between two perches (Table A21, A22, Figure 13a, b). On visiting the food cup behavior, there was a significant main effect of treatment. Control birds visited the food cup less often than Poly birds ($\beta = -16.120 \pm 1.158$, $t = -13.921$, $p < 0.05$, Table A23, Figure 13c). There was also a significant main effect of sex on visiting the food cup, as females visited food cup more often than the males ($\beta = 2.220 \pm 0.903$, $t = 2.458$, $p < 0.05$, Table A23). Finally, there was a significant interaction between treatment and sex, in which, females of Control group visited the food cup more often ($\beta = 14.726 \pm 1.017$, $t = 14.476$, $p < 0.05$). On pecking at food

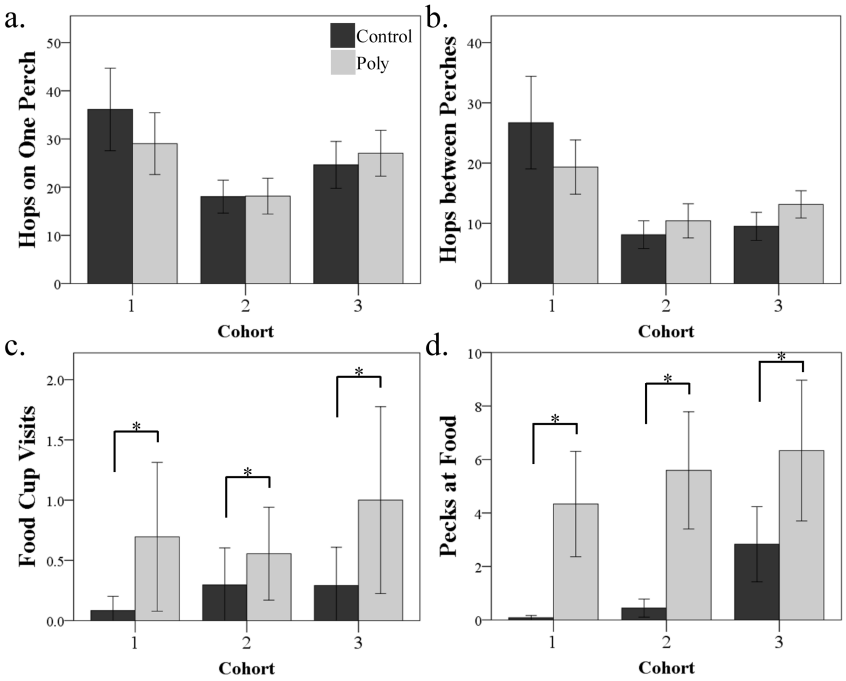

Figure 13. Behaviors of cohorts on the low-density side of the sociality condition flight cage. Number of a. Hopping on one perch, b. Hopping between perches, c. Food cup visits, and d. Pecks at food. * : $p < 0.05$. Mean values and standard errors are shown.

behavior, there was a main effect of treatment. Control birds pecked at food less often than Poly birds ($\beta = -16.400 \pm 1.052$, $t = -15.586$, $p < 0.05$, Table A24, Figure 13d). There was also a main effect of sex on pecking at food behavior. Females pecked at food more often than males ($\beta =$

2.396 ± 0.902, t = 2.655, $p < 0.05$, Table A24). Finally, there was an interaction between treatment and sex as Control group females pecked at food more often ($\beta = 15.297 \pm 1.123$, t = 13.620, $p < 0.05$).

Specific Aim 3: Does ELIC change behaviors in mate choice trials of adult zebra finches?

It was hypothesized that ELIC would change males' behavior in mate choice trials and Poly I:C injected males would behave differently from Control males according to the behavioral measurements and/or females' reaction. It was also hypothesized that ELIC would change females' behavior in mate choice trials and Poly I:C injected females would behave differently from Control females according to the behavioral measurements and/or males' reaction. The results showed that there was no difference of Control and Poly males behaviors in the presence of females. However, Control females visited Control males more often than Poly males. Below is the description of the analyses of male and female behaviors in the Y-maze.

Male Behavior During Female Sampling and Choice Periods

Hopping on one perch and hopping between two perches of 19 males were scored during the three minutes of female sampling and twelve minutes of female choice periods. GLMM were used to detect the Poly I: C's effects on both males' behavior in the Y-maze. Fixed effects were male treatment, female treatment, male treatment * female treatment, and trial number. Random effect was male id. Repeated covariance type was First-Order Autoregressive. Target behaviors distribution was selected as log linear. Degrees of freedom was varied across tests because of small sample size. For fixed effects and coefficients, robust estimations were used to handle violations of model assumptions. Finally, multiple comparisons were adjusted using sequential Bonferroni.

A GLMM analysis revealed no main effects or interactions on hopping on one perch and hopping between two perches' behaviors of Control and Poly males when females were sampling in three minutes (Table A25, Table A26, Figure 14a and b).

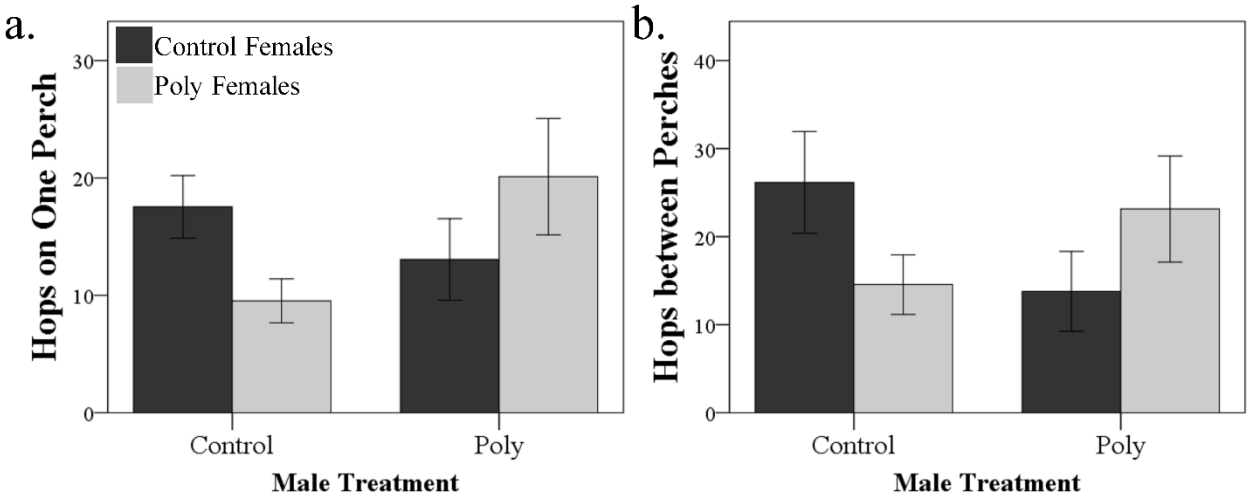

Figure 14. Behaviors of male zebra finches during 3 minutes' female sampling. Number of a. Hopping on one perch, b. Hopping between perches. Mean values and standard errors are shown.

In the 12 minutes of female choice, there were two additional fixed effects, presence of female and presence * male treatment. There was no main effect of treatment on hopping on one perch and hopping between two perches' behaviors of birds (Table A27, Figure 15a and b). There was a main effect of presence of female on hopping between two perches' behavior. When a female bird was absent (not in the arm of the subject male), males hopped between two perches more often than when female was present ($\beta = 0.631 \pm 0.184$, $t = 3.421$, $p < 0.05$, Table A28, Figure 16b).

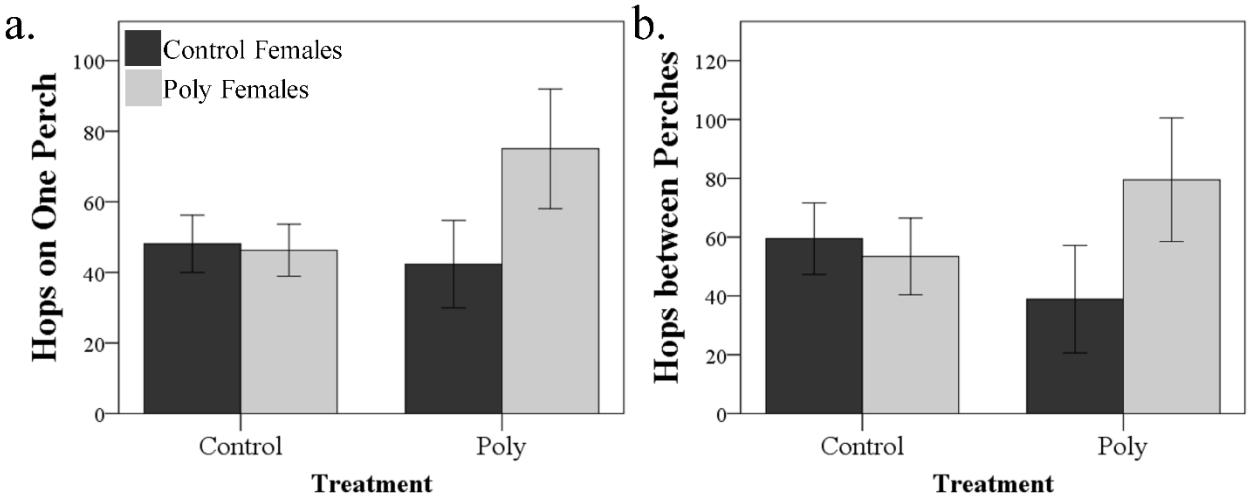

Figure 15. Behaviors of male zebra finches during 12 minutes female choice period. Number of a. Hopping on one perch, b. Hopping between perches. Mean values and standard errors are shown.

Female Behavior During Female Choice Period

The number of visits to the front perch and hops on the front perch behaviors of 14 females were scored during the 12 minutes of female choice period. GLMM were used to detect the Poly I: C's effects on females' choice in the Y-maze. Fixed effects were male treatment, female treatment, male treatment * female treatment, and trial number. Random effect was female id.

Repeated covariance type was First-Order Autoregressive. Target behaviors distribution was selected as log linear. Degrees of freedom was varied across tests because of small sample size. For fixed effects and coefficients, robust estimations were used to handle violations of model assumptions. Finally, multiple comparisons were adjusted using sequential Bonferroni.

A GLMM analysis revealed a significant interaction between male and female treatments. Control females visited the front perch more often if the male is a Control bird ($\beta = 1.401 \pm 0.362$, $t = 3.868$, $p < 0.05$, Table A29, Figure 17a). There was no main effect or interaction on number of the hops on the front perch (Table A30, Figure 17b).

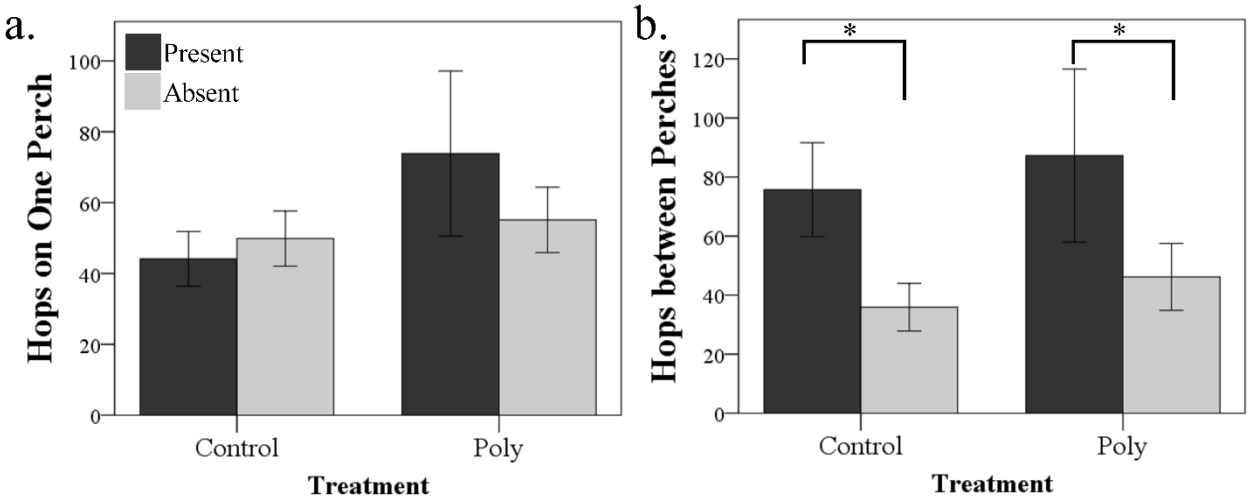

Figure 16. Behaviors of male zebra finches during 12 minutes female choice period depending on the presence of female. Number of a. Hopping on one perch, b. Hopping between perches. * : $p < 0.05$. Mean values and standard errors are shown.

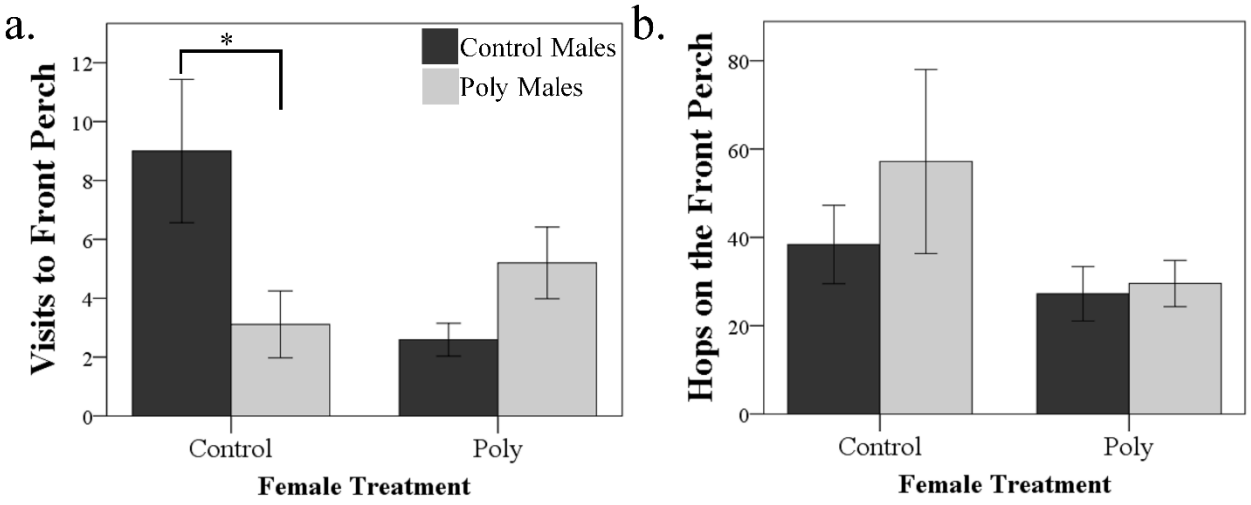

Figure 17. Behaviors of female zebra finches during 12 minutes female choice period. Number of a. Visits to the front perch, and b. Hops on the front perch. Mean values and standard errors are shown. * : $p < 0.05$.

Specific Aim 4: Does ELIC affect the brain development of zebra finches?

It was hypothesized that ELIC would impair brain development and that Poly I:C injected males would have smaller HVC, RA, and TnA. It was also hypothesized that the numbers of ZENK-positive neurons would be different between Control and Poly groups. The results

showed that Poly I:C injected males had smaller RA and TnA. However, there was no significant effect on HVC. Poly I:C injected males showed more ZENK-positive neurons in TnA while activities of HVC and RA remained similar between Control and Poly birds. Below is a detailed description of analyses for brain volume and brain activity and behaviors of birds before sacrifice.

Sixteen male zebra finches were tested in an apparatus (Figure 5 in "Methods") for their responses to a female zebra finch as a stimulus. The numbers of hopping on one perch, hopping between two perches, visiting the food-cup, and pecking at food (Table 2) of birds differed from normality as assessed by Shapiro-Wilk's test ($p < .05$). A Mann-Whitney U test revealed no significant differences in none of their behaviors between Control birds and Poly birds in the presence of females (Table A31).

Brain Volume Analysis

The brains of 19 zebra finches were processed with Nissl staining and the volumes of three brain structures were measured (Figure B1). A MANCOVA was run to determine the effect of Poly I:C injection on the volumes of three nuclei: HVC, RA, and TnA. A preliminary assumption checking revealed that data were normally distributed according to Shapiro-Wilk test ($p > 0.05$); there were no univariate outliers except two HVC volumes in the Control group. There were no multivariate outliers, as assessed by Mahalanobis distance ($p > 0.001$). There were linear relationships between nucleus volumes, as visually assessed by scatterplot. There was no multicollinearity, except a correlation between HVC and RA ($r = 0.49$, $p = 0.033$). There was homogeneity of variance-covariance matrices, as assessed by Box's M test ($p = 0.995$). The differences between the volumes of nuclei on the combined dependent variables depending on treatment was statistically significant after controlling for the telencephalon volume,

F (3,14) = 3.644, p < 0.05, Wilks' Λ = 0.562; partial η^2= 0.438 (Table A32). Follow-up univariate ANOVAs showed that neither HVC (F (1,16) =5.213, p = 0.036; partial η^2= 0.246), RA (F (1,16) =5.213, p = 0.036; partial η^2= .246) nor TnA (F (1,16) = 5.359, p = 0.036; partial η^2= 0.251) were significantly different between Control and Poly birds, using a Bonferroni adjusted α. level of 0.017 (Table A33, Figure 18).

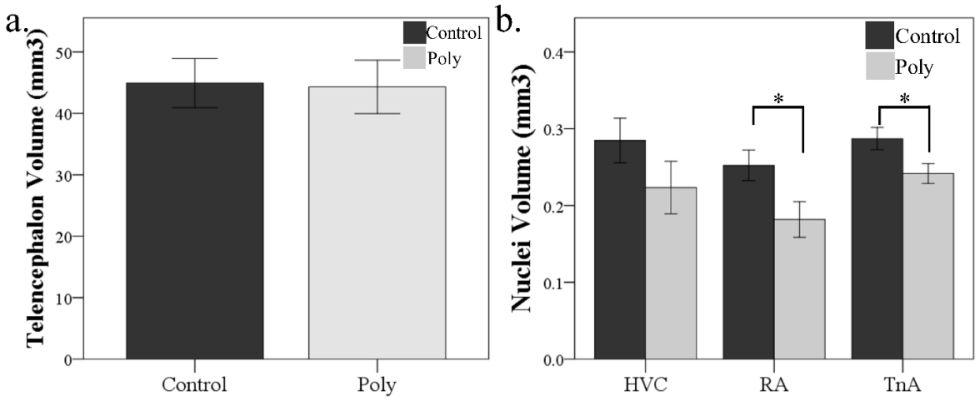

Figure 18. The effect of Poly I:C injection on brain development. Volumes of a. Telencephalon, and on b. HVC, RA, and TnA volumes. * : p < 0.05 without a Bonferroni adjustment. Mean values and standard errors are shown.

Immediate Early Gene Expression Analysis

The brains of 16 male zebra finches were processed immunohistochemically using an anti-ZENK antibody and tissues were photographed under a microscope (Figure B 2). The number of ZENK positive cells were counted in the three nuclei (HVC, RA, and TnA) as well as one control region (LSt). Although data were normally distributed, homogeneity of variance was significantly violated for RA, as assessed by Levene's test of equality of variances (F (1,12) =6.586, p<0.05). In addition, homogeneity of variance-covariance matrices was significantly violated, as assessed by Box's M test (p< 0.05). Because of these assumption violations, Mann-Whitney's U test was run to determine if there was a difference in the activity of nuclei depending on early life treatments. There was a significant difference in the activity of TnA.

Poly I:C injected birds (Mdn= 53) showed increased activity compared to Control (Mdn= 42) birds (U=50.5, z=2.014, p<0.05).

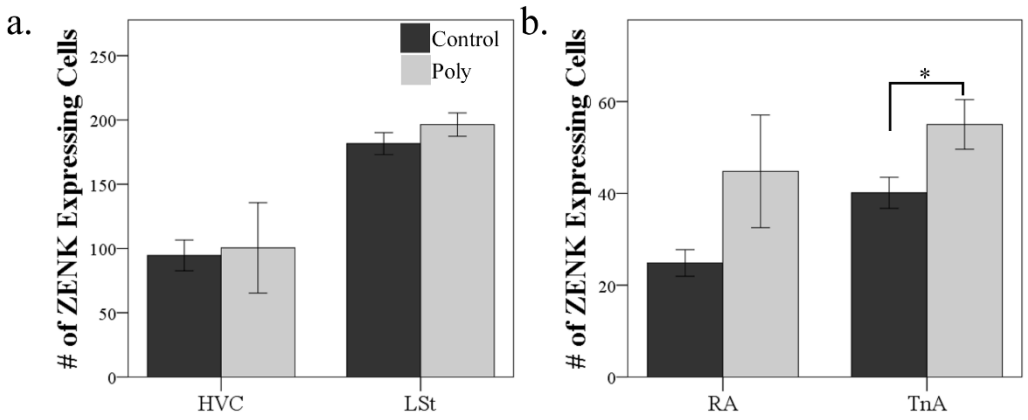

Figure 19. The effect of Poly I:C injection on brain activity. ZENK expressions of a.HVC, LSt, and b. RA, TnA in presence of a female bird. * : $p < 0.05$. Mean values and standard errors are shown.

DISCUSSION

This study aimed to determine whether Poly I:C injection affects the body growth rate in nestlings of zebra finch; ELIC affects personalities of adult zebra finches; ELIC affects behaviors of adult zebra finches in mate choice trials; and ELIC affects the brain development of adult zebra finches. In order to answer these questions, the present study used physiological and morphological measurements, behavioral observations in different conditions, volume measurements of specific brain nuclei, and histochemical analysis of neuronal activity through IEG protein expression. The results showed that nestlings' growth rates were not affected (Aim 1). Poly I:C injection had some effects on certain, but not all, personality traits observed in the study. Furthermore, such effects were found only in female zebra finches, suggesting that there was a limited sex-specific influence of ELIC on personalities of adults (Aim 2). The results also showed, although no apparent changes in the males' behaviors were observed after Poly I:C injections, females tended to choose Control males over Poly males (Aim 3). Finally, Poly I:C injection affected the overall development of targeted brain nuclei and there were changes in neuronal activity according to IEG expression (Aim 4).

Specific Aim 1: Does Poly I:C injection affect the body growth rate in nestlings of zebra finch?

In Specific Aim 1, it was hypothesized that Poly I:C injected birds would have impaired body growth and have shorter tarsus and wing lengths due to adverse effects on morphological development. The Poly birds in the present study did not show a difference in their growth rate

as measured through changes in their body weight, tarsus, and wing lengths compared to Control birds. After an early life stress, some studies showed a negative effect on growth rates (Calero-Riestra & García, 2016; Eeva & Lehikoinen, 1996; Grindstaff et al., 2012; Nowicki et al., 2002; O'Brien & Dawson, 2008; Spencer & Verhulst, 2007) while other studies showed no effect at all (Farrell, Morgan, et al., 2015; Reed et al., 2012; Shutler et al., 1999; Verhulst et al., 2005) The present results found that Poly I:C had no effects on zebra finches under these experimental conditions.

However, it is also possible that negative growth effects of ELIC was limited to only a period of Poly I:C injections and that such effects disappeared before they became juveniles when the measures were conducted in the present study. Previous studies that showed negative effects on growth also showed that such effects eventually disappeared after treatment periods (Grindstaff et al., 2012; Nowicki et al., 2002). In the present study, morphological measurements of tarsus and wing lengths were conducted twice – at 0 h (the time of an injection) and PD30 (the day nestlings were transferred to a separate flight cage). Poly I:C injection might have affected the growth rate of the animals, but only for a brief period before PD30. More frequent measurements were difficult since increased handlings could create a confounding stress.

Several physiological measurements were also conducted to examine whether Poly I:C injection in zebra finch nestlings caused APR (increased body temperature, loss of body weight, fat, and muscle load overnight). Birds did not show any APR in response to Poly I:C injection as an immune challenge. These results are similar to findings of Coon et al. (2011), who showed no change in body temperature and weight after Poly I:C injection in adult house sparrows. There are other studies that showed significant temperature effects in chicks (Kent et al., 2007) and ducks (M. Marais et al., 2011). In the present study, the dosage of Poly I:C (25 mg/kg) was at

least five times more than the dosage used in these studies (5 mg/kg for chicks and 1mg/kg for ducks), suggesting that the absence of fever should not be due to low dosage.

There are three possible explanations for not detecting a change in body temperature after Poly I:C injection. First, Poly I:C is recognized by the immune system of some bird species (e.g., fowls like chicks and ducks), but not others (e.g., passerine birds like zebra finches, house sparrows). However, Poly I:C is recognized by Toll-like receptor 3 (TLR3) (Bilbo & Schwarz, 2012) and both zebra finches and chickens are known to have clear orthologs of TLR3 in their genome (Brownlie & Allan, 2011).

The second explanation for not detecting fever after Poly I:C injection is that our measurements were conducted too late (6 hours, and 18 hours after Poly I:C injection) if the body temperature changes occurred immediately after the injections. However, in chicks, body temperature was higher than normal for at least 6 hours after Poly I:C injection, and in ducks, it remained high for over 15 hours (Kent et al., 2007; M. Marais et al., 2011).

Finally, the absence of fever could be due to the stress-induced hyperthermia. The results showed that the body temperature of birds increased at injection time point (39.6 ^{o}C at 0 h) compared to that before the injection (39.1 ^{o}C at -18 h) and sustained the higher temperature through the subsequent measurement time points (39.8 ^{o}C at 6 h and 39.6 ^{o}C at 18 h). It is known that after a stressful event, the body temperature of animals increases (Olivier et al., 2003). For example, being restrained for a minute increases the body temperature of wild blue tits (*Cyanistes caeruleus*) (Jerem, Herborn, McCafferty, McKeegan, & Nager, 2015).

There was no clear change in morphological measurements between Control and Poly nestlings. It was predicted that body weight, fat load, and muscle load would decrease overnight, due to anorexia and increased metabolic rate. Breathing rates of birds did not change either after

Poly I:C injection, suggesting no change in the metabolic rate of animals. Congruent with no change in metabolic rate, there was no difference in the body weight and fat load change of the animals, which contradicts with results of decrease in body weight after Poly I:C injection in ducks (Manette Marais et al., 2013). Overall, results of the Specific Aim 1 measurements suggest that other types of immune response measurements blood measurements such as haptoglobin assay might be useful in future studies to confirm the present results.

Specific Aim 2: Does ELIC affect personality of adult zebra finches?

In Specific Aim 2, it was hypothesized that general activity, exploratory, and social behaviors of Poly I:C injected birds would be negatively affected. The results showed that there was no main effect of Poly I:C injection on personality traits measured by these behaviors. However, females, but not males, showed effects of Poly I:C injection in visiting food-cup and pecking at food.

The lack of no main effects in the present study suggest that Poly I:C does not cause any significant effects on personality traits. This result is consistent with some previous studies by Krause, and Naguib (2011), and Kriengwatana et al. (2015), who also showed no significant effects in certain conditions. However, it is also possible that the procedures used in the present study could not capture main effects of Poly I:C. For example, timing of stressor administration, could be an important factor. In the present study, birds were injected only one time on PD14, and it could have been too early to affect their personality in adulthood. In zebra finches, LPS injection on PD5 did not affect birds' behavior in the presence of a novel object (Grindstaff et al., 2012). In ducks, injection with SRBC when they were 3 weeks old did not affected their general activity in adulthood, but ducks that were 8 weeks old were more active. (Butler et al., 2012).

Poly I:C injected females visited food cup and pecked at food less frequently compared to Control females in three of the four conditions in the present study. This result is similar to previous findings by Spencer and Verhulst (2007), who also showed that only female zebra finches exhibited less exploratory behavior after CORT admisnitration. Another study also showed sex-dependent effects of zebra finches after an administariotn of a protein-based antigen KLH although the effects were found only in males, instead of females, in learning abilities (Grindstaff et al., 2012). However, such sex-dependent results need to be further investigated since there are also reports showing that early-life stress can have effects on both males and females. Zimmer, Boogert, and Spencer (2013) reported that unexpected food removal as an early life stress increased both male and female quails exploratory behavior .

The sex-dependent effects of Poly I:C might be related to differences in sex hormones and their effects on the immune system between males and females. There is a study suggesting that a higher level of testosterone might cause weaker immune response in males compared to females (Duffy, Bentley, Drazen, & Ball, 2000; Muriel, Perez-Rodriguez, Ortiz-Santaliestra, Puerta, & Gil, 2017). However, this explanation may be unlikely because there is not a clear dimorphism in plasma level of sex hormones in zebra finches (Wade & Arnold, 2004). In addition, zebra finch nestlings did not show a sex-specific cellular immune response to phytohaemagglutinin (PHA) injection (Tobler, Hasselquist, Smith, & Sandell, 2010).

The result might be explained based on the sex difference in the tradeoff in the development of different traits after an ELIC. Food restriction during the development of song sparrows is known to reduce immune reactions to PHA in adult males, but not in females (Schmidt, Kubli, MacDougall-Shackleton, & MacDougall-Shackleton, 2015). This suggests that the development of the immune system might be more critical to females than males. After

ELIC, females in the present study might have invested more on the development of the immune system at expense of other traits, such as feeding behavior (e.g., visiting a food cup, pecking at food).

The present study also showed that there were behavioral differences between cohorts. The third and oldest cohort, compared to first and second cohorts, showed decreased activity in two of the four conditions (i.e., familiarity and exploration). It is known that the CORT level of animals is associated with general activity levels (Martins, Roberts, Giblin, Huxham, & Evans, 2007). Furthermore age-related changes in CORT levels were reported in different birds (Heidinger, Nisbet, & Ketterson, 2006). For example, stress-induced CORT levels were lower in older Florida scrub-jays (*Aphelocoma coerulescens*) (Wilcoxen, Boughton, Bridge, Rensel, & Schoech, 2011). Although it was not measured in the present study, CORT release in the present study might be the lowest at the age of the third cohort compared to first and second cohorts.

Finally, the results also showed that food cup visits were much less often when a blue meshed mosquito cup was placed in the exploratory condition compared to other novel items (i.e., pink Q letter, and yellow golf ball). It is unlikely that the color blue was a factor since Muth, Steele, and Healy (2013) showed that zebra finches did not show any avoidance to blue materials as the nesting material. It is possible that the blue meshed mosquito cup was avoided because it was simply larger than the other items in size.

Specific Aim 3: Does ELIC change behaviors in mate choice trials of adult zebra finches?

In Specific Aim 3, it was hypothesized that ELIC would negatively affect male zebra finches' behavior in mate choice trials. The present results could not show clear differences in male behaviors between Poly and Control groups. However, Control females visited Control males more often than Poly males, suggesting that these females could observe some behavioral

differences we could not capture. In contrast, Poly females did not discriminate between Poly and Control males, further suggesting that ELIC caused an effect on subject females as well.

As discussed in Introduction, MacDougall-Shackleton and Spencer (2012) suggested that there are at least two explanations for the lack of discriminability in Poly females. First, it is possible that Poly females had impaired discrimination abilities of certain (but unknown) stimuli in mate choice trials. It is also possible that their preference between these stimuli disappeared after Poly I:C injections although they could still discriminate these cues. In the present study, vocal responses including songs were not measured. The song frequency and quality of males could be critical cues for Control females to choose potential mates. However, previous studies showed that stressed females could choose males with high quality songs over those with low quality songs (Farrell, Neuert, et al., 2015; Schmidt, McCallum, et al., 2013; Woodgate et al., 2011) suggesting that stress may not impair the song discrimination ability of females.

In addition to the two possible explanations proposed by MacDougall-Shackleton and Spencer (2012), the present results suggest another potential reason for the lack of choice by Poly females. Thus, although Poly females could still discriminate and prefer certain characteristics of male signals, they might have lost motivation in mate choice trials. Malnutritioned female zebra finches were known to be less active in mate selection trials (Woodgate et al., 2010). Further studies are warranted to identify specific causes of these behavioral changes.

Specific Aim 4: Does ELIC affect the brain development of zebra finches?

In Specific Aim 4, it was hypothesized that ELIC would impair brain development and Poly I:C injected males would have smaller HVC, RA, and TnA. It was also hypothesized that the numbers of ZENK-positive neurons would be different between Control and Poly groups.

Regarding the development of brain nuclei, the MANCOVA results showed that negative effects of Poly I:C treatment on overall brain development. However, follow-up post-hoc tests did not reveal significant effect of Poly I:C treatment for the individual nuclei after Bonferroni adjustments. The overall effects on brain development are consistent with previous early life stress studies that showed adverse effects on HVC and/or RA (Buchanan et al., 2004; Honarmand et al., 2016; MacDonald et al., 2006; Nowicki et al., 2002; Schmidt, Moore, et al., 2013). Furthermore, the present study was the first study including TnA as targeted brain area to study ELIC. In zebra finches, a rapid growth of TnA was observed up to PD30 then plateaued (Ikebuchi et al., 2013). Nestlings in the present study were treated on PD14 when the TnA was developing fastest. Therefore, it is possible that Poly I:C affected TnA because of the critical timing of the treatment.

There are at least two possibilities that Poly I:C had negatively affected the brain development – either through decrease in neuron number or decrease in intracellular space. Previous studies have shown neuronal loss and decrease in neurogenesis in rats and mice after Poly I:C injection (De Miranda et al., 2010; Melton et al., 2003; Meyer et al., 2006) . However, number of neurons should be counted in future studies in order to confirm neuronal changes.

The results of the present IEG study showed that TnA was more active in Poly I:C treated males than Control males in the presence of a female; but there was no difference in the activities of HVC or RA. The TnA was selected as a targeted area for the study because it is known to be involved in sexual and social behaviors (Cheng et al., 1999; Ikebuchi et al., 2009; Thompson et al., 1998). Compared to Control males, TnA in Poly males expressed more ZENK-positive neurons. It is possible that Poly I:C treatment might have affected physiology of TnA and that TnA neurons became more reactive to environmental stimuli. As mentioned earlier, TnA is

suggested to be the homologue of part of the mammalian amygdala (Cheng et al., 1999; Patzke et al., 2011; Yamamoto et al., 2005). The mammalian amygdala plays important roles in various emotional learning and memory including fear, anxiety, and frustration (Mayes, 2006; Tavares, Judice-Daher, & Bueno, 2014). The increased TnA activity may be related to some of these amygdala functions.

Alternatively, it is possible that ZENK expression in TnA increased because of changes in the neuronal density of the entire TnA, rather than an increase of ZENK-expressing neurons. The number of ZENK neurons might be higher in brains of Poly I:C injected males due to decreased TnA volume and increased neuronal density. In that case, it would suggest that ZENK activity in TnA was similar between groups. It is also possible that the neuron number was fewer in Poly I:C injected males due to pathological effects of Poly I:C on neurons. TnA could become hyperactive to compensate for the loss of neurons. This may be similar to an increase in activity found in neurological diseases such as Parkinson's disease (Poston et al., 2016; Zigmond, Abercrombie, Berger, Grace, & Stricker, 1990).

The present study also measured males' behavior in the presence of a female prior to perfusion. Although TnA of Poly and Control males showed different ZENK activity levels, the behavioral results showed no differences between the groups. This indicates that brain activity analysis (i.e., IEG expression) could reveal subtle differences between Poly and Control groups while behavioral measurements or observations could not. It is known that in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, there is a presymptomatic phase while neuronal degeneration occurs (Dekosky & Marek, 2003). One time treatment of Poly I:C might have created a similar effect which was not enough to affect behaviors of males.

In conclusion, the present study revealed various effects of Poly I:C as an ELIC on zebra finches. Behavioral and histological findings provide new information on how developmental stress affects adulthood and will be the foundation for future studies on developmental stress with an immune challenge. The results of the current study are valuable in order to improve the predictions of DSH on the adulthood of an animal depending on the type of early life stressor.

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APPENDICES

Appendix A: Statistical Test Tables

Table A1. Generalized Linear Mixed Model of Body Temperature after Poly I:C Injection.

					95% CI	
Model Term	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	39.45	0.242	162.983	**<0.001**	38.979	39.934
Treatment						
Control	-0.088	0.245	-0.359	0.720	-0.571	0.395
Poly	0[a]					
Sex						
Female	0.449	0.301	1.493	0.137	-0.144	1.042
Male	0[a]					
Treatment * Sex						
*Control * Female*	-0.094	0.437	-0.216	0.829	-0.957	0.769
*Control * Male*	0[a]					
*Poly * Female*	0[a]					
*Poly * Male*	0[a]					
Time						
-18 h	-0.537	0.159	-3.371	**<0.001**	-0.851	-0.223
0 h	0.139	0.115	1.206	0.229	-0.088	0.366
6 h	0.104	0.128	0.815	0.416	-0.148	0.356
18 h	0[a]					

Random and Residual Effects					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.623	0.162	3.849	**<0.001**	0.375	1.037
Residual[c]						
AR1 Diagonal	0.500	0.069	7.302	**<0.001**	0.382	0.654
AR1 Rho	0.002	0.140	0.011	0.991	-0.267	0.270

[a] Set to 0 because this parameter is redundant.
[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A2. Generalized Linear Mixed Model of Breathing Rate after Poly I:C Injection

					95% CI	
Model Term	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	30.413	0.985	30.867	**<0.001**	28.465	32.361
Treatment						
Control	1.913	1.255	1.524	0.130	-0.569	4.394
Poly	0[a]					
Sex						
Females	-1.022	1.193	-0.856	0.393	-3.381	1.337
Males	0[a]					
Treatment * Sex						
*Control * Female*	-1.957	1.629	-1.202	0.232	-5.177	1.263
*Control * Male*	0[a]					
*Poly * Female*	0[a]					
*Poly * Male*	0[a]					
Time						
0 h	-0.429	0.491	-0.872	0.385	-1.400	0..543
6 h	-0.429	0.586	-0.731	0.466	-1.587	0.730
18 h						

Random and Residual Effects					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	6.416	1.075	3.764	**<0.001**	3.812	10.800
Residual[c]						
AR1 Diagonal	6.487	0.960	6.760	**<0.001**	4.855	8.669
AR1 Rho	-0.297	0.160	-1.859	0.063	-0.571	0.037

[a] Set to 0 because this parameter is redundant.
[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A3. Generalized Linear Mixed Model of Body Weight Change after Poly I:C Injection

					95% Confidence Interval	
Model Term	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	0.108	0.19	0.566	0.573	-0.270	0.485
Treatment						
Control	-0.224	0.259	-0.867	0.388	-0.739	0.29
Poly	0[a]					
Time						
0 h	0.076	0.084	0.897	0.372	-0.092	0.243
18 h	0[a]					
Treatment *Time						
*Control * 0 h*	-0.101	0.11	-0.919	0.361	-0.32	0.117
*Control * 18 h*	0[a]					
*Poly * 0 h*	0[a]					
*Poly * 18 h*	0[a]					

Random and Residual Effects					95% Confidence Interval	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.778	179.622	0.004	0.997	0	
Residual[c]						
AR1 Diagonal	0.198	179.622	0.001	0.999	0	
AR1 Rho	0.616	348.617	0.002	0.999	-1	1

[a] Set to 0 because this parameter is redundant.

[b] Covariance structure: scaled identity; subject specification: Bird id.

[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A4. Mann-Whitney U Test for Development Rate of Control and Poly Birds

	Median	Mann-Whitney U	z	Sig
Change in Body Weight (g)				
Control	2.9	305	0.362	0.718
Poly	2.9			
Change in Tarsus (cm)				
Control	0.3	289	-0.221	0.825
Poly	0.3			
Change in Wing (cm)				
Control	15	334	0.692	0.489
Poly	14			

Appendix A: Statistical Test Tables

Table A5. Generalized Linear Mixed Model of Hopping on One Perch Behavior in Familiarity Condition

					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	4.49	0.232	19.314	**<0.001**	4.032	4.948
Treatment						
Control	-0.104	0.283	-0.366	0.714	-0.66	0.453
Poly	0[a]					
Cohort						
C1	0.338	0.183	1.844	0.066	-0.023	0.699
C2	-0.146	0.188	-0.777	0.438	-0.517	0.225
C3	0[a]					
Sex						
Females	0.133	0.135	0.983	0.326	-0.133	0.399
Males	0[a]					
Treatment*Cohort						
*Control*C1*	-0.090	0.283	-0.320	0.749	-0.647	0.466
*Control*C2*	0.006	0.334	0.018	0.986	-0.652	0.664
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.3	0.242	1.237	0.217	-0.177	0.777
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.271	0.153	1.773	0.077	-0.030	0.572
2	0.170	0.155	1.093	0.275	-0.136	0.475
3	0.184	0.126	1.465	0.144	-0.063	0.432
4	0.136	0.120	1.136	0.257	-0.100	0.372
5	0.247	0.162	1.525	0.128	-0.072	0.567
6	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A5 (cont'd). Generalized Linear Mixed Model of Hopping on One Perch Behavior in Familiarity Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.184	0.053	3.503	**<0.001**	0.105	0.322
Residual[c]						
AR1 Diagonal	42.607	4.206	10.583	**<0.001**	35.404	51.276
AR1 Rho	-0.302	0.077	-3.902	**<0.001**	-0.444	-0.144

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A6. Generalized Linear Mixed Model of Hopping between Two Perches Behavior in Familiarity Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	4.066	0.339	13.307	**<0.001**	3.464	4.677
Treatment						
Control	-0.287	0.442	-0.648	0.517	-1.157	0.584
Poly	0[a]					
Cohort						
C1	0.675	0.294	2.292	**0.023**	0.095	1.254
C2	0.302	0.300	1.007	0.315	-0.288	0.892
C3	0[a]					
Sex						
Females	-0.388	0.206	-1.881	0.061	-0.794	0.018
Males	0[a]					
Treatment*Cohort						
*Control*C1*	0.172	0.483	0.356	0.722	-0.778	1.122
*Control*C2*	-0.169	0.500	-0.339	0.735	-1.153	0.815
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.756	0.362	2.086	**0.038**	0.043	1.469
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.405	0.192	2.106	**0.036**	0.026	0.784
2	0.266	0.171	1.554	0.121	-0.071	0.604
3	0.325	0.160	2.029	**0.043**	0.010	0.641
4	0.228	0.138	1.655	0.099	-0.043	0.500
5	0.352	0.209	1.687	0.093	-0.059	0.763
6	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A6 (cont'd). Generalized Linear Mixed Model of Hopping between Two Perches Behavior in Familiarity Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.456	0.113	4.040	**<0.001**	0.281	0.741
Residual[c]						
AR1 Diagonal	52.634	5.381	9.782	**<0.001**	43.077	64.310
AR1 Rho	-0.470	0.062	-7.525	**<0.001**	-0.583	-0.339

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A7. Generalized Linear Mixed Model of Visiting Food Cup Behavior in Familiarity Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	-0.133	0.557	-0.239	0.811	-1.229	0.963
Treatment						
Control	-0.073	0.452	-0.160	0.873	-0.962	0.817
Poly	0[a]					
Cohort						
C1	1.786	0.333	5.370	**<0.001**	1.131	2.440
C2	0.714	0.317	2.254	**0.025**	0.090	1.337
C3	0[a]					
Sex						
Females	-0.103	0.228	-0.454	0.650	-0.552	0.345
Males	0[a]					
Treatment*Cohort						
*Control*C1*	-0.194	0.476	-0.408	0.683	-1.132	0.743
*Control*C2*	0.009	0.483	0.019	0.985	-0.942	0.960
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.789	0.371	2.130	**0.034**	0.060	1.519
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	1.841	0.465	3.960	**<0.001**	0.926	2.756
2	-0.861	0.498	-1.729	0.085	-1.842	0.119
3	1.816	0.468	3.889	**<0.001**	0.896	2.737
4	-0.397	0.524	-0.756	0.450	-1.429	0.636
5	1.784	0.510	3.502	**<0.001**	0.781	2.788
6	`0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A7 (cont'd). Generalized Linear Mixed Model of Visiting Food Cup Behavior in Familiarity Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.427	0.117	3.655	**<0.001**	0.25	0.73
Residual[c]						
AR1 Diagonal	5.456	0.527	10.345	**<0.001**	4.514	6.594
AR1 Rho	-0.33	0.084	-3.929	**<0.001**	-0.483	-0.157

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A8. Generalized Linear Mixed Model of Pecking at Food Behavior in Familiarity Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	3.284	0.313	10.502	**<0.001**	2.669	3.900
Treatment						
Control	0.136	0.298	0.456	0.649	-0.451	0.723
Poly	0[a]					
Cohort						
C1	0.986	0.203	4.855	**<0.001**	0.586	1.386
C2	0.754	0.182	4.140	**<0.001**	0.396	1.113
C3	0[a]					
Sex						
Females	-0.555	0.154	-3.615	**0.001**	-0.857	-0.253
Males	0[a]					
Treatment*Cohort						
*Control*C1*	-0.573	0.292	-1.966	**0.050**	-1.147	0.001
*Control*C2*	-0.887	0.274	-3.236	**0.001**	-1.426	-0.347
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.436	0.214	2.401	**0.042**	0.016	0.856
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	1.575	0.266	5.917	**<0.001**	1.051	2.099
2	-0.631	0.364	-1.734	0.084	-1.348	0.086
3	1.692	0.255	6.625	**<0.001**	1.189	2.195
4	-0.415	0.274	-1.517	0.131	-0.954	0.124
5	1.651	0.267	6.176	**<0.001**	1.125	2.178
6	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A8 (cont'd). Generalized Linear Mixed Model of Pecking at Food Behavior in Familiarity Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.095	0.036	2.635	**<0.001**	0.045	0.199
Residual[c]						
AR1 Diagonal	46.847	4.376	10.704	**<0.001**	39.009	56.261
AR1 Rho	-0.273	0.103	-2.66	**<0.001**	-0.46	-0.063

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A9. Generalized Linear Mixed Model of Hopping on One Perch Behavior in Solitary Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	3.427	0.502	6.831	**<0.001**	2.435	4.419
Treatment						
Control	-0.706	0.631	-1.119	0.265	-1.955	0.542
Poly	0[a]					
Cohort						
C1	0.072	0.483	0.149	0.882	-0.884	1.028
C2	0.551	0.489	1.126	0.262	-0.416	1.518
C3	0[a]					
Sex						
Females	-0.059	0.376	-0.158	0.874	-0.802	0.683
Males	0[a]					
Treatment*Cohort						
*Control*C1*	0.778	0.698	1.114	0.267	-0.603	2.159
*Control*C2*	-0.181	0.723	-0.250	0.803	-1.610	1.248
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.773	0.556	1.391	0.167	-0.326	1.872
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.366	0.177	2.070	**0.040**	0.016	0.716
2	0.230	0.199	1.159	0.249	-0.163	0.624
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A9 (cont'd). Generalized Linear Mixed Model of Hopping on One Perch Behavior in Solitary Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.792	0.32	2.477	**0.013**	0.359	1.747
Residual[c]						
AR1 Diagonal	36.184	8.481	4.266	**<0.001**	22.857	57.283
AR1 Rho	0.07	0.226	0.312	0.755	-0.358	0.474

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A10. Generalized Linear Mixed Model of Hopping between Two Perches Behavior in Solitary Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	3.385	0.612	5.530	**<0.001**	2.174	4.595
Treatment						
Control	-1.370	0.779	-1.758	0.081	-2.912	0.171
Poly	0[a]					
Cohort						
C1	0.365	0.585	0.624	0.534	-0.792	1.523
C2	0.443	0.682	0.650	0.517	-0.905	1.792
C3	0[a]					
Sex						
Females	-0.152	0.495	-0.307	0.759	-1.130	0.826
Males	0[a]					
Treatment*Cohort						
*Control*C1*	0.688	0.842	0.817	0.415	-0.977	2.353
*Control*C2*	-0.109	0.958	-0.114	0.910	-2.003	1.785
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	1.130	0.745	1.516	0.132	-0.344	2.604
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.346	0.249	1.389	0.167	-0.147	0.840
2	0.597	0.248	2.406	**0.017**	0.106	1.087
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A10 (cont'd). Generalized Linear Mixed Model of Hopping between Two Perches Behavior in Solitary Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	1.26	0.431	2.923	**0.003**	0.644	2.464
Residual[c]						
AR1 Diagonal	55.795	10.5	5.314	**<0.001**	38.584	80.682
AR1 Rho	0.055	0.179	0.306	0.76	-0.289	0.386

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A11. Generalized Linear Mixed Model of Visiting Food Cup Behavior in Solitary Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	2.308	0.577	3.998	**<0.001**	1.167	3.450
Treatment						
Control	-1.257	0.724	-1.735	0.085	-2.689	0.175
Poly	0[a]					
Cohort						
C1	0.147	0.541	0.272	0.786	-0.924	1.218
C2	0.585	0.556	1.053	0.294	-0.514	1.684
C3	0[a]					
Sex						
Females	-0.633	0.493	-1.285	0.201	-1.607	0.341
Males	0[a]					
Treatment*Cohort						
*Control*C1*	0.837	0.810	1.033	0.303	-0.765	2.440
*Control*C2*	-0.112	0.809	-0.139	0.890	-1.711	1.487
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	1.347	0.677	1.991	**0.049**	0.009	2.686
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.175	0.208	0.842	0.401	-0.236	0.585
2	0.322	0.265	1.216	0.226	-0.202	0.846
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A11 (cont'd). Generalized Linear Mixed Model Visiting Food Cup in Solitary Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.994	0.349	2.848	**0.004**	0.499	1.978
Residual[c]						
AR1 Diagonal	12.202	2.231	5.47	**<0.001**	8.527	17.46
AR1 Rho	0.017	0.184	0.093	0.926	-0.331	0.361

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A12. Generalized Linear Mixed Model of Pecking at Food Behavior in Solitary Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	3.142	0.406	7.744	**<0.001**	2.340	3.944
Treatment						
Control	-1.463	0.656	-2.229	**0.027**	-2.761	-0.165
Poly	0[a]					
Cohort						
C1	-0.886	0.455	-1.992	**0.048**	-1.766	-0.007
C2	-0.123	0.506	-0.244	0.808	-1.124	0.878
C3	0[a]					
Sex						
Females	-0.033	0.366	-0.091	0.928	-0.758	0.691
Males	0[a]					
Treatment*Cohort						
*Control*C1*	1.991	0.735	2.708	**0.008**	0.537	3.445
*Control*C2*	1.233	0.787	1.566	0.120	-0.324	2.790
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.350	0.580	0.603	0.548	-0.798	1.497
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	-0.270	0.217	-1.245	0.215	-0.698	0.159
2	-0.028	0.273	-0.102	0.919	-0.569	0.513
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A12 (cont'd). Generalized Linear Mixed Model of Pecking at Food Behavior in Solitary Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.911	0.321	2.836	**0.005**	0.457	1.819
Residual[c]						
AR1 Diagonal	17.583	2.923	6.015	**<0.001**	12.693	24.356
AR1 Rho	-0.105	0.182	-0.575	0.565	-0.435	0.25

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A13. Generalized Linear Mixed Model of Hopping on One Perch in Exploration Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	2.478	0.332	7.464	**<0.001**	1.822	3.135
Treatment						
Control	0.213	0.647	0.329	0.742	-1.066	1.492
Poly	0[a]					
Cohort						
C1	1.722	0.328	5.241	**<0.001**	1.072	2.371
C2	1.388	0.434	3.198	**0.002**	0.529	2.246
C3	0[a]					
Sex						
Females	0.407	0.276	1.471	0.144	-0.140	0.953
Males	0[a]					
Treatment*Cohort						
*Control*C1*	-0.196	0.677	-0.289	0.773	-1.536	1.143
*Control*C2*	-0.154	0.766	-0.201	0.841	-1.668	1.360
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.073	0.451	0.162	0.871	-0.819	0.966
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.236	0.193	1.226	0.222	-0.145	0.618
2	0.283	0.188	1.505	0.135	-0.089	0.656
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A13 (cont'd). Generalized Linear Mixed Model of Hopping on One Perch in Exploration Condition

Random and Residual Effects

	Estimate	Std. error	z-Test	Sig.	95% CI Lower	95% CI Upper
Random[b]						
Var (intercept)	0.52	0.246	2.116	**0.034**	0.206	1.313
Residual[c]						
AR1 Diagonal	42.925	11.522	3.725	**<0.001**	25.364	72.644
AR1 Rho	0.244	0.218	1.119	0.263	-0.203	0.607

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A14. Generalized Linear Mixed Model of Hopping between Two Perches in Exploration Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	1.738	0.468	3.712	**<0.001**	0.812	2.663
Treatment						
Control	-0.443	0.797	-0.556	0.579	-2.019	1.134
Poly	0[a]					
Cohort						
C1	2.696	0.369	7.302	**<0.001**	1.966	3.426
C2	1.885	0.567	3.327	**0.001**	0.765	3.006
C3	0[a]					
Sex						
Females	0.329	0.489	0.672	0.503	-0.639	1.297
Males	0[a]					
Treatment*Cohort						
*Control*C1*	0.228	0.753	0.303	0.763	-1.261	1.717
*Control*C2*	0.167	0.930	0.180	0.857	-1.672	2.007
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.106	0.647	0.164	0.870	-1.173	1.385
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.237	0.247	0.959	0.339	-0.251	0.725
2	0.478	0.263	1.816	0.072	-0.043	0.999
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A14 (cont'd). Generalized Linear Mixed Model of Hopping between Two Perches in Exploration Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.827	0.301	2.744	**0.006**	0.405	1.689
Residual[c]						
AR1 Diagonal	50.759	8.602	5.901	**<0.001**	36.414	70.756
AR1 Rho	-0.019	0.18	0.106	0.915	-0.356	0.322

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A15. Generalized Linear Mixed Model of Visiting Food Cup Behavior in Exploration Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	-0.927	0.640	-1.449	0.150	-2.192	0.338
Treatment						
Control	-0.275	0.978	-0.281	0.779	-2.209	1.659
Poly	0[a]					
Cohort						
C1	2.558	0.605	4.229	**<0.001**	1.362	3.754
C2	1.892	0.664	2.851	**0.005**	0.580	3.205
C3	0[a]					
Sex						
Females	-0.278	0.362	-0.768	0.444	-0.994	0.438
Males	0[a]					
Treatment*Cohort						
*Control*C1*	-0.180	1.069	-168	0.867	-2.294	1.935
*Control*C2*	-0.880	1.089	-0.808	0.421	-3.034	1.274
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.704	0.624	1.128	0.261	-0.530	1.937
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	1.174	0.349	3.359	**0.001**	0.483	1.865
2	1.197	0.351	3.415	**0.001**	0.504	1.891
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A15 (cont'd). Generalized Linear Mixed Model of Visiting Food Cup Behavior in Exploration Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.893	0.394	2.265	**0.024**	0.376	2.122
Residual[c]						
AR1 Diagonal	5.995	1.056	5.677	**<0.001**	4.245	8.467
AR1 Rho	-0.034	0.181	-0.187	0.852	-0.37	0.31

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A16. Generalized Linear Mixed Model of Pecking at Food Behavior in Exploration Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	1.178	0.734	1.605	0.111	-0.274	2.630
Treatment						
Control	-1.700	1.035	-1.642	0.103	-3.747	0.347
Poly	0[a]					
Cohort						
C1	0.752	0.772	0.974	0.332	-0.775	2.279
C2	0.554	0.871	0.636	0.526	-1.168	2.276
C3	0[a]					
Sex						
Females	0.264	0.613	0.431	0.667	-0.949	1.477
Males	0[a]					
Treatment*Cohort						
*Control*C1*	1.135	1.098	1.034	0.303	-1.037	3.308
*Control*C2*	1.415	1.150	1.230	0.221	-0.859	3.689
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.403	0.853	0.473	0.637	-1.283	2.090
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.466	0.310	1.507	0.134	-0.146	1.079
2	0.720	0.313	2.300	**0.023**	0.101	1.338
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A16 (cont'd). Generalized Linear Mixed Model of Pecking at Food Behavior in Exploration Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	1.615	0.566	2.852	**0.004**	0.812	3.211
Residual[c]						
AR1 Diagonal	14.394	2.385	6.035	**<0.001**	10.403	19.916
AR1 Rho	-0.1	0.177	-0.564	0.573	-0.422	0.245

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A17. Generalized Linear Mixed Model of Hopping on One Perch Behavior in High-Density Side of Flight Cage in Sociality Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	3.589	0.267	13.465	**<0.001**	3.062	4.116
Treatment						
Control	-0.299	0.352	-0.849	0.397	-0.995	0.397
Poly	0[a]					
Cohort						
C1	0.321	0.243	1.321	0.189	-0.159	0.801
C2	-0.427	0.332	-1.287	0.200	-1.084	0.229
C3	0[a]					
Sex						
Females	-0.090	0.221	-0.409	0.683	-0.527	0.346
Males	0[a]					
Treatment*Cohort						
*Control*C1*	0.102	0.334	0.305	0.761	-0.559	0.763
*Control*C2*	0.405	0.414	0.979	0.329	-0.413	1.223
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.400	0.302	1.323	0.188	-0.198	0.998
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.184	0.189	0.971	0.333	-0.190	0.557
2	0.039	0.129	-0.305	0.761	-0.295	0.216
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A18. Generalized Linear Mixed Model of Hopping between Two Perches Behavior in High-Density Side of Flight Cage in Sociality Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	19.465	6.390	3.046	**0.003**	6.829	32.101
Treatment						
Control	-3.099	10.277	-0.302	0.763	-23.420	17.222
Poly	0[a]					
Cohort						
C1	13.797	9.011	1.531	0.128	-4.023	31.616
C2	-6.429	4.993	-1.288	0.200	-16.301	3.444
C3	0[a]					
Sex						
Females	-3.740	7.262	-0.515	0.607	-18.099	10.620
Males	0[a]					
Treatment*Cohort						
*Control*C1*	6.122	13.871	0.441	0.660	-21.306	33.551
*Control*C2*	4.416	8.359	0.528	0.598	-12.133	20.944
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	4.457	10.490	0.425	0.672	-16.287	25.200
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	3.184	4.824	0.660	0.510	-6.355	12.722
2	-4.776	2.615	-1.826	0.070	-9.946	-0.395
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A18 (cont'd). Generalized Linear Mixed Model of Hopping between Two Perches Behavior in High-Density Side of Flight Cage in Sociality Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	75.675	145.889	0.510	0.604	1.729	3,311.592
Residual[c]						
AR1 Diagonal	554.268	160.497	3.454	**0.001**	314.241	977.699
AR1 Rho	0.326	0.210	1.549	0.121	-0.122	0.663

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A19. Generalized Linear Mixed Model of Visiting Food Cup Behavior in High-Density Side of Flight Cage in Sociality Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	-0.972	0.588	-1.653	0.101	-2.135	0.191
Treatment						
Control	-0.121	0.860	-0.141	0.888	-1.821	1.579
Poly	0[a]					
Cohort						
C1	-0.294	0.699	-0.420	0.675	-1.675	1.088
C2	-0.927	0.665	-1.393	0.166	-2.242	0.389
C3	0[a]					
Sex						
Females	0.768	0.632	1.215	0.226	-0.482	2.017
Males	0[a]					
Treatment*Cohort						
*Control*C1*	-0.769	1.325	-0.580	0.563	-3.389	1.851
*Control*C2*	0.175	0.924	0.190	0.850	-1.651	2.002
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	-0.461	0.968	-0.476	0.635	-2.376	1.454
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.603	0.442	1.364	0.175	-0.271	1.477
2	-0.477	0.497	-0.959	0.339	-1.460	0.506
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A19 (cont'd). Generalized Linear Mixed Model of Visiting Food Cup Behavior in High-Density Side of Flight Cage in Sociality Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	1.921	0.642	2.995	**0.003**	0.999	3.697
Residual[c]						
AR1 Diagonal	1.045	0.158	6.61	**<0.001**	0.777	1.406
AR1 Rho	-0.034	0.177	-0.192	0.848	-0.364	0.303

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A20. Generalized Linear Mixed Model of Pecking at Food Behavior in High-Density Side of Flight Cage in Sociality Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	-0.034	0.876	-0.039	0.969	-1.767	1.699
Treatment						
Control	-0.398	1.113	-0.357	0.722	-2.599	1.804
Poly	0[a]					
Cohort						
C1	-0.388	0.915	-0.424	0.672	-2.196	1.421
C2	-3.012	0.807	-3.735	**<0.001**	-4.607	-1.417
C3	0[a]					
Sex						
Females	1.816	0.780	2.330	**0.021**	0.275	3.358
Males	0[a]					
Treatment*Cohort						
*Control*C1*	-0.676	1.271	-0.532	0.596	-3.190	1.838
*Control*C2*	2.465	1.074	2.294	**0.023**	0.341	4.589
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	-0.605	1.075	-0.563	0.574	-2.731	1.520
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.381	0.312	1.218	0.225	-0.237	0.999
2	0.148	0.337	0.440	0.660	-0.518	0.814
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A20 (cont'd). Generalized Linear Mixed Model of Pecking at Food Behavior in High-Density Side of Flight Cage in Sociality Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	1.894	0.563	3.364	**0.001**	1.057	3.391
Residual[c]						
AR1 Diagonal	0.214	0.052	4.116	**<0.001**	0.133	0.344
AR1 Rho	0.04	0.241	0.165	0.869	-0.408	0.472

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A21. Generalized Linear Mixed Model of Hopping on One Perch in Low-Density Side of Flight Cage in Sociality Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	3.322	0.244	13.632	**<0.001**	2.840	3.804
Treatment						
Control	-0.340	0.367	-0.927	0.356	-1.066	0.386
Poly	0[a]					
Cohort						
C1	0.048	0.218	0.220	0.826	-0.383	0.478
C2	-0.430	0.288	-1.494	0.137	-0.999	0.139
C3	0[a]					
Sex						
Females	0.064	0.235	0.272	0.786	-0.400	0.528
Males	0[a]					
Treatment*Cohort						
*Control*C1*	0.396	0.381	1.039	0.300	-0.358	1.151
*Control*C2*	0.166	0.415	0.399	0.690	-0.655	0.986
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.301	0.411	0.733	0.465	-0.512	1.114
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	-0.157	0.230	-0.681	0.497	-0.612	0.298
2	-0.013	0.179	-0.074	0.941	-0.366	0.340
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A21 (cont'd). Generalized Linear Mixed Model of Hopping on One Perch in Low-Density Side of Flight Cage in Sociality Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.128	0.134	0.959	0.338	0.017	0.992
Residual[c]						
AR1 Diagonal	22.329	4.164	5.363	**<0.001**	15.494	32.181
AR1 Rho	0.205	0.157	1.309	0.191	-0.112	0.484

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A22. Generalized Linear Mixed Model of Hopping between Two Perches in Low-Density Side of Flight Cage in Sociality Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	2.409	0.288	8.353	**<0.001**	1.839	2.979
Treatment						
Control	-0.388	0.502	-0.773	0.441	-1.381	0.605
Poly	0[a]					
Cohort						
C1	0.321	0.317	1.013	0.313	-0.306	0.947
C2	-0.364	0.370	-0.983	0.327	-1.095	0.368
C3	0[a]					
Sex						
Females	0.305	0.312	0.978	0.330	-0.312	0.922
Males	0[a]					
Treatment*Cohort						
*Control*C1*	0.588	0.542	1.084	0.280	-0.485	1.660
*Control*C2*	0.198	0.584	0.339	0.735	-0.957	1.353
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	0.007	0.543	0.013	0.990	-1.067	1.081
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	-0.210	0.272	-0.772	0.461	-0.749	0.328
2	0.083	0.188	0.442	0.659	-0.289	0.455
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A22 (cont'd). Generalized Linear Mixed Model of Hopping between Two Perches in Low-Density Side of Flight Cage in Sociality Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.463	0.224	2.072	**0.038**	0.180	1.194
Residual[c]						
AR1 Diagonal	13.527	2.664	5.078	**<0.001**	9.196	19.898
AR1 Rho	0.177	0.171	1.032	0.302	-0.166	0.481

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A23. Generalized Linear Mixed Model of Visiting Food Cup Behavior in Low-Density Side of Flight Cage in Sociality Condition

Model of Visiting Food Cup Behavior in Low-Density Side of Flight Cage in Sociality Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	-2.019	1.062	-1.901	0.059	-4.120	0.082
Treatment						
Control	-16.120	1.158	-13.921	**<0.001**	-18.409	-13.830
Poly	0[a]					
Cohort						
C1	-0.910	1.019	-0.893	0.373	-2.926	1.105
C2	-1.104	0.947	-1.165	0.246	-2.978	0.770
C3	0[a]					
Sex						
Females	2.220	0.903	2.458	**0.015**	0.434	4.007
Males	0[a]					
Treatment*Cohort						
*Control*C1*	0.104	1.386	0.075	0.941	-2.637	2.844
*Control*C2*	0.891	1.473	0.604	0.547	-2.023	3.804
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	14.726	1.017	14.476	**<0.001**	12.715	16.738
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	-0.205	0.387	-0.529	0.598	-0.971	0.561
2	-0.280	0.335	-0.835	0.405	-0.944	0.383
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A23 (cont'd). Generalized Linear Mixed Model of Visiting Food Cup Behavior in Low-Density Side of Flight Cage in Sociality Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	2.779	1.059	2.624	**<0.001**	1.317	5.866
Residual[c]						
AR1 Diagonal	0.511	0.076	6.729	**<0.001**	0.382	0.684
AR1 Rho	-0.072	0.173	-0.414	0.679	-0.391	0.263

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A24. Generalized Linear Mixed Model of Pecking at Food Behavior in Low-Density Side of Flight Cage in Sociality Condition

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	-0.933	1.032	-0.904	0.367	-2.973	1.107
Treatment						
Control	-16.400	1.052	-15.586	**<0.001**	-18.480	-14.319
Poly	0[a]					
Cohort						
C1	-0.272	1.071	-0.253	0.800	-2.390	1.847
C2	-0.622	1.082	-0.575	0.566	-2.761	1.517
C3	0[a]					
Sex						
Females	2.396	0.902	2.655	**0.009**	0.611	4.180
Males	0[a]					
Treatment*Cohort						
*Control*C1*	-2.255	1.686	-1.337	0.183	-5.589	1.080
*Control*C2*	-0.774	1.654	-0.468	0.641	-4.044	2.497
*Control*C3*	0[a]					
*Poly*C1*	0[a]					
*Poly*C2*	0[a]					
*Poly*C3*	0[a]					
Treatment *Sex						
*Control*Females*	15.297	1.123	13.620	**<0.001**	13.076	17.518
*Control*Males*	0[a]					
*Poly*Females*	0[a]					
*Poly*Males*	0[a]					
Trial Number						
1	0.029	0.295	0.097	0.923	-0.555	0.613
2	-0.352	0.485	-0.726	0.469	-1.310	0.607
3	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A24 (cont'd). Generalized Linear Mixed Model of Pecking at Food Behavior in Low-Density Side of Flight Cage in Sociality Condition

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	3.690	1.259	2.931	**0.003**	1.891	7.201
Residual[c]						
AR1 Diagonal	2.871	0.392	7.324	**<0.001**	2.197	3.751
AR1 Rho	-0.349	0.136	-2.564	**0.01**	-0.583	-0.060

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A25. Generalized Linear Mixed Model of Hopping on One Perch Behavior of Males in 3 minutes Female Sampling Period

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	2.612	0.370	7.056	**<0.001**	1.807	3.418
Male Treatment						
Control	-0.375	0.495	-0.757	0.462	-1.436	0.687
Poly	0[a]					
Female Treatment						
Control	0.142	0.179	0.792	0.437	-0.231	0.515
Poly	0[a]					
Male * Female						
*Control*Control*	0.259	0.221	1.170	0.265	-0.223	0.740
*Control*Poly*	0[a]					
*Poly*Control*	0[a]					
*Poly*Poly*	0[a]					
Trial						
1	-1.069	0.424	-2.520	**0.037**	-2.054	-0.085
2	-0.377	0.234	-1.608	0.580	-631.198	630.445
3	-0.386	0.265	-1.458	0.307	-1.807	1.034
4	0.045	0.183	0.243	0.816	-0.398	0.487
5	0.000	0.203	-0.001	0.999	-5.583	5.582
6	0[a]					

[a] Set to 0 because this parameter is redundant.

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	1.070	0.427	2.504	**0.012**	0.489	2.340
Residual[c]						
AR1 Diagonal	8.092	1.202	6.731	**<0.001**	6.048	10.827
AR1 Rho	-0.239	0.127	-1.885	0.059	-0.468	0.020

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A26. Generalized Linear Mixed Model of Hopping Between Two Perches of Males in 3-minutes Female Sampling Period

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	2.896	0.487	5.942	**<0.001**	1.862	3.929
Male Treatment						
Control	-0.123	0.573	-0.215	0.833	-1.375	1.128
Poly	0[a]					
Female Treatment						
Control	0.152	0.195	0.781	0.443	-0.251	0.555
Poly	0[a]					
Male * Female						
*Control*Control*	0.277	0.273	1.104	0.323	-0.294	0.848
*Control*Poly*	0[a]					
*Poly*Control*	0[a]					
*Poly*Poly*	0[a]					
Trial						
1	-1.582	0.456	-3.472	**0.006**	-2.590	-0.574
2	-0.740	0.236	-3.143	**0.016**	-1.297	-0.184
3	-0.596	0.332	-1.794	0.277	-3.066	1.875
4	-0.262	0.219	-1.195	0.349	-1.158	0.633
5	-0.337	0.180	-1.869	0.072	-0.706	0.032
6	0[a]					

[a] Set to 0 because this parameter is redundant.

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	1.459	0.655	2.227	**0.026**	0.605	3.517
Residual[c]						
AR1 Diagonal	12.192	2.087	5.841	**<0.001**	8.717	17.053
AR1 Rho	0.083	0.152	0.546	0.585	-0.214	0.366

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A27. Generalized Linear Mixed Model of Hopping on One Perch of Males in 12 minutes

Female Choice Period

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	3.336	0.379	8.790	**<0.001**	2.429	4.242
Male Treatment						
Control	0.163	0.503	0.325	0.753	-0.990	1.317
Poly	0[a]					
Female Treatment						
Control	-0.319	0.357	0.893	0.468	-1.253	1.891
Poly	0[a]					
Male * Female						
*Control*Control*	-0.506	0.391	-1.294	0.332	-2.293	0.567
*Control*Poly*	0[a]					
*Poly*Control*	0[a]					
*Poly*Poly*	0[a]					
Present						
Absent	0.003	0.221	0.012	0.996	-30,696	30,696
Present	0[a]					
Treatment*Present						
*Control * Absent*	-0.317	0.273	-1.160	0.691	-42,447	42,447
*Control * Present*	0[a]					
*Poly * Absent*	0[a]					
*Poly * Present*	0[a]					
Trial						
1	0.048	0.194	0.246	0.815	-0.434	0.529
2	0.013	0.176	0.074	0.951	-1.244	1.269
3	0.292	0.155	1.883	0.286	-1.172	1.756
4	0.261	0.157	1.659	0.360	-2.183	2.704
5	0.293	0.161	1.820	0.134	-0.132	0.718
6	0[a]					

[a] Set to 0 because this parameter is redundant.

Appendix A: Statistical Test Tables

Table A27 (cont'd). Generalized Linear Mixed Model of Hopping on One Perch of Males in 12 minutes Female Choice Period

Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	1.119	0.470	2.378	**0.017**	0.491	2.550
Residual[c]						
AR1 Diagonal	18.008	2.722	6.617	**<0.001**	13.391	24.217
AR1 Rho	-0.130	0.139	-0.931	0.352	-0.387	0.146

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A28. Generalized Linear Mixed Model of Hopping between Two Perches of Males in 12 minutes Female Choice Period

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	3.160	0.545	5.802	**<0.001**	2.009	4.311
Male Treatment						
Control	0.005	0.635	0.009	0.993	-1.397	1.408
Poly	0[a]					
Female Treatment						
Control	0.328	0.252	1.299	0.218	-0.222	0.877
Poly	0[a]					
Male * Female						
*Control*Control*	0.477	0.297	-1.605	0.147	-1.162	0.208
*Control*Poly*	0[a]					
*Poly*Control*	0[a]					
*Poly*Poly*	0[a]					
Present						
Absent	0.631	0.184	3.421	**0.047**	0.016	1.246
Present	0[a]					
Treatment*Present						
*Control * Absent*	-0.005	0.224	-0.022	0.983	-0.500	0.490
*Control * Present*	0[a]					
*Poly * Absent*	0[a]					
*Poly * Present*	0[a]					
Trial						
1	-0.539	0.208	-2.588	0.171	-1.900	0.821
2	-0.093	0.277	-0.336	0.834	-84.861	84.675
3	-0.252	0.251	-1.005	0.705	-7.764	7.764
4	-0.063	0.260	-0.244	0.870	-30.016	29.889
5	0.280	0.172	1.629	0.115	-0.073	0.633
6	0[a]					

[a] Set to 0 because this parameter is redundant.

Table A28 (cont'd). Generalized Linear Mixed Model of Hopping between Two Perches of Males in 12-min Female Choice Period Random and Residual Effects

					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	1.913	0.798	2.397	**0.017**	0.844	4.335
Residual[c]						
AR1 Diagonal	26.459	4.269	6.199	**<0.001**	19.287	36.299
AR1 Rho	0.015	0.137	0.109	0.913	-0.248	0.276

[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A29. Generalized Linear Mixed Model of Visits to the Front Perch Behavior of Female Birds

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	1.423	0.484	2.939	**0.022**	0.275	2.571
Female Treatment						
Control	-0.581	0.430	-1.352	0.207	-1.540	0.379
Poly	0[a]					
Male Treatment						
Control	-0.647	0.314	-2.059	0.075	-1.378	0.084
Poly	0[a]					
Female Treatment * Male Treatment						
*Control * Control*	1.401	0.362	3.868	**0.001**	0.643	2.159
*Control * Poly*	0[a]					
*Poly * Control*	0[a]					
*Poly * Poly*	0[a]					
Trial						
1	0.247	0.613	0.403	0.722	-2.138	2.632
2	0.287	0.489	0.587	0.592	-1.133	1.708
3	-0.387	0.486	-0.797	0.479	-1.865	1.090
4	0.017	0.458	0.037	0.971	-0.942	0.976
5	-0.111	0.252	-0.438	0.670	-0.670	0.449
6	0[a]					

Random and Residual Effects					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.622	0.420	1.483	0.138	0.166	2.333
Residual[c]						
AR1 Diagonal	3.441	1.073	3.207	**0.001**	1.867	6.339
AR1 Rho	0.554	0.155	3.565	**<0.001**	0.183	0.787

[a] Set to 0 because this parameter is redundant.
[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A30. Generalized Linear Mixed Model of Hops on the Front Perch Behavior of Female Birds

Model Term					95% CI	
	Coefficient	Std. error	*t*	Sig.	Lower	Upper
Intercept	3.535	0.465	7.605	**0.001**	2.300	4.770
Female Treatment						
Control	0.573	0.384	1.491	0.185	-0.361	1.507
Poly	0[a]					
Male Treatment						
Control	0.023	0.366	0.062	0.952	-0.806	0.851
Poly	0[a]					
Female Treatment * Male Treatment						
*Control * Control*	-0.033	0.562	-0.059	0.955	-1.514	1.448
*Control * Poly*	0[a]					
*Poly * Control*	0[a]					
*Poly * Poly*	0[a]					
Trial						
1	-0.697	0.585	-1.192	0.412	-5.140	4.061
2	-0.286	0.411	-0.697	0.547	-1.797	1.225
3	-0.664	0.640	-1.037	0.419	-3.743	2.414
4	-0.306	0.553	-0.553	0.620	-2.110	1.499
5	-0.368	0.243	-1.517	0.161	-0.910	0.174
6	0[a]					

Random and Residual Effects					95% CI	
	Estimate	Std. error	z-Test	Sig.	Lower	Upper
Random[b]						
Var (intercept)	0.413	0.276	1.496	0.135	0.111	1.532
Residual[c]						
AR1 Diagonal	28.735	7.286	3.994	**<0.001**	17.481	47.233
AR1 Rho	0.347	0.173	2.007	**0.045**	-0.023	0.633

[a] Set to 0 because this parameter is redundant.
[b] Covariance structure: scaled identity; subject specification: Bird id.
[c] Covariance structure: first-order autoregressive; subject specification: Bird id.

Appendix A: Statistical Test Tables

Table A31. Mann-Whitney U Test for Behaviors in Brain Activity Analysis

	Median	Mann-Whitney U	z	Sig
Hopping on One Perch				
Control	52	51.0	0.116	0.941
Poly	103			
Hopping between Two Perches				
Control	43	56.0	0.51	0.656
Poly	110			
Visiting Food Cup				
Control	3	41.5	-0.628	0.552
Poly	0			
Pecking at Food				
Control	3	44.5	-0.406	0.71
Poly	0			

Table A32. Multivariate analysis of covariance on effects of Poly I:C on Brain Development

Variables	Wilk's Lambda	F	Hypothesis df	Error df	Sig.	Partial Eta Squared
Intercept	0.25	14.016	3	14	**<0.001**	0.75
Telencephalon	0.699	2.007			0.159	0.301
Group	0.562	3.644			**0.039**	0.438

Appendix A: Statistical Test Tables

Table A33. Tests of Between-Subject Effects of Poly I:C on Brain Development

Corrected Model	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
HVC	0.053[a]	2	0.027	3.409	0.058	0.299
RA	0.028[b]	2	0.014	3.126	0.071	0.281
TnA	0.012[c]	2	0.006	3.511	0.054	0.305
Intercept						
HVC	0.012	1	0.012	1.541	0.232	0.088
RA	0.035	1	0.035	7.968	**0.012**	0.332
TnA	0.064	1	0.064	36.025	**<0.001**	0.692
Telencephalon						
HVC	0.035	1	0.035	4.531	0.049	0.221
RA	0.004	1	0.004	0.921	0.351	0.054
TnA	0.003	1	0.003	1.511	0.237	0.086
Group						
HVC	0.017	1	0.017	2.122	0.165	0.117
RA	0.023	1	0.023	5.213	**0.036**	0.246
TnA	0.01	1	0.01	5.359	**0.034**	0.251
Error						
HVC	0.125	16	0.008			
RA	0.071	16	0.004			
TnA	0.028	16	0.002			
Total						
HVC	1.419	19				
RA	1.008	19				
TnA	1.38	19				
Corrected Total						
HVC	0.178	18				
RA	0.098	18				
TnA	0.041	18				

[a] R Squared = .299 (Adjusted R Squared = .211)
[b] R Squared = .281 (Adjusted R Squared = .191).
[c] R Squared = .305 (Adjusted R Squared = .218)

Appendix A: Statistical Test Tables

Table A34. Mann-Whitney U Test for Brain Activity of Control and Poly Birds

	Median	Mann-Whitney U	z	Sig
HVC				
Control	93	19	-0.497	0.699
Poly	65			
RA				
Control	26	48	1.747	0.91
Poly	28.5			
LSt				
Control	179.5	42	1.113	0.299
Poly	193.5			
TnA				
Control	42	50.5	2.014	**0.042**
Poly	53			

Appendix B: Microscopy Pictures

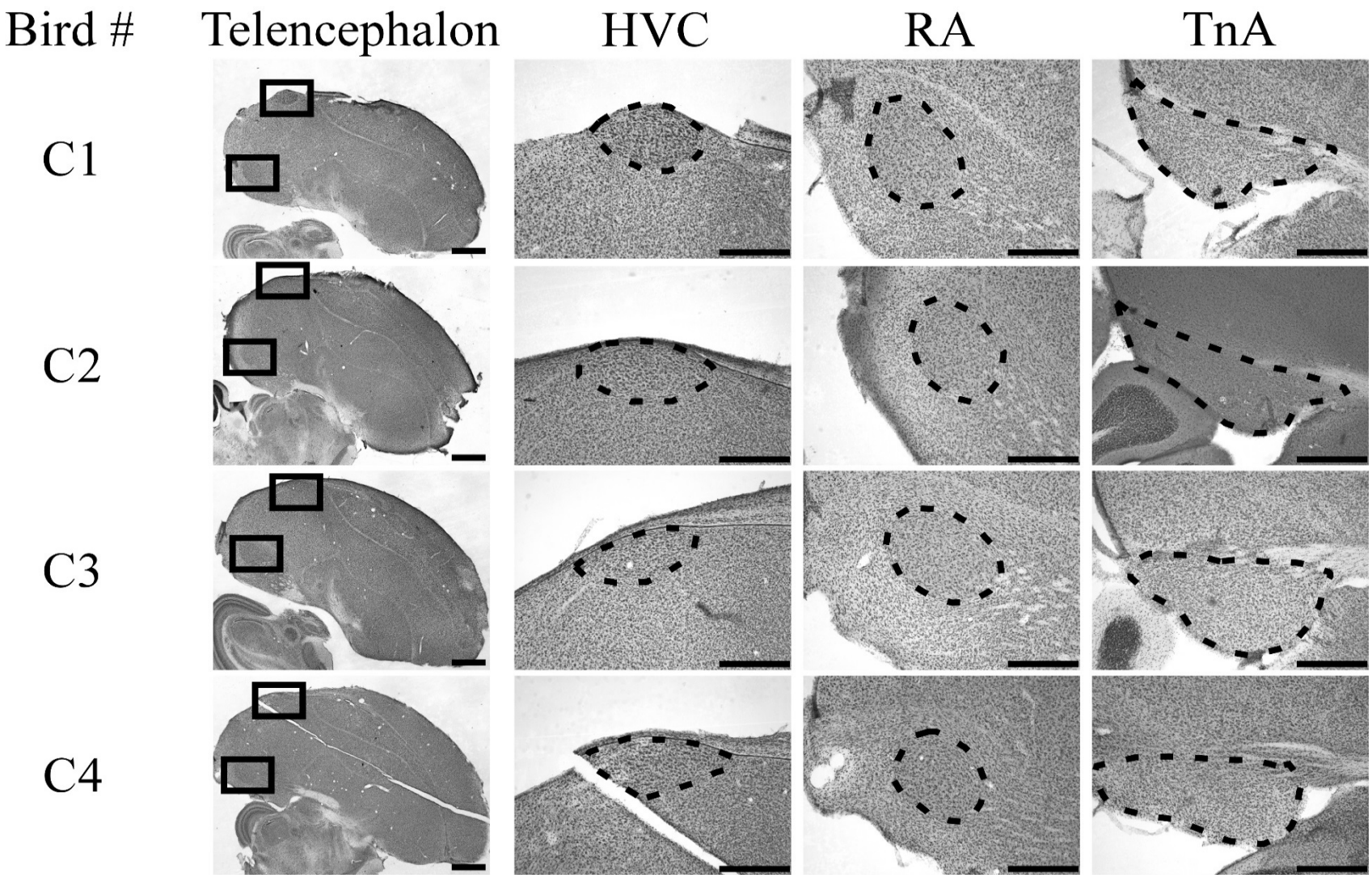

Figure B1. Microphotograph examples of brain sections used in volume analysis. Regions of interests are specified with dotted lines. Scale bars are 1 mm for Telencephalon and 0.5 mm for HVC, RA and TnA. C : Control

Appendix B: Microscopy Pictures

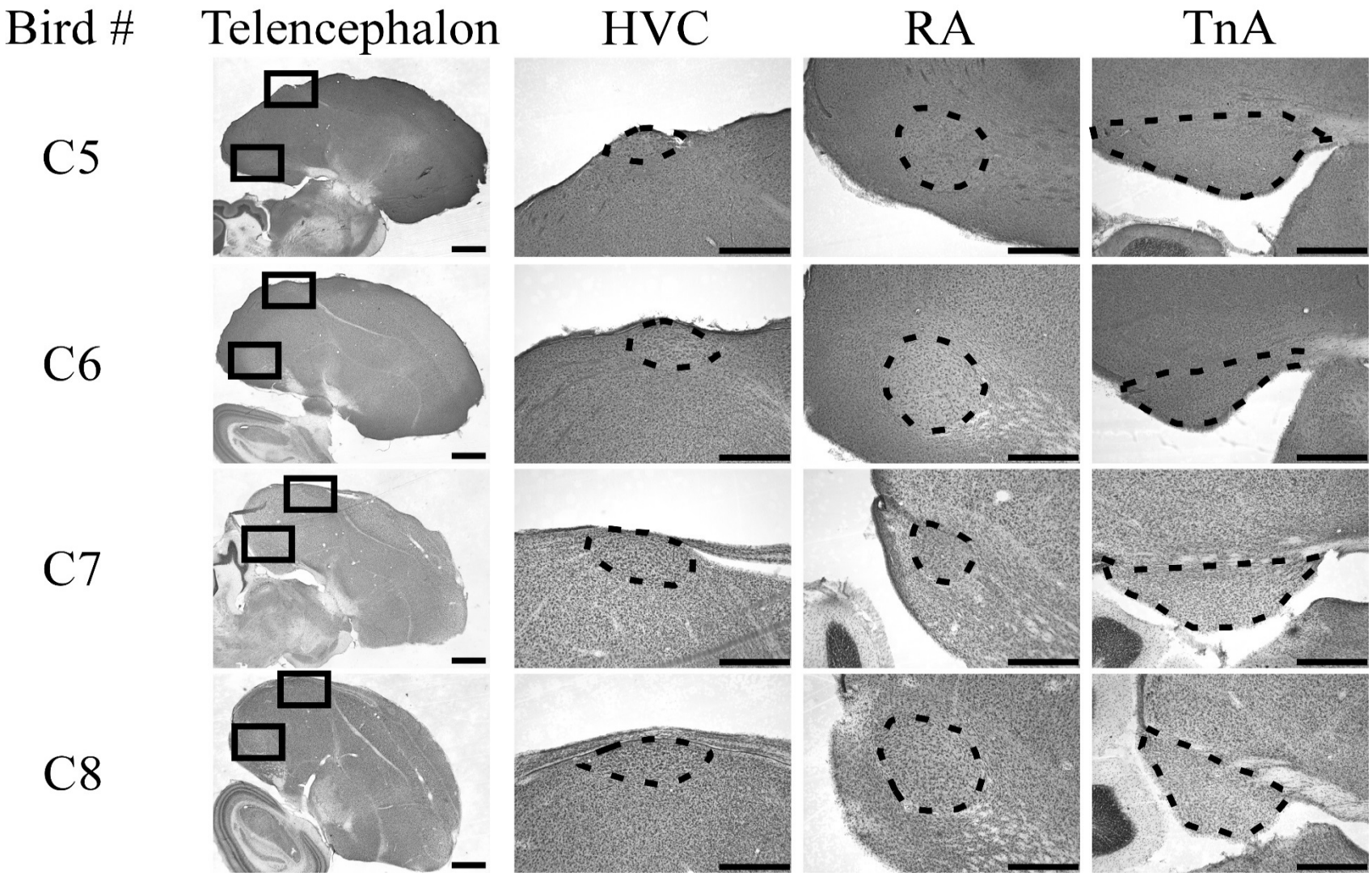

Figure B1 (cont'd). Microphotograph examples of brain sections used in volume analysis. Regions of interests are specified with dotted lines. Scale bars are 1 mm for Telencephalon and 0.5 mm for HVC, RA and TnA. C : Control

Appendix B: Microscopy Pictures

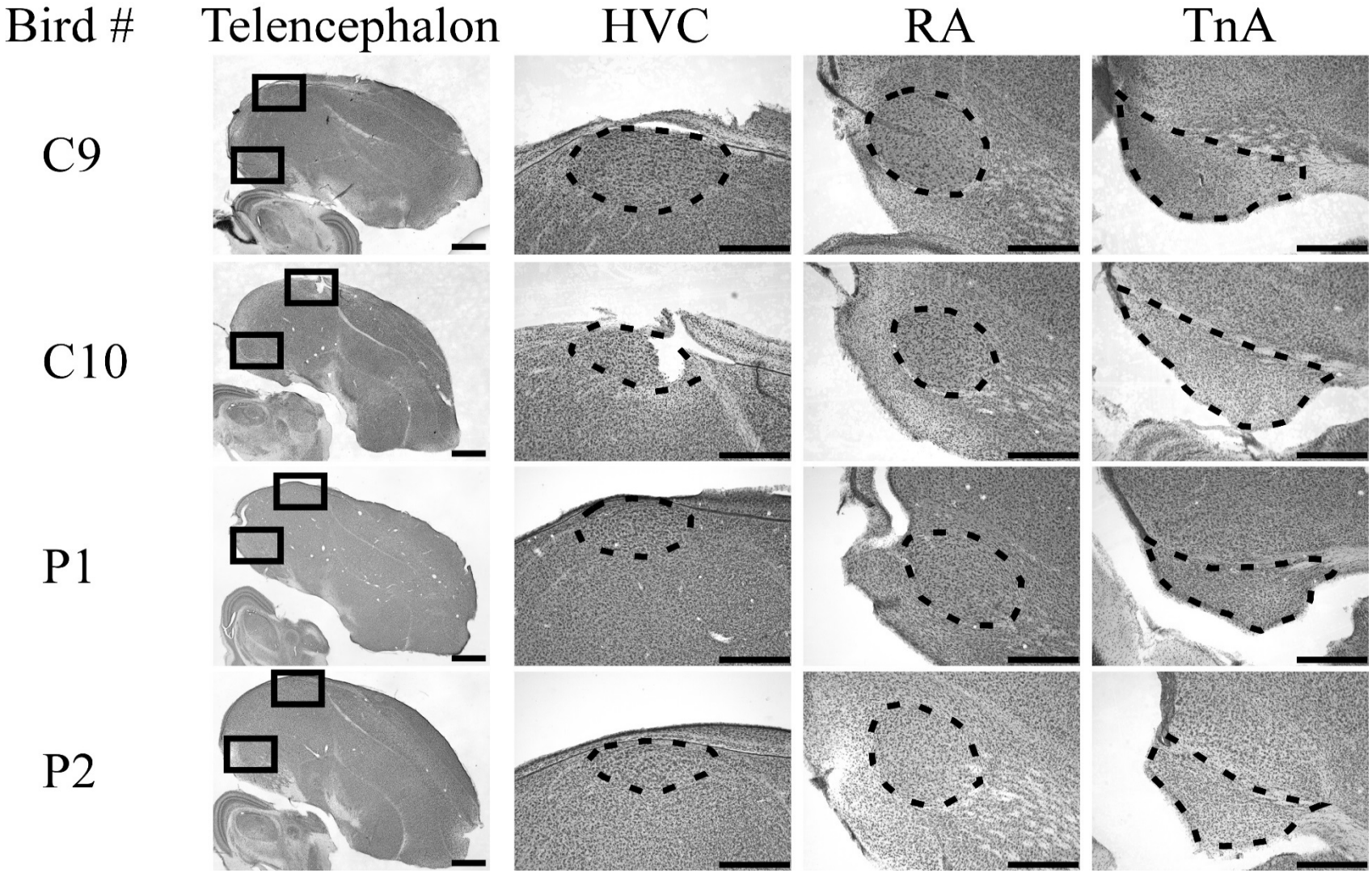

Figure B1 (cont'd). Microphotograph examples of brain sections used in volume analysis. Regions of interests are specified with dotted lines. Scale bars are 1 mm for Telencephalon and 0.5 mm for HVC, RA and TnA. C : Control, P: Poly

Appendix B: Microscopy Pictures

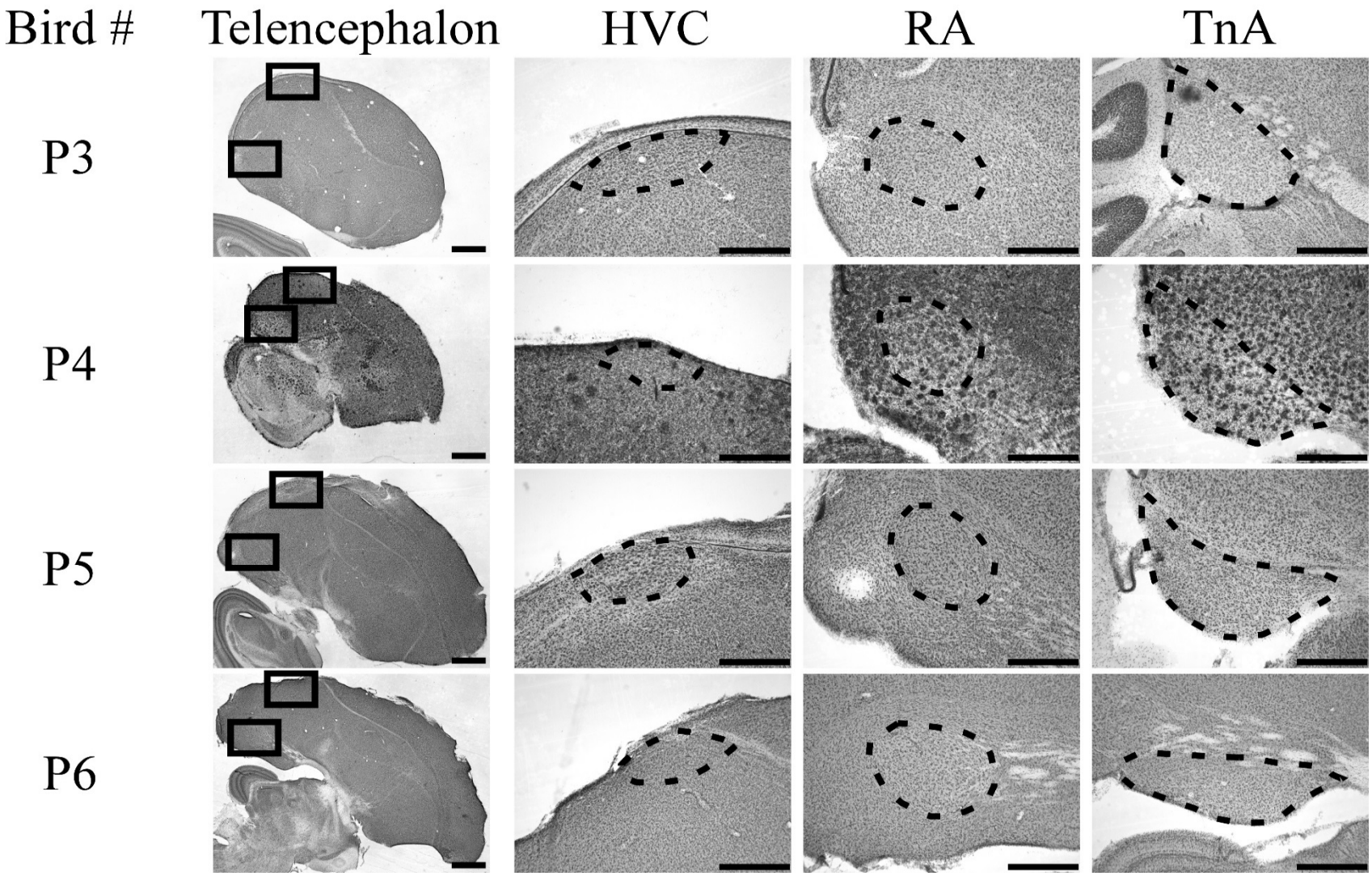

Figure B1 (cont'd). Microphotograph examples of brain sections used in volume analysis. Regions of interests are specified with dotted lines. Scale bars are 1 mm for Telencephalon and 0.5 mm for HVC, RA and TnA. C : Control, P: Poly

Appendix B: Microscopy Pictures

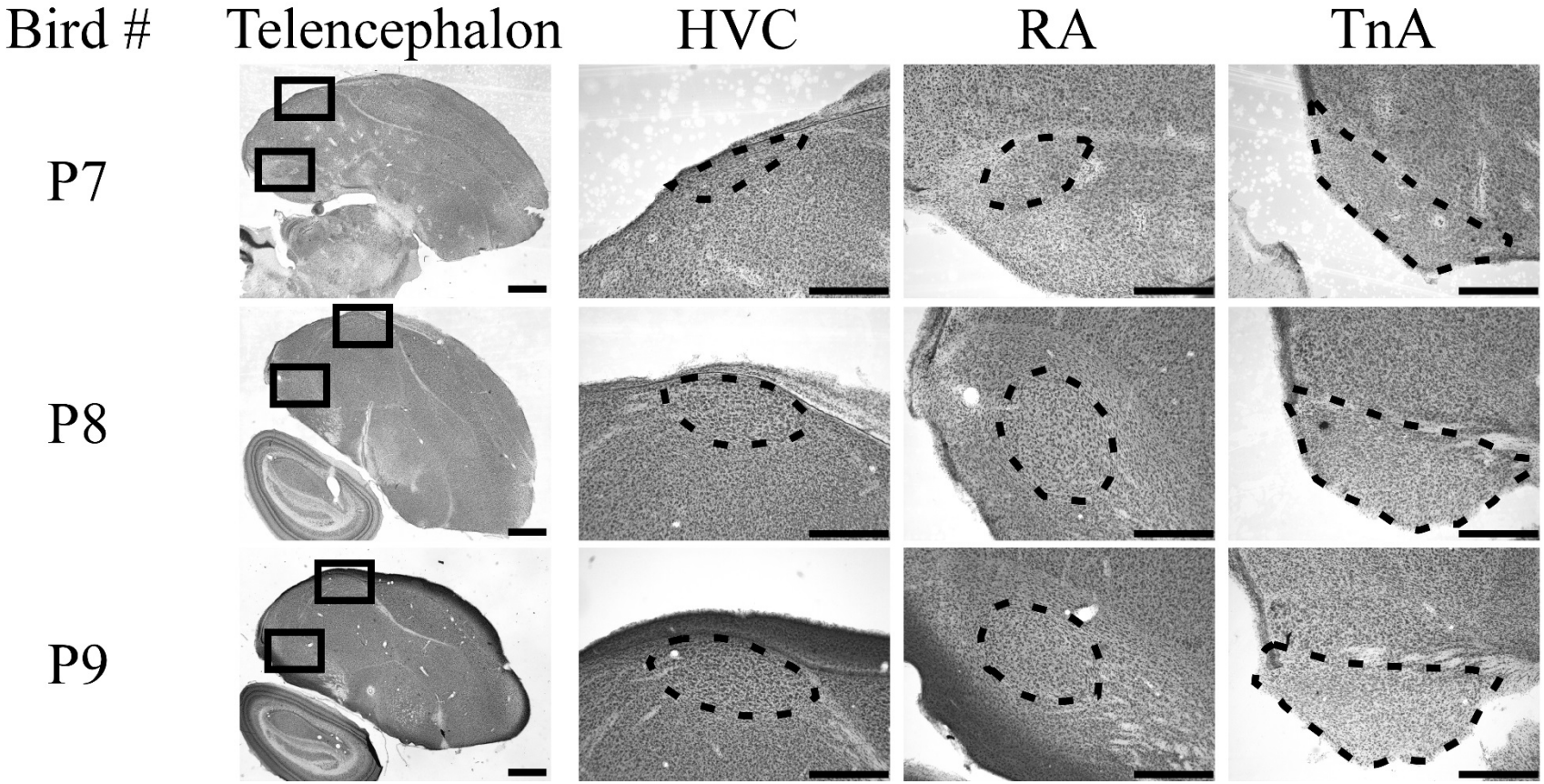

Figure B 1 (cont'd). Microphotograph examples of brain sections used in volume analysis. Regions of interests are specified with dotted lines. Scale bars are 1 mm for Telencephalon and 0.5 mm for HVC, RA and TnA. P: Poly.

Appendix B: Microscopy Pictures

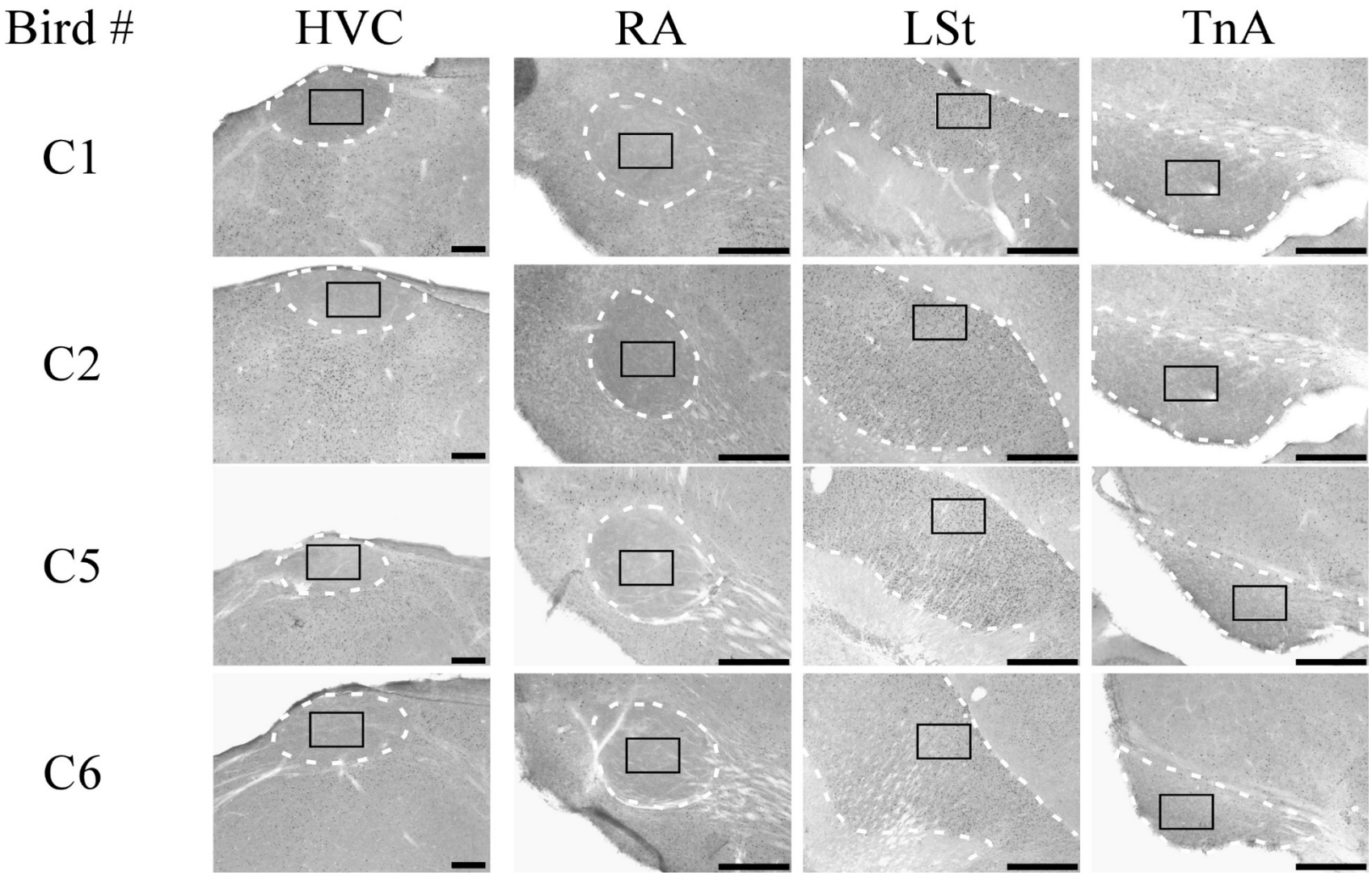

Figure B2. Microphotograph examples of brain sections used in IEG expression analysis. In each region of interest box of 0.2 mm X 0.3 mm is drawn and ZENK expressing cells were counted. Scale bars are 0.5 mm. C: Control

Appendix B: Microscopy Pictures

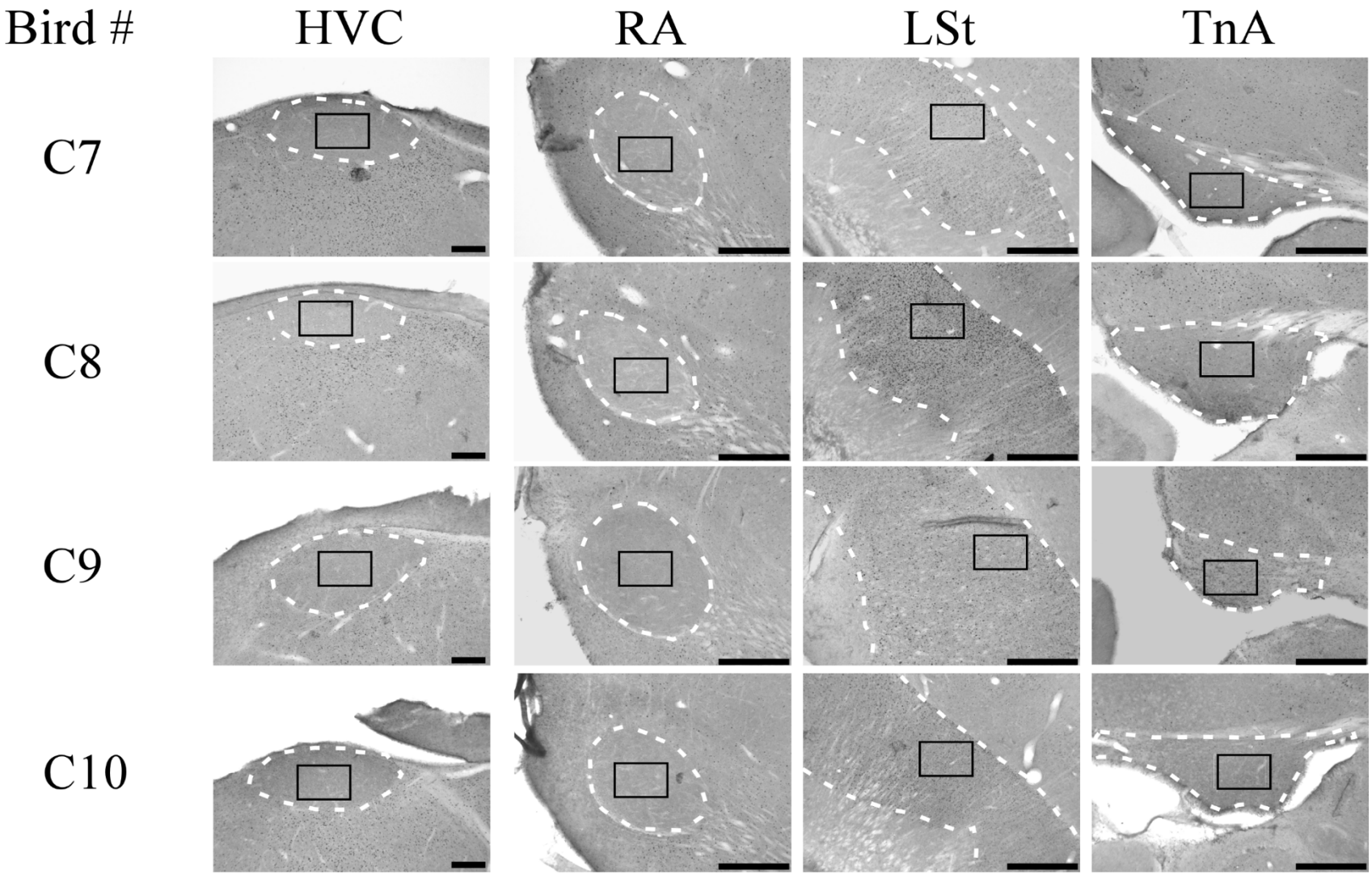

Figure B 2 (cont'd). Microphotograph examples of brain sections used in IEG expression analysis. In each region of interest box of 0.2 mm X 0.3 mm is drawn and ZENK expressing cells were counted. Scale bars are 0.5 mm. C: Control.

Appendix B: Microscopy Pictures

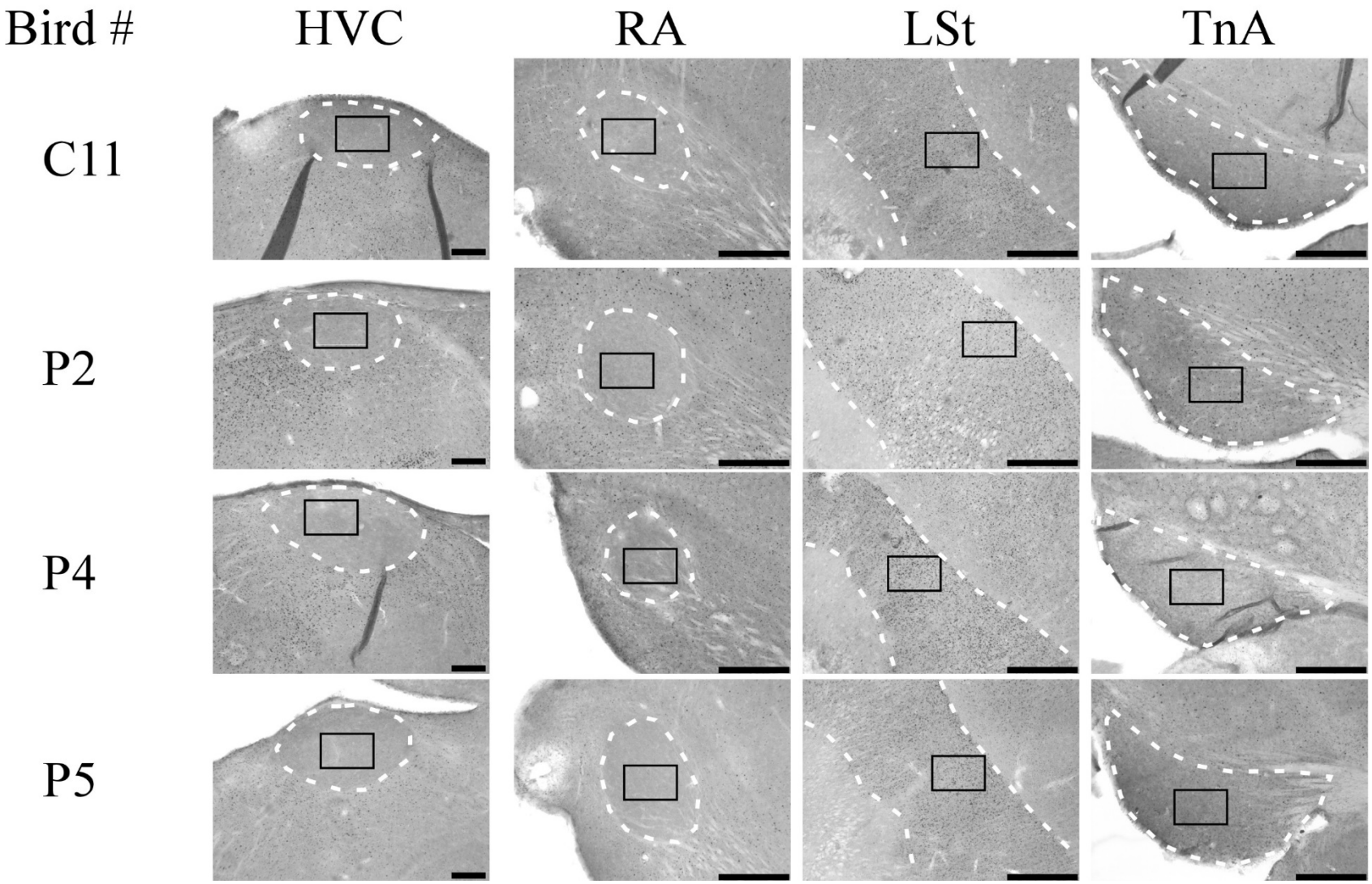

Figure B2 (cont'd). Microphotograph examples of brain sections used in IEG expression analysis. In each region of interest box of 0.2 mm X 0.3 mm is drawn and ZENK expressing cells were counted. Scale bars are 0.5 mm. C: Control, P: Poly.

Appendix B: Microscopy Pictures

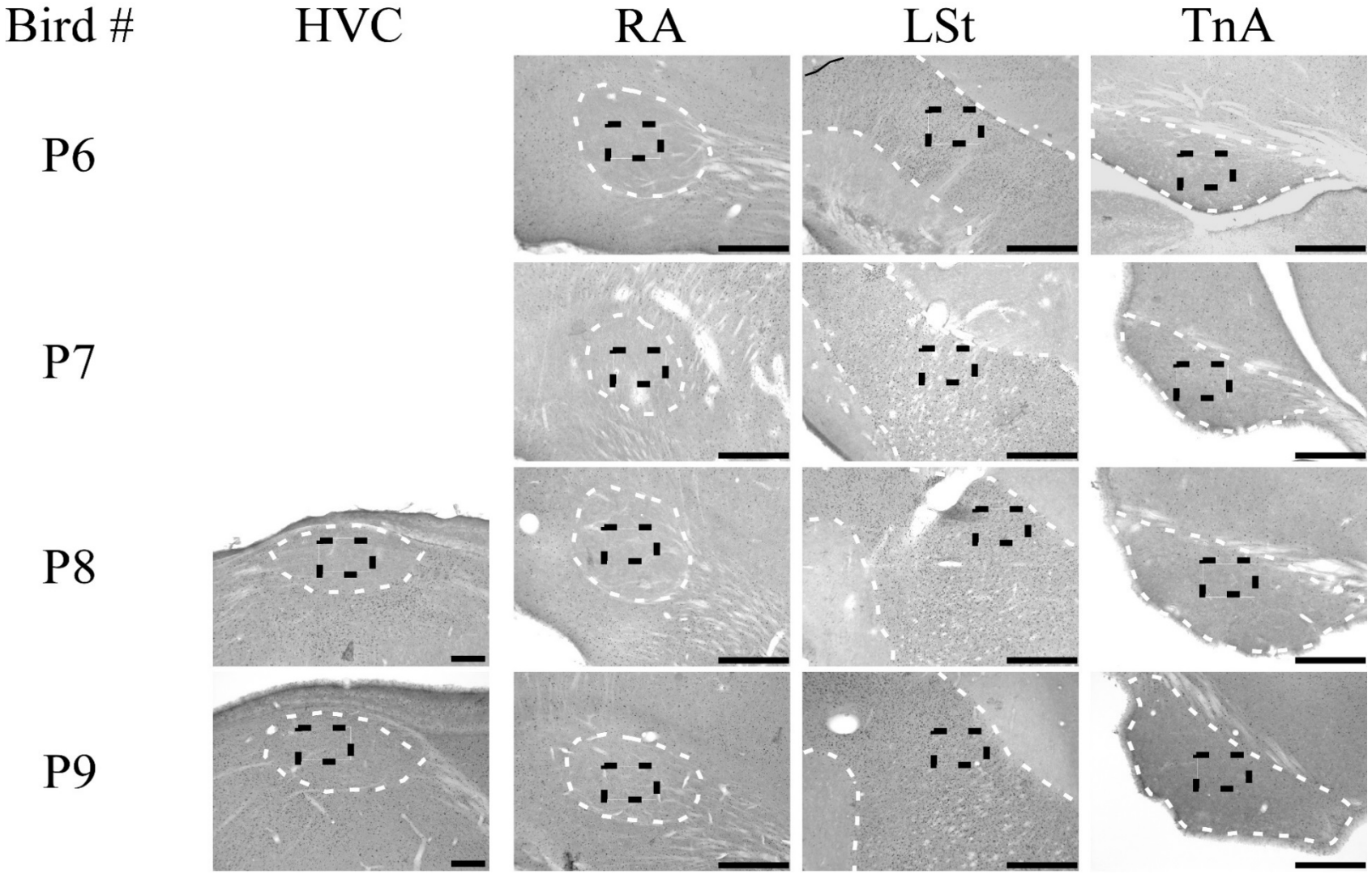

Figure B2 (cont'd). Microphotograph examples of brain sections used in IEG expression analysis. In each region of interest box of 0.2 mm X 0.3 mm is drawn and ZENK expressing cells were counted. Scale bars are 0.5 mm. P: Poly.

Appendix C: Institutional Animal Care and Use Committee for Animal Subjects Approval Letters

USF UNIVERSITY OF SOUTH FLORIDA

ESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO:	Toru Shimizu, Ph.D.
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	8/26/2014
PROJECT TITLE:	Effects of immune challenge during development on adult neuroanatomy, immunocompetence, and behavior
FUNDING SOURCE:	USF department, institute, center, etc.
IACUC PROTOCOL #:	R IS00000721
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC **APPROVED** your request to use the following animals in your **protocol for a one-year period beginning 8/26/2014:**

Finch: zebra finch (juvenile-adult/13-15g/F and M)	80

Please take note of the following:

• **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• **All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol.** Please contact the program coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.

• **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major

Appendix C: Institutional Animal Care and Use Committee for Animal Subjects Approval Letters

protocol change and requires the submission of a new application.

• **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
PHS No. A4100-01, AAALAC No.58-15, USDA No. 58-15
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