NO EVIDENCE FOR PRENATAL AUDITORY STRESS IN THE CAROLINA CHICKADEE (*POECILE CAROLINENSIS)*

A thesis presented to the faculty of the Graduate School of Western Carolina University in partial fulfillment of the requirements for the degree of Master of Science in Biology.

By

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ABSTRACT

Prenatal precocial birds have long been shown to perceive auditory signals, including stressful signals of predation, prior to hatching. Little work has been done to examine prenatal auditory stress in altricial birds. Stress, including auditory stress, may lead to the shortening of telomeres as a result of oxidative damage. I hypothesized that exposure to stress-inducing alarm calls, signals of the presence of predators, to unhatched chicks of the altricial Carolina chickadee (*Poecile carolinesnsis*) would result in the shortening of telomere length post-hatching. I measured the relative telomere length from 44 chicks in 25 Carolina chickadee nests using quantitative real-time polymerase chain reaction (qPCR) to compare the relative amplification of telomeres to the single copy control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). I found no evidence of reduction in telomere length of chicks exposed to prenatal alarm calls compared to control chicks, and thus no evidence of prenatal stress, nor were there any changes in growth metrics. I did find evidence that parental nest defense, and potentially incubation duration, were influenced by the addition of alarm calls. Future studies should explore the possibility of predator presence influencing incubation duration and the physiological processes of auditory development in altricial chicks.

INTRODUCTION

The effects of prenatal stress on embryonic development are widely observed across vertebrate taxa. (Lickliter 2000, Weinstock 2008, Henriksen *et al.* 2011). In viviparous species it is known that the health of the mother can directly affect developing offspring, for instance the transport of maternal stress hormones through the placenta causes the release of glucocorticoids in a developing fetus (Weinstock 2008). The effects of stress hormones can have similar effects in in oviparous vertebrates. Additionally, oviparous species may experience external stimuli during embryonic development. Depending on the degree of maternal investment, the post egg-laying environment can have significant impacts on the health of offspring. In this study, I investigate the impacts of external stimuli on developing birds.

The external environment can be communicated to the developing offspring via adult behavior. Incubating adults must maintain appropriate temperature during incubation to ensure normal embryonic development. Low incubation temperatures or exposure to excessive heat during incubation causes a reduction in hatchling body weight or even death (Hassan *et al.* 2004, Lay and Wilson 2002, Willemsen *et al.* 2010). Female birds deposit hormones into an egg before the shell is made and hormonal status of females can also influence embryonic development. Thus, maternal stress prior to egg laying has been shown to increase the levels of glucocorticoids in subsequently laid eggs demonstrating that developing embryos can experience external stimuli (Pitk *et al.* 2012). Other external stimuli are likely to reach the developing embryo through the thin membranes and outer layer of the eggshell. Growing evidence suggests that bird embryos are active observers of their environment while still in the egg. (Rivera *et al.* 2018). In this

study, I investigate the ability of bird embryos to perceive stressors in the environment from auditory cues produced by parents.

Because birds use acoustic signals to communicate information about threats in the environment (Gill and Bierema 2013), if embryos can perceive acoustic signals produced by adults, then they could potentially garner information about environmental stress. Prenatal auditory learning is known to occur widely in precocial species, those that hatch ambulatory and capable of feeding themselves (Carlsen and Lickliter 1999, Rivera *et al.* 2018). Prenatal auditory learning has been shown to influence post-hatching behavior in precocial birds (Grier *et al.* 1967, Gottlieb 1975). If unhatched chicks are able to both hear and learn from auditory cues, it follows that auditory stress may affect chicks *in ovo*. This appears to be true, at least for one precocial species, as the presence of alarm calls increase prenatal glucocorticoid stress hormones in prenatal yellow-legged gulls (*Larus michaehellis*) (Francisco *et al.* 2023, Noguera and Velando 2019).

Significantly less work has been done on how prenatal acoustic cues affect the development of altricial birds. Altricial birds hatch less developed than precocial chicks, with closed eyes and no down. Since they hatch less developed than precocial chicks, it is sensible to conclude that their sensory systems, including auditory sensory systems, will be less developed at hatching than precocial species. The evidence for altricial chicks being capable of auditory learning *in ovo* is mixed. Work on North American barn owls (*Tyto furcata pratincola)* and budgerigars (*Melopsittacus undulatus*) suggests that altricial birds do not fully develop hearing capabilities until post-hatching (Kraemer *et al.* 2017, Brittan-Powell and Dooling 2004). Conversely, in the superb fairy-wren (*Malurus cyaneus*) and the red-backed fairy-wren (*Malurus*

melanocephalus) developing chicks have been shown to be capable of hearing *in ovo* (Colombelli-Negrel *et al.* 2012, Colombelli-Negrel *et al.* 2014, Dowling *et al.* 2016).

This apparent contradiction may be explained by recognizing that precociality and altriciality exist on a spectrum, not as truly distinct categories. While species are typically categorized as either precocial or altricial, these are traditionally categorized based upon easily observable phenotypes such as presence or absence of down at birth and age of eye opening. These traits can actually vary significantly even amongst species considered altricial (Ducates and Field 2021). One such trait that varies significantly between the altricial chicks that have been shown to detect prenatal auditory signals and those that only develop auditory pathways much later in life is the age of fledging. Barn owls and budgerigars have relatively long nestling periods of 7-8 weeks and 30 days, respectively, while the superb fairy-wren and red-backed fairy-wren fledge at 10-14 days and 10-12 days (del Hoyo *et al.* 1999, del Hoyo *et al.* 1997). This suggests that species that fledge earlier may have more developed auditory processing *in ovo* than species that have long nestling periods, due to an overall increased development rate, and thus may be more vulnerable to prenatal auditory stress.

Alarm calls are a common response in birds to predator detection and thus an indirect measurement of predatory behavior. Alarm calls have been shown to increase stress in nestlings and may even lead to premature fledging, and thus may be expected to have a similar effect on developing embryos (Ryden 1980, Suzuki 2011, but see Rivers *et al.* 2011). This prediction is supported by a study on yellow-legged gulls, a precocial species, which found that exposure of eggs to adult alarm calls influenced two measures related to stress in chicks, glucocorticoid levels and telomere length after birth (Noguera and Velando 2019). In vertebrates the stress response is primarily controlled by the hypothalamic-pituitary-adrenal axis (HPA axis), which

controls release of glucocorticoid hormones (Haussmann 2012). While there is a baseline level of glucocorticoid hormones expected to be present, stress can spike glucocorticoid hormone levels, leading to deleterious effects on health (Constantini *et al.* 2011, Quirici 2016). Excessive glucocorticoid hormones cause oxidative stress by increasing the presence of reactive oxygen species to levels which cannot be easily removed by cellular systems, which can have various deleterious effects, such as increased risk of cancer, neurological disease and damage to telomeres (Constantini *et al.* 2011, Pizzino *et al*. 2017).

Telomeres, the repetitive regions at the end of chromosomes, are implicated with longevity across several taxa, including birds (Heidinger *et al.* 2011, Horn *et al.* 2010). Because telomeres are situated at the ends of chromosomes, they are subject to the end replication error, and each subsequent replication of a chromosome decreases the length of its telomere (Blackburn 1991, Reichert and Stier 2017). Telomere shortening been suggested to be the cause of senescence in eukaryotic organisms, as the shortening of telomeres with each replication introduces a limit to the number of times that replication can occur (Bernadotte *et al.* 2016, Stier *et al.* 2015). Prenatal oxidative stress, such as that caused by presence of glucocorticoid steroids, has been shown to reduce the length of telomeres both *in vitro* and *in vivo* across taxa, including in avians (Entringer *et al.* 2011, Reichert and Stier 2017, Quirici *et al.* 2016). As telomere length and shortening rates predict longevity in a variety of taxa this suggests that an increase in oxidative stress due to an increase in environmental stressors can directly impact an organism's longevity by shortening telomere length.

Thus far there have been no studies on the effect of prenatal predator cues on the telomere length of an altricial bird. The Carolina chickadee (*Poecile carolinensis*) is an altricial cavity nesting passerine that, like most other small passerines, produces an alarm call when

threatened by a predator such as a hawk (Bartmess-LeVasseur *et al.* 2010). Alarm calls thus serve as a proxy for the presence of predators. Similar to other altricial birds that have been found to sense prenatal auditory stimuli, the Carolina chickadee has a short nestling period of 16-19 days (del Hoyo *et al.* 2007). If environmental cues, including acoustic signals of predation, affect prenatal Carolina chickadee chicks, I expect that exposing chickadee eggs to adult alarm calls will cause oxidative stress leading to telomere damage and length reduction when compared to chicks not exposed to predatory signals.

METHODS

Species Description

The Carolina chickadee (*Poecile carolinensis*) is a small passerine in the family Paridae, which includes the tits and chickadees (del Hoyo *et al.* 2007). They weigh between 9-12 g and are between 11.5-13 cm long (del Hoyo *et al.* 2007). *P. carolinensis* live in open broadleaf woods, up to elevations of 1850 m in areas where the closely related Black-capped chickadee (*Poecile atricapillus*) are absent (del Hoyo *et al.* 2007). Breeding season begins mid-February and ends in early June. *P. carolinensis* brood singularly and are socially monogamous, with lifelong pair-bonds reported (del Hoyo *et al.* 2007). They nest in cavities, and have been reported in rotting tree stumps, woodpecker holes, pipe entrances, and human-made nest boxes (del Hoyo *et al.* 2007). Clutches are between 3-6 eggs, but clutch sizes up to 9 have been reported. Incubation is between 12 and 15 days long (del Hoyo *et al.* 2007). The species exhibits biparental care, and males often feed brooding females (Potter *et al.* 2006). Hatching success of breeding pairs is between 85-92%, and number of young fledged per nest is between 3.3-5.8 on average (del Hoyo *et al.* 2007). The offspring fledge after 16-19 days but remain near breeding territory for an additional 14-21 days (Harrap and Quinn 1995).

Data Collection

I monitored 42 nest boxes that are dispersed in and around the campus of Western Carolina University (35.3090° N, 83.1864° W) in Jackson County, North Carolina, as well as 38 nest boxes in Macon County, North Carolina. The Macon County boxes were split between 12 nest boxes at Tessentee Bottomland Preserve (35.0689° N, 83.3799° W), seven at the Cowee

Mound (35.2669° N, 83.4202° W), and 19 at Gibson Bottoms (35.2329° N, 83.3910° W). The boxes are placed primarily in woodland environments. All nest boxes in Jackson County measure 9" front height x 11 1/4" back height x 6" width x 6" depth and are erected on 5" sections of ½" aluminum conduit poles. Nest boxes in Macon County differ in construction, both between sites and within each site. Each box is assigned a unique nest identification number. The inside of each box in Macon County was lightly coated with unscented soap in order to prevent establishment of paper wasps in the nest boxes. No paper wasp infestations were observed in Jackson County; thus, no soap was applied to the interior of these boxes.

Starting in early March 2020, I checked nest boxes twice weekly for signs of nest building. Chickadee nests were recognized by nest material, as nests are primarily built with moss, which differs from the nesting material of other local cavity nesting birds in the region such as the Eastern bluebird (*Sialia sialis*) or the Northern house wren (*Troglodytes aedon)* which build nests with grass or twigs (del Hoyo *et al.* 2005, del Hoyo *et al.* 2007). Once nests were complete, I checked them twice a week to determine start of egg laying. Incubation begins after the final egg is laid and thus when no eggs were laid for two days incubation was assumed to have begun.

I randomly divided active nests into experimental and control groups. The experimental group experienced playbacks of adult Carolina chickadee alarm calls, while the control group were exposed to the song of adult male chickadees, which is expected to have no effect on prenatal stress levels. Alarm calls are expected to be a stressful auditory signal to young chicks, as it has previously been shown that in the closely related Great tit (*Parus major*), exposure of chicks to alarm calls elicits stress responses such as increased heart rate and behavioral immobility (Ryden 1980).

Playbacks were performed using an iLive ISB23 Bluetooth Wireless speaker connected to a Samsung Galaxy S20 5G at max volume. The speaker was placed approximately one foot off the ground on a Styrofoam box, directly underneath the opening to the nest box. Exact timing of playbacks varied, but all took place between 8 AM and 1 PM EDT. On days nine, ten, and eleven of incubation playbacks of adult Carolina chickadee alarm calls or song were broadcast for three minutes. This timing allows the most prenatal auditory development to occur, without risking the possibility that some nests would hatch prior to all three playbacks being performed, as chickadees can hatch as early as twelve days after incubation has begun. The playbacks were unique for each nest, containing similar but non-identical note structure, at a rate of six calls or songs per minute. To standardize the effect of human interaction and disturbance on incubating chickadees all chickadees were flushed from their nest prior to the beginning of each trial.

Three and nine days after hatching I collected small blood samples (50-100 μL) from each of two randomly selected chicks via either brachial or saphenous venipuncture and stored on cotton swabs and frozen at -20°C. This storage method has been shown to be a reliable way to store avian blood long-term for telomere analysis (Reichert *et al.* 2017). I marked the toes of each chick with a different color permanent marker, which was remarked after three days, to allow for individual identification and ensure that I collected subsequent blood samples from the same individual. At each sampling weight was measured, and tarsus and wing length were measured on the ninth day after hatching.

Nest Defense

During playbacks, I observed adult behavior from a distance of at least 10 meters. I recorded parental activity and distance to the nest box. For each trial, I placed flagging at a five-meter distance from two sides of the nest box. I recorded the approximate distance between the

parent and nest box every 15 seconds. If parents were not seen, they were recorded at five meters, the maximum distance recorded. Presence or absence of parental alarm calls were also recorded during each 15-second interval. Total duration of alarm calls was determined by the percentage of 15-second intervals for which any alarm calls were detected.

DNA Extraction

I extracted genomic DNA from all blood samples using a "Promega" gDNA kit and stored at approximately -20°C. I determined concentration and purity of samples using a NanoDrop 1000 Spectrophotometer (Thermo-Scientific), using 260/280 and 260/230 ratios.

Telomere Measurement

I analyzed blood samples for relative telomere length by a quantitative polymerase chain reaction (qPCR) method modified from Criscuolo *et al.* (2009). The repetitive nature of telomeres and their distribution throughout the genome, including interstitial (TTAGGG) sequences results in non-specific amplification that is difficult or impossible to measure with traditional qPCR methods (Criscuolo *et al*. 2009). This technique instead compares the measurement of amplified target (T) telomere sequences to measurement of single copy reference gene (S). The single copy reference gene is chosen as a gene that does not vary in copy number among individuals or over time. The amplification of the single copy reference gene can be used to normalize the amplification of the telomere sequences in each individual. The gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the single control gene copy as primers have previously been developed for usage in the closely related Great tit for telomere qPCR, and fulfills the conditions necessary for an effective single copy reference gene, being present only once in the genome (Criscuolo *et al.* 2009, Atema et al., 2013). GAPDH is a

highly conserved gene across vertebrate taxa, and Great tits are within the same family as the Carolina chickadee, thus I expected that these primers would effectively amplify the chickadee GAPDH gene (Barrett et al. 2013, Delany et al. 2003).

Measurements of relative telomere length using qPCR use the threshold cycle (C_t). The C_t is the number of cycles required to reach a threshold level of reference dye fluorescence. In other words, it is a measurement of the number of replication events required for a fluorescent signal in a reporter dye to exceed background level. C_t values were calculated via the Applied Biosystems 7500 Real-Time PCR System.

Telomere length is calculated by a ratio of telomere repeat copies (T) to a single control gene copy (S), T/S. Since there is only a single copy of GAPDH present in the genome it serves to standardize the quantity of DNA in a sample, and thus a comparison to telomeric results gives a relative estimation of telomere quantity. Relative telomere length is then determined with the formula: telomere length = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (C_tTelomere - C_tGAPDH)_{reference} - (C_tTelomere - C_tGAPDH)_{target}$ (Heidinger *et al.* 2012, Quirici *et al.* 2016).

I performed qPCR amplifications in triplicate for each sample for both telomere and GAPDH in 96-well plates. This necessitated performing three separate plates for telomere and GAPDH, six in total. I utilized a reference sample on each plate to compare the effect of different plates. Each well contained a total of 25µL: 12.5µL *Power* SYBR™ Green Master Mix (Applied Biosystems), 1µL forward primer solution, 1 µL reverse primer solution, molecular grade water, and DNA template. DNA concentrations were not constant across samples, and thus sample and water volumes varied per reaction but totaled to 10.5 µL, adjusted to contain a total of 10 ng of DNA per well, with the remainder of the volume being water. GAPDH primers were used at a concentration of 4µM, while telomere primers were used at a concentration of 2.85 µM.

GAPDH primers used were developed for the great tit: GAPDH-F (5′-TGTGATTTCAATGGTGACAGC-3′) and GAPDH-R (5′-AGCT TGACAAAATGGTCGTTC-3′) (Grunst *et al.* 2019). Telomere primers used were Tel1b (5′CGGTTTGTTTGGGTTTGGGTTTGG GTTTGGGTTTGGGTT-3′) and Tel2b (5′-GGCTTGCCTTACCCTTACCCTTAC CCTTACCCTTACCCT-3'), which amplify telomeres across avians (Criscuolo *et al.* 2009).

Cycling conditions differed for both telomere and GAPDH. Telomere cycling conditions were a 10 min initial denaturation at 95°C followed by 25 cycles of 1 min at 95°C, 1 min at 54°C, and 1 min at 60°C. GAPDH cycling conditions were 10 min at 95°C followed by 40 cycles of 1 min at 95°C, 1 min at 62°C, and 1 min at 60°C (Quirici et al. 2016). I performed melt curve analysis after amplification to confirm specificity of primer sets. Melt curve cycling parameters were the default for plates using SYBR Green reagents, recommended by the Applied Biosystems Real-Time PCR System Reagent Guide (2008). For GAPDH plates, the cycling conditions were 40 cycles of 10 sec at 95°C, 1 min at 60°C, 15 sec at 95°C, and 15 sec at 60°C. Telomere plates followed the same melt curve cycling parameters for 30 cycles.

All three telomere (T) plates formed a single peak at approximately 80°C. The three GAPDH (S) plates formed a single peak at approximately 81°C, confirming specificity of both primer sets. An adult sample was randomly selected and used as the standard sample for all 96-well plates to produce a reliable standard curve to ensure the efficiency of each PCR reaction (Quirici *et al*. 2016). This standard was run on a 1:2 dilution series with five dilution points (12ng, 6ng, 3g, 1.5ng, and 0.75ng of DNA) and in triplicates in all plates. The serial dilutions produced a reference curve used to control for the amplification within each plate to test amplification efficiencies within each reaction (accepted range 100±15%, Quirici et al. 2016).

These reference curves were also used to calculate the coefficient of variation by comparing the Ct values of each plate for both telomere and GAPDH plates, resulting in a %CV of 2.56% for the GAPDH plates and 9.72% for telomere plates. A no template control (NTC) reaction was prepared in triplicate for each of the plates to control for contamination and primer-dimer formation (Bustin et al. 2009). All other (target) nestling samples were run in triplicate (Quirici et al. 2016) and fit into each of the telomere and GAPDH plates. Reactions were centrifuged at 1,500 rpm for two minutes using a Sorvall T1 Benchtop Centrifuge (Thermo Fischer Scientific) prior to amplification. Telomere and GAPDH plates were run in an Applied Biosystems 7500 Real Time PCR instrument as a standard curve experiment.

Statistical Analysis

The average T/S for each nest were compared between control and experimental nests with a Welch two-sample t-test, as was the average T/S between three- and nine-day old chicks. In the case that multiple blood samples were taken from chicks in the same nest, their T/S ratio was averaged. The same was true of tarsus length, wing length, mass, nest defense values, and duration of incubation. To compare the change in telomere length over time between the two experimental groups the difference between day nine and day three T/S was compared using a Welch two-sample t-test.

Nest defense was scored by the average of the distance of the closest parent every fifteen seconds during the trial. In the case that no parent was seen during the entire trial, they were assigned the maximum value of five meters. Hatching success and survival until nine days of age was compared between control and experimental nests were compared using Pearson's Chi-squared test. All statistical analyses were performed in RStudio Version 2022.7.1.554.

RESULTS

I observed a total of 31 Carolina chickadee nests. Of those, six were abandoned or lost prior to blood collection, leaving 25 nests. In total 44 chicks had blood samples taken three days after hatching. Thirty-two chicks, from 19 nests, survived until an additional blood sampling occurred at day nine. I analyzed a total of 76 blood samples. Nests that failed prior to blood sampling were included in measurements of nest defense, but excluded in measurements of T/S and growth metrics.

There was no effect of playback treatment on telomere length. This held true for a comparison of nests three days after hatching (Figure 1, $t = 0.207$, $df = 22.24$ $p = 0.8381$) as well as nests nine days after hatching (Figure 2, $t = 0.49$, $df = 17.0$ $p = 0.6323$) There was no difference in telomere length between three-day old and nine-day old chicks (Figure 3, $t = 0.8671$, $df = 41.0$, $p = 0.3909$). There was also no difference in telomere degradation between day three and day nine between the two groups (Figure 4, $t = 0.66543$, $df = 13.563$, $p = 0.5169$).

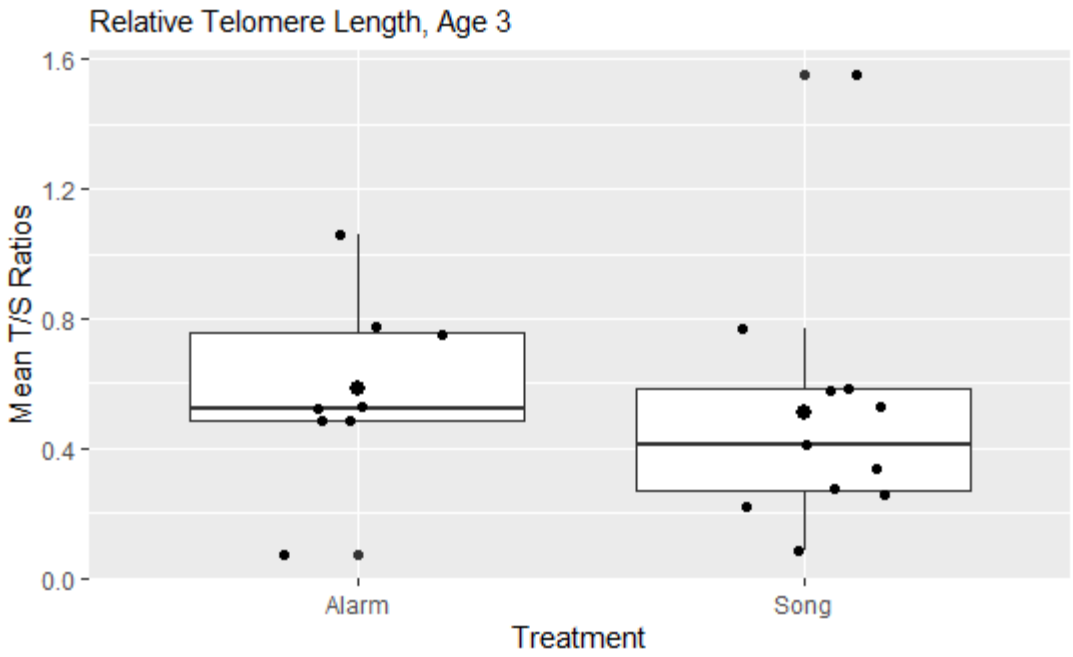

*Figure 1: Mean T/S ratio for three-day old Carolina chickadee (*Poecile carolinensis*) hatchlings that were exposed to adult alarm calls (A) and were exposed to adult song (S) prior to hatching. There was no difference in mean T/S ratios between the two treatments (t = 0.207, df = 22.24 p = 0.8381).*

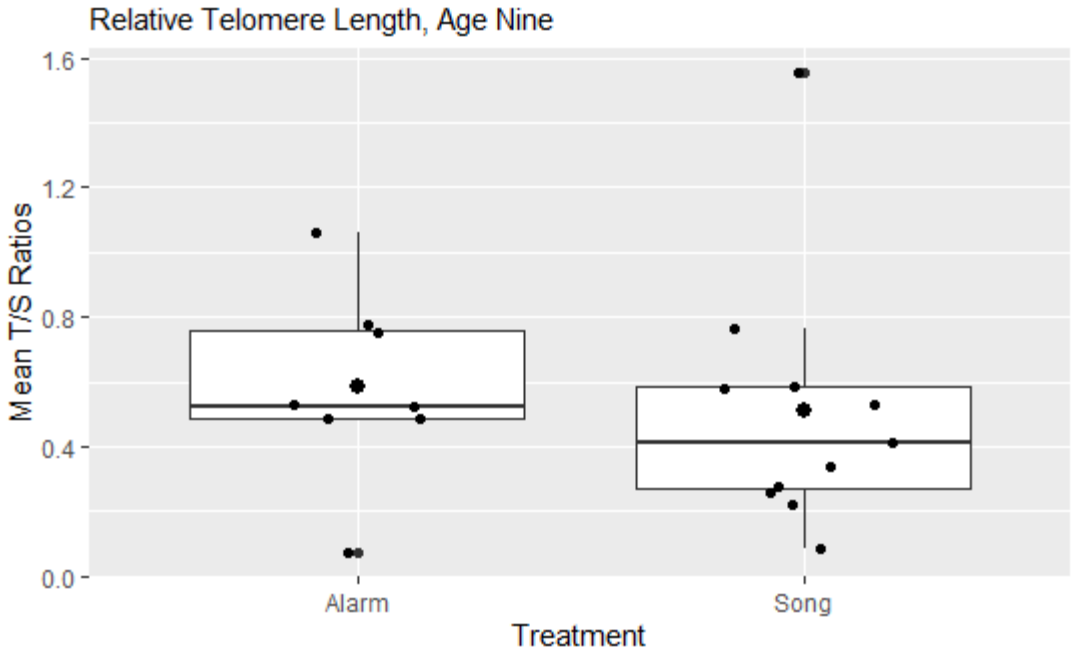

*Figure 2: Mean T/S ratio for nine-day old Carolina chickadee (*Poecile carolinensis*) hatchlings that were exposed to adult alarm calls (A) and were exposed to adult song (S) prior to hatching. There was no difference in mean T/S ratios between the two treatments (t = 0.49, df = 17.0 p = 0.6323)*

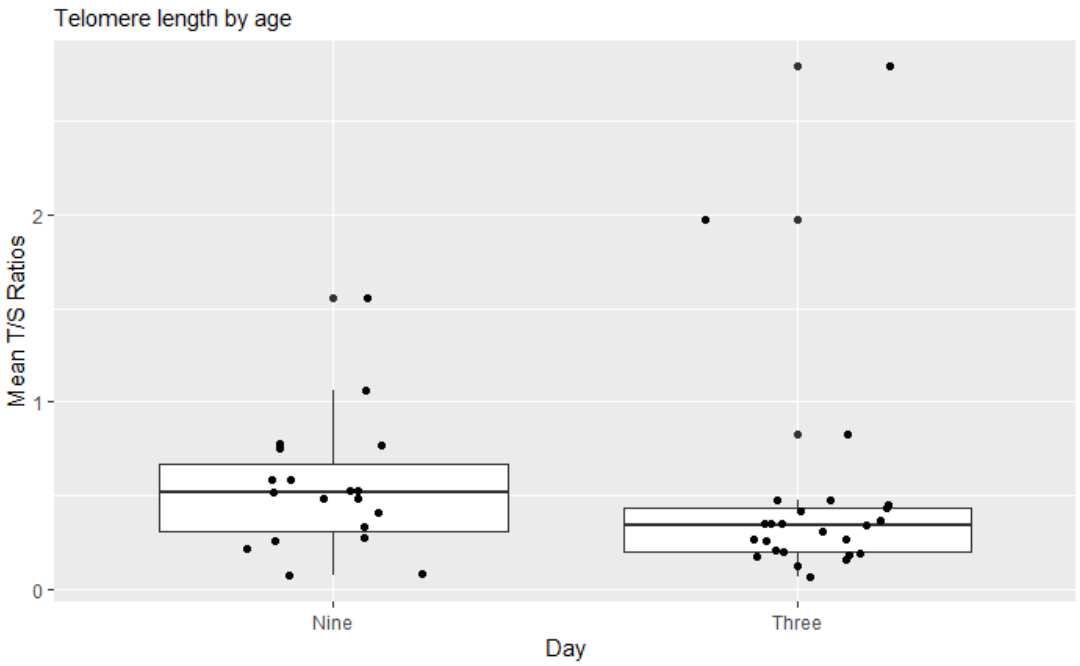

*Figure 3: Mean T/S ratio for Carolina Chickadee (*Poecile carolinensis*) chicks at three- and nine-days post-hatching. Mean T/S ratio did not differ with age (t = 0.92, df = 73.984, p-value = 0.36).*

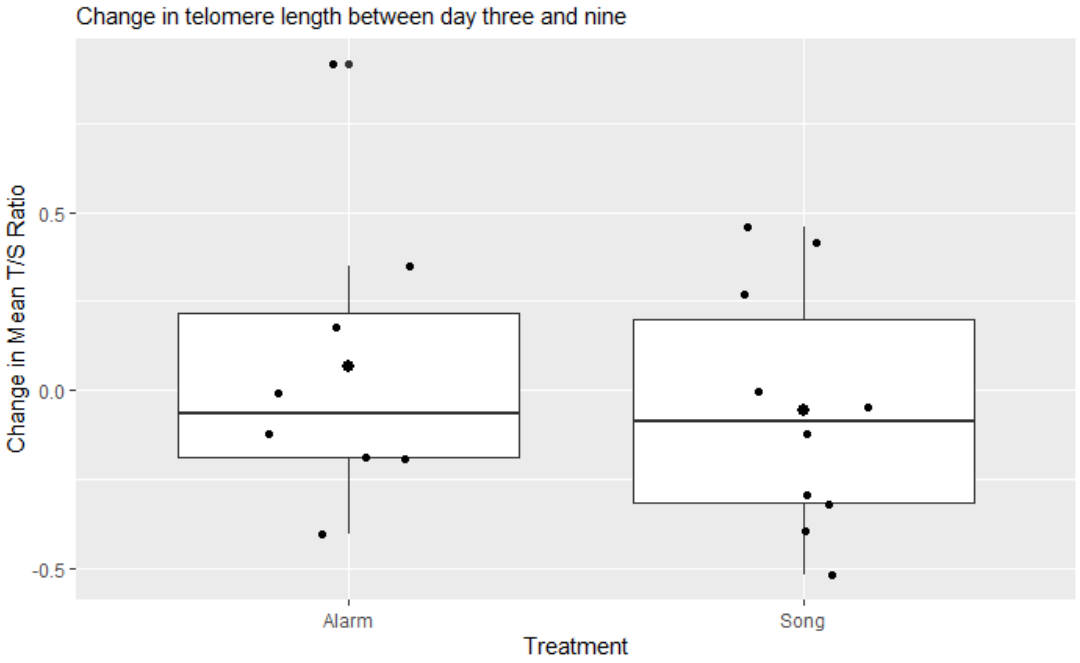

*Figure 4: There was also no difference in telomere degradation between day three and day nine in Carolina chickadee (*Poecille carolinensis*) chicks prenatally exposed to stressful adult alarm calls and non-stressful adult song. (t = 0.66543, df = 13.563, p = 0.5169).*

There was no effect of treatment on hatching success (Figure 5, $\chi^2 = 1.516$, df = 1, p-value = 0.2181) or survival to nine days old (Figure 6, $\chi^2 = 2.4425$, df = 1, p = 0.1181). Likewise, telomere length was not related to chance to survive from age three days to age nine days (t = 0.58521, df = 5.5046 p = 0.5816). Similarly, there was no effect of treatment on chick growth rate measured by either tarsus length (Figure 7, t = 0.32751, df = 16.775, p = 0.7473), wing length (Figure 8, t = - 0.36324, df = 19.222, p = 0.7204), or mass (Figure 9, t = 0.32751, df = 16.775, p = 0.7473).

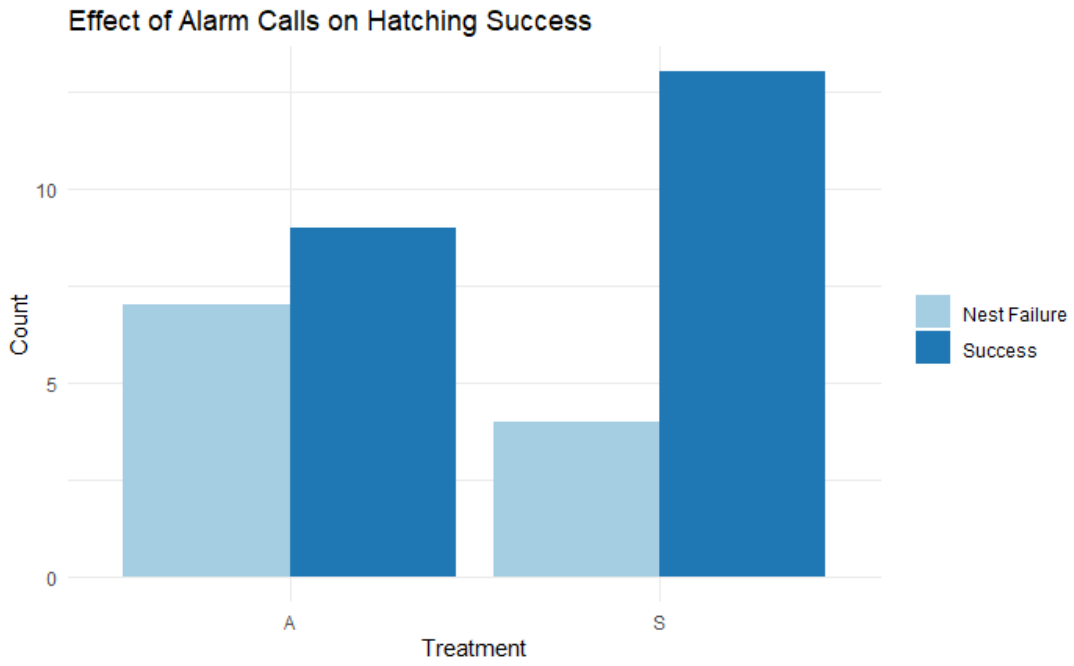

*Figure 5: The effect of adult alarm call on hatching success in the Carolina chickadee (*Poecile carolinensis*). Nests exposed to adult alarm calls (A) did not differ in successful hatch rates compared with nests only exposed to adult song (S) ($\chi^2 = 1.516$, df = 1, p-value = 0.2181)*

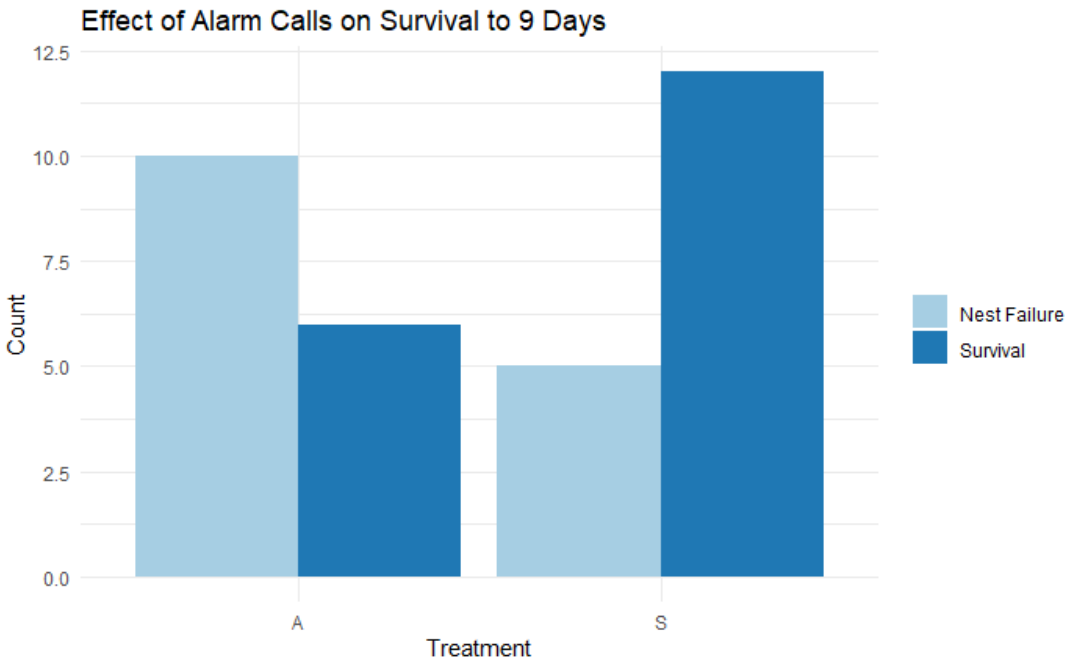

*Figure 6: The effect of adult alarm call on survival in the Carolina chickadee (*Poecile carolinensis*). Nests exposed to adult alarm calls (A) did not differ in survival until nine days of age compared with nests only exposed to adult song (S) (χ^2= 2.4425, df = 1, p-value = 0.1181)*

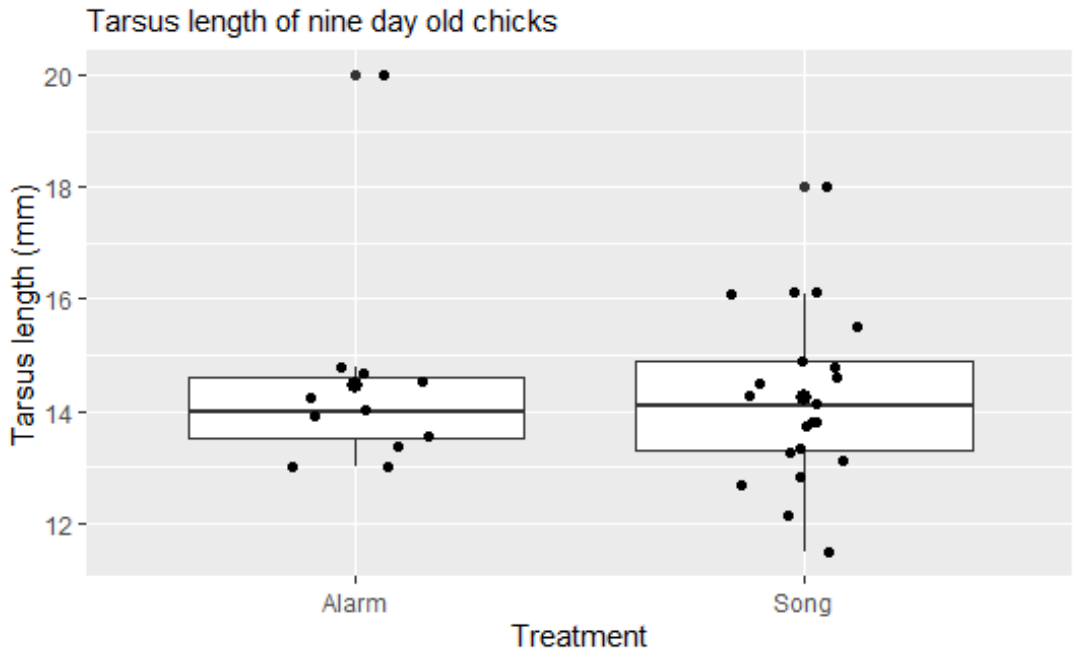

*Figure 7: The effect of prenatal exposure to adult alarm call on wing length measurements in nine-day old Carolina chickadee (*Poecile carolinensis*) hatchlings. Nests exposed to adult alarm calls did not differ in tarsus length compared with nests exposed to adult song.*

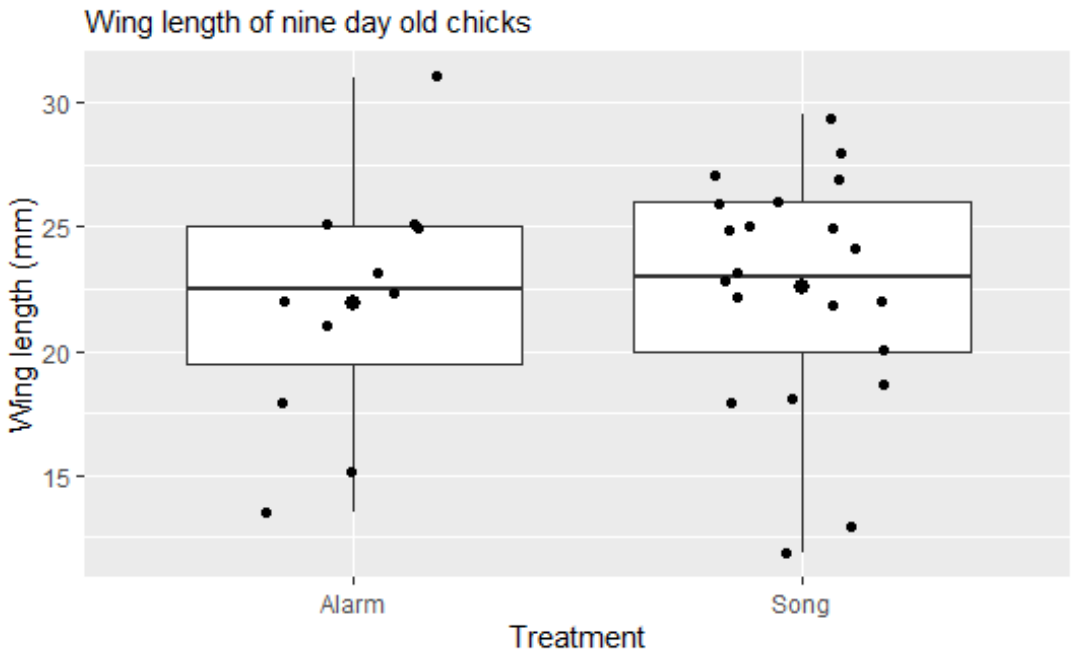

*Figure 8: The effect of prenatal exposure to adult alarm call on wing length measurements in nine-day old Carolina chickadee (*Poecile carolinensis*) hatchlings. Nests exposed to adult alarm calls did not differ in wing length compared with nests only exposed to adult song (S) (t = -0.36324, df = 19.222, p = 0.7204)*

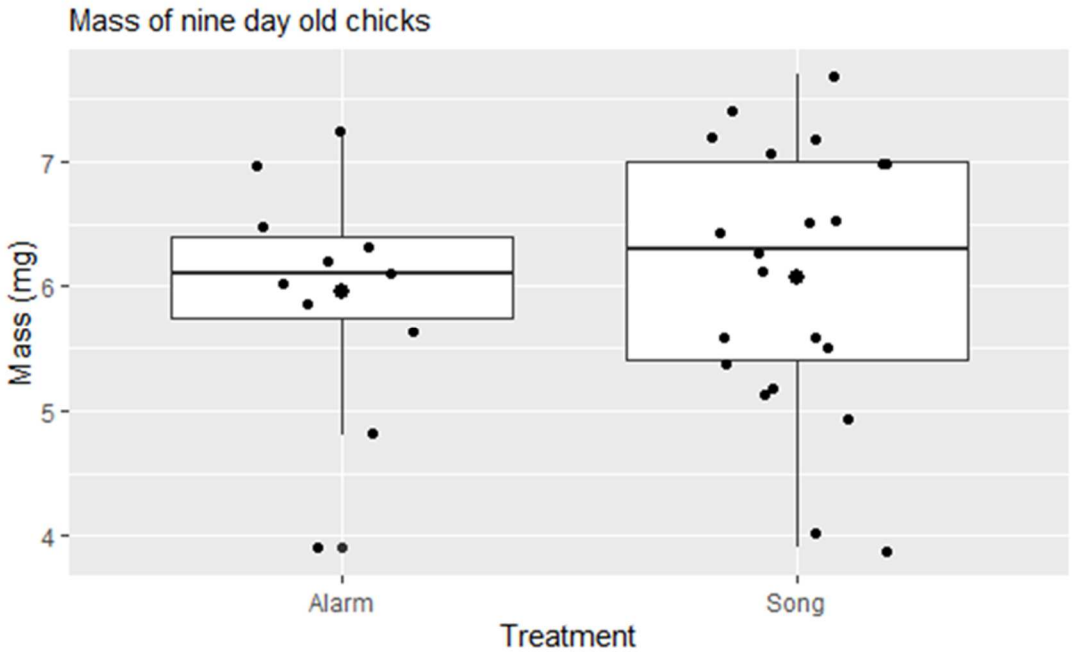

*Figure 9: The effect of prenatal exposure to adult alarm call on mass in nine-day old Carolina chickadee (*Poecile carolinensis*) hatchlings. Nests exposed to adult alarm calls (A) did not differ in mass compared with nests only exposed to adult song (S) (t = 0.32751, df = 16.775, p = 0.7473).*

Nest Defense

The average minimal distance during nest defense differed significantly between alarm (mean = 4.16 m) and control nests (mean = 4.90 m) (Figure 10, t = -3.4213, df = 17.515, p = 0.003143). Control nests experienced an average of 55.9 seconds of parental alarm calls during their trials in addition to the song playback. Amount of parental alarm calls during alarm playbacks was unable to be measured accurately due to difficulties in consistently distinguishing the parental call from the prerecorded call.

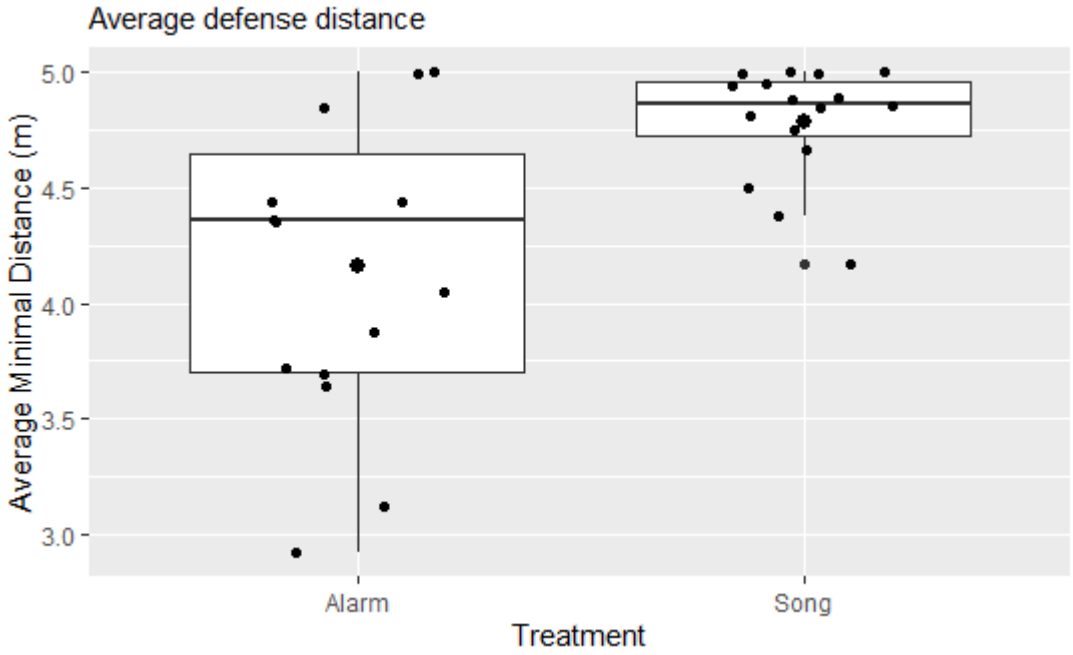

Figure 10: Average distance of parental approach to nest boxes during playbacks of either adult alarm calls or adult songs. Parents exposed to adult alarm calls approached closer (mean = 4.16 m) than parents exposed to adult song (mean = 7.79 m) (t = -3.4213, df = 17.515, p-value = 0.003143).

Duration of Incubation

The average duration of incubation was compared between alarm and song nests to test for any effect of treatment on incubation duration. The difference between average length of duration between alarm (mean = 14.5 days) and song nests (mean = 15.6 days) was not found to be significant (t = -1.9164, df = 19.766, p-value = 0.07) (Figure 11).

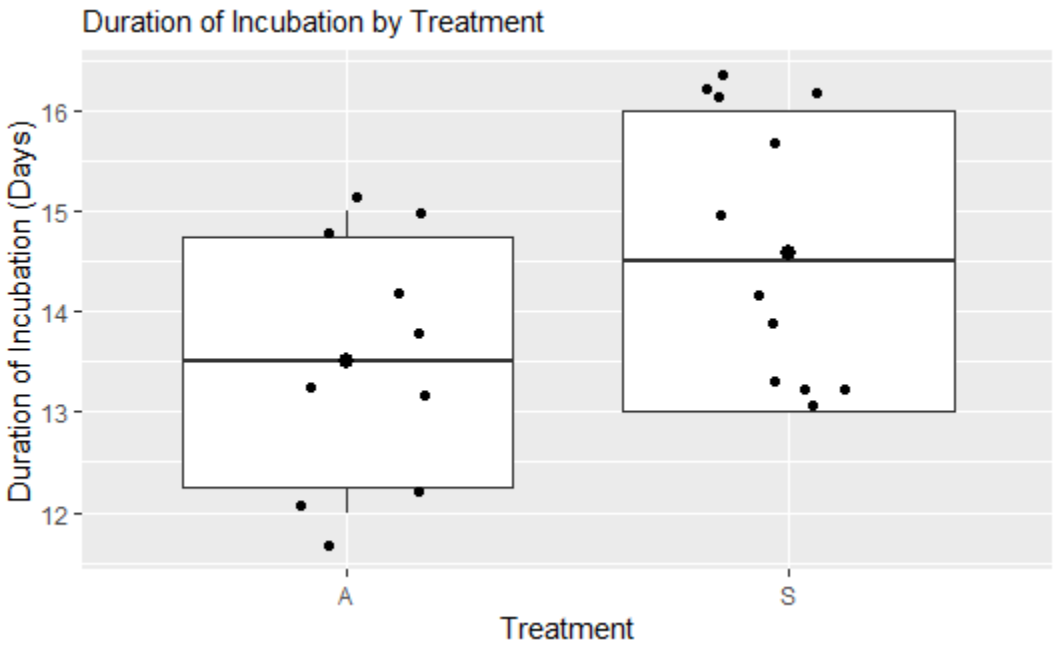

*Figure 11: Duration of incubation of Carolina chickadee (*Poecile carolinensis*) chicks for nests exposed to adult alarm calls (A) or adult song (S). There was no difference between the two groups (t = -1.9164, df = 19.766, p-value = 0.07).*

DISCUSSION

I tested the effects of adult alarm calls on developing embryos. Alarm calls are signals produced by adults that indicate predators are present in the environment. During incubation, half of the nests heard playback of alarm calls and half of the nests heard playback of adult song, a non-stressful signal. If developing embryos could perceive alarm calls as a sign of danger, I expected to find shorter telomeres in nests that heard alarm calls during incubation when compared to nestlings that heard songs during incubations, as reduction in telomere length is one result of stress. I found no evidence of reduced telomere length, and thus increased stress, in nestlings that heard alarm calls. There was no difference in reduction of telomere length in older chicks, as both three-day and nine-day old nestlings did not differ in average telomere length. There was no difference in the change in telomere length over time in nestlings of different treatment, though given the short time frame of six days between measurements, it is possible that this is simply not a long enough time period to distinguish any differences. Thus, I have found no evidence that Carolina chickadee nestlings were able to perceive alarm calls in the egg.

On possible explanation for this result is that the auditory development of altricial chicks such as the chickadee may not be advanced far enough prior to hatching to have any effect on post-hatching stress, unlike what has been shown in several species of precocial birds (Francisco *et al.* 2023, Noguera and Velando 2019). Slow development of auditory sensing has been observed before in altricial species with much longer nestling periods such as the North American barn owls and budgerigars, which take multiple weeks after hatching to fully develop auditory senses, but is unlike the fairy-wren, which has a nestling period similar to the Carolina chickadee and yet does perceive sounds *in ovo* (Brittan-Powell and Dooling 2004, Dowling *et al.*

2016, Colombelli-Negrel *et al.* 2012, Colombelli-Negrel *et al.* 2014, Kraemer *et al.* 2017). This suggests that auditory development may not proceed along the same route in altricial birds that share similar nestling periods. Alternatively, it may suggest that prenatal embryos are more severely affected by specific auditory cues. Fairy-wrens have been shown to be able to detect maternal incubation cries *in ovo*, but this does not necessarily extend adult alarm call (Colombelli-Negrel *et al.* 2012, Colombelli-Negrel *et al.* 2014). It is possible that learning maternal incubation cries, which is posited to be an adaptation to brood parasitism, is more beneficial than detecting adult alarm calls, and thus embryonic chicks may be predisposed to be affected by some sounds and not others.

I found no effect on traditional metrics of survival and growth because of the treatment. The nest failure rate during the season was relatively high (61.2% nest success rate), however treatment was not found to have a significant effect on either hatching success (Figure 5). I found no effect of exposure to alarm calls the growth metrics of weight, wing length, or tarsus length. Taken together these suggest that repeated exposure to signals of predator activity did not negatively impact chick health. This result is to be expected if prenatal chickadee auditory development is not advanced enough to detect predator cues, but it also suggests that there was no prolonged effect of predator cues on parental care. Given that I disturbed both experimental and control nests, including removal of incubating birds from the nest, it can be assumed that the addition of conspecific alarm calls did not cause any further disruption to parental care than the initial disturbance, at least not enough to result in actual differences in growth, survival, or telomere length. Survival to nine days of age had a low but non-significant p-value (Figure 6, $p = 0.1181$), thus it may be possible that the small sample size is hiding a biologically significant effect.

One potentially confounding factor is the difference in incubation time between nests. While the Carolina chickadee may hatch as early as twelve days after incubation has begun, they can take up to seventeen days to hatch. Playbacks were performed on days nine, ten, and eleven, which resulted in nests that hatched on day fifteen to not receive any alarm call playbacks on days twelve, thirteen, or fourteen. This was done so as to standardize the number of callbacks that were played to each nest, as otherwise nests that hatched later would receive playbacks for up to three days longer than nests that hatched on day twelve. However, this has the result of late hatching nests not being as developed when playbacks were performed, and may have dampened the effect of alarm calls on developing chicks. It may be prudent for future research to instead initiate playbacks at the start of incubation and continuing until hatching, though that will result in some nests receiving playbacks for a longer period of time. It is possible that there was an effect of playback on duration of incubation ($p = 0.07$), with the average date of hating for experimental nests being 13.5 days after incubation began, and control nests averaging 14.6 days. This makes intuitive sense, as it would be beneficial to reduce incubation time in a high predation environment, and previous studies have found that an increase in incubation period results in an increased risk of predation (Higgott *et al.* 2020). To my knowledge there are few previous studies that have directly examined the effect of predation risk on duration of incubation, and no evidence of an effect of predation was found on incubation duration in those that did (Basso and Richner 2015). However, studies that have looked at interspecies differences in incubation duration and have concluded that predation, parasitism, and adult mortality risk are important factors in predicting a species' length of incubation (Moller and Benton 2005, Martin 2002). If heightened predation does decrease the duration of incubation in the Carolina chickadee, given that I have obtained no evidence of chickadee embryos detecting auditory

stress, it seems more likely that this is an effect on incubation or other parental behaviors that is causing eggs to hatch sooner. Future research should examine the effects of predatory signals on incubation duration.

Nest defense was more intense for experimental nests than control nests. Parents of control nests were more likely to be absent entirely, or to keep a larger distance, during the playbacks than parents of experimental nests. Higher levels of nest defense for experimental nests should result in more signals of predator cues being broadcast to developing chicks, and if anything, should provide additional sources of stress that would have negatively impacted developing chicks and cause deleterious effects on telomere length if the embryonic chicks were perceptive of them. However, the presence of adult alarm calls at control nests does pose a problem for interpretations. Control nests received relatively few adult alarm calls compared to experimental nests, with experimental nests receiving constant alarm calls for three minutes and on average control nests receiving 56 seconds of alarm calls per trial due to parental alarm calling. These calls were less intense, as they were performed from further away, and for a shorter duration, than the alarm calls experienced by the experimental nests, but they still provide a stressful signal to the developing chicks. This means it is possible that all nests were negatively affected by exposure to adult alarm call, but if this is the case it suggests that there needs to be very minimum stress to affect chicks and that the effect is not amplified by experiencing over three times the amount of stressful auditory signals. It may be necessary in future research to capture parents prior to performing the trial to limit the amount of extraneous alarm calls that chicks are exposed to. Alternatively, it may be possible that non-conspecific songs of other local species may elicit less of a response from adult chickadees and thus be a more viable non-stressful signal.

I was unable to accurately distinguish recorded adult alarm calls from the parental alarm calls that occurred during the trial, making it impossible to accurately assess the amount of additional alarm calls that experimental nests received compared to control nests. Despite this, experimental nests did receive more stressful signals than control nests did. The addition of further alarm calls on top of the prerecorded alarm calls may cause further stress as a result of increased call frequency, or it may be possible that hearing the calls of multiple adults may be more stressful than just hearing one adult alarm call. If it is the case that additional parental alarm calling produced additional stress in the unhatched chicks, it was not reflected in telomere length reduction.

The results of this experiment, taken as a whole, are consistent with either chickadee auditory development *in ovo* being insufficient to detect alarm calls, or with chicks not yet learning the meaning of alarm calls and as a result not experiencing stress from alarm calls. Previous evidence generally suggests that responses to alarm calls in altricial birds are to a large extent innate, as cross-fostered robin and dunnock chicks were found to respond strongly to conspecific alarm calls, but not to the alarm calls of the foster species (Davies *et al.* 2004). It thus seems unlikely that the lack of response to alarm calls in chicks was due to a lack of opportunity to learn alarm calls. Instead, it may be that auditory pathways are not developed enough to respond to alarm calls in prenatal chicks. This is supported by research on secession of begging in response to alarm calls in other altricial species. Great tit and pied flycatcher (*Ficedula hypoleuca*) chicks will cease begging for food in response to adult alarm calls, but this behavior begins only several days after hatching (Korneeva *et al.* 2006, Ryden 1978). Given that responses to alarm calls seem to be innate, this suggests that the cause of these delays in responses may be due more to an underdeveloped auditory pathway, rather than any learned

behavior. This leads me to interpret the results of my experiment as indicative of Carolina chickadees not fully developing auditory sensing until after hatching.

One possibility that cannot be determined by this experiment is that auditory development of chickadees is indeed developed enough prenatally to hear the adult alarm cries, but that their fight-or-flight response pathways are not appropriately developed. Development of fear responses continues well after birth in a variety of taxa (Wiedenmayer 2009). In the pied flycatcher it has been shown that nestlings experience a hormonal stress response at least as early as nine days after hatching, but that it is underdeveloped compared to adult stress responses (Tilgar *et al.* 2009). It seems more likely, however, that if the fight-or-flight response pathways are undeveloped at this time, it is a compounding factor with an underdeveloped auditory pathway, rather than a separate reason for the lack of effect. This may be an underappreciated aspect of the difference between precocial and altricial chicks, as it seems significantly more important to have a highly developed predator response upon hatching for precocial chicks, who are able to flee and hide from predators shortly after hatching, than it does for altricial chicks, who would be limited to actions like cessation of begging to escape detection by predators. Given this complication, it may be best for future research on the onset of auditory development in altricial birds to avoid the usage of predator signals, perhaps instead favoring parental signals.

There is still much to understand about the auditory senses of prenatal altricial chicks, a topic that has been underappreciated and understudied compared to precocial chicks. My study is to my knowledge the first to examine potential impacts of prenatal auditory stress on an altricial bird, the Carolina chickadee. I found no evidence that prenatal auditory stress is an important factor on chick health post-hatching in terms of survival, growth, or telomere length, and conclude that this may be due to lack of auditory development prior to hatching. I did find

evidence that parents were affected by auditory stress, increasing measures of nest defense in response to the addition of alarm calls. I have also identified duration of incubation as a trait potentially affected by the presence of signals of predation, though I am unable to definitively draw a conclusion on this. The results of this experiment emphasize the importance that parents have in the early life of altricial birds and suggest that the largest impact of predatory stress on developing chicks is likely to be caused indirectly by the effects it has on parents, rather than directly affecting the embryos themselves. Future research on the study of predatory stress in altricial chicks should focus on how such stress affects parents during the incubation period and how this affects the duration of incubation, as well as identifying mechanisms that may cause differential incubation differences. There is also much still to be learned about the physiological development of auditory systems in altricial avians, a topic that has been neglected in favor of research on precocial avians, especially with regards to how altricial chicks with differing incubation and nestling durations develop, and further research on this will do much to elucidate the effects of auditory stress on developing altricial chicks.

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