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Of Mites and Brains: Ectoparasitism in Nestlings Alters the Development of Song Control Nuclei in European Starlings

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OF MITES AND BRAINS: ECTOPARASITISM IN NESTLINGS ALTERS THE DEVELOPMENT OF SONG CONTROL NUCLEI IN EUROPEAN STARLINGS

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The developmental stress hypothesis suggests that birdsong can reflect the current condition and developmental history of a songbird. Developmental stressors such as food restriction or ectoparasitism can hinder normal song development, leading to altered song quality in adulthood. Previous research has demonstrated that early food restriction results in reduced growth of song control nuclei, which may account for the effects of early food restriction on adult song quality. However, the neural mechanisms underlying the effects of early ectoparasitism on birdsong have not been directly investigated. In this study, we examined the development of song control nuclei in European starling (*Sturnus vulgaris*) nestlings under varying levels of ectoparasitic infestation. We subjected nests to either the addition of Northern fowl mites (*Ornithonyssus sylviarum*) or the use of a miticide, Permethrin, to reduce ectoparasites. We followed nestlings throughout pre-fledging development and assessed physical growth and hematological titers on brood-days 10 and 20. On brood-day 20, we harvested nestling brains for later histological analysis. We found that nestlings from nests with added mites had significantly shorter wing chord and lower body mass on brood-day 10, but on brood-day 20, structural growth was similar to that of nestlings in miticide-treated nests. Our analyses revealed that, relative to miticide-treated nests, hematocrit, brain mass, and the volumes of two song control nuclei (HVC and the robust nucleus of the archipallium) were significantly reduced in nests with added ectoparasites on brood-day 20. These findings suggest that early ectoparasitism can affect the development of song control nuclei, potentially resulting in altered song quality in adulthood.

KEYWORDS: Developmental stress hypothesis, European starling, song control nuclei, ectoparasitism, birdsong, nestling

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Abstract

The developmental stress hypothesis suggests that birdsong can reflect the current condition and developmental history of a songbird. Developmental stressors such as food restriction or ectoparasitism can hinder normal song development, leading to altered song quality in adulthood. Previous research has demonstrated that early food restriction results in reduced growth of song control nuclei, which may account for the effects of early food restriction on adult song quality. However, the neural mechanisms underlying the effects of early ectoparasitism on birdsong have not been directly investigated. In this study, we examined the development of song control nuclei in European starling (*Sturnus vulgaris*) nestlings under varying levels of ectoparasitic infestation. We subjected nests to either the addition of Northern fowl mites (*Ornithonyssus sylviarum*) or the use of a miticide, Permethrin, to reduce ectoparasites. We followed nestlings throughout pre-fledging development and assessed physical growth and hematological titers on brood-days 10 and 20. On brood-day 20, we harvested nestling brains for later histological analysis. We found that nestlings from nests with added mites had significantly shorter wing chord and lower body mass on brood-day 10, but on brood-day 20, structural growth was similar to that of nestlings in miticide-treated nests. Our analyses revealed that, relative to miticide-treated nests, hematocrit, brain mass, and the volumes of two song control nuclei (HVC and the robust nucleus of the archipallium) were significantly reduced in nests with added ectoparasites on brood-day 20. These findings suggest that early ectoparasitism can affect the development of song control nuclei, potentially resulting in altered song quality in adulthood.

1. Introduction

1.1 Birdsong

For the nearly 5,000 species of songbirds that constitute over half of all bird species, song is a vital signal used in intra- and inter-sexual communication (Prum et al., 2015; Searcy et al., 1996). Many female songbirds preferentially mate with males who sing more frequently or with greater complexity, which are traits that are thought to have evolved and persisted due to sexual selection (Searcy & Yasukawa, 2015). The various qualities of birdsong, such as complexity, repertoire size, song output, and duration of song, that songbirds produce are typically honest indicators of a bird's viability which provides potential mates with an assessment of their quality. This, in turn, gives choosy females who use the honest indicator of a potential mate's song an advantage in reproductive fitness for selecting partners with higher song quality (Nowicki et al., 2000).

As songbirds mature throughout the first few months of life, they form auditory memories on which they will model their song and undergo the growth and development of brain regions necessary for the sensory-motor learning that eventually leads to adult song production (Nowicki et al., 1998). This learning and production of birdsong is controlled by a network of song control nuclei that form the neural song system (Brenowitz et al., 1997). HVC is a critical brain region that is an important part of the avian song system that links the song-learning and song-production circuits in male songbirds (Nottebohm, 2005). HVC is the formal name of this region, not an abbreviation – this nomenclature is used to correct an early naming error (Anton et al., 2004). Song production arises from the song motor pathway that includes projections

from HVC neurons directly onto the robust nucleus of the arcopallium (RA), which sends axons that terminate in brain stem nuclei that then innervate the syrinx and muscles of the respiratory system that aid in inhalation, exhalation, and in turn, singing. Song learning requires the anterior forebrain pathway which also receives afferent input from HVC neurons and provides RA with an additional source of afferent input (Fortune & Margoliash, 1995; Nottebohm et al., 1976; Mooney, 2009).

The ability to produce song varies greatly in female songbirds from inability to produce learned songs in female zebra finches (*Taeniopygia guttata)* (Shaughnessy et al., 2016) to production of similar amounts of song with similar complexity as their male counterparts in female African bush shrikes (*Laniarius funebris)* (Gahr et al., 1998). Female songbirds have similar 'song systems' to males, even in species in which females do not produce song, but the neural regions are typically significantly smaller than those of conspecific males (MacDougall-Shackleton & Ball, 1999). Despite these relatively diminished HVC volumes in female songbirds, they still possess male-typical patterns of gross HVC neural connectivity (Casto, 2001; Shaughnessy et al., 2016). In female songbirds, HVC may not assist with song learning and production to the same degree as in male songbirds, but instead, HVC can aid in the recognition and discrimination among conspecific songs (Brenowitz, 1991; Del Negro et al., 1998; Leitner & Catchpole, 2002) as well as the perception of song quality (Del Negro et al., 2000), processes that are critical to mate choice (Rebel, 2000).

As early as day 20, the HVC of male zebra finches contains significantly more neurons than in females (Kirn & DeVoogd, 1989) and in 20-day-old European starlings (*Sturnus vulgaris),* sex differences in HVC volume are also present (Casto, 2001).

Together, these findings highlight that the process of sexual differentiation and general post-hatching production and growth of HVC neurons in songbirds is well underway by 20 days of age. This growth continues throughout much of juvenile development (Casto & Ball, 1996; Casto, 2001).

Relative to precocial birds, altricial birds, such as songbirds, exhibit relatively small brain sizes prior to hatching and undergo a rapid period of brain growth after hatching (Bennett & Harvey 1985). In canaries, this early and rapid post-hatching development includes the birth of HVC projection neurons that eventually grow to innervate RA, a connection crucial for song production. These HVC_{RA} neurons reach a peak of production around 60 days of age in canaries (*Serinus canaria***)** with production tapering off at about 120 days of age (Alvarez-Buylla et al., 1988). In male and female European starlings, the song control nucleus RA, is relatively uninnervated by HVC_{RA} neurons at 20 days of age, as axons of HVC neurons appear to terminate at the borders of RA; however, by 50 days of age RA is substantially innervated by HVC_{RA} neurons (Casto, 2001). The early post-hatching period is a critical time for the production and development of the neural structures necessary for song production later in life.

1.2 The developmental stress hypothesis provides an explanation for why male birdsong remains an honest indicator of male quality

In both male and female songbirds, the early growth of HVC can be affected by developmental perturbation (Nowicki et al., 1998; MacDonald et al., 2006). The developmental stress hypothesis (Buchanan et al., 2003), a more general version of the

earlier nutritional stress hypothesis (Nowicki et al., 1998) posits that adult song remains an honest indicator of male quality because it not only can reflect current condition, but also can signal developmental history. Developmental stressors can interfere with the normal growth of song control nuclei which, in turn, can alter the quality of the song produced in adulthood (Nowicki et al., 2002). More resources allocated to the development of HVC may allow male songbirds to recruit new neurons into HVC to promote its behavioral functions in adulthood, but this development is costly and can be diminished or curtailed in response to developmental stressors (DeVoogd et al., 1993; Catchpole, 1996). Thus, the primary emphasis of the developmental stress hypothesis has been to explain why females assess and choose males as mates based on song quality — because it reflects the development and individual condition of prospective mates.

As research on the developmental stress hypothesis has progressed, the methods and foci of the research have broadened. A variety of developmental perturbations such as reduced nutrition, direct manipulation of the endocrine stress response, and endoparasitism have now been directly linked to decreased adult male song quality as well as reductions in the size of HVC; other critical song regions have also been examined, but those analyses have not produced as clear and consistent results. (Nowicki et al., 2002; Spencer et al., 2011; Spencer et al., 2005). Additionally, despite the common sex differences found in HVC size in many songbird species, females, like males, are sensitive to effects of developmental stress on HVC volume; although, the effects of reduced HVC volume on female song preferences are likely both subtle and complex (Buchanan, 2011). In addition to further research into the types

of stressors that impair HVC development, a more complete understanding of the role that HVC plays in song preferences of female songbirds should help us to better predict the potential fitness consequences of reduced HVC volume in response to developmental stress in female songbirds.

The effects of developmental stressors on HVC growth has been investigated largely in captive, often hand-reared, songbirds. Given the challenges associated with conducting carefully controlled experimental studies of developmental stress on brain development in the wild, studies of the developmental stress hypothesis in free-living birds have tended to be correlative, usually relating the degree of developmental stress to adult song characteristics, without assessment of neural development (Spencer et al., 2011). Studies that can incorporate experimental exposure to natural developmental stressors and assess song system growth during development in free-living songbirds help extend ecological relevance while maintaining carefully controlled field setting when compared to that of findings of captive research.

1.3 Ectoparasitism as a developmental stressor

In addition to nutritional stress, developmental perturbation by early infestation of nestlings with ectoparasitic hen fleas (*Ceratophyllus gallinae*) have been shown to decrease song duration and competitive song overlap in adult male great tits (*Parus major*), but neural changes associated with this delayed behavioral response to ectoparasitism have yet to be explored (Bischoff et al., 2009). Ectoparasitism also induces other important developmental consequences in nestlings such as increases in

levels of the pro-inflammatory cytokine interleukin 6 (IL-6) (McDonald et al., 2019) as well as decreases in body mass, aspects of beak development, hemoglobin production, hematocrit levels, and bacterial killing ability (Pryor & Casto, 2015; Kleindorfer et al., 2019; Pryor & Casto, 2017). These developmental consequences might not be solely direct effects of the ectoparasites as alterations in parental behavior in response to ectoparasites may have indirect phenotypic effects. Parents could increase the amount of food delivered to the nest, possibly to help offset any energetic costs that ectoparasites can produce (Tripet & Richner, 1997). Alternatively, parents may pursue the opposite strategy and withhold provisioning as the amount of time devoted to brooding or otherwise spent at the nest may be negatively impacted by the presence of ectoparasites, thus indirectly impacting nestling development (Kleindorfer et al., 2021, Avilés et al., 2009), or parents may opt for neither strategy (Hornsby et al. 2013). While many of these ectoparasite-induced trade-offs have been well studied, trade-offs between growth and development of brain regions related to song learning, production, and perception have received comparatively little attention to date (Spencer & MacDougall-Shackleton, 2011), especially in free-living birds.

1.4 Research objectives

In this research, we studied a natural developmental stressor, ectoparasite infestation, in nestling European starlings, a cavity-nesting songbird species in which males have been previously shown to produce songs that honestly signal exposure to early developmental stress (Buchanan et al., 2003). By experimentally altering the magnitude of northern fowl mite (*Ornithonyssus sylviarum*) infestations in nests, we

investigated whether ectoparasitism influences the volume of the developmentally sensitive song control region, HVC. Northern fowl mite infestations during development are common among many birds, especially cavity-nesting species and those that nest in colonies (Murillo & Mullens, 2017), and regularly occur in the local starling populations of central Illinois (Pryor & Casto, 2015; Pryor & Casto, 2017). This, coupled with the extensively studied song and mating behavior of starlings, where adult female starlings select mates based on the quality, complexity, and length of the mate's learned songs (Mountjoy & Lemon, 1996; Gentner & Hulse, 2000), allow us to determine the effects of ectoparasitism on brain development associated with a sexually selected courtship signal, birdsong.

This study aims to investigate the effects of ectoparasitism on the size of two song control nuclei, HVC and RA, in male and female starlings. We hypothesize that ectoparasites act as developmental stressors that alter the growth of HVC and RA in both male and female songbirds. Specifically, we predict that nestlings experiencing more intense ectoparasite infestations will exhibit smaller HVC and RA volumes compared to those with reduced ectoparasitism, while still showing a species-typical sex difference (Casto, 2001). This study builds on previous research showing that ectoparasites can induce developmental trade-offs in birdsong (Bischoff et al., 2009), which is regulated in part by HVC and is a sensitive indicator of male quality (Nowicki et al., 2002). By analyzing the brains of starlings from nests with varying levels of mite infestation, this study can shed light on the effects of ectoparasitism on brain development and related mechanisms of song learning in birds.

2. Methods

2.1 Study population and general procedures

During the 2020 breeding season**,** April 29th through July 13th, we studied 83 European starling nestlings born to adults breeding in 19 out of our lab's 146 nest boxes located across four field sites at Illinois State University (ISU) in McLean County, Illinois. We monitored nests for egg-laying and hatching, and after egg-laying had ceased, the nest boxes were alternately assigned to an experimental treatment to maintain relatively equivalent sample sizes. Then, we followed chicks throughout their nestling growth and assessed their structural and physiological development at brood-days10 and 20. The first day on which the majority of the eggs in a nest had hatched was designated as brood-day 0 for the nest (A summary nestling cycle and our field experimental design is outlined in Figure 1).

2.2 *Nest treatments*

In order to experimentally induce increased and reduced Northern fowl mites (NFM; the most common ectoparasite in our starling colonies) infestations, after the female laid the final egg of a clutch (by noting the first morning after the start of egg laying on which no new egg was added to the nest), we randomly assigned nests to treatments and treated nests with NFM to create a NFM infestation or miticide to reduce any existing NFM populations.

Mite-treated nests: We added approximately 50 unfed NFM collected from locally abandoned starling nests or nest material from nests of the prior breeding

season. On brood-days 0, 5, and 10, we temporarily removed nestlings and sprayed those nests with ≈ 5 mL of distilled water to control for the disturbance associated with miticide application. 38 nestlings across 10 nests received the mite-treatment.

Miticide-treated nests: On brood-days 0, 5, and 10 we temporarily removed nestlings before we applied ≈ 5 mL of a 0.5% solution of the miticide permethrin (FarmGard™, Country Vet, Atlanta, GA) in distilled water. 45 nestlings across 9 nests received miticide-treatment.

2.3 *Nestling marking and somatic growth*

We monitored nest boxes for hatching daily and using a nail clipper, clipped a unique single toenail per hatchling to serve as a within-nest identifier. On brood-days 10 and 20, we measured the structural growth using a stopped wing rule to measure wing length, digital calipers to measure tarsus length, and a digital scale to weigh body mass of nestlings from both treatments, and on brood-day 10, nestlings were also banded using a banding pliers with individually numbered U.S.G.S. aluminum leg bands, which then served as their permanent unique identifier.

2.4 Blood collection and analysis

Across both treatments, on brood-days 10 and 20, we collected approximately 100 µL of blood from each nestling into heparinized microcapillary tubes via brachial

venipuncture with a 26G needle and stored it on ice for up to several hours prior to processing. When we returned to the lab, the microcapillary tubes were centrifuged at 17 g for 10 min to separate blood plasma and red blood cells. We then measured hematocrit using a micro-capillary reader (I.E.C 2201, Damon IEC, Needham Heights, Ma), harvested the plasma and stored it at -80°C for purposes unrelated to this study.

2.5 *Brain collection and histology*

On each nest's brood-day 20, starling nestlings from both treatments were collected from the nestbox and euthanized by carbon dioxide asphyxiation followed by decapitation, and their brains were immediately harvested and promptly frozen on pulverized dry ice using an unfixed, fresh-frozen method for processing and preservation (Paletzki & Gerfen 2019). After this rapid freezing, we wrapped these brains in aluminum foil and stored them at -20°C until processing. In order to reduce bias, throughout all stages of histological analysis, each brain was identified only by a unique identity code devoid of information about the treatment group or sex of the nestling from which it came. Prior to cryosectioning, we measured frozen brain mass to the nearest hundredth gram on a digital balance. We then sagittally bisected the brains and preferentially mounted the left hemisphere, but if there was substantial damage to the left hemisphere we mounted the right hemisphere to a cryostat using O.C.T compound (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan). We sectioned these frozen brains on a cryostat chuck (Microm Hm 550, Microm Inc Minneapolis, MN) and mounted every third 40 µm-thick sagittal section on gelatin-subbed microscope slides. We stained the resulting brain sections with a 1% thionin solution, cover-slipped them,

and digitally photographed them using a dissecting microscope (Leica DFC300 FX, Leica Microsystems, Wetzlar, Germany) equipped with a CCD camera (Lecia DFC320, Leica Microsystems, Wetzlar, Germany). From these digital images, contrast and brightness were adjusted to better visualize the borders of song control nuclei of interest (LAS V4.0 software, Leica Microsystems, Wetzlar, Germany). With the aid of several oscine brain altlases, (Nixdorf-Bergweiler & Bischof, 2007; Stokes et al., 1974; De Groof et al. 2016) we identified images of brain sections containing HVC and RA. Using ImageJ software (LOCI, Madison, WI), we scaled pixels to millimeters by calibrating the measurement function with a digital image of a stage micrometer that was collected at the same magnification and in the same manner as the images of brain sections. We then estimated the volume of two brain regions, HVC and RA by tracing the perimeter of each nucleus on the digitized images to determine their cross-sectional area, and then multiplied each area measurement by the distance between successive section images (usually 120 µm) to obtain a nucleus volume estimate for that sampling interval, and finally summed all volume estimates from a nucleus to yield an overall nucleus volume estimate. If a section was lost or damaged, we used a subsequent section to estimate the missing area and adjusted sampling intervals accordingly. In cases where the cross-sectional area was unmeasurable due to tissue damage or staining anomaly, we estimated the area by averaging the measured areas of the sections collected before and after them. Overall, this method allowed us to estimate the volumes of HVC and RA despite occasional section loss or damage.

Sexing of Nestlings: Concurrent with brain collection, we dissected the body cavity of each nestling, and used the presence of bilateral testes as a positive identifier of male nestlings; in the absence of testes, nestlings were sexed as female.

2.6 Mite load assessment

After some time without an available host, NFM often tend to become inactive, but commence movement in response to vibration, and exhibit positive thermotaxis (Owen & Mullens, 2004; McDonald et al., 2018). To quantify mite load in a nest once nestlings had been removed on brood-day 20, we waited 24 hours, knocked twice on the wall of the nestbox to stimulate NFM locomotion, waited 10 seconds, then placed a warmed, reusable, water-filled, plastic ice cube in the center of the nest depression, and closed the nestbox door for one minute to attract and sample mites. Using forceps, we then transferred the cube and any mites on it into a jar of 70% ethanol to kill and preserve the mites (Schauff, 1986) and later we filtered the ethanol and counted and categorized NFM as either fed or unfed by the presence or absence of reddish coloration, an indication that a blood meal had been taken. To date, in our nestboxes, this sampling method has only led to the collection of NFM, and no other arthropods.

2.7 Statistical analysis

We used linear mixed models utilizing a Kenward-Rodger correction of degrees of freedom associated with the F–statistic in order to minimize type-1 errors (McNeish 2017) with a random effect of nest, when applicable, to analyze nest treatment effects,

sex effects, and any interaction of sex and nest treatment where applicable on the dependent variables of HVC volume, RA volume, body mass regression-adjusted brain mass, somatic growth parameters, mite load, and blood parameters using Statistical Analysis System software (SAS Institute, Cary, NC). Nestling survival between nest treatments was compared using a log-rank test.

3. Results

Because gonadal sexing was performed on brood-day 20 after some attrition of nestlings that were included in analyses of data collected on brood-day 10, for which nestling sex was unknown, we separately analyzed data collected from nestlings on brood-day 10 and brood-day 20. Furthermore, we harvested the nestlings' brains at brood-day 20, so any data on brain mass or volume of song control nuclei was only collected on nestlings surviving to brood-day 20 and analyzed with the rest of the brood-day 20 somatic growth parameters.

3.1 Experimental nest treatments

The experimental nest treatments (*i.e.,* mite-treated and miticide-treated) were successful in creating a significant difference in total sampled mite loads between treatments ($F_{17} = 9.29$, $p = .0458$, figure 2). Mite-treated nests had significantly higher sampled mite-loads than miticide-treated nests. This overall effect held true for both mites that had taken blood meals ($F_{17} = 6.52$, $p = 0.0441$) and those that had not ($F_{17} = 17.9$, $p = 0.00409$).

3.2 Nestling survival analysis

There was no significant effect of nest treatment on the post-hatching survival of nestlings in our treatments ($\chi^2 = 0.949$, DF = 1, $p = 0.329$, figure 3). Seven nestlings from mite-treated nests and five nestlings from miticide-treated nests died before brood-day 20 (figure 3).

3.3 Nestling somatic growth and physiological development on brood-day 10

There was a significant effect of nest treatment on wing length ($F_{1,81} = 2.17$, $p = 0.0237$, figure 4A) and body mass ($F_{1,81} = 4.55$, $p < 0.0001$, figure 4B) of nestlings on brood-day 10, but no significant effect of nest treatment on tarsus length ($F_{1,81} = 1.38$, $p = 0.404$, figure 4C). Wing length was shorter and body mass was lighter were in mite-treated nests when compared to miticide-treated nests. There was also a significant effect of sex on body mass with female nestlings weighing approximately 5% less than male nestlings ($F_{1, 81} = 6.44$, $p = 0.0237$). There were no significant effects of sex on wing length ($F_{1,81} = 1.62$, $p = 0.208$) or tarsus length ($F_{1, 81} = 1.2$, $p = 0.279$) on brood-day 10 and there was no significant interaction of nest treatment and sex for wing length ($F_{1,79} = 1.08$, $p = 0.397$), body mass ($F_{1, 79} = 1.08$, $p = 0.393$), and tarsus length ($F_{1,79} = 1.2$, $p = 0.747$).

There were no significant effects of nest treatment ($F_{1, 81} = 3.47$, $p = 0.138$, figure 4D), sex ($F_{1, 81} = 0.43$, $p = 0.5142$), or interaction of nest treatment and sex ($F_{1,79} = 1.68$, $p = 0.12$) on hematocrit levels on brood-day 10.

3.4 Nestling somatic growth and physiological development on brood-day 20

On brood-day 20, there was no significant effect of nest treatment on wing length ($F_{1,70}$ = 2.66, p = 0.157), body mass ($F_{1,73}$ = 1.24, p = 0.456), and tarsus length ($F_{1,72}$ = 1.23, p = 0.464) and no interaction of nest treatment and sex on wing length ($F_{1,68}$ = 1.97, p = 0.066), body mass ($F_{1,71}$ = 0.92, p = 0.519), and tarsus length ($F_{1,70}$ = 1.03, p = 0.432) either. However, there was a significant effect of sex on wing length ($F_{1,70}$ = 6.44, p = 0.0147, figure 5A), body mass ($F_{1,73}$ = 9.04, p = 0.0043, figure 5B), and tarsus length ($F_{1,72}$ = 14.5, p = 0.0004, figure 5C) with males having significantly longer wing and tarsus length and greater body mass than females.

There was a significant effect of nest treatment on hematocrit ($F_{1,72}$ = 12.78, p = 0.0028, figure 6) on brood-day 20 with nestlings in mite-treated nests having significantly less hematocrit than those in miticide-treated nests. There was no significant effect of sex ($F_{1,72}$ = 2.65, p = 0.109) on hematocrit and no significant interaction between nest treatment and sex $F_{1,70}$ = 0.12, p = 0.735) for hematocrit on brood-day 20.

3.5 Brain growth, HVC and RA volume

We found a significant effect of nest treatment on body mass regression-adjusted brain mass ($F_{1,73}$ = 6.66, p = 0.0463, figure 7A) with nestlings in mite-treated nests having significantly less brain mass than miticide-treated nests. There was also an effect of sex on brain mass ($F_{1,73}$ = 34.2, p < 0.0001), with female nestlings having less

brain mass than male nestlings. There was no interaction of nest treatment and sex for brain mass ($F_{1,71}$ = 0.01 p = 0.943). We found a significant effect of nest treatment on the volumes of RA ($F_{1,62}$ = 10.27, p = 0.0063, figure 7C) and HVC ($F_{1,63}$ = 7.81, p = 0.0154, figure 7B), with RA and HVC volumes of nestlings in miticide-treated nests being significantly greater than those in mite-treated nests. We also found a significant effect of sex on HVC and RA volumes ($F_{1,63}$ = 32.08, p < 0.0001 and $F_{1,62}$ = 25.19, p < 0.0001, respectively); with female nestlings having smaller HVC and RA volumes than male nestlings. There was no interaction of nest treatment and sex on HVC or RA volume ($F_{1,61}$ = 1.06, p = 0.308, and $F_{1,60}$ = 0.71, p = 0.402, respectively).

4. Discussion

This study aimed to investigate the role of ectoparasites as developmental stressors and their impact on the development of critical song control nuclei in male and female songbirds. We hypothesized that nestlings experiencing more intense ectoparasite infestations would exhibit reduced volumes of HVC and RA, and that this effect would be observed in both sexes. We found that this prediction was supported, with nestlings from miticide-treated nests showing significantly greater volumes of HVC and RA than those from mite-treated nests, and this effect was observed in both male and female nestlings. Furthermore, there were differences in physical and physiological development observed in nestlings on brood-day 10 between treatment groups that were not present at brood-day 20, suggesting that aspects of physical growth may be related to decreases in the volumes of song control nuclei in parasitized nestlings. The

data suggest that ectoparasites do act as developmental stressors and alter the development of HVC and RA in songbirds.

4.1 Efficacy of experimental treatments

The experimental treatments were successful in creating differences in mite load and these differences likely contributed to the observed phenotypic differences. Specifically, the miticide treatment yielded lower mite loads, while the mite treatment yielded higher mite loads. Despite these differences, however, survival of nestlings was not statistically different between the mite-treated and miticide-treated nests, and there was no significant difference in mortality between the experimental nest treatments. Given that survivor bias was mitigated and there was a clear difference in mite load, it is reasonable to conclude that the observed phenotypic differences were likely due to variation in experimentally induced mite infestation.

4.2 Retarded brain development as an associated cost of compensatory somatic growth

When normal growth rates are suppressed by developmental perturbation, such as occurs in response to ectoparasitism of European starlings (Pryor & Casto, 2015), organisms may undergo a subsequent period of rapidly increased structural growth to match the physical requirements of a developmental benchmark (Wilson & Osborn,1960). This abnormally rapid growth to reach a stage of maturation is known as compensatory somatic growth and has been widely observed across taxa. In nestling birds, a major developmental benchmark is that of fledging, which requires advanced

physical maturation (Heers 2016). Not surprisingly, ectoparasite-induced compensatory somatic growth has been associated with reduced physiological development in the form of anemia (Pryor & Casto, 2015) as erythropoiesis is a developmental process that overlaps temporally with somatic growth (Cornell et al., 2017) and brain growth, including the relatively late development of the song system in young songbirds (Alvarez-Buylla et al. 1994). Since brain development in altricial songbirds overlaps with somatic growth, it might be reduced when compensatory somatic growth becomes necessary. Measures of somatic growth were significantly smaller in nestlings from mite-treated nests on brood-day 10, but these same measures were statistically indistinguishable from nestlings of miticide nests on brood-day 20, just prior to fledging, providing strong evidence for compensatory somatic growth. The smaller mean brain masses and volumes of HVC and RA in nestlings from mite-treated nests at brood-day 20 suggests that reduced or delayed brain development is likely an associated cost of compensatory somatic growth in nestlings, but just like compensatory somatic growth, compensatory brain growth may not be as effective as the stressor-free ontogenetic growth it replaces (Metcalfe & Monoaghan, 2001). Further research is needed to determine if a form of compensatory brain development could ameliorate some of the early effects of ectoparasitism on HVC and RA volume that we report here, and whether such later compensatory development of the song system has associated behavioral consequences.

4.3 Effects of developmental stressors on starling song, its perception, and the underlying neural mechanisms

Developmental stress has previously been shown to have negative effects on birdsong in male European starlings. Food restriction during development has been found to be correlated with repertoire size in male starlings. Specifically, birds subjected to an unpredictable food supply early in life produced a significantly smaller repertoire of song phrases than those with a constant food supply (Buchanan et al., 2003). Additionally, juvenile male starlings raised in captivity, who experienced unpredictable short-term food deprivation, spent less time singing, sang fewer song bouts, took longer to begin singing, and had significantly shorter song bouts during the following spring (Spencer et al., 2004). Taken together, these earlier studies suggest that developmental stress, in the form or food limitation, significantly affects the quality and complexity of male song in European starlings, but as song system growth was not assessed in these studies, the underlying mechanisms for these effects remained unresolved. The current study is the first to demonstrate that developmental stress reduces growth of the song system in male starlings (but see Farrell, 2015, chapter 5, a dissertation chapter comparing the effects of developmental food limitation on song control nuclei volume in male starlings at juvenile and adult stages of development). Adult song was not assessed in the males studied here due to the early age at which their brains were collected for analysis. However, had the reduced growth of HVC and RA in males experiencing high levels of ectoparasitism persisted through the remainder of song system development, which continues throughout much of the first year of life (Casto and Ball, 1996), we might expect to find negative effects on their songs similar to those

mentioned above; although there is evidence to suggest that male European starlings given an unpredictable food schedule had smaller song-control regions than control birds when juveniles, but not as adults. The treatment, however, did not affect song bout length (Farrell 2015; findings also demonstrated in song sparrows, Schmidt et al. 2014).

Research has also indicated that developmental stress can have analogous effects on the song preferences of female birds. Specifically, female European starlings exposed to unpredictable food stress during their early months of life displayed a reduced preference for songs of their own species compared to those raised in control conditions. Additionally, when listening to conspecific song, control females exhibited significantly higher levels of Zenk (an immediate early gene) induction in auditory forebrain areas than food-restricted females. However, no significant differences in the sizes of the song-control regions were observed between the two groups (C.F. Farrell et al., 2015). When considered along with these previous data, our results suggest that developmental perturbations during the very early post-hatching period, prior to parental independence, significantly retard the growth of the female song system, investigations of later effects on song preferences are warranted.

4.4 *Summary, conclusion, and future directions*

This research found significant effects of ectoparasitism on development of critical song control nuclei as predicted by the developmental stress hypothesis. Previous studies have established ectoparasites as mediators of developmental trade-offs between structural growth and immune function (Pryor & Casto, 2015) as well as

the effects of ectoparasites on aspects of song quality (Bischoff et al. 2009), but none have examined their effects on the volume of brain regions involved in song learning, production, perception, and discrimination. We provide evidence of ectoparasite-induced changes in the volume of song regions in free-living nestlings. This evidence could help to link the proximate responses to ectoparasitism with the sexual selection of male song quality. Furthermore, this appears to be the first study to test the effect of ectoparasitism on brain development and it extends examination to both sexes. We also demonstrated that although sex differences exist in brood-day 20 nestling somatic growth, brain weight, HVC volume, and RA volume, these do not appear to be due to sex-specific responses to ectoparasite-induced developmental stress. Similar experimental manipulations of ectoparasite removal have been shown to have a sex-specific effect in nestling tree swallows (*Tachycineta bicolor*), in which males had longer telomeres than females at 12 days of age under experimental ectoparasite removal (Wolf et al., 2023) and in great tits, in which females had longer telomers than males in deparasitized nests, but not in parasitized nests(Tschirren et. al 2021). It is imperative that research on animal signaling, especially birdsong in which mechanisms for female song production and song perception- are often understudied or neglected in comparison to male songbirds and male birdsong, is sex inclusive in order to promote understanding of the sex specificity, function, and evolution, of these signals (Reibel, 2019). Until the functions of female song system are better understood, we will not be able to fully appreciate the likely implications of the effects of ectoparasitism on the female song system development, but the effects appear similar in their relative

magnitude to those seen in males suggesting perhaps similar underlying developmental mechanisms.

In addition, this study investigated nestlings at developmentally sensitive ages while in the field, shedding light on critical periods of song system development in a natural setting. Ectoparasitism at the nestling stage might allow parents the opportunity to intervene on behalf of their young with additional provisioning that might offset energetic deficits associated with ectoparasitism, due to blood cell replacement and immune system activation (Tripet & Richner, 1997).

The choice of developmental stressors used in studies such as this often involves trade-offs between ecological relevance and experimental control (MacDougal-Shackleton 2015). Our experimental manipulation of NFM infestations in treated nests resulted in arguably high ecological relevance as mite infestations might have been somewhat limited in the current study as ectoparasites could influence both nestlings and the parents attending the manipulated nests, so not all similarly intense infestations would be expected to have similar effects on nestling development as parental response could vary.

Alternatively, ectoparasites might reduce parental investment through actions like provisioning at the nest and set up an indirect form of early nutritional stress and although it has been shown in Darwin's Finch species that female, but not male parental visits and time spent on the nest was negatively impacted by ectoparasite prevalence (Avilés et al. 2009; Kleindorfer et al., 2021). Nevertheless, ectoparasites might not affect the rates of parental investment (Hornsby et al. 2013) and further research on the

effects of ectoparasites on parenting is warranted to better understand the specific nature of the stresses that were induced.

Future studies can explore the mechanisms of the induced trade-off of HVC volume and immune function, examine the acoustic behavior responses of female songbirds under ectoparasitic infestation, and investigate other neural developmental sexual differences in response to ectoparasitic perturbation. Most research on the developmental stress hypothesis has utilized food restriction in captive males and females as a developmental stressor beginning at around the time of fledgling independence to study the underlying neural development of song (Nowicki et al., 2002), this research extends our understanding of the factors that may influence early brain development to other natural developmental perturbations, in both sexes at earlier developmental ages, and in more natural developmental environments.

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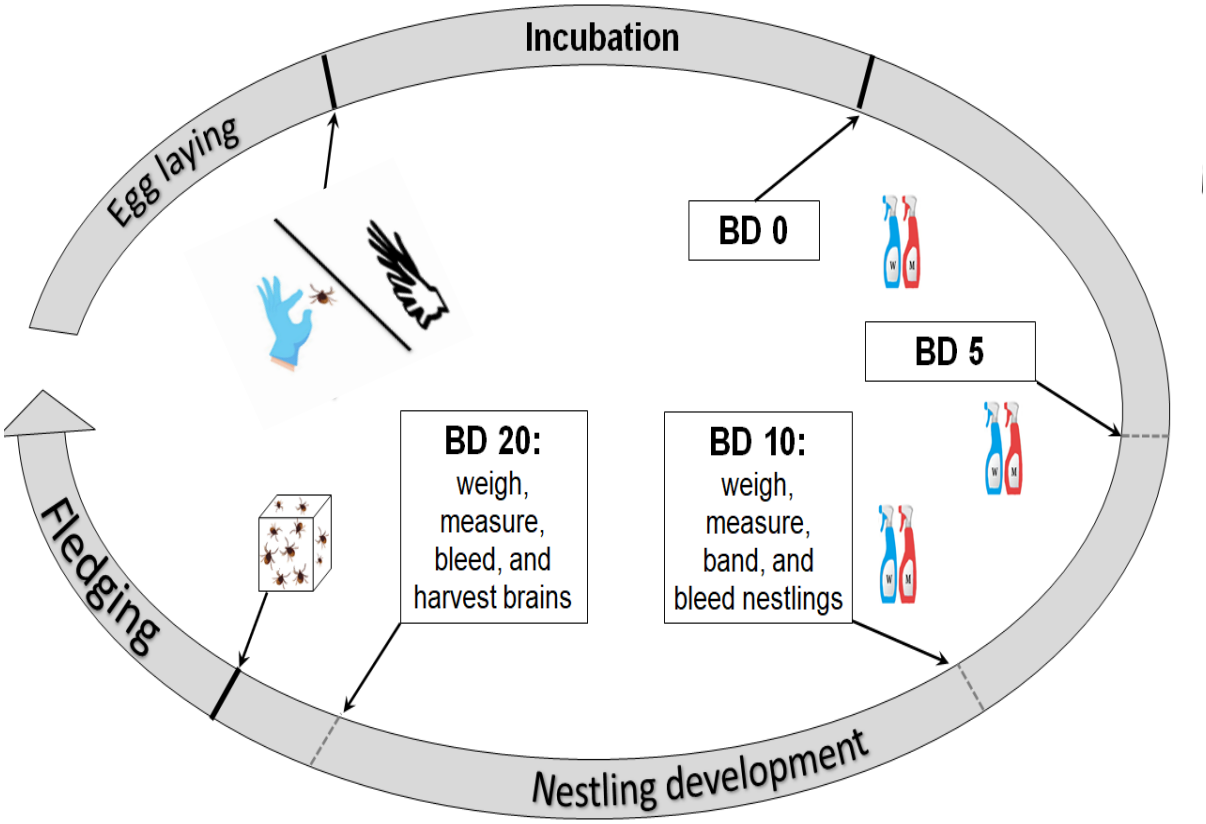
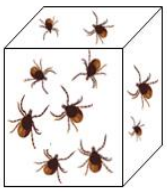
Figures:



Add ≈50 unfed mites to *mite-treated* nests or create a nest disturbance for *miticide treated* nests

Apply treatments to nests, ≈ 5 mL of either:
Distilled water (mite-treated nests)
Or
Miticide (0.5% permethrin solution)



Sample mite abundance using a warmed reusable water-filled plastic ice cube 24 hours after nestling removal on BD 20.

Figure 1 the experimental design and set-up for the field collection portion of this research. The gray circular arrow illustrates a typical nesting cycle. The stages of nesting and the relative timing of manipulations and measurements carried out during the experiment are depicted. The key below the graphic describes the treatments applied to the nests. These treatments were administered at the various times depicted along the nesting cycle. The boxes within the nesting cycle indicate the dependent variables that were measured/assessed/collected on specific brood-days.

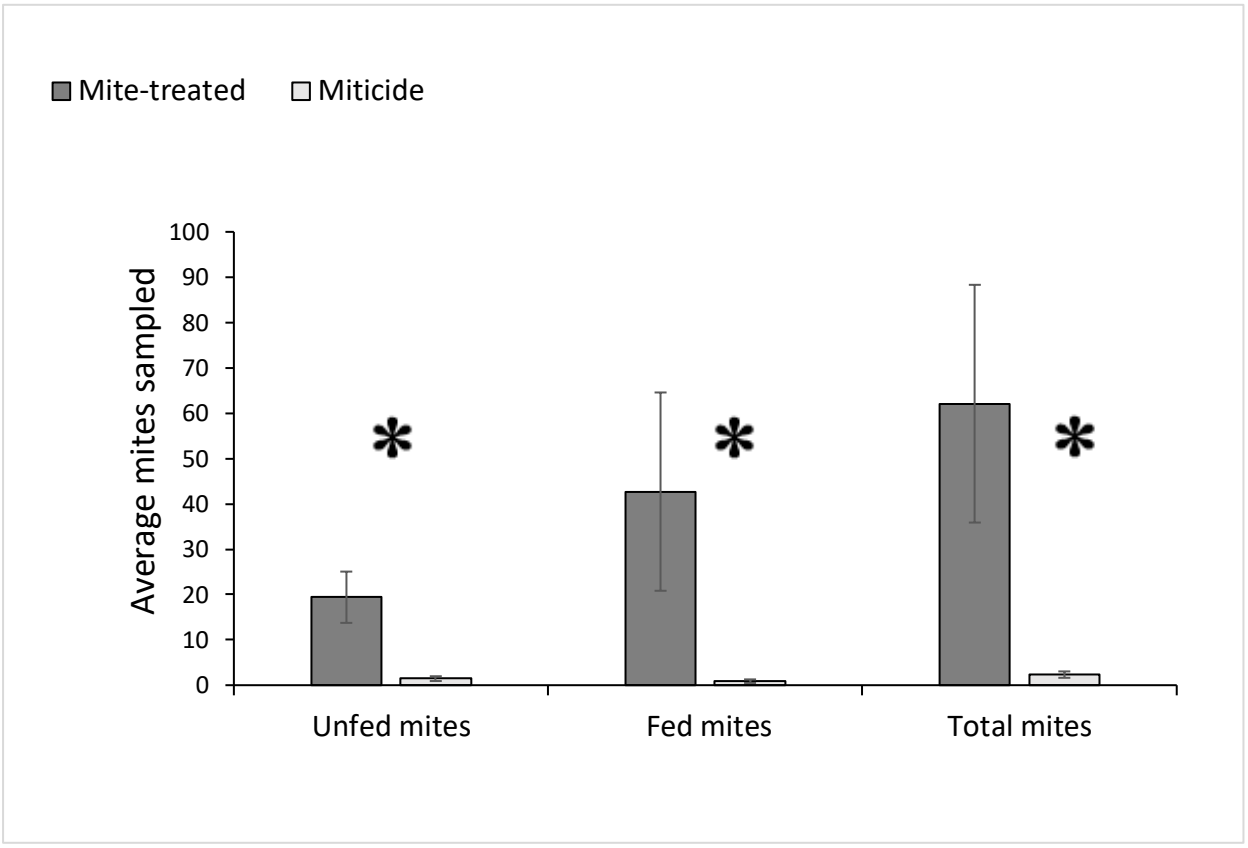

Mite-treated
Miticide
Average mites sampled
100
90
80
70
60
50
40
30
20
10
0
*
*
*
Unfed mites
Fed mites
Total mites

Figure 2 the average number of mites sampled per nest for each treatment. The average number of mites is separated into those that have not taken a blood meal (unfed), those that have taken a blood meal (fed) and then these two groups are summed to yield average total number of mites in a nest. The asterisks denote a significant difference.

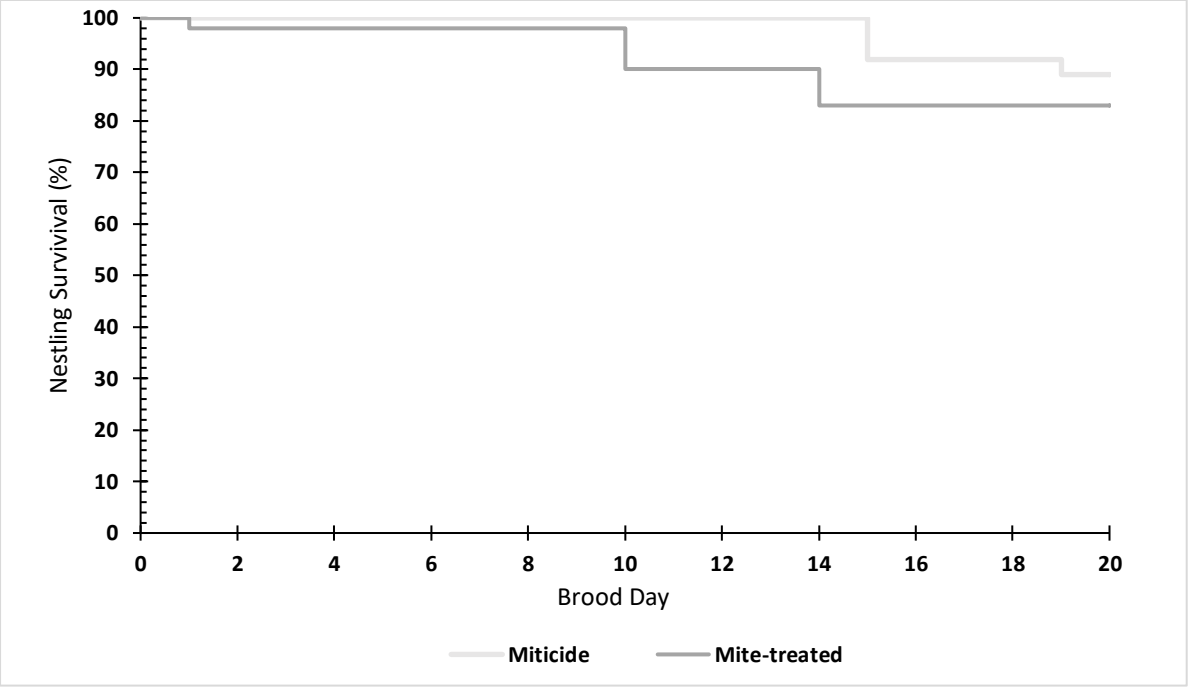

100
90
80
70
60
50
40
30
20
10
0
Nestling Survivival (%)
0
2
4
6
8
10
12
14
16
18
20
Brood Day
Miticide
Mite-treated

Figure 3 a Kaplan-Meier plot illustrating the survival of nestlings from both treatments across the first 20 days of nestling development.

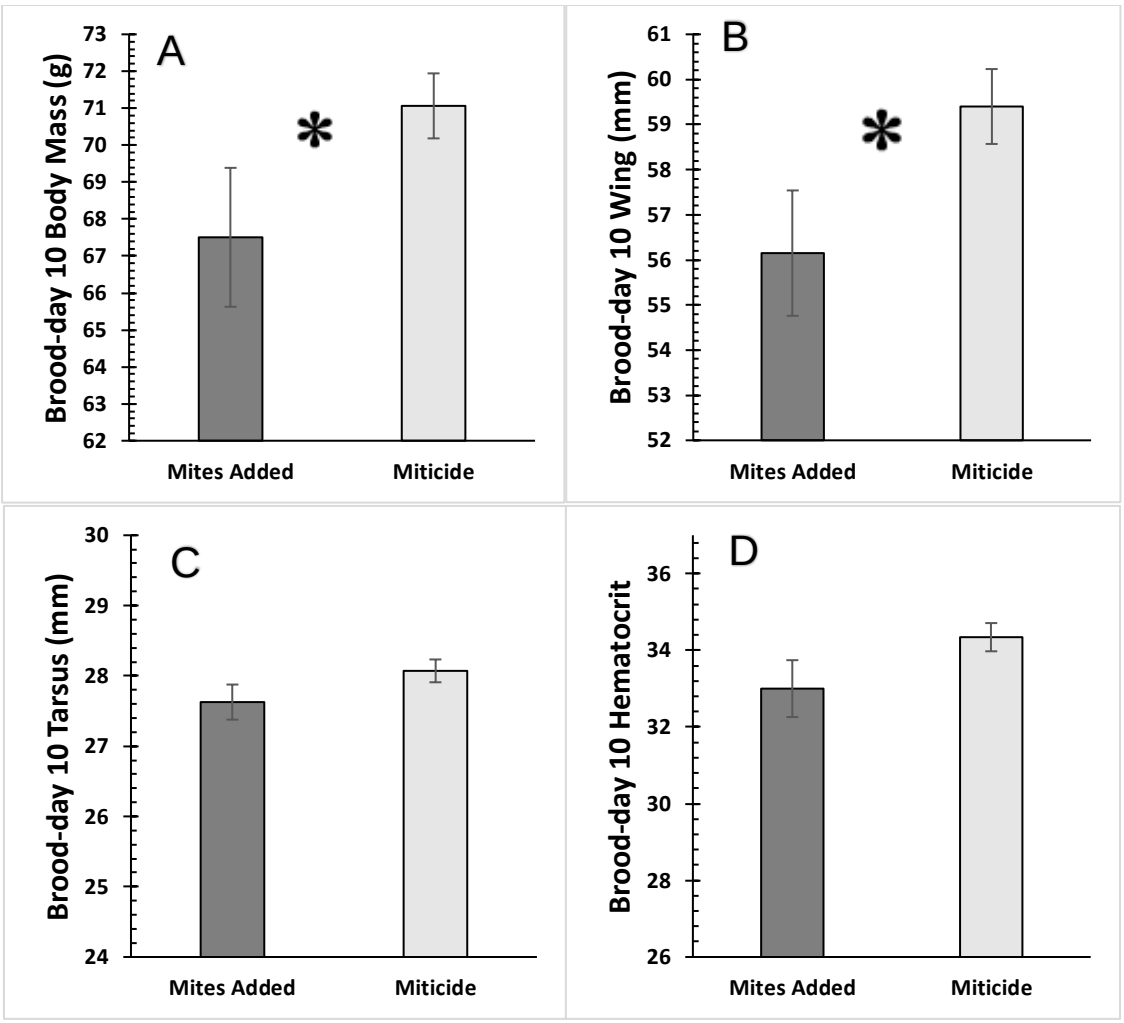

A
Brood-day 10 Body Mass (g)
73
72
71
70
69
68
67
66
65
64
63
62
*
Mites Added
Miticide
B
Brood-day 10 Wing (mm)
61
60
59
58
57
56
55
54
53
52
*
Mites Added
Miticide
C
Brood-day 10 Tarsus (mm)
30
29
28
27
26
25
24
Mites Added
Miticide
D
Brood-day 10 Hematocrit
36
34
32
30
28
26
Mites Added
Miticide

Figure 4 (A-D) the physical parameters recorded on brood-day 10 of our experiment separated by treatment. (**A**) The average nestling body mass in grams; (**B**) represents the average wing cord length in millimeters; (**C**) the average nestling tarsus length; (**D**) the average hematocrit levels. The asterisks denote a significant difference.

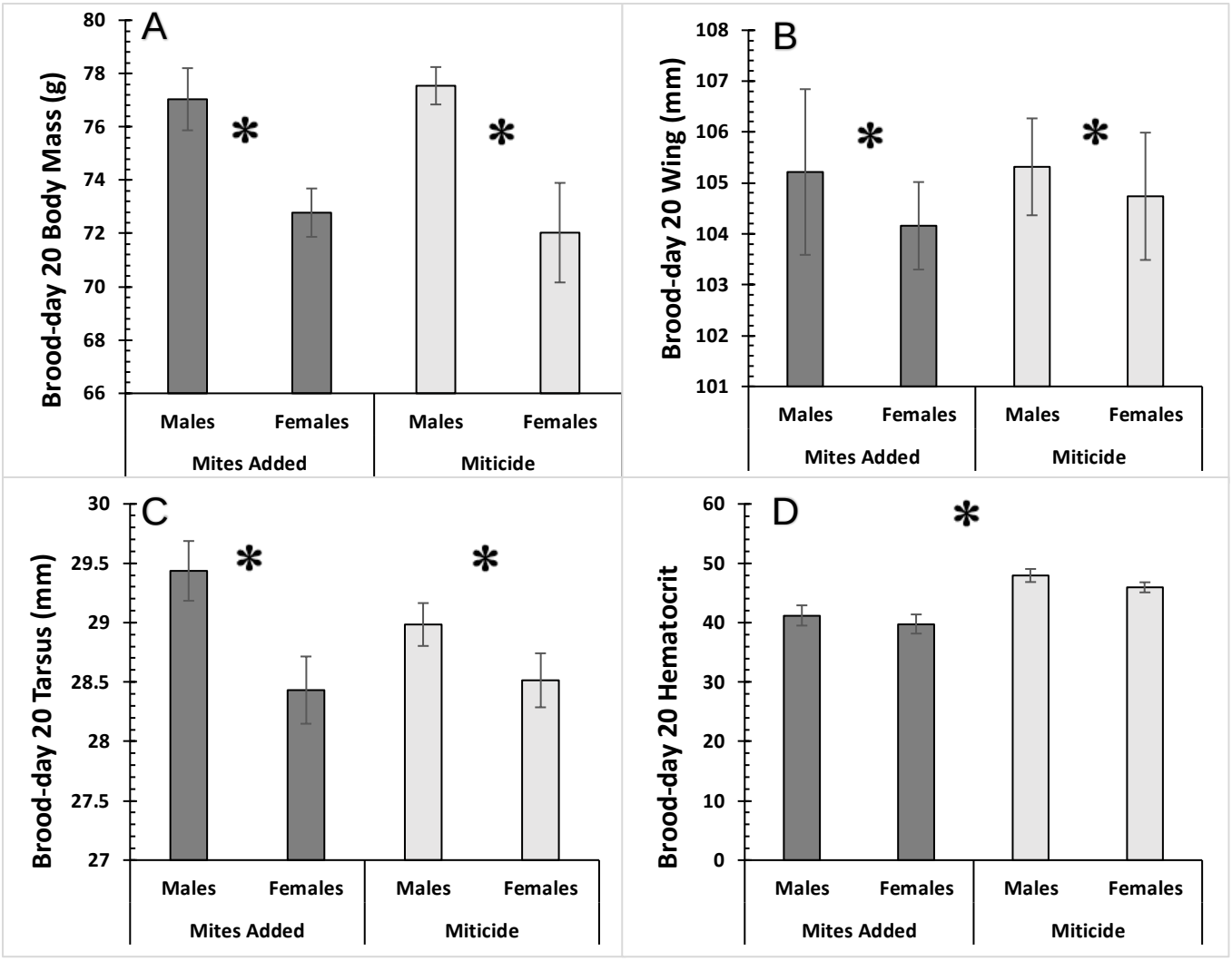
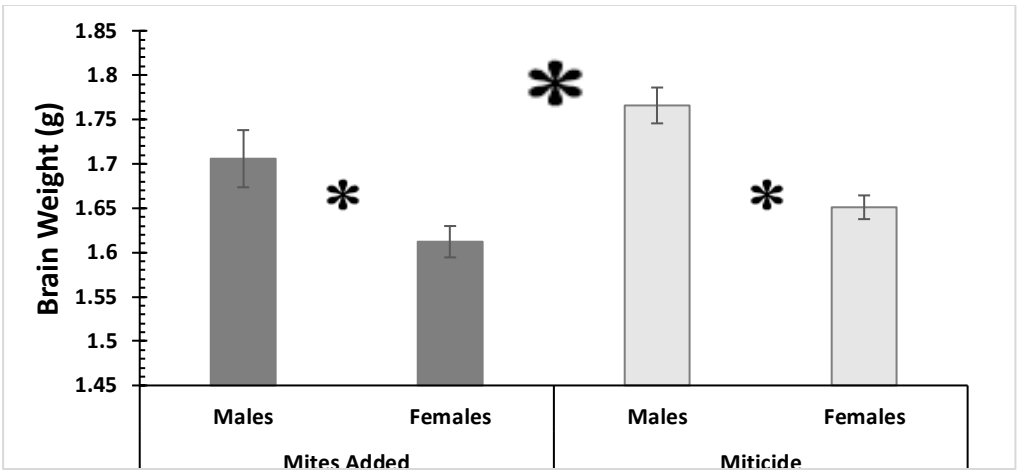
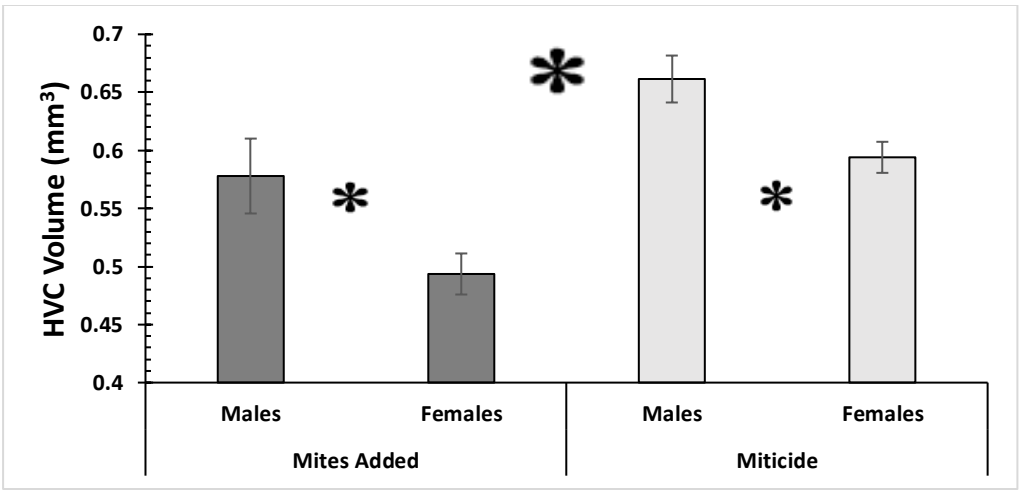

A
Brood-day 20 Body Mass (g)
80
78
76
74
72
70
68
66
*
*
Males
Females
Males
Females
Mites Added
Miticide
B
Brood-day 20 Wing (mm)
108
107
106
105
104
103
102
101
*
*
Males
Females
Males
Females
Mites Added
Miticide
C
Brood-day 20 Tarsus (mm)
30
29.5
29
28.5
28
27.5
27
*
*
Males
Females
Males
Females
Mites Added
Miticide
D
Brood-day 20 Hematocrit
60
50
40
30
20
10
0
*
Males
Females
Males
Females
Mites Added
Miticide

Figure 5 (A-D) the physical parameters recorded on brood-day 10 of our experiment separated by treatment and sex within each treatment. (**A**) The average nestling body mass in grams; (**B**) represents the average wing cord length in millimeters; (**C**) the average nestling tarsus length; (**D**) the average hematocrit levels. The asterisks denote a significant difference either between sex or between treatment.


Brain Weight (g)
1.85
1.8
1.75
1.7
1.65
1.6
1.55
1.5
1.45
Males
Females
Males
Females
Mites Added
Miticide


HVC Volume (mm³)
0.7
0.65
0.6
0.55
0.5
0.45
0.4
Males
Females
Males
Females
Mites Added
Miticide

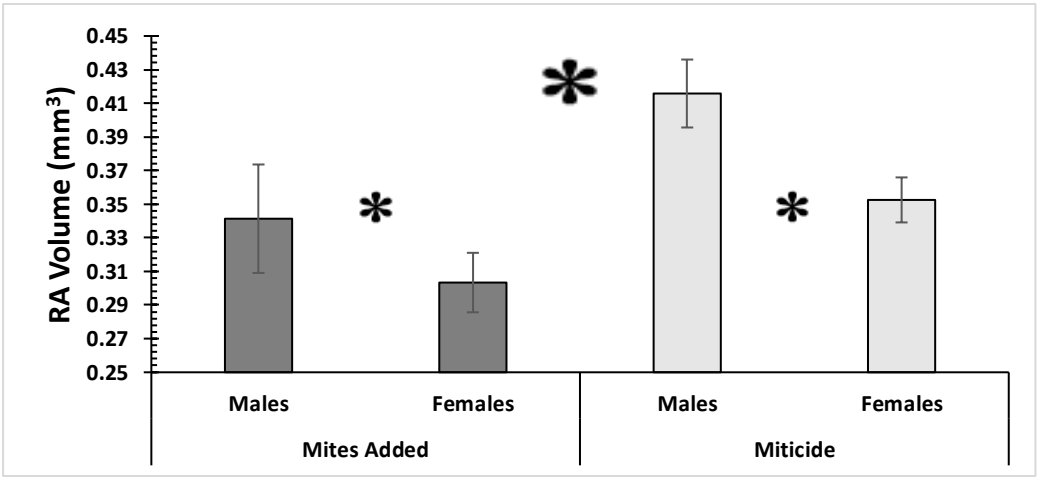

RA Volume (mm³)
0.45
0.43
0.41
0.39
0.37
0.35
0.33
0.31
0.29
0.27
0.25
Males
Females
Males
Females
Mites Added
Miticide

Figure 6 (A-C) the parameters relating to brain weight and song control nuclei volumes in the brain separated by treatment and sex within each treatment; (**A**) the average nestling brain weight in grams on day 20. These are the raw brain weights are not regressed onto body mass to give a more tangible measurement; (**B**) the average nestling volume of HVC in millimeters cubed on day 20; (**C**) the average nestling volume of RA in millimeters cubed on day 20. The larger asterisks represent a statistically significant difference between treatments, while the smaller asterisks represent a statistically significant difference between sex.