

HOST RECOGNITION AND FITNESS COSTS OF AVIAN BROOD PARASITISM

BY

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DISSERTATION

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ABSTRACT

One of the richest opportunities for studying coevolution comes from obligate brood parasitism, a unique reproductive strategy performed by only 1% of the world's bird species. Brood parasites are birds that lay their eggs into the nest of another species. They never care for their own young, leaving the host to care for the unrelated, parasitic offspring. Assessing and documenting how hosts are affected by and respond to brood parasitism are critical in understanding this coevolutionary process. The brown-headed cowbird (*Molothrus ater*) is an obligate generalist brood parasite that uses over 200 host species and shares the nest with host nestmates. Using integrative approaches, I characterized two components of coevolution between cowbirds and their hosts – the hosts' recognition of parasites, and the effects of the parasite on the fitness of host nestlings. In an egg-rejector host, the American robin (*Turdus migratorius*), I determined whether robin recognition and rejection of parasitic eggs involved lateralized visual processing. Critically, robins were more lateralized in their eye use when viewing mimetic eggs compared to non-mimetic eggs, and more highly lateralized robins rejected more model eggs. In contrast, the prothonotary warbler (*Protonotaria citrea*) never rejects cowbird eggs, though it is frequently parasitized. It is possible that warblers perceive the cowbird eggs as foreign, but simply do not have the ability to physically remove these eggs from their nest. However, through an experimental egg addition, I documented no difference in the circulating corticosterone levels between females given either a mimetic or a non-mimetic type of model egg or control females given no model egg, which suggests that warblers do not perceive and recognize parasitic eggs, potentially due to evolutionary lag as they are a historically recent cowbird host. To assess recognition at the nestling stage, I compared the antipredator responses of breeding warbler

parents through playback of cowbird nestlings' alarm calls, warbler nestlings' alarm calls, or bluebird nestlings' alarm calls as controls. Warbler parents responded most strongly to warbler nestling alarm calls but also responded more strongly to cowbird compared to bluebird nestlings' alarm calls. These results imply that as a generalist brood parasite, cowbirds employ a general alarm call that elicits host antipredator responses better than other heterospecifics, but not as well as the host's own species. To investigate physiological effects of cowbirds on host warbler young, nests were experimentally parasitized and physiological measurements of immune (PHA injection) and stress axis (corticosterone) responses were taken from the host nestlings. Parasitized warbler host nestlings exhibited decreased immune responses and higher mortality relative to non-parasitized controls, while their corticosterone levels and body condition were similar. These results suggest that host nestlings incur some adverse effects from parasites but are resilient in other physiological aspects. Finally, I found that when raised with a cowbird, warbler nestlings were smaller, fledged at older ages, and had greater overall fledging latency compared to nests parasitized with an additional warbler chick. Because fledging is such a critical transition between life history stages for birds, these effects could further negatively influence the fitness outputs of parasitized broods. In summary, my dissertation provides a multi-level integrative perspective on host recognition and the fitness costs of brood parasitism. This research explores the physiological understanding of species interactions and has raised exciting questions for future studies on brood parasites and coevolution.

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In loving memory of Mom and Grandpa, who always believed in me.

I hope I have made you proud.

To Dad and Grace, who supported me all the way through.

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INTRODUCTION

Parental care is a nearly ubiquitous trait among the more than 10,000 described bird species. One or both parents participate in a series of behaviors from selecting the nest location, building a nest, incubating the eggs, and feeding the chicks until independence. However, with an abundance of birds providing care to their offspring, there has also evolved a group of birds that exploit this behavior. Obligate avian brood parasitism, which has evolved independently seven times in five families and four orders, is a reproductive strategy performed by 1% of bird species (Soler 2017). These birds do not provide care to their young, and instead, lay their eggs into the nests of another species. Obligate brood parasites have many unique adaptations to avoid host detection and solicit host parental care. This rare reproductive strategy also provides an opportunity to investigate the dynamics of coevolutionary parasite-host interactions in the contexts of fitness and recognition.

Brood parasitism is undesirable for hosts as it causes resources to be diverted from their own young or future reproductive events to caring for unrelated young that do not increase their reproductive fitness. In addition to this, hosts may suffer from a myriad of fitness costs due to the act of parasitism itself as well as the presence of the parasitic chick(s). In many species, the female parasite may destroy one or more of the host's eggs before, during, or after she lays the egg in the nest to increase the chances of her offspring's survival (Sealy 1992; Davies 2000; Fiorini et al. 2014). In addition, adults may also destroy whole host broods during farming behaviors, where the parasite destroys an unparasitized brood in an attempt to cause the host to re-nest so that the new clutch can be parasitized (Arcese et al. 1996; Swan et al. 2015), or through

mafia behavior, where parasites destroy the nests of hosts that reject parasitic eggs in order to enforce acceptance and cause renesting (Hoover and Robinson 2007; Soler et al. 1995).

Brood parasitic chicks may be either evictor or non-evictors. In evictor species, the parasitic chick destroys the entirety of the host brood, whether it be through ejecting eggs or nestlings from the nest (Davies 2000) or through bludgeoning host chicks to death with a hooked beak (Spottiswoode and Koorevaar 2011). These parasites are especially costly to host fitness as their presence results in the total failure of the host brood. Non-evictor parasites, on the other hand, do not directly kill their host nestmates, and coexist alongside them in the nest. These parasites are still be harmful, however, as the typically larger, more competitive chick usurps food from host chicks or require more than a host-typical per capita rate of provisioning (Lorenzana and Sealy 1999; Hauber 2003a; Hoover and Reetz 2006). Additionally, parasitic chicks may also indirectly harm host fitness by making nests more conspicuous to predators (Dearborn 1999; Hannon et al. 2009).

To avoid detection, some brood parasites, such as the common cuckoo (*Cuculus canorus*), lay eggs that are mimetic to the host's eggs. Although many hosts of parasitic cuckoo species reject non-mimetic eggs from their nest, the majority of hosts of brown-headed cowbirds (*Molothrus ater*), which do not produce mimetic eggs, accept the cowbird eggs (Davies 2000). The two main hypotheses behind why hosts accept costly brood parasitic eggs are evolutionary equilibrium or evolutionary lag (Takasu 1998). Evolutionary equilibrium assumes that the costs of rejecting a parasitic egg outweigh the costs of accepting. Costs of rejection can include discrimination errors, in which the host mistakenly removes one of their own eggs instead of a parasitic egg.

Although recognition errors could potentially be costly, cowbird eggs do not resemble the eggs of many of their hosts, and therefore acceptance due to the costs of recognition errors may be unlikely (Rothstein, 1982; but see Rutledge et al., 2021). Reduced reproductive success could also occur due to rejection errors, in which host eggs are damaged during the removal of the parasitic egg. Indeed, cowbird eggs are thicker and harder to pierce than the eggs of other passerines (Spaw and Rohwer 1987). Brown-headed cowbirds do not evict their nestmates and are thus less virulent than evictor brood parasites, giving hosts the ability to fledge some of their own young from a parasitized nest. If the costs of raising cowbird young are small enough, and potential damage to host eggs from the ejection of cowbird eggs large enough, host acceptance could be favored. Indeed, rejector hosts of cowbirds are larger than the average acceptor host, which could make it easier for them to carefully reject a cowbird egg (Rothstein 1975a; Peer and Sealy 2004). However, even small hosts can successfully reject cowbird eggs. Warbling vireos (*Vireo gilvus*), which are typically puncture ejectors, were still able to grasp eject eggs despite their small size, suggesting that bill size may not be a constraint on grasp ejection for hosts that do not have strong enough bills to puncture cowbird eggs (Underwood and Sealy 2006). Acceptor hosts can also have other defenses against cowbirds besides egg or nestling discrimination by employing frontline defenses (Feeney et al. 2012). For example, yellow warblers nesting synchronously and near red-winged blackbirds experience less brood parasitism (Clark and Robertson 1979).

Evolutionary lag suggests that the reason that many cowbird hosts accept is because there has not been sufficient time for rejection behavior to evolve in response (Davies 2000). Hosts of cuckoos have had more time to evolve cognitive and behavioral strategies to resist cuckoo parasitism

(Davies 1999). Rothstein (1975) suggested that this is the most likely explanation for host acceptance, and there is some evidence to support this. Hosts which have been parasitized by cowbirds for longer are more likely to desert their nests after natural parasitism than hosts that have more recently come into contact with cowbirds (Hosoi and Rothstein 2000). A similar analysis investigating evolutionary lag and equilibrium in cowbird hosts also found evolutionary lag to be the best supported (Peer and Sealy 2004). However, some typically egg-acceptor hosts have still evolved cowbird-specific behaviors, like the functionally referent anti-parasitic “seet” call by yellow warblers (*Setophaga petechia*), which is produced when a cowbird is heard or spotted and results in female warblers flying back to sit on their nest, presumably to prevent cowbird parasitism (Gill and Sealy 2003). Thus, evolutionary lag cannot explain the lack of all anti-parasite behaviors by cowbird hosts.

These questions surrounding what the fitness costs brood parasites impose on their hosts are, and in turn why some hosts may accept, illustrate why avian obligate brood parasitism is a powerful system for integrative studies, as it represents a coevolutionary arms race between host recognition and defenses and parasitic evasiveness. Chapters 1 and 2 in this dissertation focus on recognition of mimetic and non-mimetic model eggs in an egg-rejector species, the American robin (*Turdus migratorius*), and an egg-acceptor species, the prothonotary warbler (*Protonotaria citrea*) through cognitive and physiological lenses. Chapter 3 investigates recognition at the nestling stage, through an audio playback experiment presenting parasitic and non-parasitic nestling alarm calls to warbler parents. Finally, chapters 4 and 5 focus on the fitness costs warbler nestlings experience when sharing the nest with a cowbird parasite, analyzed through physiological and life history changes. Together, this dissertation contributes to aspects of

parasitism that are not well understood and investigates these questions using an integrative perspective and suite of experimental methodologies.

CHAPTER 1: MIMICRY-DEPENDENT LATERALIZATION IN THE VISUAL INSPECTION OF FOREIGN EGGS BY AMERICAN ROBINS¹

1.1 ABSTRACT

Brain lateralization, or the specialization of function in the left versus right brain hemispheres, has been found in a variety of lineages in contexts ranging from foraging to social and sexual behaviors, including the recognition of conspecific social partners. Here we studied whether the recognition and rejection of avian brood parasitic eggs, another context for species recognition, may also involve lateralized visual processing. We focused on American robins (*Turdus migratorius*), an egg-rejecter host to occasional brood parasitism by brown-headed cowbirds (*Molothrus ater*) and tested if robins preferentially used one visual hemifield over the other to inspect mimetic versus non-mimetic model eggs. At the population level, robins showed a significantly lateralized absolute eyedness index (EI) when viewing mimetic model eggs, but individuals varied in left versus right visual hemifield preference. By contrast, absolute EI was significantly lower when viewing non-mimetic eggs. We also found that robins with more lateralized eye usage rejected model eggs at higher rates. We suggest that the inspection and recognition of foreign eggs represent a specialized and lateralized context of species recognition in this and perhaps in other egg-rejecter hosts of brood parasites.

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1.2 INTRODUCTION

Cerebral lateralization, first studied in detail over 150 years ago and thought to be exclusive to humans until only around 25 years ago, has since been described in a diversity of vertebrate and invertebrate lineages (Frasnelli et al. 2012; Rogers et al. 2013; Versace and Vallortigara 2015; Rogers and Vallortigara 2015; Vallortigara and Versace 2017). Left or right eye and appendage use biases commonly appear in natural behaviors including foraging, prey discrimination, predator detection, agonistic responses and courtship and mating (Rogers et al. 2013) (Table 1). Sidedness can differ in separate contexts for the same individual and these biases are also not always shared between individuals of the same species (Vallortigara and Rogers 2005). Thus, although the term ‘lateralization’ is used to describe side-based biases in function at the individual, population and species level, these biases might have evolved independently and convergently (Vallortigara and Rogers 2005). Understanding the presence and variation in lateralized behaviors at multiple levels can give a clearer picture of its adaptive functions and evolutionary pathways.

Table 1. Non-exhaustive, representative classification of lateralization in a range of taxa and biological contexts, illustrating the conceptual organization of laterality patterns in nature. Lateralization in eye and hand/paw use is shown at the individual, population, and inter-population levels.

species	behavioral context	outcome	reference
individual			
common octopus (<i>Octopus vulgaris</i>)	predatory	left (5 individuals) or right (3 individuals) eye preference when looking at a crab model	Bryne et al. 2002
Campbell's monkeys (<i>Cercopithecus c. campbelli</i>)	foraging	left (6 individuals) or right (8 individuals) eye preference looking through a tube at a seed	Chapelain & Blois-Heulin 2008
New Caledonian crow (<i>Corvus moneduloides</i>)	foraging	stick tool use to left (2 individuals) or right (2 individuals) of head while foraging	Rutledge & Hunt 2004
population			
black-winged stilt (<i>Himantopus himantopus</i>)	predatory and sexual	majority of right eye use for predatory pecking and majority of courtship displays when female was seen with left visual hemifield	Ventolini et. al. 2005
Australian green tree frog (<i>Litoria caerulea</i>)	agonistic	majority of strikes per frog when competitor is in left visual hemifield	Rogers & Rogers 2006
honeybee (<i>Apis mellifera</i>)	foraging	bees entering an open cavity have a strong rightward turning bias but no bias when entering a sequential choice maze	O'Shea-Wheller 2019
domestic chick (<i>Gallus gallus domesticus</i>)	social recognition	chicks using the left eye discriminate between stranger and companion better than chicks using the right eye	Vallortigara & Andrew 1994
inter-population			
poeciliid spp. (<i>Brachyraphis episcopi</i>)	anti-predator vigilance	fish from high predation regions preferred right eye to view a live predator, whereas fish from low predation regions had no preference	Brown et al. 2004

The lateralization of visual inspection has been studied extensively in a variety of social contexts relating to species recognition, but it has yet to be studied in the context of host responses to avian brood parasitism, where both population- and individual-level variation exists in parasite-rejection decisions. For hosts, the acceptance of foreign eggs is costly, as brood parasitism can result in a lower hatching rate, decreased nestling growth and increased nestling and fledgling mortality (Payne and Payne 1998; Hauber 2003a; Hoover 2003a; Hoover and Reetz 2006). American robins (*Turdus migratorius*; hereafter robin), an occasional host of the brown-headed cowbird (*Molothrus ater*; hereafter: cowbird), are rejecters of most cowbird eggs and thereby eliminate the recoverable costs of brood parasitism (Briskie et al. 1992; Rasmussen et al. 2009). Previous experiments using various model egg colors produced extensive variability in the rejection behavior by robins, and these responses were highly repeatable within individuals (Croston and Hauber 2014). Therefore, robins are an ideal species to study the proximate basis of the potentially lateralized assessment and discrimination of parasitic eggs.

To determine how robins make their egg-rejection decisions, the process of visual clutch inspection must be understood. Ground foraging birds, like the American robin, tend to have a single center of acute vision (i.e. fovea) in each eye that projects laterally given the positioning of the eyes in the skull (Moore et al. 2015). This center of acute vision has the highest densities of cone photoreceptors involved in color vision (Baumhardt et al. 2014). In birds, the right eye is often preferred for visual discrimination in foraging tasks, whereas the left eye is used for viewing familiar conspecifics (Mench and Andrew 1986; Güntürkün and Kesch 1987; Vallortigara and Andrew 1991; Vallortigara 1992; Alonso 1998; Vallortigara et al. 2001; Deng and Rogers 2002; Valenti et al. 2003; Zucca and Sovrano 2008). Few studies have investigated

lateralization in avian color discrimination, but in conditioning experiments, the right visual hemifield, and therefore left brain hemisphere, has been shown to be typically dominant (Diekamp et al. 1999; Skiba et al. 2000). Furthermore, in vertebrates, the left brain hemisphere is preferentially involved in focused attention to repeated stimuli and is important in categorization, while the right hemisphere is characterized by responding to novelty and is important in processing stress cues (Rogers et al. 2013). Robins can respond to multiple features of foreign eggs in their rejection decisions, including background color, size and maculation (Luro et al. 2018), which could favor the use of the right eye. However, non-mimetic parasitic eggs are a known physiological stressor for hosts of avian brood parasites (Ruiz-Raya et al. 2018), which could predict the biased use of the left eye for rejection responses. Here, we investigate whether lateralized eye use is present in robins when examining mimetic and non-mimetic model eggs in the nest.

1.3 METHODS

1.3.1 Experiments

We conducted this experiment during May and June of 2017 and 2018, using a total of 26 American robin nests in ornamental tree farms, apple orchards and suburban backyards around Urbana, Illinois. All nests used were active at the incubation stage and had a natural clutch size of at least three eggs. For experimental parasitism, we purchased commercially three-dimensional printed eggs (Model no: Cow Bird; Shapeways.com) in the size and shape of brown-headed cowbird eggs and painted robin-mimetic blue or cowbird-like white ground colors (for manufacture and painting details, see Igc et al., 2015).

In 2017, either an immaculate blue or white egg was placed into an active robin nest (Igic et al. 2015) and recorded with a GoPro Hero Camera (Los Angeles, USA) until its maximal battery life on a single charge. The camera was secured above the nest and set up on a camouflaged extended flexible gooseneck and wrapped around a nearby branch, ideally positioned 20–80 cm away from the nest with the clamp of the gooseneck latched to a nearby branch, depending on the environment. In 2018, each active robin nest received both a blue and white egg added to their natural clutch for consecutive 1-h trials (one egg at a time), in a randomized order. The 2018 nests were recorded simultaneously with two custom cameras (Sony 1.8 mm high-definition cameras connected to a Brandoo two-channel digital video recorder) placed on either side of the nest to gain more angles at the returning and incubating female robin.

All videos were reviewed and the number and direction of each head orientation towards the nest for every visit by a robin were recorded and categorized as either a left or a right head tilt when discernible. These head tilts were counted only when the robin was looking primarily with one eye down at the nest, from the time a robin stood on the edge of the nest until it either (1) rejected the egg, (2) began incubating or (3) flew off. For the trials in which two cameras were on either side of the nest, the numbers of left and right head tilts observed from both videos were averaged to minimize bias from the camera angle. In robins, only females incubate the eggs (Vanderhoff et al. 2016) and so our observed head tilts counted should represent maternal behaviors. To assess the replicability of our methods, all the videos were viewed and scored separately by two people (H.M.S. and K.S.).

1.3.2 Statistical analysis

We used two indices to characterize eye bias for each nest. An eyedness index (EI, range: -1 to 1) was calculated using the formula: $(R - L)/(R + L)$, with R representing the number of right head tilt counts per video recording, and L representing the number of left head tilts (Chapelain and Blois-Heulin 2008). Negative values indicate a bias towards left eye use, while positive values indicate a bias towards right eye use. A second index is the absolute value of EI [ABS(EI)], which is used to characterize the strength but not the direction of the side bias (Chapelain and Blois-Heulin 2008).

To determine the repeatability of observations between the two observers, two-tailed paired Spearman's rank correlations were used for both the total number of head tilts counted and the EI calculated by the observers. To assess whether there was an effect of video types between years, general linear mixed models (GLMM) were used for the number of head tilts and the EI metric, with the nest ID as a random effect. To determine if EI and ABS(EI) biases were present when robins examined blue (mimetic) or white (non-mimetic) eggs, one-sample Wilcoxon tests were used (with the random expectation of 0). GLMMs with nest ID as a random effect were used to assess the effect of treatment type on EI, ABS(EI) and total number of head tilts, as well as the effect of the number of head tilts on ABS(EI). Finally, GLMMs with nest ID as a random effect were used to assess the effect of EI, ABS(EI), and number of head tilts on whether the egg was accepted or rejected in 2018, while accounting for treatment effects. JMP 12.0.0 (SAS Institute Inc., Cary, NC) was used to analyze linear mixed models, and R v. 3.5.1 was used for non-parametric tests and figures.

1.4 RESULTS

We found positive interobserver repeatability for the number of head tilt counts recorded ($\rho = 0.87$, $p < 0.0001$) and for the calculated EI metric ($\rho = 0.50$, $P = 0.01$). In addition, there was no statistical difference between 2017 (single camera footage, $n = 15$ nests) and 2018 (double camera footage, $n = 12$ nests) in either the head tilt counts detected ($F_{1,22} = 1.05$, $P = 0.32$) or in the calculated EI metric ($F_{1,20} = 0.79$, $P = 0.39$).

When viewing mimetic eggs, robins had a lateralized bias in eye use, as shown by ABS(EI) ($\mu = 0.86$, $W_z = -1.80$, $P = 0.036$). However, the direction of this bias varied at the nest level and was not significantly skewed towards either the left or right eye preference overall in the population, as shown by the unbiased EI metric ($\mu = -0.12$, $W_z = 1.22$, $P = 0.89$). By contrast, when viewing non-mimetic eggs, robins did not show lateralized eye use, as indicated by both the ABS(EI) ($\mu = 0.45$, $W_z = -1.34$, $P = 0.09$) and the EI metrics ($\mu = 0.21$, $W_z = 0.70$, $P = 0.76$). There was no significant difference in EI when robins inspected mimetic versus non-mimetic eggs ($F_{1,4} = 0.67$, $P = 0.46$), whereas ABS(EI) shifted from more lateralized to less biased scores when robins examined mimetic versus non-mimetic eggs ($F_{1,14} = 11.56$, $P = 0.004$) (Figure 1a).

We examined whether our analyses showed a stronger absolute lateralized bias when viewing eggs with fewer total head tilt counts. Overall, there was no significant difference in the number of head tilts that robins used when looking at mimetic versus non-mimetic eggs ($F_{1,10} = 1.65$, $P = 0.23$). However, there was a negative relationship of the number of head tilts on ABS(EI) ($F_{1,12} = 12.56$, $P = 0.042$). Yet, ABS(EI) was still significantly greater for mimetic versus non-mimetic model eggs ($F_{1,16} = 5.38$, $P = 0.034$), even when accounting for covariation with head tilt counts ($F_{1,13} = 7.24$, $P = 0.019$).

During the 1-h exposure of the paired-design 2018 experiments ($n = 12$ nests), non-mimetic eggs were rejected more often than mimetic eggs (8% mimetic eggs rejected versus 33% non-mimetic eggs rejected) ($\chi^2 = 4.5$, $P = 0.034$). In turn, ABS(EI) was higher for nests in which experimental eggs were rejected versus nests where eggs were accepted ($\chi^2 = 7.8$, $P = 0.0052$), irrespective of the order in the stimulus presentation ($\chi^2 = 2.3$, $P = 0.13$) (Figure 1b).

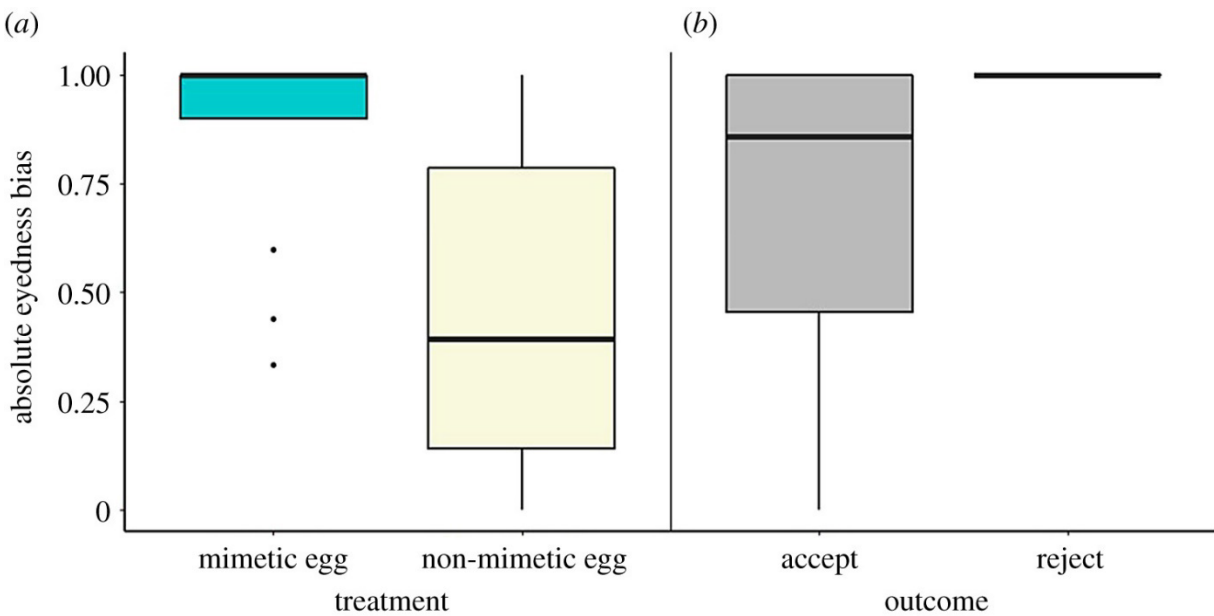


Figure 1. (a) Median and quartiles for difference in absolute eyedness index [ABS(EI)] in robins when observing a mimetic egg and when observing a non-mimetic egg ($P = 0.004$). (b) Median and quartiles for difference in ABS(EI) in robins when they accepted the experimentally added egg and when they rejected it ($P = 0.005$).

1.5 DISCUSSION

These results show that when robins examine an experimentally added mimetic egg in their nest, they apply a stronger bias to which eye they use to inspect that egg. By contrast, when robins inspect experimentally added non-mimetic eggs, they use both eyes in a more balanced way. This outcome was constant across the two different years of treatments in the study. Thus, the

results are consistent with a context-dependent strategy for lateralized robin eye use when observing eggs in their nest. In addition, the increased rejection of eggs when eye-use bias was higher suggests that the lateralization of eye use may also affect the success of antiparasitic egg-rejection decisions in this host species. Population-level eye-use lateralization biases reported here have also been found in other social recognition contexts: for example, domestic chicks (*Gallus domesticus*), adult hens and Japanese quail (*Coturnix japonica*) are better at discriminating familiar individuals from unfamiliar individuals with their left eye (Vallortigara and Andrew 1991; Vallortigara 1992; McKenzie et al. 1998; Vallortigara et al. 2001; Deng and Rogers 2002; Valenti et al. 2003).

The recognition and rejection of brood parasitic eggs can be considered a type of species recognition, in which birds must distinguish between their own eggs and the eggs of heterospecifics. However, the elimination of a parasitic egg could also be considered an agonistic response (Ruiz-Raya et al. 2018). Similar to social recognition, aggressive responses are usually more frequent when the stimulus is viewed with the left eye, as has been found in frogs (Robins and Rogers 2006), lizards (Hews et al. 2004) and birds (Krakauer et al. 2016), although all in the context of aggression towards conspecifics.

In our study, the stronger bias in lateralized eye use when examining mimetic eggs was not due to consistent use of the left or right eye across the population, but rather strong bias at the nest level with interindividual variability in which eye is preferred. Directional asymmetries occurring at the individual level, also known as antisymmetry, are hypothesized to occur to increase brain efficiency (Rogers et al. 2013). Population-level lateralization has been

hypothesized to occur as a result of interindividual interaction, in which individuals must align their asymmetries with other individuals in the group (Frasnelli and Vallortigara 2018). When viewing eggs, however, robins do not have to coordinate their behavior with other individuals, which might explain why there was no population-level trend in the direction of eye-use bias in the context of nest visual exploration to discriminate brood parasite eggs.

Robins that rejected our model eggs were more biased in their eye use than accepters. It is possible that stronger lateralization in eye use can promote better discrimination between eggs and aid in more successful rejection decisions for hosts. In the domestic chick, strongly lateralized individuals are faster at detecting model predators and are better at distinguishing between grain and pebbles (Rogers 2000; Rogers et al. 2004). In parrots, strongly lateralized individuals performed better at problem-solving tasks using their eyes and feet than less lateralized individuals (Magat and Brown 2009). The addition of non-mimetic eggs can be a physiological stressor for avian hosts, so it is possible that non-mimetic eggs are a source of stress for robins and that the shift in the laterality of eye use is mediated hormonally (Ocklenburg et al. 2016; Ruiz-Raya et al. 2018; Abolins-Abols and Hauber 2018).

In sum, these experiments show that lateralization in the eye use of robins inspecting eggs is context-dependent, with changes in lateral bias according to the mimicry of a foreign egg. The patterns in eye-use sidedness reported here thus span multiple tiers of biological organization, with a population-level bias in the strength of lateralization and variation in individuals' left versus right preferences. More importantly, these eye-use biases have potential impacts on the rejection rates of brood parasitic eggs, with more highly lateralized individuals rejecting model

eggs at higher rates. Consequently, it is conceivable that lateralization in eye use could contribute as an additional element in the coevolved defensive repertoire for hosts to aid in egg-rejection against avian brood parasites.

**CHAPTER 2: EXPOSURE TO A MIMETIC OR NON-MIMETIC MODEL AVIAN
BROOD PARASITE EGG DOES NOT PRODUCE DIFFERENTIAL
GLUCOCORTICOID RESPONSES IN AN EGG-ACCEPTOR HOST SPECIES²**

2.1 ABSTRACT

Avian obligate brood parasitism, a reproductive strategy where a parasite lays its egg into the nest of another species, imposes significant fitness costs upon host parents and their offspring. To combat brood parasitism, many host species recognize and reject foreign eggs (rejecters), but others are accepters that raise the parasitic progeny. Some accepter hosts may be unable to grasp or pierce parasitic eggs even if they recognize them as foreign eggs in the clutch, whereas other accepters may not have evolved the cognitive skillsets to recognize dissimilar eggs in the nest. Here we assessed the endocrine responses of an accepter host species to model parasitic eggs to address these two alternatives. We experimentally parasitized nests of a locally common host of the brood-parasitic brown-headed cowbird (*Molothrus ater*), the prothonotary warbler (*Protonotaria citrea*; a cowbird-egg accepter), with a mimetic or non-mimetic model cowbird-sized egg. Our goal was to determine whether they perceived the non-mimetic egg as a greater stressor by measuring circulating corticosterone levels. We added eggs to nests during the incubation stage and obtained blood plasma samples from females on the nest 2 h later, using females with unmanipulated clutches as controls. Incubating females showed no differences in

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baseline plasma corticosterone levels between our different treatments. We conclude that exposure to foreign eggs does not activate the hypothalamic-pituitary-adrenal axis of prothonotary warbler hosts in this experimental paradigm.

2.2 INTRODUCTION

Coevolutionary arms races, in which competing species evolve adaptations and counter-adaptations against each other, describe many of the interspecific interactions of avian obligate brood parasites and their hosts (Rothstein 1990). Avian obligate brood parasitism is a reproductive strategy in which the parasite lays its egg into the nest of another species (Soler 2017). Some brood parasites' hatchlings evict or otherwise kill the entire host clutch within days, and thus cause reproductive failure for hosts (Moskát et al. 2017). Alternatively, nest-sharing parasitic young can also impose fitness costs on their hosts by causing decreased hatching rate (Hauber 2003b), slower nestling growth (Lorenzana and Sealy 1999), increased chick mortality (Hauber 2003a), reduced recruitment (Hoover and Reetz 2006), and delayed or decreased future re-nesting for the foster parents (Mark and Rubenstein 2013).

To combat these costs, some host species have evolved egg rejection behaviors, whereby they puncture- or grasp-eject foreign eggs (rejecters: (Moksnes et al. 1991). Other host species, however, never reject brood parasitic eggs (accepters: (Medina and Langmore 2016).

Nevertheless, acceptor hosts can still reduce the cost of brood parasitism in other ways through employing frontline defenses (Feeney et al. 2012), including making nests less conspicuous (Davies 2000), mobbing brood parasites that come close to the nest (Davies and Welbergen

2009; Louder et al. 2020), or abandoning nests that are parasitized and re-nesting elsewhere (Hosoi and Rothstein 2000).

Despite the wealth of literature on the evolution of these host counter-defenses, less is known about the physiological, including hormonal, responses of hosts exposed to brood parasitism (Avilés 2018). Reproductive behaviors have a strong endocrine basis, suggesting that hormonal mechanisms may also regulate antiparasitic responses (Abolins-Abols and Hauber 2018). In the handful of studies that assessed hormonal responses of hosts to avian brood parasitism, parasitized parents had higher baseline circulating corticosterone (CORT) levels than non-parasitized parents (natural parasitism: (Mark and Rubenstein 2013); experimental parasitism: (Ruiz-Raya et al. 2018), and these responses were sex-specific in some species (experimental parasitism: (Antonson et al. 2020). CORT, the product of the hypothalamic–pituitary-adrenal (HPA) endocrine axis is one of the main glucocorticoid hormones in birds (Romero and Butler 2007; Herman et al. 2016; MacDougall-Shackleton et al. 2020). Environmental stressors can induce elevated levels of CORT (Remage-Healey et al. 2003; Jones et al. 2016), which is considered one of the principal mediators of physiological allostasis in birds as it promotes immediate survival and self-maintenance at the expense of other behaviors, such as reproduction (Wingfield and Hunt 2002; McEwen and Wingfield 2003; Angelier and Wingfield 2013). Thus, brood parasitism could affect host reproductive fitness through hormonal mediators like CORT. Accordingly, a recent experimental study demonstrated that individuals of a rejecter host species with reduced a priori CORT levels showed diminished egg-ejection responses to model non-mimetic brood parasite eggs, relative to sham manipulated subjects (Abolins-Abols and Hauber 2020a). Heightened CORT may aid in antiparasitic strategies by improving host discrimination

of foreign eggs and aiding in rejection, or by suppressing maternal behavior and promoting the abandonment of the nest (Abolins-Abols and Hauber 2018, 2020a).

In accepter species that do not respond behaviorally to parasitism, studying hormonal mechanisms may elucidate cognitive aspects of the recognition (i.e., perception and processing: (Mendelson et al. 2016; Ruiz-Raya et al. 2018) of foreign egg cues. Acceptor species may not reject eggs because they simply do not perceive or recognize the brood parasite egg as different from their own. This could result from evolutionary lag wherein hosts that have only recently come into contact with brood parasites have not yet evolved antiparasitic defenses (Davies 1999). However, the acceptance of brood parasitic eggs by hosts may also result from various physical constraints, such as small or weak beaks of some hosts that constrain grasp- or pierce-ejection of the brood parasitic egg (Underwood and Sealy 2006; Antonov et al. 2006); but see (Rasmussen et al. 2010), or the potential of the host harming its own eggs during rejection attempts of the parasitic egg (Rohwer et al. 1989). To our knowledge, only one study has investigated host CORT levels in response to experimental brood parasitism during incubation. In their study, Ruiz-Raya et al. (2018) found that after three days, female Eurasian blackbirds (*Turdus merula*; a rejecter species) experimentally parasitized with a large and non-mimetic foreign egg had higher baseline corticosterone levels than did non-parasitized females.

Here, we studied aspects of the endocrine responses of an accepter host species to model eggs to determine if non-mimetic eggs, or the addition of model eggs in general, resulted in elevated CORT levels. We experimentally parasitized the prothonotary warbler (*Protonotaria citrea*), a locally common host of the obligate brood parasitic brown-headed cowbird (*Molothrus ater*),

nests with mimetic beige or non-mimetic blue painted cowbird-sized model eggs, with non-parasitized nests serving as a control. We predicted that if female prothonotary warblers recognize non-mimetic eggs as foreign, they should have higher baseline CORT levels than females given a mimetic model egg or no model egg.

2.3 METHODS

Trials were conducted from May-July 2019 in three separate study sites within the Cache River watershed in southern Illinois, USA (Hoover 2003a). Prothonotary warblers are socially monogamous, cavity-nesting, neotropical migrants that breed in bottomland swamps and are a locally common host of brown-headed cowbirds at our sites, with up to 60% of nests parasitized with at least one cowbird egg (Hoover and Hauber 2007). This host readily nests in double-walled recycled milk cartons serving as nestboxes, which are painted black and affixed to two greased metal poles to prevent predation (Hoover 2003b). Only females incubate the eggs, but both sexes provision young. Nestboxes were monitored every 1–4 days depending on the sites and subsequent experiments, with the females flushed to check nest contents.

For experimental parasitism, we commercially sourced 3D printed eggs [Model no: “cow bird”; Shapeways.com] in the size, shape, and weight of brown-headed cowbird eggs, and painted them with a non-mimetic, robin-blue or a more mimetic, cowbird-like beige background color (for manufacture and dimensions, see (Igic et al. 2015); for painting instructions, see (Canniff et al. 2018); for degree of mimicry, see Results below; Figure 2). Though both the host and parasite eggs are spotted, we did not paint maculation patterns on the model eggs in this study (following (Hanley et al. 2019b, a)). Non-parasitized warbler nests in the incubation stage were chosen and

randomly assigned a treatment of either a blue painted model egg ($n = 16$), a beige painted model egg ($n = 17$), or a control treatment ($n = 8$) where no model egg was added. Cowbirds typically do not remove a host egg when they parasitize a nest, so our addition resembled natural parasitism (Hoover 2003a).

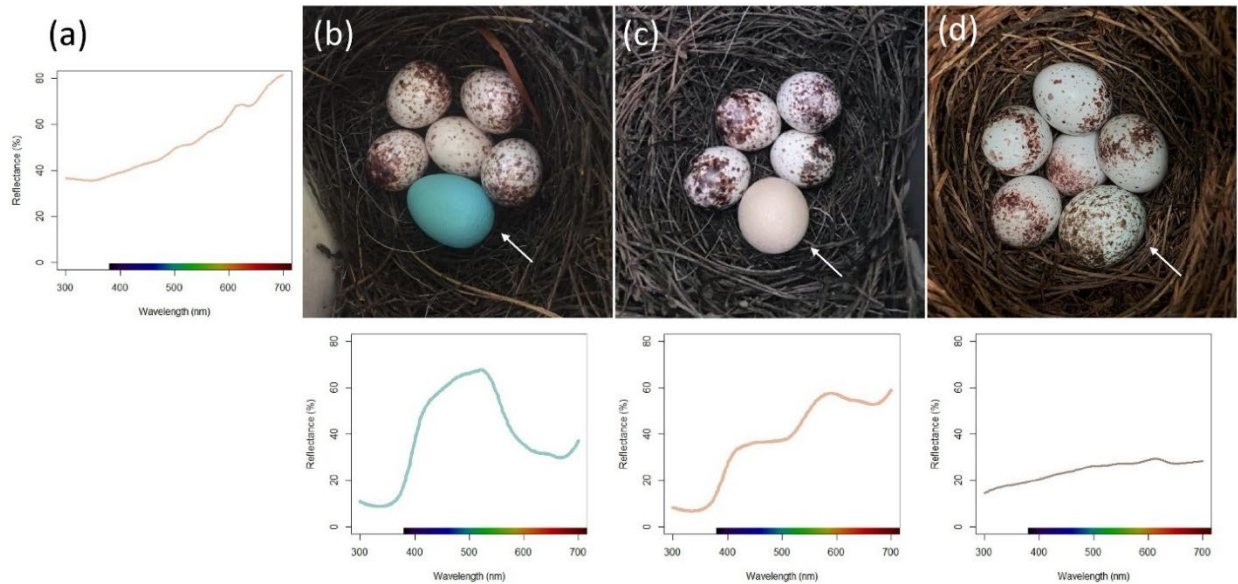


Figure 2. Reflectance spectra across the wavelengths of light visible to ultra-violet sensitive birds of (a) natural prothonotary warbler egg background color, (b) blue painted model egg color, (c) beige painted model egg color, and (d) natural brown-headed cowbird egg background color.

For each trial, the female incubating her complete clutch was initially flushed off her nest. In the blue or beige egg treatments, a model egg was added to her clutch, and in the control treatment, her eggs were touched but nothing was added. Two hours after these manipulations, incubating females were flushed again and caught with a bag placed over the hole as they exited the nestbox upon our arrival. The two-hour capture timing was chosen because in songbirds CORT concentration in blood typically increases rapidly in the first half an hour following exposure to a stressor (Romero and Romero 2002). In many bird species, corticosterone levels tend to stabilize an hour after a continuous exposure to a stressor (Wingfield et al. 1982; Romero and Romero

2002; Reneerkens et al. 2002; Breuner et al. 2006). Given that we did not record the duration spent incubating by the females at their nests after our initial flushing, catching females at the two-hour mark made it more likely that we caught most of the females at a point at which any CORT increase in response to viewing the parasitic eggs had stabilized (Moore et al. 1991). In addition, delaying capture until 2 hrs made it more likely that CORT levels did not reflect a possible stress-response in response to our initial nest disturbance (Rich and Romero 2005).

Starting from this point of capture, blood from most birds ($N = 36/41$) was obtained in under 180 s (median: 165 s, range: 110 – 195 s). We did not find a significant relationship between CORT and time elapsed between capture and blood collection ($\beta = 0.021$, $t_{36} = 0.029$, $P = 0.467$), so we included the samples taken slightly over 180 s in our study. Birds were bled from the brachial vein via heparinized microcapillary tubes, with blood transferred and stored in 1.5 mL Eppendorf tubes that were placed on ice. Within 2 h, all samples were centrifuged at 5,000 rpm for 10 min, with the plasma then drawn off and frozen at -40 degrees C until analysis later.

2.3.1 CORT Assays

To extract CORT from the defrosted plasma, we suspended 10 μL plasma in 200 μL double-distilled water and mixed with 1 mL diethyl ether, following the protocol described by Abolins-Abols and Hauber (2020b). We used a commercial enzyme immunoassay (Cayman Chemical Company, Item no. 501,320 Ann Arbor, MI, USA, sensitivity: 10.72 pg/mL) to analyze CORT concentrations across 2 plates (within-plate CV: 11.47%, between-plate CV: 9.90%) Our assay had good parallelism (slope = 1.01, $R^2 = 0.994$), calculated recovery (93.5–123.4%), and extraction efficiency: (96%). Samples were randomized so that all treatments were represented

across both plates. CORT levels from these samples were determined in relation to an eight-point standard curve using a curve-fitting program (Cayman Chemical).

2.3.2 Avian visual modelling

We measured the reflectance spectra from the avian-perceivable portion of light relevant for ultraviolet-sensitive (UVS) bird species (300–700 nm) from our model non-mimetic blue ($n = 3$) and mimetic beige ($n = 3$) eggs. These spectra were measured with a 400 μm fiber optic reflection probe fitted with a rubber stopper. The probe was placed at 3 mm away from the egg's surface at 90 degrees, with an area of 2 mm^2 of the egg's surface measured. A JAZ spectrometer with a pulsed-xenon light source (Ocean Optics, Dunedin, USA) took measurements which were made relative to a diffuse reflectance white standard (Spectralon WS-1-SL, Labsphere, North Sutton, USA). We obtained host and parasite ground color reflectance spectra from museum-specimens of each species (courtesy of D. Hanley from Hanley et al. 2015).

The spectra obtained were plotted in R 3.5.1 (R Core Team, 2020) using the pavo package (v.2.4–0 Maia et al. 2013) and with spectra lines colored using an RGB transform. The spec2rgb function was used for plotting graphs. Differences between these model eggs and prothonotary warbler eggs were calculated as just noticeable differences (JNDs) using a receptor-noise limited model with the pavo function “coldist” of the ultra-violet sensitive (UVS) blue tit (*Cyanistes caeruleus*) from the pavo function “vismodel” (Manna et al. 2020). We chose the UVS perceptual modelling option because of the known UVS status of another, related parulid, the yellow warbler (*Setophaga petechia*) (Aidala et al. 2012).

2.3.3 Statistical analyses

All analyses were run in R v 3.5.1; we used the package “ggplot2” for figures. To analyze the effect of treatment on plasma corticosterone concentration of the incubating females, a linear model containing treatment, ordinal date of blood collection, and time (s) elapsed between capture and blood collection as predictors was used with concentration of corticosterone (ng/mL) as the response variable. Response variables were a priori checked for homogeneity of variance and residuals of all response variables were inspected for normality.

2.4 RESULTS

Avian perceptual modelling demonstrated that the beige painted model eggs were perceptibly more similar to the ground coloration of prothonotary warbler eggs (chromatic JND = 2.98) relative to the greater dissimilarity of the blue model eggs (chromatic JND = 6.26, Figure 2). The beige eggs were also perceptibly more similar to the ground coloration of brown-headed cowbird eggs (chromatic JND = 3.65) relative to the greater dissimilarity of the blue model eggs (chromatic JND = 5.01, Figure 2).

There was no significant difference of the circulating corticosterone plasma concentrations at the time of the females’ capture across the different treatments (blue – beige: $\beta = -1.369$, $t_{36} = -1.001$, $P = 0.323$, control – beige: $\beta = -3.353$, $t_{36} = -1.986$, $P = 0.055$, Figure 3). There was also no significant effect of either seasonal variance (date on which blood was taken) ($\beta = -0.029$, $t_{36} = -0.778$, $P = 0.442$) or the time elapsed between capture and blood collection ($\beta = 0.021$, $t_{36} = 0.029$, $P = 0.467$).

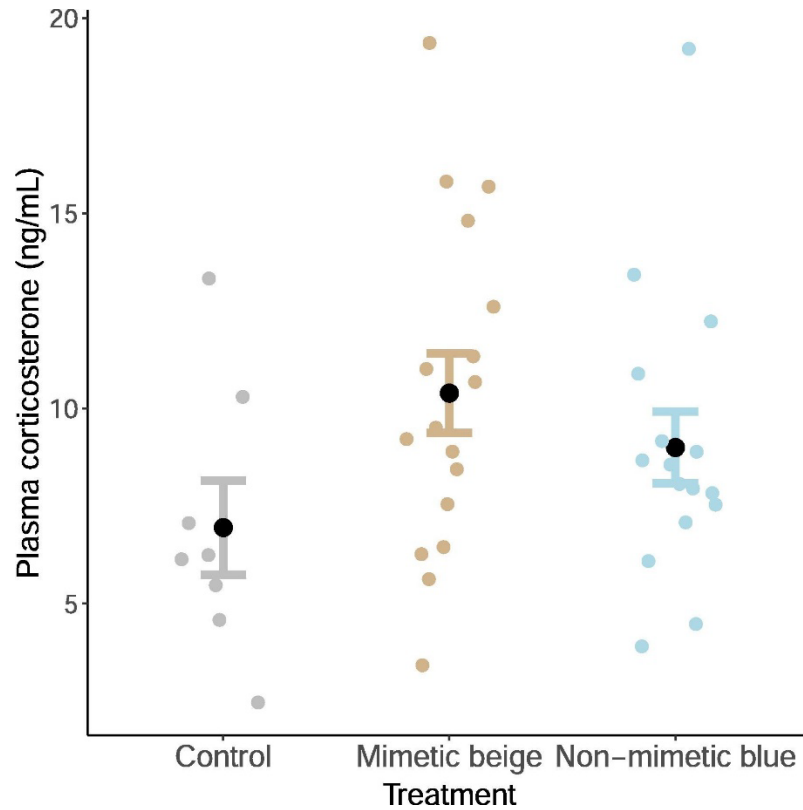


Figure 3. Plasma CORT levels of incubating female prothonotary warblers 2 h after experimental parasitism with a beige or blue model egg, or 2 h after being flushed off the nest only (control). Means are shown with the black circle and whiskers represent standard errors. No significant differences of the CORT levels were found between the treatments.

2.5 DISCUSSION

We detected no statistical effect of the addition of either a mimetic or non-mimetic model egg, relative to each other and to control where no egg was added, on the baseline CORT of incubating female prothonotary warblers at the 2 hr timepoint following the initial manipulation. This is in contrast to findings regarding Eurasian blackbirds, which had higher baseline CORT levels when parasitized for three days with a non-mimetic egg (Ruiz-Raya et al. 2018). However, unlike prothonotary warblers, which never naturally eject parasitic cowbird eggs (Hoover 2003b), Eurasian blackbirds are robust rejecters of foreign eggs (Hauber et al. 2014; Ruiz-Raya et al. 2016). Additionally, in our study, blood samples from incubating females were taken 2 h

after parasitism, whereas the blood samples of the Eurasian blackbirds were taken 3 days after parasitism as the authors were also testing the effects of CORT on prolactin levels and body condition. Finally, our experiments were conducted with a cavity-nesting species, whereas blackbirds are open-cup nesters, so these two species assess the presence of foreign (model) eggs in different visual milieus.

Because the control birds were also disturbed through flushing 2 h before plasma collection, this treatment may not represent a true baseline if CORT levels were still elevated 2 hrs after the initial disturbance. However, the CORT concentrations measured in this study are similar to other studies from our system where birds had been not disturbed 2 h prior (Davros 2014; Antonson et al. 2020), and in other, related parulid warbler species (e.g., Wilson and Holberton 2004). In addition, because our sites are visited ca. every other day, the females may have been already acclimated to human disturbance and attenuated their CORT response to being flushed off the nest as a result (Walker et al. 2006).

Elevated CORT levels in response to brood parasitism could be beneficial to hosts by promoting antiparasitic response either by aiding in the discrimination of a parasitic egg, or by suppressing maternal behavior and promoting egg rejection or abandonment of the nest (Abolins-Abols and Hauber 2018, 2020a). However, elevated CORT levels can also be costly, especially during the incubation period, as females with higher baseline CORT levels can suffer from lower reproductive success (Vitousek et al. 2018). Alternatively, if tolerance and acceptance of a brood parasite are less costly than resistance of parasitism, it might be advantageous for the host to not

elevate CORT levels in response to parasitism, and instead adjust life history traits to minimize parasitism costs (Medina and Langmore 2016).

As accepters, it is possible that prothonotary warblers are not hindered by physical constraints to egg rejection, such as weak beak strength or gape size, or the difficulty of removing a parasitic egg from deep inside a cavity, but rather do not identify and recognize cowbird eggs as foreign.

Parasitism by cowbirds is costly to prothonotary warblers, resulting in decreased clutch size, hatching success, and nestling survival (Hoover 2003a), as well as reduced first-year survival (McKim-Louder et al. 2013), so selection pressure for antiparasitic responses is likely.

Prothonotary warblers are habitat specialists that breed in bottomland and swamp forests, and widespread brood parasitism by cowbirds on this warbler may be an evolutionarily recent phenomenon resulting from anthropogenic fragmentation of bottomland swamp woodlands within the past 200–300 years (Rothstein 1975b; Hosoi and Rothstein 2000; Hoover 2003a).

Thus, incubating females of this host species might not recognize (i.e., perceive and process) parasitic eggs as different from their own eggs and, in turn, mount no hormonal responses.

Prothonotary warblers do not behaviorally respond to cowbird parasitism through egg rejection, nest abandonment, or a priori choice of cavities with smaller entrances inaccessible to cowbirds (Hoover 2003b), so it follows that they may also not have a circulating CORT response when exposed to foreign eggs.

Alternatively, it is possible that incubating female prothonotary warblers do exhibit increased CORT in response to a parasitic egg, but that our experimental protocol did not allow us to detect it. For example, because we could not standardize and did not record the amount of time females

were exposed to eggs at the entrance or inside the nest boxes, the CORT measurements might have instead reflected variation in exposure time, despite catching females at a timepoint when CORT levels should show a low rate of change. Furthermore, individual females may differ in the strength of negative feedback in their HPA axis (Zimmer et al. 2019), which might obscure CORT increase two hours following the instruction of the stimulus. On the other hand, it is possible that a hormonal response to the eggs takes longer than two hours, as exemplified by Ruiz-Raya et al. (2018) who still found increased baseline CORT levels in hosts 3 days after continuous exposure to non-mimetic parasitism. The addition of a parasitic egg could increase energetic demands of incubating females and affect CORT levels over longer periods of time (DuRant et al. 2013); however, previous research in our study system failed to find increased CORT in females that had incubated and raised a brood parasite to day eight of the nestling period (Antonson et al. 2020).

Finally, although we calculated the avian-perceivable JNDs of the model egg colors for this experiment, we do not know for certain if these eggs looked perceptibly different to incubating female warblers in the putatively variable light milieus of the different nest boxes, even though we modelled the JNDs of the egg colors using the visual system of the blue tit, which is also an obligate cavity nester species. Other cavity nesters can and do reject parasitic eggs both based on chromatic cues and independent of nest box light-levels (e.g. Manna et al. 2020), so prothonotary warblers may be able to discriminate our model eggs' colors as well, even within variably low-light cavity conditions. Prothonotary warblers could also use the number of eggs as a parasitism cue (Lyon 2003), although in another parulid warbler host species, acceptance rates were the same whether the cowbird removed a host egg or not (Sealy 1992).

In sum, this experiment contributes to the long-studied system of host-parasite interactions in general (Soler 2017) and between prothonotary warblers, an acceptor host, and brown-headed cowbirds, their brood parasites, in particular (Hoover and Robinson 2007). It lends support to the hypothesis that prothonotary warblers do not recognize cowbird eggs, potentially due to evolutionary lag. This study also adds to earlier evidence that female prothonotary warblers lack costly anti-parasite responses, including showing no increase in CORT levels in response to experimentally parasitized broods at the nestling stage (Antonson et al. 2020), and that in contrast to other parulid warbler species, such as the yellow warbler, prothonotary warblers lack defenses against cowbird parasitism (Gill and Sealy 1996). Future studies incorporating physiological data from other acceptor hosts could shed further light on the causes of parasitic egg-acceptance, distinguishing between a lack of perception of a foreign egg, and the physical or fitness constraints to rejecting it. Such studies should especially consider collecting plasma at multiple or a diversity of timepoints after the onset of experimental parasitism.

**CHAPTER 3: HOST PARENT RESPONSES TO HETEROSPECIFIC PARASITE
NESTLING ALARM CALLS ARE INDEPENDENT OF PAST AND CURRENT
EXPERIENCE WITH EXPERIMENTAL BROOD PARASITISM³**

3.1 ABSTRACT

Communication between parents and dependent offspring is critical not only during provisioning, but also in antipredator contexts. In altricial birds, a top cause of reproductive failure is nest predation, and alarm calls both by parents and chicks can serve to alert others and increase the likelihood of offspring escaping predation. Understanding the factors that determine the strength of parental antipredator responses to different nestling alarm calls can provide insight into parent–offspring recognition. The prothonotary warbler (*Protonotaria citrea*), a host of the obligate brood parasite, the brown-headed cowbird (*Molothrus ater*), never rejects cowbird young and raises the parasite together with its own offspring. To determine whether warbler parents learn cowbird nestling alarm calls, we presented experimentally parasitized or non-parasitized parents with playbacks of conspecific warbler, parasitic cowbird, and a harmless heterospecific control, eastern bluebird (*Sialis sialis*), nestling alarm calls. We recorded the latency to respond and the number of chips given by members of the resident warbler pair. We found that parents were most likely to respond to warbler nestling alarm calls, least likely to respond to bluebird calls, with a statistically intermediate likelihood of responding to cowbird

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<https://doi.org/10.1007/s10071-022-01612-w>

calls. Critically, current and past parasitism status did not affect the likelihood of response to any playback or the number of chips given, however, currently parasitized parents had greater response latencies to playbacks than non-parasitized parents. These results suggest that warbler parents do not learn cowbird alarm calls from breeding experiences and, in turn, that cowbirds may employ a generalized, bet-hedging alarm call.

3.2 INTRODUCTION

Parent–offspring communication is a critical component of parental care for parents to appropriately respond to offspring needs. In many species that provide parental care, offspring have specialized signal(s) to indicate distress. Accordingly, offspring may communicate to their parents that they are hungry (Grodzinski and Lotem 2007), cold (Evans 1994), or in danger (Magrath et al. 2010), which may elicit parental provisioning, brooding, or defensive behaviors, respectively. In some species, such as invertebrate treehoppers (*Umberia crassicornis*), offspring use tactile, vibrational signals to alert their mother of nearby danger (Cocroft 1999). In contrast, many vertebrates use auditory signals, like black caimans (*Melanosuchus niger*, Vergne et al. 2011) and California towhees (*Pipilo crissalis*, Benedict 2007), which have unique calls to indicate that predators are close.

Antipredator signals are especially important for nesting birds, because the major cause of reproductive failure in birds is nest predation (Martin 1995; Chiavacci et al. 2015). To help counteract this, nestlings of many altricial bird species give “screaming” vocalizations in response to a predatory stimulus (Roulin 2001). These screams attract parents, who can distract predators through parental injury displays to give nestlings time to force-fledge, or can directly

attack predators, deterring them from the nest (Magrath et al. 2010). This has been shown experimentally in the Eurasian magpie (*Pica pica*), where playbacks of nestling scream calls induced parents to increase nest defense (Redondo and Carranza 1989). Thus, parental antipredator responses to nestling alarm calls are a useful framework to provide insight into cognitive processes in general, and in particular, parent–offspring communication.

In birds, evidence is mixed as to whether parents exhibit individual recognition of their own nestling’s alarm calls. In the California towhee, parents do not have different responses between playbacks of offspring vs non-offspring nestling alarm calls, and also did not respond differently depending on the age of their brood (Benedict 2007). However, European starling (*Sturnis vulgaris*) parents were more likely to give diving attack responses when nestling alarm calls were given by their own offspring compared to a neighbor’s (Chaiken 1992). These contrasting results may lie in differences between the breeding ecology of these birds. Individual recognition may be more important for European starlings, which are semicolonial breeders, than for California towhees, which breed on distinct territories and may rely indirectly on the origin location of the alarm call rather than directly on its acoustic structure (Benedict 2007).

One aspect of nestling alarm call recognition that has yet to be explored is the context of brood parasitism. Avian obligate brood parasitism is a reproductive strategy in which females lay their egg into the nests of other bird species, relinquishing parental care duties onto the host (Soler 2017). While some host species recognize and eliminate the foreign egg or young, others show no apparent discrimination between their own and foreign progeny. In fact, brood parasitic chicks can manipulate parental behavior, such as provisioning, by mimicking the begging calls

of their host nestmates (Langmore et al. 2008) or by successfully competing for the majority of the delivered feeds (Lichtenstein and Sealy 1998; Kilner et al. 2004). While it may be beneficial for a parent to discriminate against the begging calls of parasitic young and feed their own young instead, alarm calls present a different scenario in that most predation events affect more than one chick in the brood. Thus, for parents that are the host of a nest-sharing parasite, responding to alarm calls of parasitic young would be advantageous if it helps protect their own biological young. Many birds eavesdrop on and respond to alarm calls from heterospecifics given in response to predators and can even distinguish between predator types or threats (Rainey et al. 2004; Templeton and Greene 2007). However, whether host parents respond to the alarm calls of parasitic chicks, and how these responses compare to their own chicks' calls, remain unstudied.

To investigate the effect of brood parasitism on parental responses to nestling alarm calls, we experimentally parasitized prothonotary warbler (*Protonotaria citrea*) nests with eggs of the obligate brood parasitic brown-headed cowbird (*Molothrus ater*). We hypothesized that as acceptor hosts of a nest-sharing brood parasite, parasitized warbler parents learn brown-headed cowbird nestling alarm calls during current or previous broods and respond to these calls as they would to their own species' nestling alarm calls. We predicted that (a) parasitized warbler parents will show similar responses (numbers of chips or latency to respond) to warbler and cowbird nestling alarm calls, relative to a heterospecific, harmless, non-parasitic control, (b) non-parasitized warbler parents will show the strongest responses to warbler nestling alarm calls, with weaker responses to the cowbird and the control calls, and (c) parents currently non-parasitized but parasitized in the previous breeding season should show stronger responses to cowbird nestling alarm calls than parents non-parasitized in both years.

3.3 METHODS

3.3.1 Study area and species

All playbacks were completed across 5 separate study sites within the Cache River Watershed in southern Illinois, USA between May and July of 2021. Playbacks were presented to breeding pairs of prothonotary warblers, which are migratory, neotropical songbirds and obligate secondary-cavity nesters in swamp habitats, readily using nest boxes made from 1.9L beverage cartons deployed through these sites (Petit 1999; Hoover and Schelsky 2020). Prothonotary warblers are socially monogamous and typically have 2 broods per breeding season, with the first clutch consisting of ~ 5 eggs and second clutches typically consisting of ~ 4 (Petit 1999). Prothonotary warblers are habitat specialists and only breed in bottomland swamp habitat, but because these sites are interspersed with farmland at our study location, they are regularly parasitized by brown-headed cowbirds, with up to 60% of nests naturally parasitized with one or more cowbird eggs (Hoover 2003a). While our nest boxes are most commonly used by prothonotary warblers, they are also occasionally used by eastern bluebirds (*Sialis sialis*).

For this study, we experimentally parasitized warbler nests with one cowbird egg. Unincubated cowbird eggs were removed from naturally parasitized warbler nests to make these nests non-parasitized, and randomly moved to a naturally non-parasitized warbler nest before incubation (Antonson et al. 2020; Scharf et al. 2021b). The majority of playbacks (N = 283/336) were given to birds that were banded with a federal aluminum band, a colored passive integrated transponder (PIT, Eccel Technology, UK) tag, and additional color bands to generate unique color combinations. Past parasitism status was determined for banded birds which had bred on these

sites previously and were known to have either been experimentally parasitized or non-parasitized the 2020 breeding season with the same methods as stated above.

3.3.2 Playback stimulus construction

Playback files were generated for three different treatments: (1) prothonotary warbler nestling alarm calls, (2) brown-headed cowbird nestling alarm calls, and (3) eastern bluebird nestling alarm calls (a harmless sympatric heterospecific: control). Recordings of warbler (Figure 4a) and cowbird nestling alarm calls (Figure 4b) were taken in 2020 and early 2021 from southern Illinois from nests not used in this study. Bluebird nestling alarm calls (Figure 4c) were taken in early 2021 from North Carolina (at the study site of Stanback and Dervan 2001). In total, 6 warblers, 4 cowbirds, and 4 bluebirds were recorded. All nestlings recorded were close to fledging age and from separate nests; warblers and cowbirds were around 9 days old and bluebirds were 15 days old. Recordings were taken via iPhone 8 and XR (© Apple Inc.) as mp4 files. All species' nestlings were taken from their nest and handled to generate the alarm call used for these playbacks.

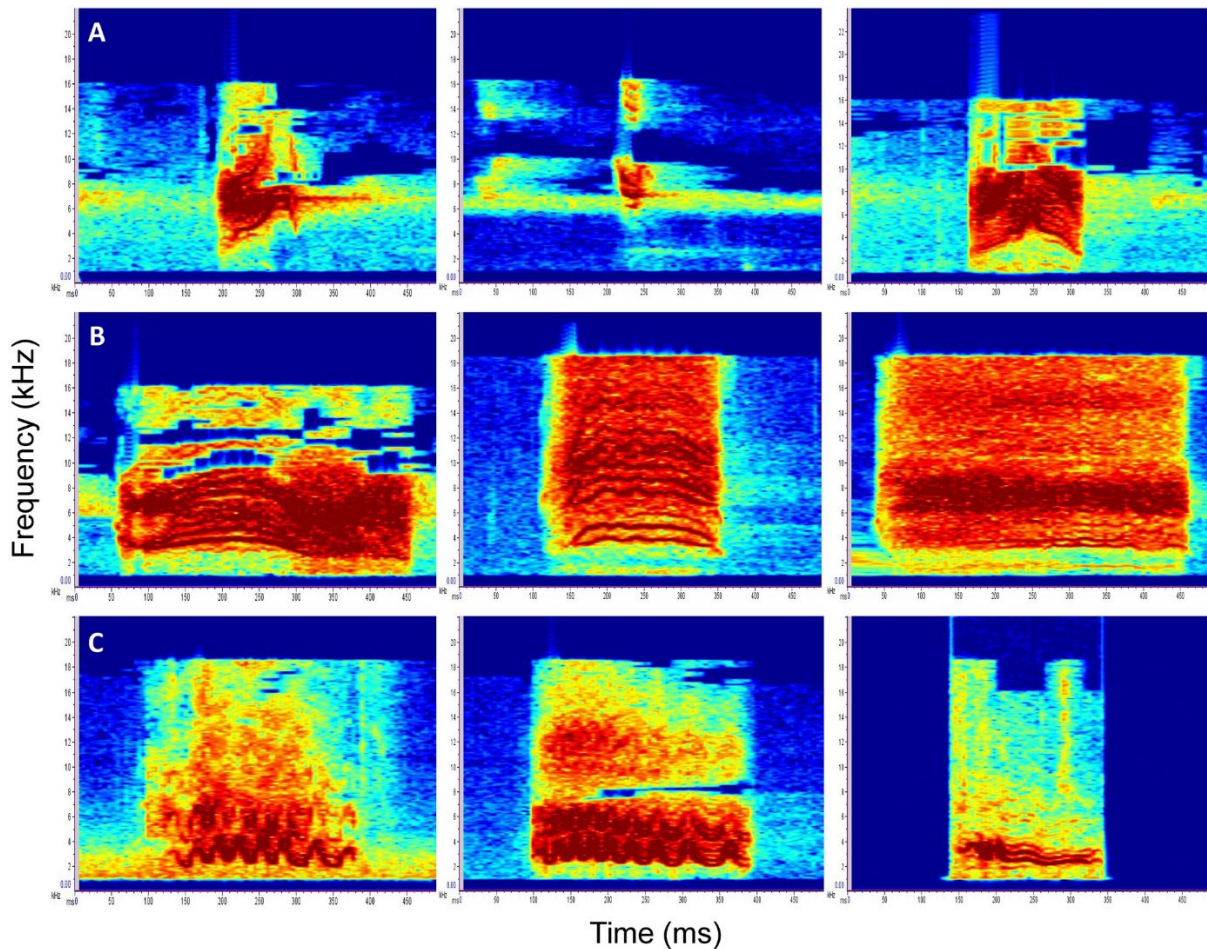


Figure 4. Exemplar spectrograms depicting alarm calls of three different (a) prothonotary warbler nestlings, (b) brown-headed cowbird nestlings, and (c) eastern bluebird nestlings used for this study. Spectrograms were generated in Raven version 2.0.

Playlists were created using Adobe Audition 2021. Frequencies below 1000 Hz, well below our range of alarm call stimuli, were filtered out to minimize signal-to-noise ratio. Five playback files for warblers, four playback files for cowbirds, and four playback files for bluebirds were generated, with 4 unique calls from at least 3 unique individuals on each playback. If available, for individuals represented on multiple playbacks, different calls were used for each playback file. These calls were assorted randomly for each playback. Each call was repeated 4 times in a row separated by 1 s of silence, followed by 10 s of silence before the next unique call. Each set of four unique calls was separated by 20 s of silence, and each set was repeated 3 times for a

total of 3 min of alarm call playback. No individuals were represented on every playback; each playback excluded at least one individual represented on another playback to create unique sets. Although alarm calls can change depending on urgency (Leavesley and Magrath 2005; Suzuki 2014), chicks were handled similarly and our playbacks capture natural variation in alarm call sounds. The randomized balanced design of our playback trials should help eliminate spurious results from any outliers (see Playback Trials). Each playback began with two minutes of silence to observe and record the bird's baseline behavior, and one minute of silence after to account for responses that continued after the playback stopped, for a total of 6 min of behavior recorded per playback trial.

3.3.3 Playback trials

Playbacks were presented on a FOX-PRO2 speaker broadcast at ~ 55 dB SPL (measured 1.0 m from speaker) and averaging all calls per playback, playbacks were within 5 dB SPL of each other. This volume is similar to what could realistically be produced by a nestling bird, and generally was not detectable by human ears from the distance of most neighboring territories. The speaker was secured directly to the nestbox poles or to the back of battery boxes already present on the poles with a bungee cord. To ensure that responses were due to the playback, and not the novel addition of the speaker, every playback started directly following the successful provisioning of the male and female after leaving the nestbox, when at least one parent was likely still in the immediate area. If only one parent showed up after 20 min, the trial began with the successful provisioning of that parent while they were still on the territory.

All playbacks occurred between May–July 2021. Although most playbacks occurred in the morning (60% before 12:00), playbacks were presented anytime between 8:00 and 18:00 CT due to heavy workloads and playback schedules. Playbacks that were conducted at neighboring territories on the same day were separated by at least 1 h to avoid carryover effects (Lawson et al. 2020). The order and identity of each playback was chosen in a balanced random design, so all playbacks were represented equally in number and in order.

Only one playback per day was given to a pair, with each pair receiving all 3 playback treatment types over a period of 3 consecutive days. If a pair was tested during their first brood and again for their second, they were given playback files that they did not experience during their first brood. Generally, the first playback day occurred when the majority of nestlings were 6 days old, and the last playback day occurred when the majority of nestlings were 8 days old ($N = 148/158$), although due to weather or other constraints the full range is 5–10 days old. Prothonotary warbler nestlings generally fledge at 10 days old, and as early as 9 days old, so playbacks were given at the above ages so that each nest would receive all playback treatment types before fledging. Warblers have the ability to force-fledge at 8 days old and can make chip vocalizations to parents when removed for banding from the nest starting at 6 days old (Petit 1999).

Two observers were present for each playback, with observers alternately assigned to monitor either the male or female parent to record individual responses. Prothonotary warblers are sexually dichromatic and sex is easily visually determined with females being darker olive above on the head and back. Both observers were always together for playbacks and shared a timer, to ensure synchronous observations. During the playback, for the first 2 min of silence, the number

of chips from each parent was counted separately from the chips counted within last 4 min of playback. Male and female prothonotary warblers give chip calls in aggressive responses to nest predators, and we considered a bird “responding” to the playback if they chipped after we started the playback (Petit 1999). If a bird was chipping in the absence of a researcher setting up the speaker within the first 2 min of silence of the playback, the number of chips per minute was subtracted from the number of chips per minute during the last four minutes of the playback to account for this baseline. The response latency, measured in seconds, was recorded from the start of playback sound as when a bird first chipped at the playback. Birds that were never heard or sighted in the area during the playback were still included in analysis, as including only birds visually or auditorily detected resulted in a bias with females being more responsive. This is likely because males are more conspicuous in general and were often heard singing on the territory or seen because of bright plumage irrespective of their response, whereas females were seen or heard typically because they chipped during the playback. Observations of heterospecifics and warbler conspecifics apart from the focal parental pair were recorded, however, no neighboring pairs or other bird species ever clearly responded to any alarm call playbacks.

3.3.4 Statistical analyses

We used R version 3.5.1 (R Core Team 2017) with packages “nlme” (Pinheiro et al. 2022), “lme4” (Bates et al. 2015), and “emmeans” (Lenth 2021) for statistical analyses and packages “ggplot2” (Wickham 2009) and “ggsignif” (Ahmann-Eltze and Patil 2021) for figures. To determine the factors that influenced whether a bird responded to the playback (i.e., the likelihood of response, 0 vs > 0 chips), binomial models were used. To determine the factors that

influenced the intensity of the response, Gaussian models were used only for the subset of birds that did respond (> 0 chips). The number of chips was transformed with a \log_{10} transformation to adhere to normality assumptions. We used these binomial and Gaussian linear mixed models to determine the effects of treatment, current parasitism status, sex, clutch (brood) number (1st or 2nd per season), observer identity, and trial order on likelihood of responding, number of chips given during the playback, and latency to respond. Band number was used as the random effect to account for multiple trials per bird. Any birds not banded in this study were given a unique ID based on their nestbox and sex and were only tested during one nesting attempt. For each of these three models, we included an interaction between treatment and current parasitism status. If this interaction was not significant, it was taken out and the models were re-analyzed with the additive version. Post hoc Tukey pairwise comparisons were used between warbler vs cowbird, cowbird vs bluebird, and warbler vs bluebird treatments to account for multiple comparisons between these playbacks. For a smaller subset of trials in which the parasitism experience for the previous year was known, these same models were run with past parasitism experience, sex, and brood number as predictor variables for cowbird playbacks only, with study site as the random effect. Site was used as a random effect as most birds were not tested with the cowbird playback more than once in this subset. For the model(s) in which brood number was significant, we ran a secondary analysis with only the responses from second broods to determine if there was a difference in the responses of birds tested during their first brood and again for their second vs birds hearing the playbacks for the first time during their second brood. These included the same predictor variables as the original analysis, but with experience substituted for brood number. In total, there were 336 responses/non-responses recorded from males and females during 168

playbacks. There were 56 playbacks per treatment at 56 nests. Of these, 12 nests were experimentally parasitized, while 44 were non-parasitized.

3.4 RESULTS

There was no significant interaction between treatment and current parasitism status ($\chi^2 = 2.472$, $P = 0.291$) for the likelihood of a bird responding to the playback, so these interactions were removed for the rest of the analyses. The likelihood of responding (0 vs > 0 chips) was significantly different between treatments ($\chi^2 = 21.621$, $P < 0.0001$). Based on post hoc analyses, we found that warbler parents were more likely to respond to warbler nestling alarm playbacks than cowbird nestling alarm playbacks ($\beta = 0.683$, $P = 0.0497$), more likely to respond to cowbird than bluebird playbacks ($\beta = 0.930$, $P = 0.024$), and more likely to respond to warbler than bluebird playbacks ($\beta = 1.613$, $P < 0.0001$, Figure 5a). In contrast, sex ($\chi^2 = 0.057$, $P = 0.811$), current parasitism status ($\chi^2 = 0.460$, $P = 0.498$, Figure 6a), and brood number ($\chi^2 = 0.116$, $P = 0.733$) did not affect the likelihood of warbler parents responding to playbacks. Trial order ($\chi^2 = 1.815$, $P = 0.178$) and observer identity ($\chi^2 = 2.278$, $P = 0.131$) also had no effect on response likelihood.

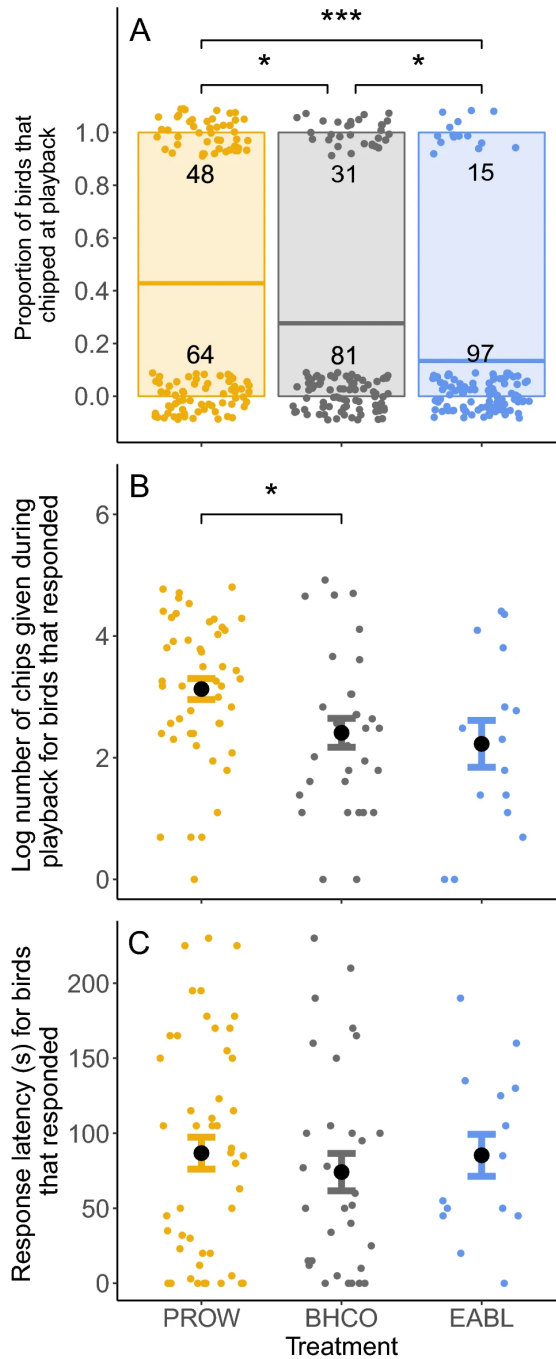


Figure 5. Effect of playback treatment on (a) likelihood of parent response, (b) number of chips from only parents that responded (\log_{10} transformed), and (c) latency (s) of the responses for only parents that responded. Bird Banding Laboratory abbreviations for species names are used, with PROW representing prothonotary warbler, BHCO representing brown-headed cowbird, and EABL representing eastern bluebird. For (a), bars represent the proportion of birds that responded to the playback for each treatment. For (b) and (c), black points represent the mean, with bars representing standard error. Comparisons with $P < 0.05$ are represented with “*”, comparisons with $P < 0.001$ are represented with “***”. Each point denotes the response of an individual bird.

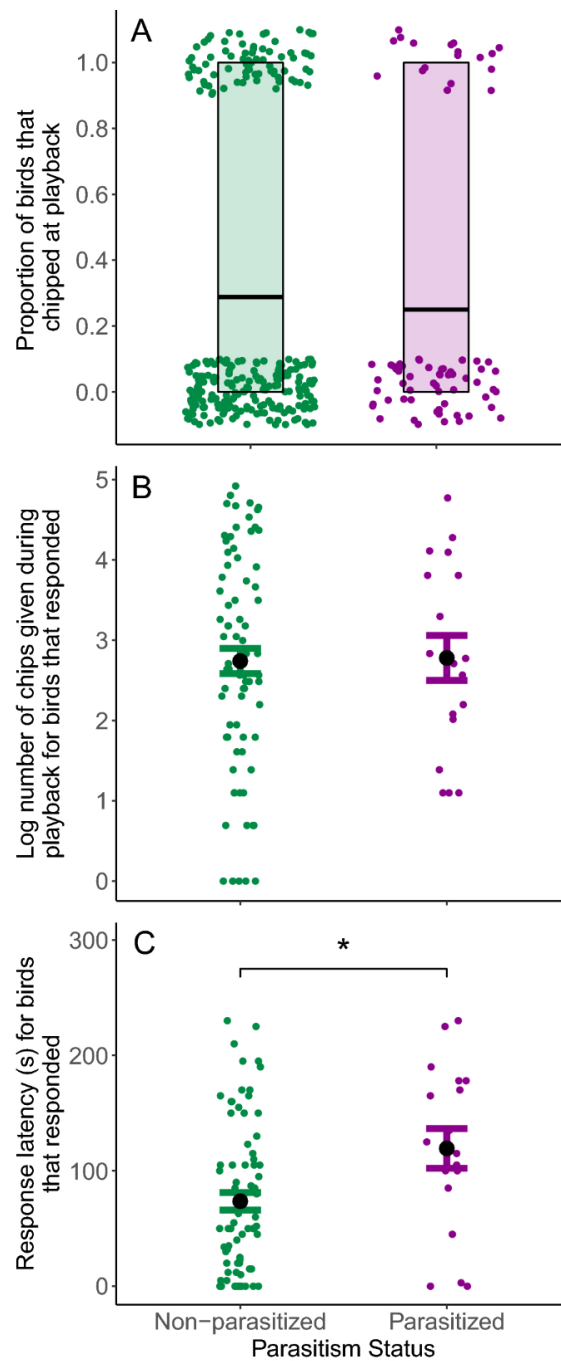


Figure 6. Effect of current parasitism status on (a) likelihood of parent response, (b) number of chips from only parents that responded (\log_{10} transformed), and (c) latency (s) of the responses for only parents that responded. As there was no significant interaction between treatment and parasitism status for these metrics, all playback treatments are represented. For (a), bars represent the proportion of birds that responded to the playback for each treatment. For (b) and (c), black points represent the mean, with bars representing standard error. Comparisons with $P < 0.05$ are represented with “*”. Each point denotes the response of an individual bird.

For only birds that chipped at the playback ($N = 94/336$ individual birds per trial), there was no significant interaction between treatment and current parasitism status ($\chi^2 = 1.759$, $P = 0.415$) for the number of chips given, so these interactions were removed for the rest of the analyses. The number of chips given was significantly different between treatments ($\chi^2 = 9.259$, $P = 0.010$). More chips were given at warbler nestling alarm playbacks than cowbird ($\beta = 0.775$, $P = 0.039$), however, there was no statistical difference in the number of chips given between cowbird and bluebird ($\beta = 0.069$, $P = 0.985$) or warbler and bluebird ($\beta = 0.775$, $P = 0.093$, Figure 5b). There was also no difference between sexes ($\chi^2 = 0.090$, $P = 0.764$) or current parasitism status ($\chi^2 = 0.176$, $P = 0.675$, Figure 6b) in the number of chips given for playbacks. However, birds with their first brood chipped significantly more at playbacks than birds with their second brood ($\chi^2 = 4.592$, $P = 0.032$, Figure 7). This did not appear to be experience-dependent, as birds that had experienced playback trials previously did not chip significantly more or less than birds experiencing the trial for the first time during their second brood ($\chi^2 = 1.524$, $P = 0.217$). Trial order ($\chi^2 = 0.485$, $P = 0.486$) and observer identity ($\chi^2 = 0.224$, $P = 0.636$) had no effect on the number of chips given.

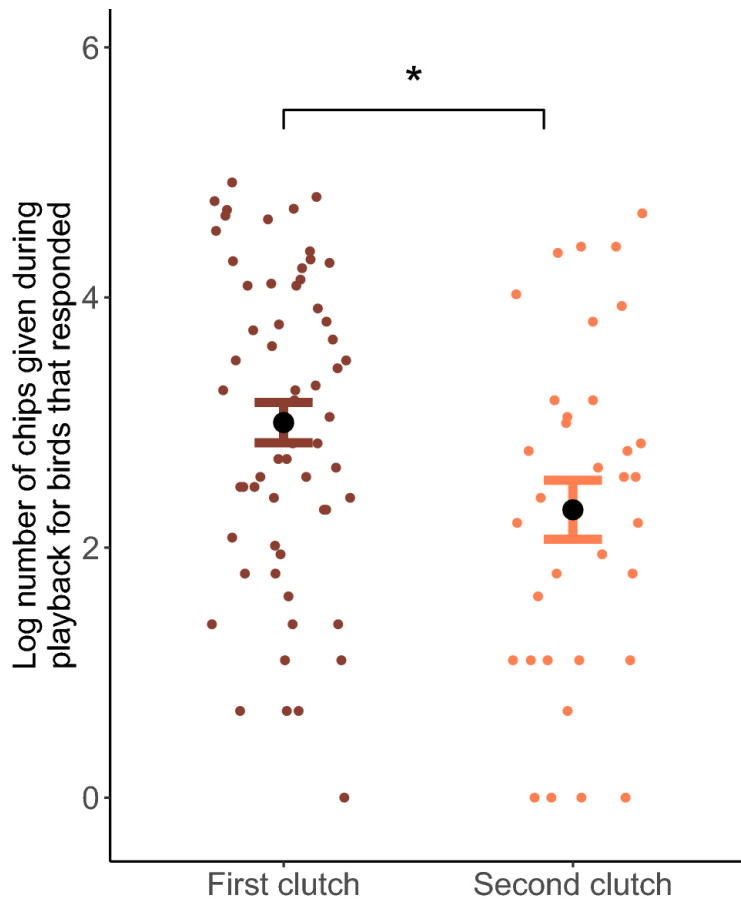


Figure 7. Comparison of the number of chips from birds that responded (\log_{10} transformed) between first clutches and second clutches. Comparisons with $P < 0.05$ are represented with “*”. Each point denotes the response of an individual bird.

For only the birds that responded to the playback ($N = 94/336$ individual birds per trial), there was no significant interaction between treatment and current parasitism status for the latency (s) of response ($\chi^2 = 0.665$, $P = 0.717$), so this interaction was removed. There was no difference in response latency (s) between the treatments ($\chi^2 = 1.667$, $P = 0.435$, Figure 5c), the sexes ($\chi^2 = 3.095$, $P = 0.079$), or between brood numbers ($\chi^2 = 0.619$, $P = 0.432$). However, birds that were currently parasitized had significantly higher response latencies than birds that were non-parasitized ($\chi^2 = 6.887$, $P = 0.009$, Figure 6c). Trial order ($\chi^2 = 0.231$, $P = 0.631$) and observer identity ($\chi^2 = 1.335$, $P = 0.248$) had no effect on response latency.

Past parasitism experience ($N = 38$ birds with known past parasitism status) did not affect the likelihood of parents responding to the cowbird playback ($\beta = -0.024$, $P = 0.978$). For the subset of birds that did chip to the cowbird playback, past parasitism experience also did not affect the number of chips given ($\beta = -0.648$, $P = 0.662$) or the response latency to cowbird playbacks ($\beta = 7.583$, $P = 0.913$). Sex and brood number were also not significant for all these models ($p > 0.05$).

3.5 DISCUSSION

Our results revealed that, contrary to our predictions, neither past nor current parasitism experience affected host parent responses to cowbird nestling alarm calls. Parents that had been experimentally parasitized the previous year did not respond differently in any metric compared to parents that had not been parasitized the previous year. Current parasitism status only affected response latency, in a way that was opposite to what we had predicted: parasitized parents had a higher response latency to nestling alarm calls in general. However, we did find that although adult prothonotary warblers were more likely to respond to nestling alarm calls of their own species and chip more at them relative to the brood parasite, parents were more likely to respond to brown-headed cowbird nestling alarm calls than the alarm call of another sympatric and non-parasitic heterospecific. Thus, in our study, it does not appear that warbler parents learn cowbird nestling alarm calls through experience raising them. Instead, cowbirds, as a generalist brood parasite, may employ a generalized nestling alarm call that any host species can recognize as a distress call. This hypothesis should be tested further by conducting parallel studies to ours in

other hosts of this generalist brood parasite throughout its range, and comparing these calls acoustically.

Brown-headed cowbird nestlings could employ at least two strategies to manipulate parental behavior through calls: cowbirds could use cues of host identity to develop alarm calls specific to their hosts, or they could have a fixed, generalist, bet-hedging call that is used no matter the host. Many bird species respond to heterospecific alarm calls as a learned behavior, but birds may also respond to unfamiliar alarm calls that are acoustically similar to conspecific alarm calls (Fallow et al. 2013). Although we are aware of no other studies focused on cowbird nestling alarm calls in different host nests, studies on begging behavior of brood parasites in different host environments may help elucidate alternative pathways. While some brood parasites, like the Horsfield's bronze-cuckoo (*Chalcites basalis*) modify their begging calls through experience with shaping by host parents (Langmore et al. 2008), brown-headed cowbirds do not appear to have plastic begging calls based on host identity, and may rely on greater begging intensity instead to compete effectively with host chicks (Hauber 2003a; Rivers 2007). Although the selective pressures on parasitic begging calls and alarm calls are likely different, parasites benefit if they are able to generate an antipredator response from their host parents that incites an urgent and strong antipredator response for them to escape predation. If cowbirds do indeed have bet-hedging alarm calls, this could explain why, in the apparent absence of learning by host parents, warblers were more likely to react to cowbird nestling alarm calls than to another sympatric heterospecific, the eastern bluebird.

Although we did not test individual nestling recognition by warbler parents, it is likely that because parents did not change responses to cowbird alarm calls depending on parasitism status, they do not learn the alarm calls of their chicks each brood. Warblers may have an innate knowledge of alarm calls, and aspects other than individual structure of alarm calls may be more reliable or cost-effective to assess threat. California towhees, which are similar to prothonotary warblers in that they breed on non-overlapping territories with nestling periods of less than 2 weeks, showed no discrimination between alarm calls of their own nestlings and nearby nestlings (Benedict 2007). Instead, their responses were location-based, with adults much more likely to approach alarm calls originating from near their nest rather than calls originating elsewhere on their territory (Benedict 2007). Even if nestling alarm calls are not given by the parent's own offspring, responding to alarm calls on a location basis may offer parents important information about predator threats in the area. This may be why warbler parents sometimes responded to eastern bluebird nestling alarm calls, a species that they are sympatric with, but would never experience raising in the warbler nest. Alternatively, we may not have found an effect of past or current parasitism status on responses because this system has very high parasitism rates, and most parents have likely experienced parasitism at some point in their life (Hoover et al. 2006). Thus, alarm calls could have been learned prior to one known year of parasitism or birds may have been parasitized while nesting in natural cavities, which would be unknown to us during this study.

Although currently parasitized parents were not more likely to respond to nestling alarm calls, when they did respond, they took longer to respond than non-parasitized parents. Brown-headed cowbird nestlings are 2–2.5 times larger than prothonotary warbler nestlings, and accounting for

broods of the same total mass, parents still have higher provisioning rates at parasitized nests than non-parasitized nests (Hoover and Reetz 2006). With this increased demand for provisions, parents may be spending more time and energy on foraging, and thus are not as receptive to nestling alarm calls, as foraging and nest defense are mutually exclusive (Mutzel et al. 2013). Parasitized nests with cowbird chicks are often reported to have higher predation rates (Payne and Payne 1998; Dearborn 1999; Hauber 2000; Hannon et al. 2009; Stumpf et al. 2012), however, a direct comparison of foraging time, nest defense, and predation is still necessary in future research.

We did not find parental sex differences in any playback responses that we measured. In the prothonotary warbler, females build the majority of the nest and are the sole incubators (Scharf et al. 2021a), but provisioning duties are split evenly between the parents (Hoover and Reetz 2006). While male and female prothonotary warblers are sexually dichromatic, they are similar in size, although males have longer wings on average and females are heavier during the breeding season (Petit 1999). It is reported that in response to predators at the nest, males may be slightly more likely to attack predators, and females may be more likely to give broken-wing distraction displays (Petit 1999). However, our playbacks did not include any predator stimulus to avoid confounds during our treatments and may explain why there were no sex differences seen in the likelihood of responding, number of chips given, and response latency.

Lastly, although there was no change in the likelihood of responding or the latency of responses, we found a decrease in the number of chips given from parents during first broods compared to second broods. This is in contrast to the reproductive value hypothesis, which predicts that birds

breeding late in the season should have higher levels of nest defense because there is little opportunity to reneest (Montgomerie and Weatherhead 1988; Thornhill 1989; Redondo and Carranza 1989; Pavel and Bureš 2008). However, some field experiments show no change in nest defense from one brood to the next (Hatch 1997; Tryjanowski and Goławski 2004), and some even show a decrease in defense intensity (Halupka and Halupka 1997). Nest defense may decrease throughout the season because of lower brood survival (Wiklund 1990; Halupka and Halupka 1997), as warblers from late season nests are known to have lower recruitment rates into the adult population in our system (McKim-Louder et al. 2013). Alternatively, warbler parents may have decreased the intensity of their responses through habituation to playbacks, however, we found no evidence that parents who had been tested during their first brood chipped less than parents who had not been tested during their first brood. Thus, the change in intensity of response is related to seasonality, although more manipulations would need to be done to show that this is indeed related to reproductive value.

This study is the first to examine parental responses to nestling alarm calls in an obligate brood parasitic context. For brood parasites to be successful, communication with host parents and manipulation of their behavior is essential. Much of this focus on the behavioral manipulation of host parents by brood parasites has been in a provisioning context, with emphasis on the begging calls of parasitic nestlings (Gabriel and Kilner 2017). Brood parasites benefit from increased food from parents (Kilner et al. 2004), however, other crucial impactors of survival, such as predation, have largely been understudied from a communication perspective (but see Dearborn 1999). Future research focusing on alarm calls of generalist brood parasites in different hosts, the alarm calls of specialist brood parasites compared to their host, and comparisons of the acoustic

structure of these calls, would aid our understanding of how brood parasites manipulate antipredator responses in their hosts.

CHAPTER 4: THE EFFECT OF AVIAN BROOD PARASITISM ON PHYSIOLOGICAL RESPONSES OF HOST NESTLINGS⁴

4.1 ABSTRACT

Avian obligate brood parasites lay their eggs in the nests of other species that may provide care for the foreign offspring. Brood parasitism often imparts substantial fitness losses upon host nestlings when they are raised alongside the typically more competitive, larger, and older parasitic chick(s). Whereas fitness costs due to reduced host offspring survival in parasitized broods have been studied in detail, the physiological changes in host nestlings caused by parasitic nestmate(s) are less well known. We compared prothonotary warbler (*Protonotaria citrea*) nestlings, a host of the nest-sharing brown-headed cowbird (*Molothrus ater*), in experimentally parasitized vs. non-parasitized broods. Our aim was to determine whether cohabitation with brood parasitic young impacted host nestling baseline corticosterone plasma concentrations, immune responses, body condition, and mortality. Corticosterone levels and body condition of host nestlings were similar between nests with or without a cowbird nestmate, whereas host immune responses were lower and nestling mortality was greater in parasitized broods, irrespective of variation in brood size or total brood mass. We detected no trade-offs of baseline corticosterone levels with either immune responses or with body condition. These results suggest that this host species' nestlings experience some adverse fitness-relevant

⁴ This work was published in the journal *Oecologia*.

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physiological effects in parasitized broods, but are also resilient in other aspects when coping with brood parasitism.

4.2 INTRODUCTION

Avian obligate brood parasitism, in which the brood parasite lays its egg into the nests of other species, is a reproductive strategy present in only ~1% of birds (Davies 2000). Brood parasitism can pose significant energetic, temporal, and other fitness costs for the host species (Soler 2017) and may, therefore, act as a substantial stressor on both progeny and parents. Parasite-host competition is mostly limited to non-evictor parasites, because they do not forcibly remove host young and rather coexist alongside them (Moskát et al. 2017). In turn, detrimental effects of non-evictor parasitic nestmates on host eggs/young include decreased hatching rate (Hauber 2003b), slower nestling growth (Lorenzana and Sealy 1999), increased nestling mortality (Hauber 2003a), and reduced nestling recruitment (McKim-Louder et al. 2013). Numerous studies have looked at adult hosts' responses to brood parasites such as mobbing (Gloag et al. 2013), egg rejection (Manna et al. 2017), nestling discrimination (Grim 2017), and fledgling discrimination (De Mársico et al. 2017), and host parents may even compensate when exposed to parasitism by being more likely to have a second brood (Louder et al. 2015a). However, how antiparasitic strategies are regulated hormonally in host adults and nestlings, especially when parasitic young do not eliminate all host chicks (Moskát et al. 2017), still remains poorly known (Abolins-Abols and Hauber 2018; Antonson et al. 2020).

To better understand the proximate factors of how cohabitating host chicks manage costly interspecific brood parasitism, we set out to assess physiological metrics, focusing on endocrine

levels and immune activation, that are already known to be influenced by many environmental and social challenges, including social competition in the brood (Glennemeier and Denver 2002; Smiseth et al. 2011). For example, corticosterone (CORT), the main glucocorticoid hormone in birds, is involved in the hypothalamic–pituitary–adrenal cortex (HPA) axis, wherein various stressors activate the HPA axis, causing elevated levels of circulating glucocorticoid hormones, including CORT in birds (Herman et al. 2016; MacDougall-Shackleton et al. 2020). While most knowledge of the effects of stressors in birds is derived from studies on adults, altricial nestlings also react to a variety of stressors, including ectoparasites, food restriction, and distress calls by upregulating CORT (Kitaysky et al. 1999; Raouf et al. 2006; Tilgar et al. 2010), especially with increasing age in the nest (Bebus et al. 2020). In young birds, the HPA axis develops as the nestlings' age: 6-day-old house wren (*Troglodytes aedon*) nestlings already show a detectable rise in CORT in response to handling and restraint (Strange et al. 2016). In turn, older nestlings typically have higher baseline CORT levels compared to younger nestlings (Wada et al. 2007; Bebus et al. 2020). CORT acts as a regulator of multiple behaviors in early life and can mediate the physiological impacts of brood competition (Smiseth et al. 2011). For instance, higher levels of CORT can induce exaggerated begging and nestmate aggression (Kitaysky et al. 2001, 2003). Thus, nestlings may elevate CORT levels in stressful situations that also elicit compensatory behaviors to cope with social competition, including those posed by non-evictor brood parasitic nestmates. Inversely, chicks may instead reduce their CORT plasma levels in response to social factors, like perceived risk of predation, potentially as a mechanism to reduce begging activity and thus dampening auditory cues for predators (Ibáñez-Álamo et al. 2011).

In the handful of host species with published data on hormones, parasitized parents have higher baseline corticosterone levels than non-parasitized parents during the incubation, nestling, and fledgling stages (Mark and Rubenstein 2013; Ruiz-Raya et al. 2018; Antonson et al. 2020), and may be able to respond to brood parasitism by investing more in offspring through increased yolk androgen deposition (Hahn et al. 2017; Hauber et al. 2020). Less is known about obligate brood parasitism's influence on host nestling CORT levels. In the one published study that investigated the effect of a non-evictor obligate brood parasite, great spotted cuckoo (*Clamator glandarius*), on developing host nestlings, Ibáñez-Álamo et al. (2012) reported that brood parasitism increased baseline corticosterone levels in host nestlings of both European magpies (*Pica pica*) and carrion crows (*Corvus corone*), although only at certain stages of development—the mid- and late-nestling period for magpies and the mid-nestling period only for crows. Beyond this pioneering study, however, the endocrine and other physiological responses of host nestlings reacting to experimental brood parasitism are not well characterized (Abolins-Abols and Hauber 2018; Bebus et al. 2020).

Enlarged conspecific brood sizes per se may affect endocrine responses and other aspects of nestling physiology by increasing sibling competition. Experimental brood enlargement increased baseline CORT levels in nestlings of multiple species of songbird, including tree swallows (*Tachycineta bicolor*), violet-green swallows (*T. thalassina*), and barn swallows (*Hirundo rustica*) (Saino et al. 2003; Vitousek et al. 2017; Hardt et al. 2018). Other means of food limitation have also been shown to increase nestling plasma CORT levels (Kitaysky et al. 1999). However, this effect is not universal since nestlings in other species, such as the spotless starling (*Sturnus unicolor*) and the eastern bluebird (*Sialia sialis*), did not have increased CORT

levels in response to brood enlargement (Gil et al. 2008; Kozłowski and Ricklefs 2011). Static baseline CORT levels in nestlings confronted with brood enlargement may be a byproduct of how provisioning parents respond. Adults may increase provisioning to enlarged broods, including those that are parasitized (Hoover and Reetz 2006), thereby taking on the cost themselves and potentially dampening stress responses in their nestlings.

Physiological trade-offs may also result in negative relationships between fitness-relevant physiological and phenotypic traits. For example, high circulating levels of CORT can decrease T-cell-mediated and cutaneous immune function in nestlings (Saino et al. 2003; Rubolini et al. 2005; Loiseau et al. 2008; Butler et al. 2010). In addition to immunosuppression, CORT can negatively affect growth, resulting in lower nestling body mass (Spencer and Verhulst 2007; Loiseau et al. 2008). For host chicks, decreased immune function could be a major cost at such a vital developmental stage, as pathogens and ectoparasites in the nest are known to reduce body mass, increase sibling competition, and cause higher chick mortality (Christe et al. 1996; Gwinner and Berger 2005). Decreased body condition and growth for passerine nestlings in general could also be very costly, resulting in greater mortality prior to fledging (e.g., Harriman et al. 2017) and reduced recruitment to breeding (Mitchell et al. 2011). These costs to nestlings may be exacerbated in hosts of brood parasites, as parasites typically hatch earlier, and are often already larger, beg more intensively, and act as superior competitors (Hauber 2003a, c; but see Rivers et al. 2013).

Testing the mechanisms of how brood parasitism affects physiological aspects of host nestlings is also important in understanding the evolution of host tolerance vs. host resistance of brood

parasitism (Avilés 2018) and the co-evolutionary cycles between parasites and hosts (Rothstein 1990), as less virulent brood parasites can select for host acceptance (Medina and Langmore 2016). Here, we investigated the effect of experimental brood parasitism on host nestling baseline CORT levels and immune responses by experimentally manipulating the parasitism status of host prothonotary warbler (*Protonotaria citrea*) nests with parasitic brown-headed cowbird (*Molothrus ater*; hereafter: cowbird) eggs. We also collected novel experimental data to complement previously published correlational patterns on prothonotary warbler nestlings' condition and mortality associated with natural instances of cowbird parasitism (Hoover 2003a). We predicted that a cowbird in the nest would represent a competitive stressor for warbler nestlings, so that host chicks with a parasitic nestmate should have (i) higher levels of baseline plasma CORT, and (ii) decreased immune responses. Based on past observational research in our system, we also expected (iii) lower condition, and (iv) increased mortality (Hoover 2003a) when parasitized. In addition, we predicted that we would find a physiological trade-off between plasma corticosterone levels and (i) host nestling T-cell-mediated immune response and/or (ii) host nestling condition.

4.3 METHODS

4.3.1 Study area and species

This study was conducted from May 2006 to July 2006 at 10 separate study sites within the Cache River watershed in southern Illinois, USA (Hoover 2003a). These sites consisted of discrete forested wetland habitat patches each separated by at least 0.5 km of agricultural or upland forest habitat not suitable for breeding by prothonotary warblers. Prothonotary warblers are neotropical migrants to eastern North America that are cavity nesters and breed as socially

monogamous pairs, and serve as a locally common cowbird host, with up to 60% of their nest cavities naturally parasitized in these field sites, and around half of those receiving two or more cowbird eggs (Hoover 2003a; Hoover and Hauber 2007). They typically initiate multiple nesting attempts within a breeding season, with first clutches usually containing 5 eggs and subsequent clutches containing fewer (Hoover 2003a). The female incubates the eggs, both sexes provision the young, and pairs often fledge two broods in a single breeding season.

Prothonotary warblers are the only cavity-nesting host species that is frequently parasitized by brown-headed cowbirds (Louder et al. 2019) and readily nest in artificial nest boxes made from modified 1.9 L beverage cartons that are ideal for experimental manipulations and monitoring parasitism (Louder et al. 2015b). All manipulated and measured nests from this study were built in these artificial nest boxes. Prothonotary warblers readily accept experimentally introduced cowbird eggs (see below), with only 6% of naturally parasitized nests being deserted (Hoover 2003b).

4.3.2 Field procedures

Warbler pairs were randomly selected to incubate eggs and raise broods with or without cowbirds by flipping a coin for the first pair encountered on each study site and then alternating treatment with each additional pair found nesting on each respective site thereafter. All adults were caught via mist net at the nest box or while incubating and banded with USFWS aluminum bands and unique color band combinations; accordingly, the identity of each nesting pair was known. Cowbird eggs were collected elsewhere within the Cache River watershed at additional warbler nest box sites, and two cowbird eggs not yet incubated were placed in the treatment

clutches prior to the onset of incubation by host pairs assigned to raise mixed broods. Two cowbird eggs per nest were used as insurance in case one did not hatch, and in most cases, only one egg hatched ($n = 21$ nests). Natural parasitism rates within this system vary, but about half of warbler nests that are parasitized receive one cowbird egg, while the other half receive two or more (Hoover 2003a). We excluded nests from all analyses whenever both cowbird eggs hatched, due to their low sample size ($n = 7$ nests), and because it was not the intended outcome of our experimental manipulations. When a host nest selected to be non-parasitized was naturally parasitized by cowbirds, any naturally laid cowbird egg(s) was (were) removed prior to incubation. In prothonotary warblers, clutch size is determinate, with a typical first clutch containing 5 eggs and subsequent clutches containing fewer, and manipulations of egg numbers during laying do not have an effect of reducing or increasing the final natural clutch size (Petit 1999). We did not find any difference between clutch sizes of the parasitized and non-parasitized warblers in this study (two-sample t-test; $t_{32} = 0.712$, $P = 0.481$). The average original clutch size for warblers in the treatment group was 4.57 eggs (SE: 0.130), and the average clutch size for warblers in the control group was 4.47 eggs (SE: 0.071).

We did not add conspecific warbler egg(s) to non-parasitized broods or remove a warbler egg from parasitized broods, because in our study system under natural conditions, the majority (~75%) of parasitized prothonotary warbler broods simply receive a brown-headed cowbird egg without having one of the host eggs removed (Hoover 2003a). Thus, our experimental treatment was modelled after natural conditions, and our non-parasitized nests were also experimentally assigned and manipulated to maintain non-parasitism status. Finally, we minimized the chance of potential retaliatory mafia behaviors (*sensu* Hoover and Robinson 2007) from female cowbirds

whose eggs were removed by the experimenter through narrowing the nest box opening's size to exclude the adult cowbirds' entry once incubation began. Nests were monitored every three days, so nestlings were directly observed upon hatching or within 1–2 days of hatching. Nestlings were also aged with our custom-made visual guides of known-aged chick photographs or by calculating hatching date 13 days from the onset of incubation.

At an average of 8 days post-hatching (range: ± 1 day), two host nestlings randomly selected from each nest had blood samples ($\leq 100 \mu\text{l}$) taken from the brachial vein via heparinized microcapillary tubes within 3 min of removal from the nest (Newman et al. 2017). Whole blood was stored in 1.5 ml Eppendorf tubes, immediately placed on ice, and later (i.e., within 8 h of collection) centrifuged at 2000 rpm for 3 min to separate out the plasma. Plasma was drawn off, stored in 500 μl of 98% ethanol, and frozen at -20°C until later hormone analyses.

Immune response was measured by performing a phytohemagglutinin (PHA) skin test on the nestlings 8 days post-hatch (range: ± 1 day), which measures acquired T-cell-mediated immune response in birds (Tella et al. 2008). For this aim, 0.1 ml of 1 mg ml^{-1} PHA (PHA-P, Sigma L-8754, Sigma-Aldrich, St. Louis, MO) dissolved in cell-culture grade (e.g., pyrogen free) phosphate-buffered saline (PBS, Sigma-Aldrich P-9453, St. Louis, MO) was injected (UltiCare insulin syringe 28G, 0.5 cc, 0.5 in, model ULT08258) into the wing web not used for blood sampling. Three measures of wing web thickness in mm were taken before and 24 h after injection using a pressure-sensitive spessimeter (Käfer pocket dial thickness gauge, Model JZ 15) (Martin et al. 2006). Nestlings were banded using USFWS aluminum bands and were measured for tarsus length (mm) and mass (g) on day 8 before PHA injection and weighed again

on day 9 (i.e., 24 h post-injection). Measurements, CORT samples, and PHA injections were conducted on day 8 post-hatch to prevent nestlings from force-fledging the nest. Nestlings were weighed to the nearest 0.01 g on a digital scale (Myweigh Flip125, HBI Technologies, Phoenix, AZ) and tarsus measurements were taken to the nearest 0.1 mm using a dial caliper (Polymid Dial Caliper 150 mm, Swiss Precision Instruments, La Palma, CA). Body condition was calculated as the residuals of mass regressed against tarsus length (Gosler et al. 1998; Dodson et al. 2016). Nestling mortality per nest was calculated as a proportion, by taking the number of prothonotary warbler chicks that died during the entirety of the nestling stage divided by the total number of host hatchlings in that brood.

4.3.3 Corticosterone assays

Plasma samples were thawed 2 h before analysis and vacuum centrifuged to remove ethanol, and then, deionized water was used to reconstitute to the original plasma volume. Samples were treated with 1:100 dilution of steroid-displacement reagent in deionized water to free corticosterone from its binding proteins, diluted to 1:50 with assay buffer, and divided in half to be measured as duplicates. Samples and standards were prepared in duplicate on 96-well corticosterone Enzyme Immunoassay (EIA) plates (Enzo Life Sciences, Plymouth Meeting, PA). Absorbance was read at 405 nm on a FilterMax F3 microplate reader (Molecular Devices, Sunnyvale, CA) and averaged across duplicate samples with Multi-Mode Analysis software (Molecular Devices version 3.4.0.25). We used the manufacturer's recommended protocol of a four-factor polynomial equation that we derived from each plate's five-point standard curve (as in Mommer and Bell 2013) to derive baseline corticosterone concentration (ng/ml) of each warbler's original plasma sample from the EIA optical density. Plasma samples of both

parasitized and non-parasitized broods, differing levels of brood size, brood mass, nestling age, and ordinal date were represented across each of the six plates. The inter-assay coefficient of variation between all 6 EIA plates was 3.18% and the intra-assay coefficients of variation averaged $2.24 \pm 1.22\%$ (mean \pm standard deviation).

4.3.4 Statistical analyses

Analyses excluded the subset of the treatment groups with small sample sizes, that is, nests with more than one cowbird chick ($n = 7$) and a pair with a third clutch ($n = 1$). Plasma samples that showed evidence of ethanol evaporation were also excluded from analyses due to possible measurement error ($n = 20$). Models were run in R (version 3.5.1) using packages “nlme”, “MuMIn”, and “lme4”. All variables were checked for collinearity using Pearson’s correlation tests, and all were $|r| < 0.7$, so they were retained in the full models. Response variables were checked for homogeneity of variance and residuals of all response variables were inspected for normality. Baseline CORT level residuals were natural log transformed to meet assumptions of normality. Thirteen out of the 239 values for change in wing-web swelling after PHA injection were negative (i.e., showing no increase), so these values were set to zero.

We used an information-theoretic approach (corrected Akaike information criterion, AIC_C) to evaluate the combined effects of the randomized experimental treatment on our physiological and survival response metrics and the role of additional, non-experimental, life history, and seasonality traits that had been known to effect host nestlings’ fitness in this system (sensu Hoover 2003a). We used general linear mixed models (GLMM) to create our model set for this analysis, with mother identity nested within site as a random effect. The physiological and

survival response metrics used were warbler nestlings' baseline CORT levels, immune responses, body condition, and the proportion of nestling mortality in each nest. The predictor variables used, in addition to cowbird presence in the nest (yes/no), included either the experimentally altered total brood size (number of warbler plus cowbird nestlings, 2–6, continuous) or the brood mass (total mass of all nestlings, including cowbirds if present, on day 8), ordinal date of the onset of incubation (with January 1 = day 1; Muck et al. 2009), and nestling age (in days, 7–9) as additional fixed effects. Brood size, brood mass, and nestling age were included as fixed effects for the CORT, immune response, and body condition models only.

Ordinal date was used instead of brood number, because it had higher explanatory power and encompassed variation from first and second broods as well as seasonal components that affect nestling body condition (McKim-Louder et al. 2013), and we included the metric of brood mass to help disentangle the effects of brood parasitism vs. brood enlargement, as cowbirds are 2.0–2.5 times larger than prothonotary warbler nestlings (Hoover 2003a). Combinations of fixed effect predictors were modeled and ranked using AIC_C (Burnham and Anderson 2002). Three interactions were also included in these model sets. These included the interaction of cowbird presence with ordinal date, cowbird presence with brood mass, and cowbird presence with brood size, to investigate if the costs of cowbird parasitism vary with seasonality and with host competition (Hoover 2003a; Antonson et al. 2020). Cases with missing values of any variable were removed from the data set before comparing models with AIC_C.

Model averaging was used to calculate β coefficients and 95% confidence intervals by using weights from all models adding to a cumulative AIC_C weight of 0.95 and taking the average

across these models (Burnham et al. 2011; Symonds and Moussalli 2011). Effect sizes were calculated by applying conditional model averaging using Akaike weights from AIC_C with a partial standard deviation standardization for models within 0.95 of W_i (Cade 2015). The resulting sample sizes were as follows: 120 nestlings with baseline CORT from 68 nests, 233 nestlings with change in wing-web swelling after PHA injection from 69 nests, 337 nestlings with body condition from 73 nests, and 90 nests with the proportion of nestling mortality. Of these nests, 48 were first brood nests and 42 were second brood nests. Twenty-five percent of first brood nests and 14% of second brood nests were parasitized experimentally. Fewer nests were parasitized later in the season as there is a seasonal drop in parasitism rates and the availability of experimental cowbird eggs (Hoover and Hauber 2007), so we did not always have cowbird eggs available for experimental parasitism. This also led to one egg being placed into each experimental nest later in the season instead of two. For the AIC analyses where ordinal date was an important predictor, we expanded our analyses in the Supplementary Materials where we truncated the data to exclude all late-season host-only nests after the last parasitized nest was measured.

Finally, the effect of CORT levels (ln transformed) on the immune response and body condition of host nestlings were modeled separately using linear mixed models to detect possible physiological trade-offs. The mother's identity nested within site was included as a random effect for all models to account for the biological non-independence of siblings.

4.4 RESULTS

4.4.1 Host nestling plasma corticosterone levels

In our AIC_c analysis, the top model explaining differences of baseline CORT levels in nestlings was an additive model containing ordinal date and nestling age and did not include cowbird presence (hereafter: cowbird; Table 2; Supplementary Table 1). However, neither ordinal date nor nestling age affected nestling plasma CORT levels as the 95% confidence intervals (CI) overlapped zero ($\beta = -0.143$, 95% CI = $-0.293, 0.007$; $\beta = 0.131$, 95% CI = $-0.012, 0.273$, respectively, Table 3). The 95% CIs for cowbird presence also overlapped zero ($\beta = 0.084$, 95% CI = $-0.074, 0.241$, Figure 8a).

Table 2. AIC_c tables of models investigating the importance of cowbird parasitism with non-experimental factors including total brood size (a–c), brood mass (a–c), ordinal date (a–d), and nestling age (a–c) on (a) prothonotary warbler nestling baseline plasma corticosterone levels, (b) warbler nestling immune response as measured by wing-web swelling 24 h after injection of PHA, (c) warbler condition calculated as the residuals of mass on tarsus, and (d) the proportion of warbler nestlings that died per nest.

	k	AIC_c	ΔAIC_c	W_i
(a) BASELINE CORTICOSTERONE LEVELS				
ordinal date + nestling age	6	283	0	0.338
ordinal date	5	284.6	1.64	0.149
(b) IMMUNE RESPONSE				
cowbird * ordinal date + brood size	8	-227	0	0.672
(c) CONDITION				
cowbird * brood mass	7	736.9	0	0.228
cowbird * brood size	7	738	1.1	0.132
brood size	5	738.1	1.19	0.126
(d) NESTLING DEATH				
cowbird	5	-71.2	0	0.648

Table 3. 95% confidence intervals and model averaged coefficients for models summing to a cumulative AIC_C weight of 0.95 investigating the importance of cowbird parasitism with non-experimental factors including total brood size (a–b), brood mass (a–c), ordinal date, and nestling age (a–c) on (a) prothonotary warbler nestling baseline plasma corticosterone levels, (b) warbler nestling immune response as measured by wing-web swelling 24 h after injection of PHA, (c) warbler condition calculated as the residuals of mass on tarsus, and (d) the proportion of warbler nestlings died per nest. Variables with 95% confidence intervals that do not overlap zero are bolded.

	95% Confidence Interval	β
(a) BASELINE CORTICOSTERONE LEVELS		
ordinal date	-0.293, 0.007	-0.143
nestling age	-0.012, 0.273	0.131
cowbird	-0.074, 0.241	0.084
cowbird * ordinal date	-0.255, 0.029	-0.113
brood mass	-0.085, 0.223	0.069
brood size	-0.152, 0.173	0.011
(b) IMMUNE RESPONSE		
cowbird	-0.092, -0.021	-0.057
ordinal date	-0.018, 0.039	0.010
brood size	-0.061, -0.006	-0.033
cowbird * ordinal date	0.020, 0.090	0.055
brood mass	-0.051, 0.010	-0.021
(c) CONDITION		
cowbird	-0.207, 0.251	0.022
brood mass	-0.009, 0.217	0.104
cowbird * brood mass	-0.275, -0.010	-0.143
brood size	-0.170, 0.051	-0.060
cowbird * brood size	-0.259, 0.006	-0.126
ordinal date	-0.043, 0.147	0.052
cowbird * ordinal date	-0.077, 0.184	0.054
nestling age	-0.072, 0.147	0.038
(d) NESTLING MORTALITY		
cowbird	0.005, 0.082	0.044
ordinal date	-0.037, 0.034	-0.001
cowbird * ordinal date	-0.048, 0.023	-0.013

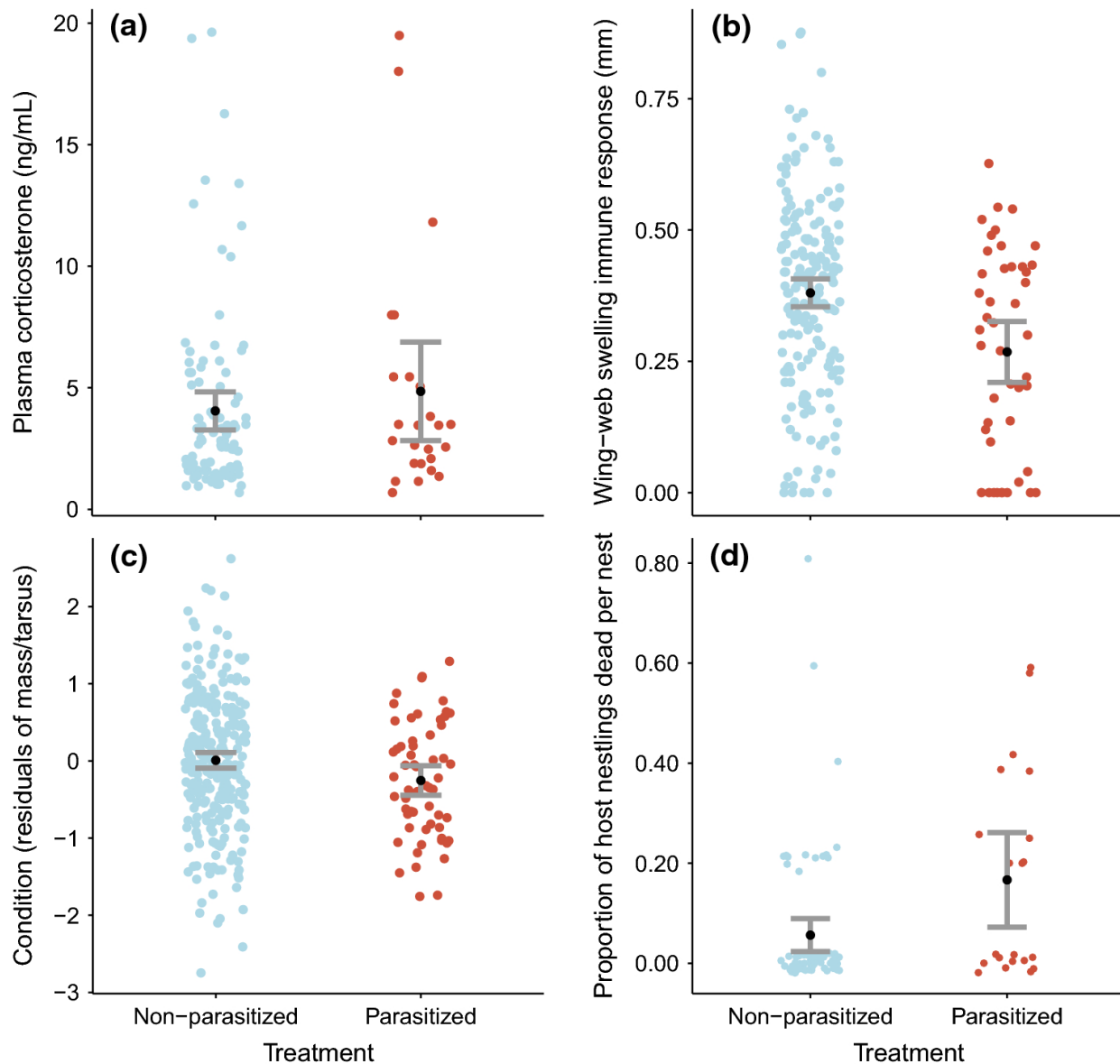


Figure 8. The effect of experimental brown-headed cowbird parasitism on different aspects of prothonotary warbler nestling phenotype and survival, including host nestling (a) plasma corticosterone levels (ng/mL), (b) immune response, as measured through wing-web swelling 24 h after injection of PHA, (c) condition, calculated as the residuals of mass on tarsus, and (d) the proportion of host nestlings that died in each nest. Means are shown with the black circle and lines represent 95% confidence intervals.

4.4.2 Host nestling T-cell-mediated immune response

The top AIC_c model for immune response of the warbler nestlings contained the interaction between cowbird presence and ordinal date, and the additive effect of brood size (Table 2;

Supplementary Table 2). No other models were within $\Delta AIC_c \leq 2.00$. Host nestling immune response was lower with cowbird parasitism overall ($\beta = -0.057$, 95% CI = $-0.092, -0.021$, Figure 8b; Table 3), but the immune function of parasitized warblers increased across ordinal date more than non-parasitized warblers ($\beta = 0.055$, 95% CI = $0.020, 0.090$, Figure 9, Supplementary Figure 1). Immune response also decreased with increasing overall brood size ($\beta = -0.033$, 95% CI = $-0.061, -0.006$). Immune response was not affected by ordinal date alone ($\beta = 0.010$, 95% CI = $-0.018, 0.039$). Nests with a cowbird did not extend as far into the breeding season as host-only nests because of a natural decline in parasitism rates throughout the season, but truncating the samples to not include host-only nests after the last parasitized nest's date yielded the same top model (Supplementary Table 5). Experimental cowbird parasitism by itself now had a CI for the immune response that overlapped zero ($\beta = -0.045$, 95% CI = $-0.103, 0.012$, Supplementary Table 6), but the interaction between cowbird presence and ordinal date remained important as it still did not overlap zero ($\beta = 0.054$, 95% CI = $0.012, 0.096$).

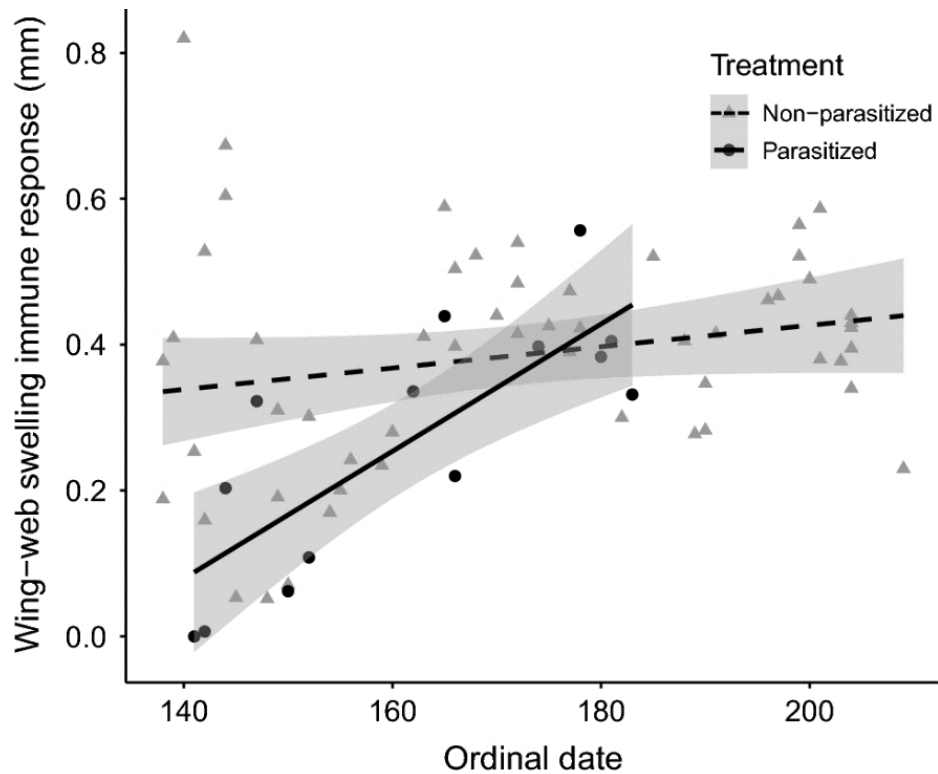


Figure 9. The effect of brood parasitism on T-cell-mediated immune response in prothonotary warbler nestlings and its interaction with ordinal date. Each point represents the averaged measurements of the offspring of a single brood. Lines of best fit for each treatment are indicated by the solid and dashed lines. The shaded area represents the 95% confidence intervals of the fitted lines.

4.4.3 Host nestling body condition

The top model for host nestling body condition in the AIC_c ranking was a model that contained the interaction of cowbird presence and brood mass (Table 2; Supplementary Table 3). Host nestling body condition was not lower with cowbird presence overall ($\beta = 0.022$, 95% CI = -0.207, 0.251, Figure 8c; Table 3), but because of the interaction term, the body condition of parasitized warblers decreased with increasing brood mass whereas the body condition of non-parasitized warblers increased with increasing brood mass ($\beta = -0.143$, 95% CI = -0.275, -0.010, Figure 10; Supplementary Figure 2). The interaction between cowbird presence and brood size, as well as brood size alone, were both within $\Delta\text{AIC}_c \leq 2.00$, but unlike brood mass,

the body condition of parasitized warblers did not decrease with increasing brood size ($\beta = -0.126$, 95% CI = $-0.259, 0.006$) and the body condition of host nestlings did not change across different brood sizes ($\beta = -0.060$, 95% CI = $-0.170, 0.051$).

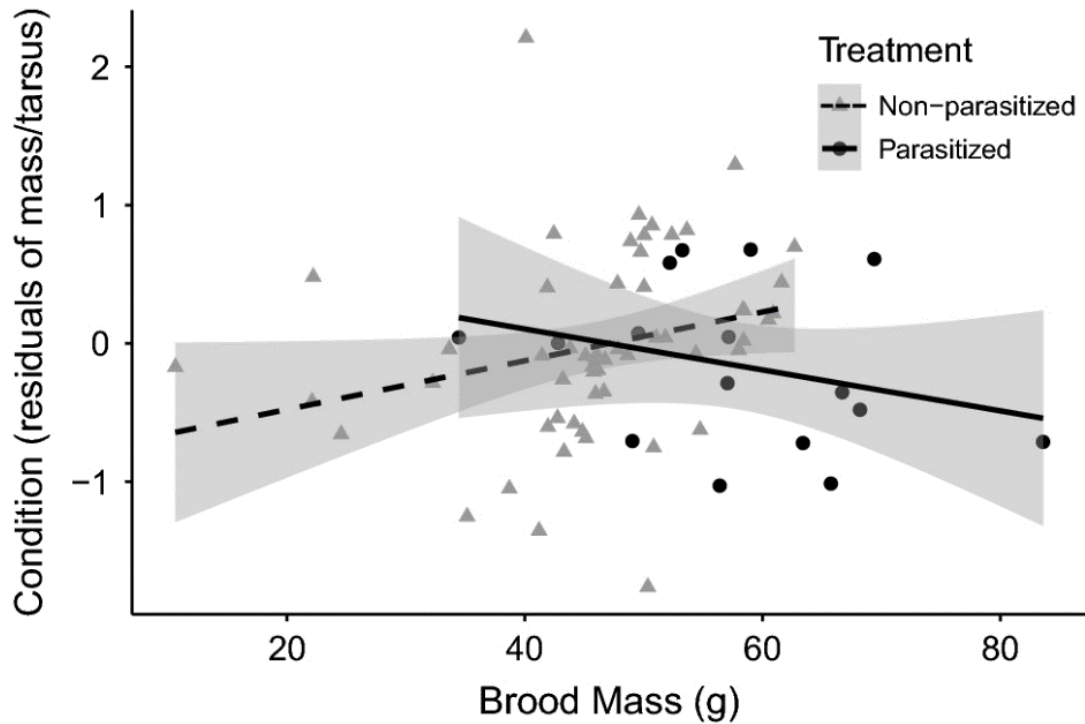


Figure 10. The effect of parasitism on nestling condition, calculated as the residuals of mass on tarsus and its interaction with brood mass. Because our analyses used female ID as a random effect, each point represents the averaged measurements of the offspring of a single female, apart from females that had different treatments between broods, in which case the measurements of the offspring are averaged within that brood only. Lines of best fit for each treatment are indicated by the solid and dashed lines. The shaded area represents the 95% confidence intervals of the fitted lines.

4.4.4 Host nestling mortality

For the proportion of host nestlings that died in a nest, the top AIC_C model contained only cowbird presence (Table 2; Supplementary Table 4), which increased nestling mortality ($\beta = 0.044$, 95% CI = $0.005, 0.082$, Figure 8d; Table 3). No other models were $\Delta\text{AIC}_C \leq 2.00$.

4.4.5 Physiological trade-offs

Corticosterone levels did not covary significantly with immune response in host nestlings ($F_{1,36} = 0.013$, $P = 0.908$, Figure 11a; Supplementary Figure 3a), or with their body condition ($F_{1,54} = 0.121$, $P = 0.730$, Figure 11b; Supplementary Figure 3b).

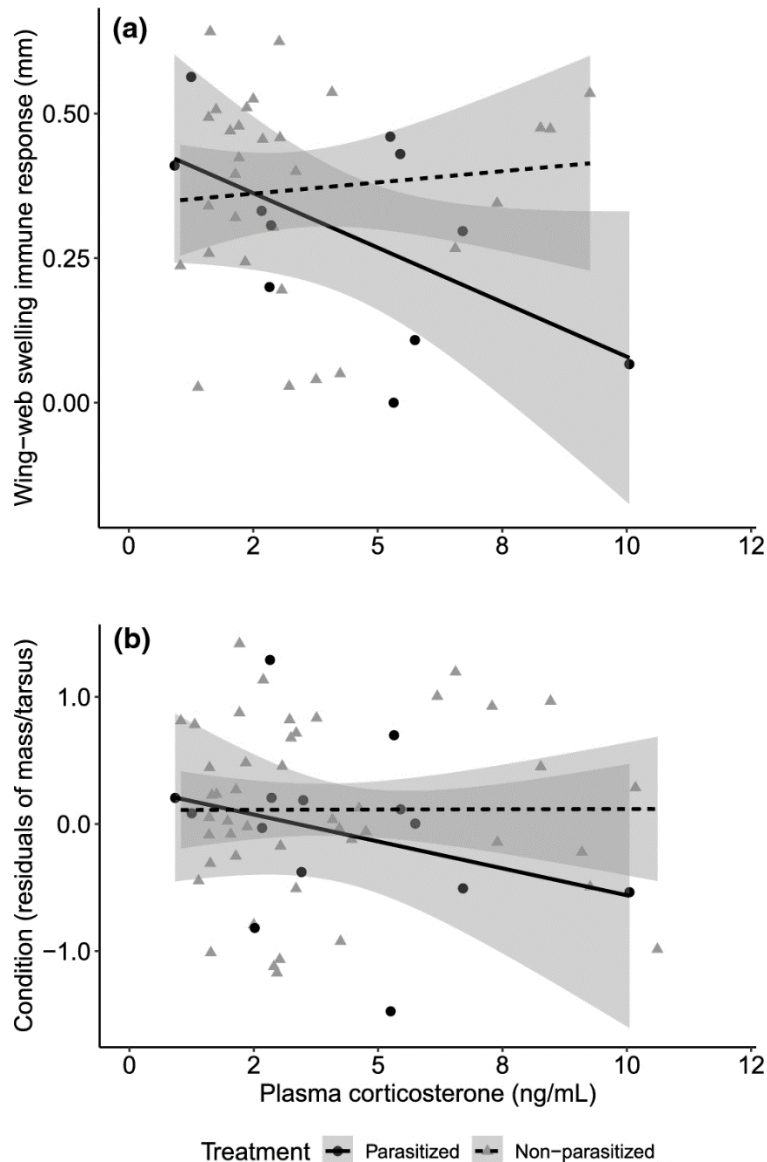


Figure 11. Plot showing the lack of relationships between raw plasma corticosterone levels of prothonotary warbler nestlings and (a) their immune response as measured by wing-web swelling in response to PHA injection in parasitized and non-parasitized broods, and (b) nestling condition, calculated as the residuals of mass on tarsus, in parasitized and non-parasitized

Figure 11. (Cont.) broods. Because we used female ID as a random effect, each point represents the averaged measurements of the offspring of a single female, apart from females that had different treatments between broods, in which case the measurements of the offspring are averaged within that brood only. The shaded area represents the 95% confidence intervals of the fitted lines.

4.5 DISCUSSION

Our results show that brood parasitism by the brown-headed cowbird causes detrimental effects on some, but not all, aspects of host nestling physiology in the prothonotary warbler.

Specifically, we did not find an effect of experimental parasitism on prothonotary warbler nestlings' baseline plasma CORT levels or body condition. In contrast, we found that the parasitism treatment caused decreased immune responses to PHA, as well as higher host mortality across the nestling stage, relative to the non-parasitized treatment. These results may have arisen due to the combination of increased brood competition through brood enlargement, as well as the competition specifically with a larger parasitic chick. In our study system, prothonotary warblers usually incur these costs concurrently, as brown-headed cowbirds rarely remove host eggs when they parasitize nests (Hoover 2003a).

Our experimental findings that warbler nestlings have decreased immune responses and increased mortality in the presence of cowbirds demonstrates that cowbird parasitism does indeed have a detrimental effect on warbler nestling phenotypes and survival, in agreement with previous observational research in this system (e.g., Hoover and Reetz 2006). Obligate brood parasitic and nestmate non-evictor brown-headed cowbirds are often larger, beg more vigorously, and are superior competitors compared to the host nestlings with which they are raised (Hauber 2003a; but see Rivers et al. 2013). Cowbirds can thus pose a significant threat to the development of smaller warbler nestlings, as one cowbird chick represents the mass

equivalent of 2.0–2.5 prothonotary warbler nestlings (Hoover 2003a). Decreased host nestling survival was likely due to the increased rivalry imposed by competitively begging cowbird chicks (e.g., Hauber 2003c), possibly as a cost of reduced parental food provisions received per host chick (e.g., Kilner et al. 2004). Although previous research showed that warbler parents are able to increase their provisions in response to increasing brood mass and cowbirds (Hoover and Reetz 2006), it is possible that host nestlings do not compete equitably for these increased provisions (e.g., Lichtenstein and Sealy 1998). Broods of equivalent masses with cowbirds (rather than without cowbirds) require higher provisioning than nests without cowbirds, and cowbirds could potentially be monopolizing parental provisions (Kilner et al. 2004). This could also explain why parasitism did not affect body condition alone in prothonotary warbler nestlings. Body condition decreased with increased brood mass in parasitized nests but increased with brood mass in non-parasitized nests (Figure 10).

In response to the likely increased competition imposed by a parasitic chick in the host nest environment, we predicted that host nestlings would have higher baseline CORT levels, because the cowbird nestmate could present as a stressor. If correct, higher levels of CORT are associated with exaggerated begging and could help the smaller warblers compete with their large, parasitic nestmate (Kitaysky et al. 2001, 2003; Loiseau et al. 2008). In contrast, we found that warbler nestlings have statistically similar CORT plasma levels regardless of parasitism status. This is contrary to Ibáñez-Álamo et al. (2012), who found increased baseline CORT levels in both host European magpies and carrion crows as a result of brood parasitism by the non-evictor great spotted cuckoo. However, not all nestling stages showed this difference between parasitized and non-parasitized broods. Larger crow chicks only had increased CORT levels during the mid-

nestling period, whereas the smaller magpie chicks had higher CORT levels during both the mid- and late-nestling period. Thus, stronger effects may be seen in some developmental stages but not in others, and it is possible that warbler nestlings do respond to cowbird parasitism by having higher CORT levels during their last 3–4 days in the nest (after our day 8 post-hatch measurement) due to stronger HPA axis response or higher nestmate competition during this stage (Roncalli et al. 2020). Conversely, it is also possible that warbler nestlings respond to cowbird parasitism with increased stress-induced CORT levels, rather than through elevated baseline CORT levels. Finally, the CORT responses of parasitized warbler nestlings could have been buffered through the behavior or physiology of parents (Antonson et al. 2020). Cowbird parasitism negatively affected another physiological metric, immune response, as well as survival in nestling warblers, but the proximate mechanisms for these relationships, including a potential trade-off between these and other physiological traits throughout development, need further studies at diverse nestling and fledgling ages, as well as comparatively in species with different life-histories.

Warbler nestlings raised with cowbirds and in larger broods, regardless of parasitism, had lower immune responses via T-cell-mediated immunity. T-cell-mediated immune response has been shown to be lower in nestlings raised in larger broods in other bird species such as barn swallows, great tits (*Parus major*), and pied flycatchers (*Ficedula hypoleuca*) (Saino et al. 1997; Hõrak et al. 1999; Ilmonen et al. 2003), which suggests that in our study, competition with cowbirds may simply be a proxy for larger brood sizes (i.e., number of nestlings present) and not specific to the brood parasite per se. Indeed, brood mass, which accounts for the larger size of cowbird nestlings, was not important in explaining immune response in warbler host nestlings.

In turn, the immune responses of warbler nestlings in parasitized nests early in the season were weaker than warbler nestlings in parasitized nests later in the season, potentially as a result of the larger brood sizes in first broods compared to the smaller and later second broods (Hoover 2003a). It is possible that immune system responsiveness is simply a function of the overall brood size regardless of parasite/host composition of the brood and future studies will be needed to adequately discriminate between these two hypotheses. However, because cowbirds rarely remove host eggs in our system, the majority (~75%) of prothonotary warbler nests that are naturally parasitized will incur the costs of cowbird parasitism and brood enlargement in tandem (Hoover 2003a). Competition with a parasitic nestmate vs. competition with an additional host (conspecific) nestmate might have costs that vary differentially for hosts of different sizes, so it is possible that in hosts smaller than prothonotary warblers, brood mass and parasite species might be more important than brood augmentation (Rivers 2007).

Higher CORT levels in multiple species of avian nestlings have been found to negatively covary with immune responses (Saino et al. 2003; Loiseau et al. 2008; Butler et al. 2010). We expected to see this trade-off in the immune response of the host nestlings as a result of higher CORT levels, but did not find a significant relationship between these two metrics. The interactions between glucocorticoids and immune response are complex, however, and can be affected by multiple environmental factors (Buchanan 2000). Inhibition of T-cell-mediated immunity as a result of stress can occur independently of variation in plasma CORT (Gao and Deviche 2019), which may explain why we did not find a significant negative relationship between immune function and CORT in this study. The effect of stressors on immunity may also vary according to

the sensitivity of different components of immunity, so CORT may affect immune components other than T-cell-mediated immunity in this study system (Martin 2009). In addition, baseline corticosterone concentration and H/L-ratio differ in sensitivity to various stressors (Müller et al. 2011). Similarly, we also expected to find a trade-off between CORT levels and body condition of warbler nestlings, as CORT is known to negatively affect growth in young birds (Spencer and Verhulst 2007; Loiseau et al. 2008). However, we did not find statistical support for this correlation. Again, the relationship between CORT levels and body condition is likely not a simple trade-off in prothonotary warbler nestlings, but rather the interaction of many environmental and physiological factors outside of those measured in this study, such as ectoparasite load (Raouf et al. 2006) or maternal investment (Rubolini et al. 2005).

Understanding the physiological effect of brood parasitism on the nestlings of acceptor hosts is important in determining how host reproductive success is influenced by brood parasitism and how host nestlings may tolerate the detrimental effects of brood parasitism (Avilés 2018). We report here that although experimental brood parasitism of brown-headed cowbirds reduced the host nestlings' immune responses and survival, this treatment did not affect their baseline corticosterone levels or body condition. Additionally, we found no relationship between the levels of corticosterone, immune response, or body condition in the host nestlings, suggesting that these pathways are likely affected by many variables in this system. Although prothonotary warbler nestlings do suffer from decreased immune function and survival when raised alongside the larger, more competitive cowbird chick, they are resilient against brood parasitism in at least some other fitness-relevant physiological aspects in the late nestling stage. Thus, the complex effects of cowbird parasitism on the physiological responses and the resulting fitness of host

nestlings, as well as the overall reproductive success of foster parents, can interact and impact the proximate mechanisms underlying the coevolutionary interactions between brood parasites and hosts and the evolutionary path of these host defenses.

CHAPTER 5: SHOULD I STAY OR SHOULD I GO: THE EFFECT OF AVIAN BROOD PARASITISM ON HOST FLEDGING DYNAMICS⁵

5.1 ABSTRACT

Transitions between life history stages are fitness-limiting events that depend on environmental and individual characteristics. For altricial birds, fledging from the nest is a critical shift in development with direct impacts on survival, yet it remains one of the most understudied components of avian ontogeny. Even less is known about how brood parasitism affects the fledging process in host nestlings. The prothonotary warbler (*Protonotaria citrea*) is a host of the obligate brood parasitic brown-headed cowbird (*Molothrus ater*). We tested whether the presence of parasitic nestlings negatively alters host fledging by experimentally parasitizing nests with a cowbird (heterospecific parasite) or a warbler (conspecific parasite) egg, comparing them to non-manipulated control nests, and monitoring them using radio-frequency identification (RFID) systems. As expected, in heterospecifically parasitized nests, warblers were smaller, fledged at older ages, and had greater overall fledging latency compared to conspecifically parasitized nests. There was no such impact of conspecific parasitism relative to controls. Warbler nestling size predicted the age and order of fledging, with larger nestlings fledging earlier. Nestlings fledging at earlier ages fledged later during daytime hours. Cowbirds and last-fledged, smaller warbler chicks spent the most time in the nest entrance before fledging. Finally, although male warbler nestlings were larger than females, there were no sex effects or effects of

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extra-pair status on fledging. Our study shows that while conspecific parasitism has no detectable effect on host nestmates, heterospecific parasitism impacts host size and fledging phenology, which may influence post-fledging survival of parasitized broods.

5.2 INTRODUCTION

In many animals, major transitions, or phase shifts, between life history stages are critical, fitness-limiting events that are affected or determined by characteristics of the previous life stage. Environmental and individual characteristics can affect life stage transitions, such as hydroperiods, food availability, and the number of eggs laid in their cohort affecting the length of the larval period in frogs (Berven 1990; Rudolf and Rödel 2007; Amburgey et al. 2012), food concentrations affecting the age of metamorphosis in copepods (Twombly 1996), and learning ability and fearfulness affecting the weaning age in dairy calves (Neave et al. 2019). These transitions are especially important for juveniles, as many species experience high mortality early in ontogeny (Doherty et al. 2004; Smith et al. 2013; Jones et al. 2017).

Fledging, or the act of leaving the nest, is a crucial transition of the reproductive cycle of altricial birds. During fledging, dependent young transition from the nestling stage, a largely sedentary lifestyle confined to the nest structure, to a much more active post-fledging stage when they are typically still dependent on their parents for food and predator warnings, but also begin to independently move around in the environment (Jones et al. 2020). The process and outcome of fledging represent some of the most understudied components of both avian breeding biology and ontogeny (Martin et al. 2018), and for most species, little is known about fledging phenology beyond the average nestling period length (Mainwaring 2016). Despite this, fledging has been

identified as a critical period in the avian lifecycle that serves as a bottleneck for survival, with 40–60% of birds dying within the first 4 days of fledging (Naef-Daenzer et al. 2001; Jones et al. 2017). Thus, fledging is a key phase shift event linking characteristics of the nestling period with post-fledge survival and subsequent recruitment into the adult population (Radersma et al. 2015).

Competition and the nest environment can be important predictors of fledging characteristics. In tree swallows (*Tachycineta bicolor*), brood size explained a significant proportion of the variance in fledging age independent of development, although in some years this relationship was positive, whereas in others it was negative (Michaud and Leonard 2000). Increased competition for parental provisions between nestmates in larger broods could result in fledging at younger ages, as nestlings may depart the nest to obtain additional feedings (Nilsson and Svensson 1993), particularly if parents feed fledglings more than nestlings (Lemel 1989). Alternatively, more competition could delay fledging because nestlings may suffer from slower development (De Kogel 1997), or the nest may be too crowded for nestlings to effectively stretch their wings and exercise their flight muscles (Michaud and Leonard 2000). For cavity-nesting species, time spent in the nest entrance before fledging could also be impacted by competitive ability, and in mountain bluebirds (*Sialia currucoides*), nestlings that spent the longest amount of time in the cavity entrance typically fledged first (Johnson et al. 2013).

Similarly, metrics of body size, condition, and their components can covary with fledging characteristics within a brood. For example, wing length (Michaud and Leonard 2000; Radersma et al. 2011; Johnson et al. 2013) and body mass (Radersma et al. 2011) predict the age at which a brood fledges, with heavier and longer-winged broods fledging at earlier ages in comparison to

lighter and shorter-winged broods. Similarly, within broods, nestlings with longer wings fledge before their shorter-winged nestmates (Johnson et al. 2004; Radersma et al. 2011), whereas the relationship with body mass may be more complex (Lemel 1989; Johnson et al. 2004). For species with sex differences in nestling size or competitiveness, sex ratio could also be an important predictor of fledging characteristics; however, Radersma et al. (2011) did not find an effect of brood sex ratio on fledging time or asynchrony, and fledging order was not affected by chick sex.

The first few days after fledging are associated with high mortality for young birds, as survival during the first week out of the nest is substantially lower than the nestling period and later post-fledging in many altricial species (Naef-Daenzer and Gruebler 2016). Development not only affects fledging phenology but post-fledge survival as well. For example, great tits (*Parus major*) and coal tits (*P. ater*) that were heavier at fledging had higher overwinter recruitment rates (Radersma et al. 2015; Naef-Daenzer and Gruebler 2016). Wing development has also been shown to be important for fledgling survival, as fledglings that leave the nest earlier with more poorly developed wings are worse at flying and have higher mortality (Martin et al. 2018; Jones and Ward 2020). Predation is the largest source of mortality for recently fledged birds (Naef-Daenzer and Gruebler 2016), so enhanced flying ability could help fledglings escape predators.

Post-fledge survival and recruitment may also be affected by fledging phenology of the nestlings; for example, great tit nestlings that fledged early in the morning were more likely to be recruited into the adult population (Radersma et al. 2015). For many altricial species, fledging typically occurs within 6 h of sunrise and the nestmates often fledge within hours of each other

(Lemel 1989; Johnson et al. 2004; Chiavacci et al. 2015; Santema et al. 2021). According to the maximum-time hypothesis, earlier fledging may reduce predation risk by extending the daylight hours a fledgling has to find a safe location to stay before dark (Johnson et al. 2004; Radersma et al. 2015; Chiavacci et al. 2015). Fledging during the dark may be more dangerous than fledging during the day, due to increased risk of predation and losing sight of parents (Johnson et al. 2004; Radersma et al. 2015; Chiavacci et al. 2015). In contrast, a recent study on blue tits (*Cyanistes caeruleus*) found no relationship between the timing of fledging and post-fledging survival and, instead, detected that this timing was related to a developmental threshold (Santema et al. 2021). Nestlings may tend to fledge early in the day because they reach this developmental threshold required for fledging overnight (Santema et al. 2021).

One aspect of the nestling period that has yet to be studied in-depth in the context of fledging ecology is avian brood parasitism. Brood parasitism is a reproductive strategy in which a female bird lays her egg(s) into the nest of other(s), relinquishing parental duties upon the host (Soler 2017). The parasite may be the same species as the host, known as conspecific brood parasitism, or a different species than the host, known as heterospecific brood parasitism. Brood parasitism can impose many fitness costs by causing egg or offspring death as well as negatively impacting future fitness of host parents (Mark and Rubenstein 2013; Moskát et al. 2017; Antonson et al. 2020). In our study system, the obligate brood parasitic brown-headed cowbird (*Molothrus ater*) parasitizes nests of the prothonotary warbler (*Protonotaria citrea*), which accept cowbird young and raise them alongside their own offspring. Cowbird nestlings cause reduced growth, survival, and recruitment of warbler nestlings (Hoover 2003a; McKim-Louder et al. 2013; Scharf et al. 2021b). Conspecific parasitism between prothonotary warbler nests can also be detrimental, as

although hosts fledge more total young, they fledge fewer biological offspring (Tucker et al. 2016). Despite this, very little is known about how either heterospecific or conspecific parasitism mediates fledging dynamics of the host nestlings. A study on the parasitic, nestmate-evictor American striped cuckoo (*Tapera naevia*), and its host, the rufous-and-white wren (*Thryophilus rufalbus*), found that mean age at fledging did not differ between parasitized and non-parasitized nests, although cuckoo chicks were more variable in their fledge age than wren chicks (Mark and Rubenstein 2013). Another study found that nestmate-evictor common cuckoo (*Cuculus canorus*) chicks fledged at older ages when forced to cohabitate with common redstarts (*Phoenicurus phoenicurus*) (Grim et al. 2009). However, Kysučan et al. (2020) found no difference in fledging age between cuckoos in mixed or solitary broods, and also found no difference in the age of fledging for redstart hosts from parasitized vs. non-parasitized broods. We are aware of no studies that have investigated the effects of conspecific parasitism on the fledging behavior of host nestlings.

In our experiment, we set out to determine (i) how parasitism status affects fledging phenology, (ii) whether nestling size is a predictor of fledging characteristics, (iii) how these fledging characteristics interact, and (iv) if the chicks' sex or extra-pair status affects fledging characteristics. We also examined (v) the effect of parasitism treatment on nestling survival and size. For our manipulations, we used nests of the prothonotary warbler, an acceptor host of the larger, obligate brood parasitic brown-headed cowbird, and added a cowbird egg (heterospecific parasitism), an unrelated warbler egg (conspecific parasitism) or kept the nest non-parasitized as a control. Even though it is already well known in our system that cowbirds negatively affect the survival, growth, and recruitment of prothonotary warbler host chicks (Hoover 2003a; McKim-

Louder et al. 2013; Scharf et al. 2021b), we have not yet analyzed these effects in the context of experimental conspecific brood parasitism. Because of these known negative effects of cowbird parasitism on warbler nestlings, we predicted that warbler nestlings parasitized by cowbirds should experience altered fledging. We predicted that cowbird parasitism should cause (A1) increased fledging latency between all nestlings, (A2) increased age of hosts at fledging, (A3) altered fledging time of day for hosts, and (A4) decreased duration of time spent in the nest entrance compared to conspecifically parasitized nests. We also predicted that larger nestlings should be (B1) younger in age at fledging, (B2) earlier in fledging order, (B3) fledge later in the day, and (B4) spend more time in the nest entrance. We also were interested in whether there were negative relationships between (C1) age and fledging time of day, (C2) fledging order and fledging time of day, and (C3) fledging order and time spent in the nest entrance. Finally, we looked at the effect of sex and extra-pair status of the warbler chicks on the size and fledging characteristics listed above, and sex effects of the cowbird nestlings on size.

5.3 METHODS

5.3.1 Study area and species

All manipulations were conducted and data collected between May and August 2020 and 2021 from two separate study sites within the Cache River watershed in southern Illinois, USA. The prothonotary warbler is a socially monogamous, neotropical migrant to eastern North America that specializes in bottomland swamp habitats (Petit 1999). Prothonotary warblers are obligate secondary cavity-nesting species, and readily nest in artificial boxes constructed from 1.9 L beverage cartons. At our sites and habitat, prothonotary warblers are a common cowbird host, with up to 60% of these nest boxes naturally parasitized with one or more cowbird eggs (Hoover

2003a). Critically for the design of our manipulations, in this system, the majority (~ 75%) of naturally parasitized nests simply receive a brown-headed cowbird egg without having one of the host eggs removed (Hoover 2003a). Parasitism rates peak early in the season, during first broods, and decline throughout the summer (Hoover et al. 2006). Warblers typically have two nesting attempts in a single summer, with the majority of first clutches comprising 5 eggs and clutches thereafter having fewer. Incubation periods typically last 12 days (Petit 1999). During first clutches, all eggs hatch the same day, whereas during second clutches, females usually incubate on the penultimate laid egg and one egg hatches a day later than the rest. Cowbird eggs have rapid embryonic development (McMaster and Sealy 1998) and typically hatch one day earlier than the warbler eggs. Although conspecific parasitism does occur in prothonotary warblers (Tucker et al. 2016; Tucker and Bulluck 2018), we do not see any evidence of it in our population, and did not have any instances of it naturally occurring during our study.

5.3.2 Experimental manipulations

Active warbler nestboxes were randomly selected for each treatment prior to incubation. All experimentally added eggs were unincubated or incubated for less than 1 day before transfer. For the heterospecific parasitism treatment, warbler clutches were experimentally parasitized with a cowbird egg during the laying stage or within 1 day of the onset of incubation. For the conspecific parasitism treatment, warbler pairs were experimentally parasitized with a warbler egg sourced from another study site to ensure that the offspring would be unrelated to the social pair, also during the laying stage or within 1 day of incubation. Finally, for the control treatment, the nest was checked regularly but no eggs were added, or experimentally added eggs did not hatch. In all treatments, any naturally laid cowbird eggs were removed. To distinguish between

the experimentally added eggs and the host eggs and/or naturally laid cowbird eggs, a small dot was placed on the sharp pole of the added egg with a felt-tip pen. These treatments were randomly distributed across the first and second clutches (Fisher's exact test, $P = 0.678$).

Nests were monitored every other day to determine the day of hatching, age of the nestlings, and mortality. All adults were captured and banded with a USA federal aluminum band, a colored passive integrated transponder (PIT, Eccel Technology, UK) tag, and additional color bands to generate unique band and color combinations. Adult males were caught at mist nets via playback, and females were caught during incubation by placing a transparent bag over the nest entrance. Nestlings were banded with an aluminum band and PIT-tag at an average of 6 days (range: 5–8 days) of age to prevent force-fledging. Measurements, including weight (g), tarsus length (mm), and first primary feather length (mm), were also taken at this time. The initial sample sizes were as follows: 47 nests experimentally parasitized with cowbird eggs, 31 nests experimentally parasitized with warbler eggs, and 66 nests that were non-parasitized controls. Due to nest (e.g., predation) and technology (e.g., battery death) failure, the final sample sizes were 12 heterospecifically parasitized nests, 11 conspecifically parasitized nests, and 47 non-parasitized control nests that had all fledging information recorded. It was not possible to use blinded methods when recording size measurement data because the treatment status of each nest was always known, however, fledging metrics were recorded blindly to treatment because they were only taken by RFID readers.

5.3.3 RFID systems

We used radio-frequency identification systems (RFID) to record when a bird was in the nest entrance and the identity of the bird. This RFID system comprised an Arduino circuit board (Bridge et al. 2019), 12 V battery, and 12 V to 5 V converter stored in a waterproof battery box, with a copper antenna extending out of the battery box into the nestbox, forming a loop around the entrance hole. The RFID reader checked for the presence of a bird with a passive integrated transponder (PIT) tag in the entrance every 0.2 s and recorded the identity and time of every individual in the entrance. If an individual was present for more than one 0.2 s check, the reader would record the tag every 8 s. If an individual was present for less than 8 s, it was recorded once. The RFID reader can only record one tag at a time; however, the antenna is 42 mm, so it would be difficult for two birds to be fully in the entrance at once. These readers were set to sleep between sunset (22:00) and local sunrise (5:00). Readers were checked and data downloaded every week and batteries replaced as necessary. Twenty-five nests had nestlings PIT-tagged and measured but did not include the time of fledging for all nestlings, 14 of these were functional but did not record every nestling fledging, whereas 11 were excluded because of battery deaths, reader malfunctions, accidental force fledging, or total brood death. Thus, it is possible that for 14 nests, nestlings could have fledged between 22:00 and 5:00, or there may have simply been PIT-tag interference. Only nests that detected all nestlings ($N = 70$) were included in the analysis. RFID systems have previously been used successfully to measure fledging behaviors (Radersma et al. 2011, 2015; Johnson et al. 2013; Santema et al. 2021).

5.3.4 DNA analysis

As we were not present when the conspecific parasitic warbler egg hatched, to determine its identity, as well as the sex of all chicks in the study, a blood sample (< 100 µl) was taken from each nestling from the brachial vein via heparinized microcapillary tubes at an average of 6 days of age (range: 5–8 days) at the same time the nestlings were measured. These blood samples were stored at room temperature in 100% ethanol until extraction. The samples were extracted using Qiagen DNeasy Blood & Tissue kits (Qiagen Inc., Valencia, CA, USA).

For assessing the identity of the conspecific parasite chick, the PCR reactions were run with a set of 8 microsatellite primers using a Bio-Rad® T-100 thermal cycler (Bio-Rad, Hercules, CA USA, for detailed methods and primer sets, see Schelsky 2010). Microsatellite primers had been screened for polymorphism from closely related species and optimized for prothonotary warblers (Schelsky 2010). We used three primers (Dpu01, Dpu03, Dpu16) from yellow warbler (*Setophaga petechia*) (Dawson et al. 1997), four (VeCr04, VeCr05, VeCr06, VeCr07) from golden-winged warbler (*Vermivora chrysoptera*) (Stenzler et al. 2004), and one (Lsw12) from Swainson's Warbler (*Limnothlypis swainsonii*) (Winker et al. 1999). Forward primers were labelled with 5'-fluorescent labels (NED, HEX, 6-FAM, Applied Biosystems, Foster City, CA, USA). The cycling profile was 1 cycle at 95 °C for 3 min, 30 cycles of 30 s at 95 °C, 45 s at the locus-specific annealing temperature (55 °C for Lsw12 and VeCr06 and 60 °C for remaining markers), and 60 s at 72 °C followed by a final extension cycle of 10 min at 72 °C. Each 15 µl reaction contained 15–100 ng of DNA, DreamTaq Master Mix containing DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA), 2X DreamTaq buffer which contains dATP, dCTP, dGTP, and dTTP, 0.4 mM each, 4 mM MgCl₂ and 0.6 uM of the forward and

reverse primers each. Fragment sizes for all PCR products were analyzed by the University of Illinois at Urbana-Champaign Core Sequencing Facility using an ABI 3730xl (Applied Biosystems® Inc., Foster City, CA, USA). Fragment data were sized and scored using GeneMapper® Software (Applied Biosystems® Inc., Foster City, CA, USA). Parentage analyses were done using CERVUS 3.0.7 (Field Genetics Ltd) (Kalinowski et al. 2007). We used a simulation with both parents' identities and sexes known to determine which warbler chick was the parasite. In all cases, the warbler parasite was identifiable at 95% confidence.

We genotyped all warbler and cowbird nestlings at the CHD gene using the 1237L forward and P2 reverse primers (Griffiths et al. 1998; Kahn et al. 1998). The CHD gene is present on the Z and W chromosomes (e.g., female birds are heterogametic ZW) and yields different-sized fragments depending on which chromosome the amplicon(s) reside(s). We used DNA from known-sex adult warblers and cowbirds as positive controls.

5.3.5 Statistical analysis

We used R version 4.1.1 (R Core Team 2017) for all statistical analyses and figures with packages “ggplot2” (Wickham 2009), “nlme” (Pinheiro et al. 2022), “FactoMineR” (Lê et al. 2008), and “missMDA” (Josse and Husson 2016). When comparing treatment groups, we had two a priori comparisons of interest—the first between the hetero- and conspecific parasitism treatments to test for effects specific to parasite identity, and the second between the conspecific parasitism and control treatments to test for effects specific to brood enlargement with an unrelated egg/chick. All models containing treatment were analyzed accordingly.

For nestling size, the first principal component (PC1) from a principal component analysis (PCA) was used with mass, first primary feather length, and tarsus length each loading positively and explaining greater than 80% of the variance. Whenever this principal component was used in a model, nestling age at measurement was used as a covariate. Fledging latency was calculated as the time elapsed since the fledging of the first nestling in a nest, regardless of species identity. The number of fledglings per nest was included as a covariate for models investigating fledging latency. Fledging order is fledging rank linearly transformed to compare broods of different sizes, with values ranging from 0 (first fledged in a brood) to 1 (last fledged in a brood) (Radersma et al. 2011). Fledging time of day was calculated as the hours elapsed since sunrise that a nestling fledged. For all linear mixed models, year was also included as a covariate because samples were taken from 2020 and 2021.

Two-tailed Wilcoxon's tests were used to compare survival via natural (host-only, before manipulations) clutch size, natural host brood size at banding age, and natural number of fledglings between the three treatments, as well as experimental (including hetero- and conspecific parasites) brood sizes at banding age (Table 4).

We used general linear mixed models (GLMMs) with nest attempt ID as a random effect to determine the effects of treatment, species, and conspecific parasite identity on nestling size, fledging latency, fledging age, fledging time of day, and time spent in the nest entrance as approximated by the number of RFID reads (Table 5). We used GLMMs to determine the effect of nestling size on fledging age, fledging order, fledging time of day, and time spent in the nest entrance (Table 6). To determine the effects of fledging characteristics on each other, the effect

of fledging age on the time of day of fledging, the effect of fledging order on the time of day of fledging, and the effect of fledging order on time spent in the nest entrance were analyzed using GLMMs (Table 7).

Table 4. List of models used in the statistical analysis for this paper. Table 4 corresponds to the “Treatment effects on survival” section in the results.

<i>Treatment effects on survival:</i>				
Response variable	Data included	Sample Size	Predictor	Statistical test
Number of natural warbler eggs	All	N = 95	Treatment	Wilcoxon
Total (experimental) brood size	All	N = 95	Treatment	Wilcoxon
Natural brood size	All	N = 94	Treatment	Wilcoxon
Natural number of fledglings	All	N = 94	Treatment	Wilcoxon

Table 5. List of models used in the statistical analysis for this paper. Table 5 corresponds to the “Treatment effects on fledging characteristics” section in the results. Predictor variables of interest are shown in bold, with covariates non-bolded. Nestling size PC1 was generated from a PCA of nestling mass, wing length, and tarsus length at 6 days old. Fledging order is the relative fledge order with 0 being first fledged. Fledging time is measured as the hours since sunrise that a nestling fledged.

<i>Treatment effects on fledging characteristics:</i>					
Response variable	Data included	Sample size	Predictor + covariates	Model type	Random effect
Nestling size PC1	All host warblers	N = 346	Treatment Nestling age Year	GLMM	Nest ID
Nestling size PC1	Heterospecifically parasitized nests	N = 61	Species Nestling age Year	GLMM	Nest ID
Nestling size PC1	Conspecifically parasitized nests	N = 67	Conspecific parasite identity Nestling age Year	GLMM	Nest ID

Table 5. (Cont.)

Response variable	Data included	Sample size	Predictor + covariates	Model type	Random effect
Fledging latency between all nestlings	All warblers + cowbirds	N = 268	Treatment Species Number of fledglings/brood Year	GLMM	Nest ID
Fledging latency between warblers	All host warblers	N = 246	Treatment Number of fledglings/brood Year	GLMM	Nest ID
Fledging latency between warblers	Conspecifically parasitized nests	N = 50	Conspecific parasite identity Number of fledglings per brood Year	GLMM	Nest ID
Fledging age (days)	All host warblers	N = 246	Treatment Year	GLMM	Nest ID
Fledging age (days)	Heterospecifically parasitized nests	N = 44	Species Year	GLMM	Nest ID
Fledging age (days)	Conspecifically parasitized nests	N = 50	Conspecific parasite identity Year	GLMM	Nest ID
Fledging time	All host warblers	N = 246	Treatment Year	GLMM	Nest ID
Fledging time	Heterospecifically parasitized nests	N = 44	Species Year	GLMM	Nest ID
Fledging time	Conspecifically parasitized nests	N = 50	Conspecific parasite identity Year	GLMM	Nest ID
Log ₁₀ number of RFID reads	All host warblers	N = 246	Treatment Year	GLMM	Nest ID
Log ₁₀ number of RFID reads	Heterospecifically parasitized nests	N = 44	Species Year	GLMM	Nest ID
Log ₁₀ number of RFID reads	Conspecifically parasitized nests	N = 50	Conspecific parasite identity Year	GLMM	Nest ID

Table 6. List of models used in the statistical analysis for this paper. Table 6 corresponds to the “Impact of nestling size on fledging characteristics” section in the results. Predictor variables of interest are shown in bold, with covariates non-bolded. Nestling size PC1 was generated from a PCA of nestling mass, wing length, and tarsus length at 6 days old. Fledging order is the relative fledge order with 0 being first fledged. Fledging time is measured as the hours since sunrise that a nestling fledged.

<i>Impact of nestling size on fledging characteristics:</i>					
Response variable	Data included	Sample size	Predictor + covariates	Model type	Random effect
Fledging age	All host warblers	N = 246	Nestling size PC1 Nestling age at measurement Year	GLMM	Nest ID
Fledging order (0-1)	All host warblers	N = 245	Nestling size PC1 Nestling age at measurement Year	GLMM	Nest ID
Fledging order (0-1)	Conspecifically parasitized nests	N = 50	Conspecific parasite identity Year	GLMM	Nest ID
Fledging time	All host warblers	N = 246	Nestling size PC1 Nestling age at measurement Year	GLMM	Nest ID
Log ₁₀ number of RFID reads	All host warblers	N = 246	Nestling size PC1 Nestling age at measurement Year	GLMM	Nest ID

Table 7. List of models used in the statistical analysis for this paper. Table 7 corresponds to the “Relationships between fledging characteristics” section in the results. Predictor variables of interest are shown in bold, with covariates non-bolded. Fledging order is the relative fledge order with 0 being first fledged. Fledging time is measured as the hours since sunrise that a nestling fledged.

<i>Relationships between fledging characteristics:</i>					
Response variable	Data included	Sample size	Predictor + covariates	Model type	Random effect
Fledging time	All host warblers	N = 246	Fledging age Year	GLMM	Nest ID
Fledging time	All host warblers	N = 245	Fledging order (0-1) Year	GLMM	Nest ID
Log ₁₀ number of RFID reads	All host warblers	N = 245	Fledging order (0-1) Year	GLMM	Nest ID

Finally, we used Wilcoxon tests to determine if sex ratios of natural warbler nestlings were different in the three treatments and between years, with sex ratio represented by the number of natural warbler female nestlings in a nest divided by the total number of natural nestlings in a nest. Linear mixed models were used to determine if male and female cowbird and warbler nestlings were different in size (PC1 from a PCA of mass, tarsus, and wing length), and if sex and extra-pair status affected the fledging latency, fledging age, fledging time of day, fledging order, and time spent in the nest entrance as approximated by the number of RFID reads (Table 8).

Table 8. List of models used in the statistical analysis for this paper. Table 8 corresponds to the “Nestling sex and extra-pair status effects” section in the results. Predictor variables of interest are shown in bold, with covariates non-bolded. Nestling size PC1 was generated from a PCA of nestling mass, wing length, and tarsus length at 6 days old. Fledging order is the relative fledge order with 0 being first fledged. Fledging time is measured as the hours since sunrise that a nestling fledged. Sex ratio represents the number of females in each brood divided by the total number of nestlings in a brood.

<i>Nestling sex and extra pair status effects:</i>					
Response variable	Data included	Sample size	Predictor + covariates	Model type	Random effect
Sex ratio of each nest (0-1)	All host warblers	N = 95	Treatment	Wilcoxon	-
Sex ratio of each nest (0-1)	All host warblers	N = 95	Year	Wilcoxon	-
Nestling size PC1	All host warblers	N = 346 (Sex), 342 (EPY)	Sex or EPY status Age at measurement Year	GLMM	Nest ID
Nestling size PC1	Cowbirds	N = 15	Sex Age at measurement Year	GLM	-
Fledging latency between warblers	All host warblers	N = 246 (Sex), 242 (EPY)	Sex or EPY status Number of fledglings/brood Year	GLMM	Nest ID
Fledging age	All host warblers	N = 246 (Sex), 242 (EPY)	Sex or EPY status Year	GLMM	Nest ID
Fledging time	All host warblers	N = 246 (Sex), 242 (EPY)	Sex or EPY status Year	GLMM	Nest ID
Fledging order	All host warblers	N = 245 (Sex), 241 (EPY)	Sex or EPY status Year	GLMM	Nest ID
Log ₁₀ number of RFID reads	All host warblers	N = 246 (Sex), 242 (EPY)	Sex or EPY status Year	GLMM	Nest ID

5.4 RESULTS

5.4.1 Treatment effects on survival

The hetero- and conspecifically parasitized nests had similar natural (host-only, before manipulations) warbler clutch sizes ($W_z = 116$, $P = 0.892$; $\bar{x} = 4.200 \pm 0.243$ (SE) and 4.267 ± 0.153 , respectively) as did the conspecifically parasitized and control nests ($W_z = 544.5$, $P = 0.438$; $\bar{x} = 4.267 \pm 0.153$ and 4.385 ± 0.087 , respectively). As planned in our experimental design, conspecifically parasitized nests had significantly larger experimental brood sizes (including parasites) on banding day (~ 6 days post-hatch) than did control nests ($W_z = 281.5$, $P = 0.007$; $\bar{x} = 4.467 \pm 0.307$, 3.831 ± 0.119 , respectively), whereas experimental brood sizes (including parasites) between hetero- and conspecifically parasitized nests were not significantly different ($W_z = 83$, $P = 0.200$; $\bar{x} = 4.067 \pm 0.284$, 4.467 ± 0.307 , respectively).

Survival to banding day (6 days old post-hatch) was not statistically different between natural host warbler nestlings in hetero- vs. conspecifically parasitized nests ($W_z = 68$, $P = 0.087$; $\bar{x} = 3.067 \pm 0.284$ (6/47 deaths) vs. 3.714 ± 0.194 (2/52 deaths), respectively). There was also no difference in the number of natural host warbler chicks between conspecifically parasitized and control nests on banding day ($W_z = 490.5$, $P = 0.632$; $\bar{x} = 3.714 \pm 0.194$ (2/52 deaths), 3.831 ± 0.119 (4/248 deaths), respectively). However, at fledging, the number of natural host warbler chicks in heterospecifically parasitized nests was lower than in conspecifically parasitized nests ($W_z = 60$, $P = 0.039$; $\bar{x} = 2.933 \pm 0.284$ (8/47 deaths), 3.714 ± 0.194 (4/52 deaths), respectively), but there was no difference between conspecifically parasitized and control nests ($W_z = 471.5$, $P = 0.826$; $\bar{x} = 3.714 \pm 0.194$ (4/52 deaths), 3.708 ± 0.140 (7/248 deaths), respectively).

5.4.2 Treatment effects on fledging characteristics

Warbler host nestlings at ~6 days of age post-hatch were significantly smaller in the heterospecifically parasitized nests compared to warblers in conspecifically parasitized nests ($\beta = -0.828$, $t_{90} = -2.417$, $P = 0.018$, Figure 12a). In contrast, there was no significant difference in the size of host nestlings between the conspecific parasitism and control treatments ($\beta = -0.065$, $t_{90} = -0.232$, $P = 0.817$, Figure 12a). While cowbirds were significantly larger than their host nestmates ($\beta = -3.018$, $t_{44} = -14.018$, $P < 0.0001$, Figure 12a), conspecific parasite warbler chicks were not different in size compared to their host nestmates ($\beta = -0.216$, $t_{50} = -0.671$, $P = 0.505$).

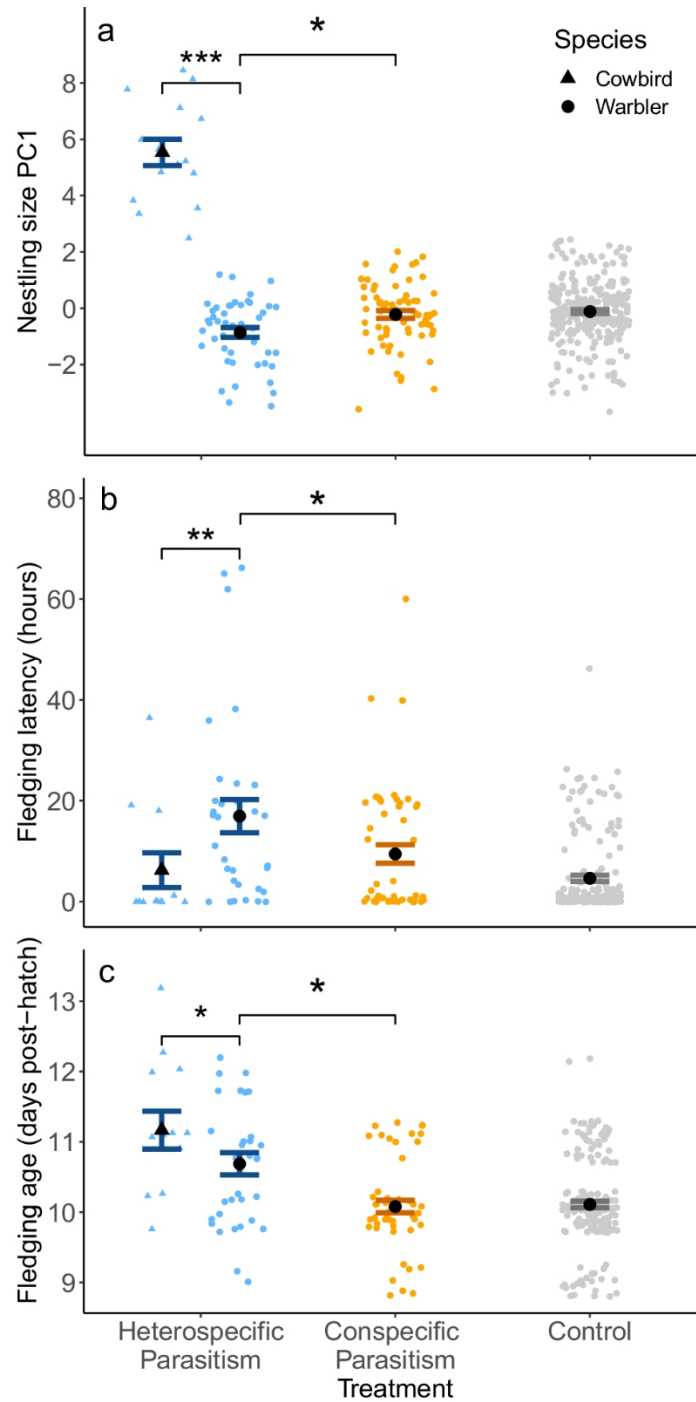


Figure 12. Effect of experimental heterospecific parasitism compared to conspecific parasitism, and conspecific parasitism compared to non-parasitized controls, on (a) nestling size, PC1 from a PCA of nestling mass, tarsus length, and wing length at 6 days of age posthatch, (b) fledging latency including cowbirds, calculated as the time elapsed between the first cowbird or warbler nestling in a brood fledging and each subsequent nestling fledging, and (c) age at fledging. Black points represent the mean, with bars representing standard error. Comparisons with $P < 0.05$ are represented with “*”, comparisons with $P < 0.01$ are represented with “**”, and comparisons with $P < 0.001$ are represented with “***”. Each point denotes an individual nestling.

Fledging latency of chicks was significantly greater in heterospecifically parasitized nests compared to conspecifically parasitized nests ($\beta = 8.798$, $t_{65} = 2.509$, $P = 0.015$, Figure 12b) with warblers fledging later than cowbirds ($\beta = 9.543$, $t_{197} = 3.029$, $P = 0.003$, Figure 12b). Cowbirds fledged first in 58% of nests and fledged last in 33% of nests. Fledging latency did not increase with number of fledglings ($\beta = 1.771$, $t_{65} = 1.572$, $P = 0.121$). However, when only considering the fledging latency between host warblers and not including cowbirds in the fledging latency calculation, there was no difference in fledging latency between warblers in the two parasitized nest treatments ($\beta = -2.383$, $t_{65} = -0.812$, $P = 0.420$). There was no difference in fledging latency between the conspecifically parasitized and control treatments ($\beta = -3.686$, $t_{65} = -1.445$, $P = 0.153$, Figure 12b). Conspecific warbler parasites had similar fledging latencies compared to their host nestmates ($\beta = -2.517$, $t_{38} = -0.700$, $P = 0.488$).

Host warblers fledging from heterospecifically parasitized nests were significantly older than in conspecifically parasitized nests ($\beta = 0.563$, $t_{66} = 2.153$, $P = 0.035$, Figure 12c), whereas there was no difference in the age at which host warblers from conspecifically parasitized versus control nests fledged ($\beta = -0.070$, $t_{66} = -0.317$, $P = 0.753$, Figure 12c). While cowbirds fledged at older ages compared to their host nestmates ($\beta = -0.465$, $t_{31} = -2.121$, $P = 0.042$, Figure 12c), conspecific parasites did not fledge at different ages compared to their host nestmates ($\beta = 0.015$, $t_{38} = 0.103$, $P = 0.919$).

There was no significant difference in the time of day of fledging between host warbler nestlings of the two parasitized nest treatments ($\beta = 1.521$, $t_{66} = 1.324$, $P = 0.190$) or the conspecifically

parasitized and control nests ($\beta = 0.957$, $t_{66} = 1.001$, $P = 0.321$). There was also no difference between the time of day when warbler and cowbird chicks fledged ($\beta = 1.710$, $t_{31} = 1.323$, $P = 0.196$). Conspecific warbler parasites did not fledge at different times compared to their host nestmates ($\beta = 0.090$, $t_{38} = 0.090$, $P = 0.929$). Fifty-two percent of offspring fledged before solar noon (13:00 local time) and the mean fledging time was 13:26 local time.

Finally, there was no difference in the \log_{10} number of RFID reads between host warblers in the two parasitized nest treatments ($\beta = 0.324$, $t_{66} = 0.765$, $P = 0.447$), or between host warblers in the conspecifically parasitized and control nests ($\beta = -0.418$, $t_{66} = -1.202$, $P = 0.234$).

Cowbirds spent significantly more time in the nest entrance than warblers, as approximated by the \log_{10} number of RFID reads per bird ($\beta = -1.484$, $t_{31} = -2.945$, $P = 0.006$). There was no difference in the amount of time conspecific parasites and their hosts spent in the nest entrance ($\beta = -0.406$, $t_{38} = -0.816$, $P = 0.420$).

5.4.3 Impact of nestling size on fledging characteristics

Prothonotary warbler host nestlings that were larger in size on banding day fledged at significantly earlier ages ($\beta = -0.194$, $t_{174} = -6.840$, $P < 0.0001$, Figure 13a). Larger host warbler nestlings also fledged significantly earlier in relative order compared to their smaller nestmates ($\beta = -0.113$, $t_{174} = -6.067$, $P < 0.0001$, Figure 13b). There was no difference in fledge order between conspecific parasites and their host nestmates ($\beta = 0.094$, $t_{38} = 0.711$, $P = 0.482$). There was no effect of host warbler size on the time of day of fledging ($\beta = 0.339$, $t_{174} = 1.751$, $P = 0.082$). Lastly, smaller host warbler nestlings spent more time in the nest

entrance, as approximated as the \log_{10} number of RFID reads ($\beta = -0.349$, $t_{174} = -4.420$, $P < 0.0001$, Figure 13c).

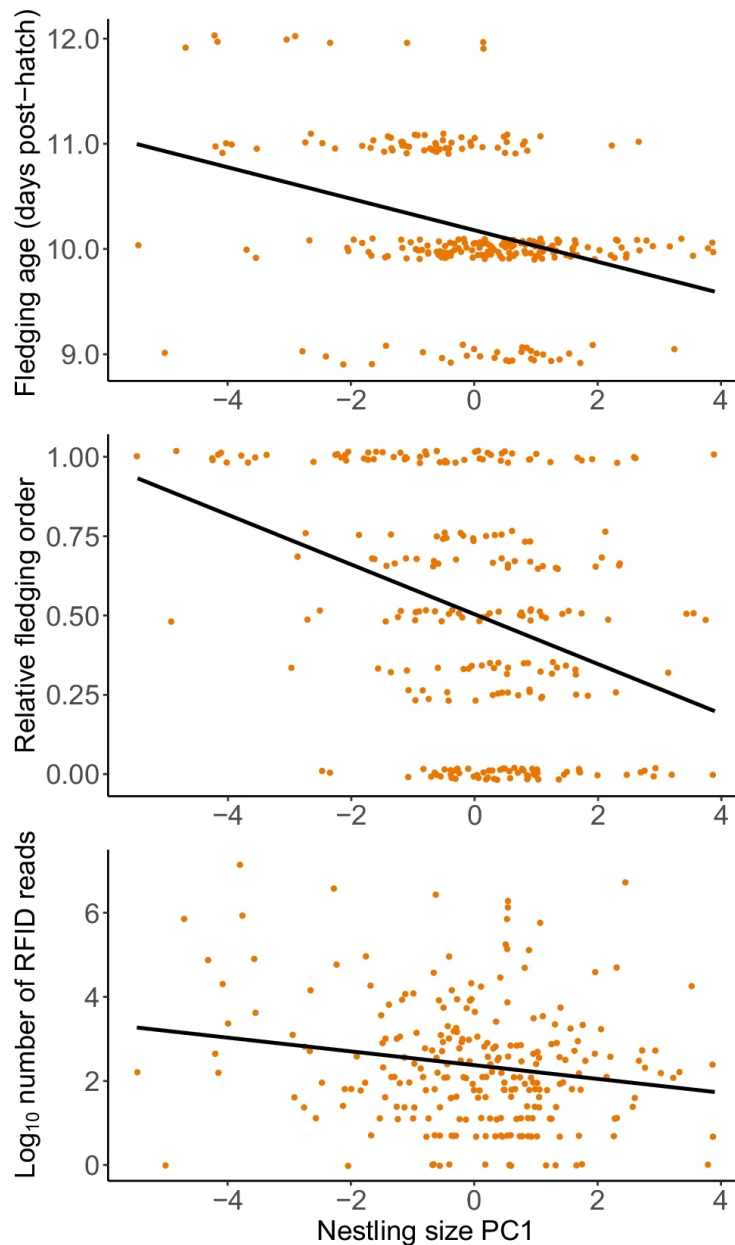


Figure 13. The relationship between prothonotary warbler nestling size, calculated as PC1 from a PCA of nestling mass, tarsus length, and wing length at 6 days of age posthatch, on (a) the age at fledging, (b) the relative fledging order within a brood (with 0 being first fledged), and (c) the number of radio-frequency identification (RFID) reads for each nestling with a \log_{10} transformation, as an approximation of time spent in the nest entrance. Each point denotes an individual nestling and the regression line represents significant relationships.

5.4.4 Relationships between fledging characteristics

Host nestlings that fledged at older ages fledged at significantly earlier times in the day ($\beta = -3.911$, $t_{175} = -11.768$, $P < 0.0001$, Figure 14). In contrast, fledging order did not impact the time of day that host nestlings fledged ($\beta = -0.802$, $t_{175} = -1.622$, $P = 0.107$). Host nestlings that fledged later in order spent more time in the nest entrance than nestlings that fledged earlier in brood order, approximated as the \log_{10} number of RFID reads ($\beta = 0.990$, $t_{175} = 4.698$, $P < 0.0001$, Figure 15).

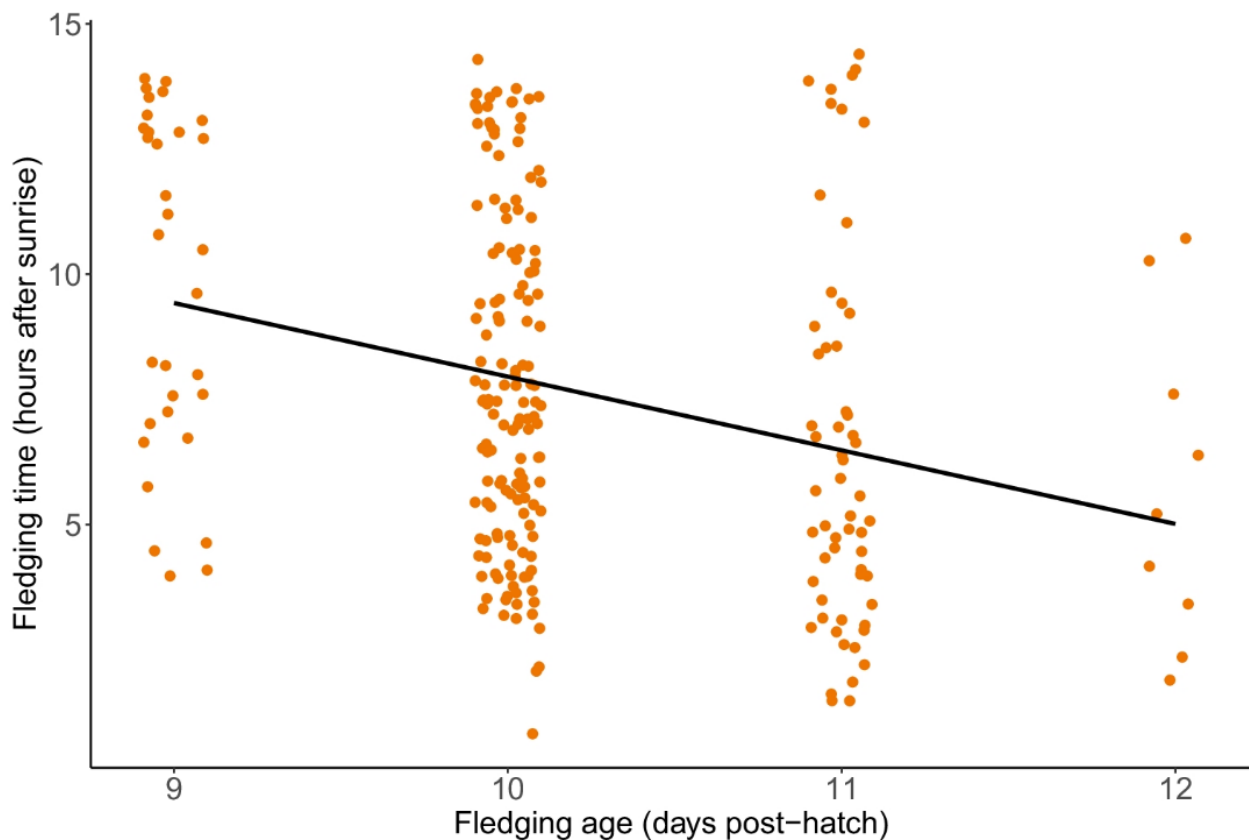


Figure 14. The relationship between the age at which prothonotary warbler nestlings fledge and the time of day they fledge. Time is shown as the hours after sunrise on the day of fledging. Each point represents an individual nestling, and the regression line shows the significant relationship between fledging age and time.

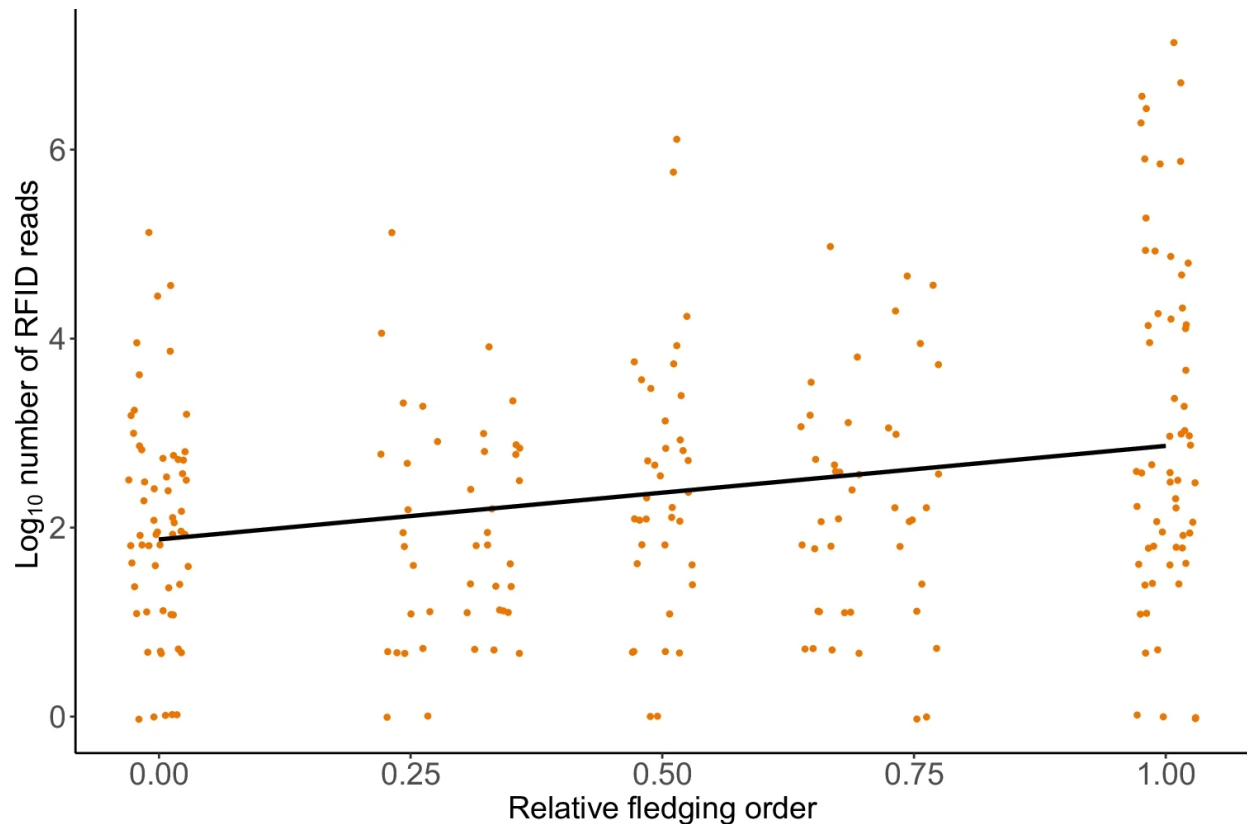


Figure 15. The effect of relative fledging order of prothonotary warbler nestlings within a brood (with 0 being first fledged) on the number of radio-frequency identification (RFID) reads for each nestling with a \log_{10} transformation, as an approximation of time spent in the nest entrance. Each point represents an individual nestling, the regression line shows the significant relationship between order and number of RFID reads.

5.4.5 Nestling sex and extra-pair status effects

The sex ratio of natural warbler nestlings was not different between hetero- and conspecifically parasitized broods ($W_z = 103.5$, $P = 0.721$; 50.9% (SE) $\pm 8.8\%$ and $57.2\% \pm 7.0\%$ female, respectively) or between conspecifically parasitized and control nests ($W_z = 601.5$, $P = 0.158$; $57.2\% \pm 7.0\%$ and $46.5\% \pm 3.4\%$ female, respectively). There was a significant difference in the sex ratios between our 2 years ($W_z = 1486.5$, $P = 0.007$); as in 2020, 56% of warbler nestlings were female, whereas in 2021, 40% of warbler nestlings were female. In a PCA of mass, tarsus, and wing length, male warbler nestlings were larger than females ($\beta = 0.218$, $t_{250} = 2.036$, $P =$

0.043). This trend was largely driven by wing length, with males having longer wings than females ($\beta = 0.644$, $t_{250} = 2.559$, $P = 0.011$). We did not detect a significant difference in size between male and female cowbirds as measured on day 6 ($\beta = 1.127$, $t_{11} = 1.435$, $P = 0.179$). We detected 4 extra-pair young in heterospecifically parasitized nests, 4 in conspecifically parasitized nests, and 21 in control nests. Extra-pair status did not affect warbler nestling size ($\beta = 0.075$, $t_{247} = 0.334$, $P = 0.738$). There were no sex differences in any fledging characteristics for the warblers, or between warbler nestlings that were extra-pair and not extra-pair ($P > 0.05$ for all results, Supplementary Table 7).

5.5 DISCUSSION

Our results show that while conspecific parasitism did not affect survival up to fledging, body size, or any fledging characteristics documented here for host warbler nestlings, heterospecific parasitism by cowbirds caused warbler nestmates to experience increased mortality up to fledging, decreased growth, increased total fledging latency for the brood, and increased fledging age relative to conspecific parasitism. Cowbirds and last-fledged, smaller warbler nestlings in a brood spent consistently more time in the nest entrance than warblers and earlier, larger fledged nestmates. Additionally, nestlings that were larger during the mid-nestling period fledged at earlier ages and earlier in order, and nestlings that fledged at older ages fledged earlier in the day. Although male warbler nestlings were larger, they were not different in any fledging characteristics than female warblers. The extra-pair status also did not affect any size or fledging characteristics. Together, these results demonstrate the importance of nestling development in determining aspects of fledging events and expand our knowledge of the impact of brood parasitism on host fledging dynamics.

In our host-parasite system, cowbird parasitism causes host warbler nestlings to have reduced survival and growth (Hoover 2003a; Scharf et al. 2021b). However, we have not yet tested the effects of experimental conspecific brood parasitism (Tucker et al. 2016) on brood survival to fledging. Our results on host nestling survival were consistent with an observational study on conspecific parasitism in prothonotary warblers (Tucker et al. 2016), although that study did not report larger clutch sizes in naturally parasitized nests. In contrast, our experimental conspecific brood parasitism treatments generated a brood enlargement scenario. Nevertheless, even with an enlarged brood, warbler nestlings did not experience decreased survival or growth in conspecifically parasitized nests, and the conspecific parasites are indistinguishable from host nestlings in all our metrics of fledging. According to David Lack's food limitation hypothesis, natural selection should favor birds that produce the highest number of nestlings that they can successfully feed and fledge (Lack 1947, 1948). It is possible, however, that the years in which this experiment was conducted were more productive than average, and therefore parents were able to support an added, conspecific chick in not only first but also second clutches (Perrins and Moss 1975). Alternatively, extra nestlings may have been supported by the generally high foraging productivity of the bottomland forest ecosystem in which this species nests, which was evident in a prior study that failed to find the effects of population density on warbler productivity (Hoover et al. 2020). The costs of an added cowbird chick and the costs of an added warbler chick are unequal, as cowbirds are 2–2.5 times larger than warbler chicks (Hoover 2003a), and may act as superior competitors (Hauber 2003a). Thus, it is possible that warbler parents might have been able to adjust their care to account for an added warbler chick, but not enough for an added cowbird.

Differences in nestling development between parasitism treatments likely account for the effect of increased fledging age in warblers parasitized with a cowbird compared to warblers in conspecifically parasitized nests. Although we measured the size of nestlings several days prior to fledging, which could leave time for compensatory growth (Anderson et al. 2009), we found that size at 6 days old post-hatch predicted the age and order at which nestlings fledged, with smaller warblers fledging at older ages and after their larger nestmates. Other studies have found strong negative relationships between metrics of size, like wing length and body mass, with characteristics like fledging age and order of fledging within a brood (Michaud and Leonard 2000; Johnson et al. 2004; Radersma et al. 2011). Thus, warblers in nests parasitized with cowbirds may have to spend extra time in the nest to compensate for reduced growth. Flight ability is important for post-fledging survival (Jones et al. 2017), and may be especially important to warblers, as many of their nesting cavities are typically directly over water, where there is a lower risk of nest predation (Hoover 2006) but a higher risk of drowning. In addition, compared to open-cup nesting species, cavity-nesting species move farther distances daily after fledging and stay more aggregated as a family group, and thus, wing development may be especially important (Şahin Arslan and Martin 2021). Warblers raised with cowbirds have 40% lower first-year survival compared to non-parasitized warbler broods (McKim-Louder et al. 2013), which may be caused at least in part by the decreased growth and extended nestling periods they experience due to cowbird parasitism affecting post-fledging behavior.

We found that in most heterospecifically parasitized nests, the cowbird fledged first (58%) or last (33%), increasing the interval of fledging for the whole brood. Cowbirds may be able to fledge

earlier than host nestmates because they have rapid embryonic development (McClelland et al. 2021) and typically hatch 1 day before their host nestmates (McMaster and Sealy 1998). However, cowbirds exhibit host-specific growth rates (Winnicki et al. 2021), and the age at which they fledge is largely influenced by their nestmates (Jones et al. 2020; Jones and Ward 2021). Cowbirds may leave before nestmates to secure more provisions from parents as fledglings and avoid costly competition within the nest with several warbler nestmates (Nilsson and Svensson 1993; Kilner et al. 2004; Jones et al. 2020). Alternatively, some cowbirds may fledge last because cavity nests are much safer from predation than many of the other ground-nesting species they parasitize, as cowbirds have longer nestling periods in safer nests (Jones and Ward 2021). This gives them more time to develop their wings, which is an important predictor of their post-fledging survival (Jones and Ward 2021), and may be especially important for cowbirds in warbler nests over water, as unlike warblers (Petit 1999), cowbird fledglings cannot swim and will drown if they fall into deep water. While the cowbird's presence increased fledging latency for the whole brood, fledging latency between the host warblers themselves was not significantly different between heterospecifically and conspecifically parasitized broods. Fledging latency between the warblers themselves may not be altered because they are still competitively similar to each other. Warbler parents and offspring may suffer from increased overall fledging latency because the attention of the parents is split between fledglings and nestlings (Lemel 1989).

In contrast to several other studies which found that nestlings preferentially fledge in the morning (Lemel 1989; Johnson et al. 2004, 2013; Chiavacci et al. 2015; Santema et al. 2021), we found that both warblers and cowbirds fledged throughout the day with 52% of fledging events

before and 48% after solar noon. The earliest fledging was at 6:40, and the latest at 19:55. At our sites, the longest daylight hours span from 5:35 to 20:20, and the shortest daylight hours span from 6:13 to 19:58. This is contrary to what might be expected from the maximum-time hypothesis, which posits that earlier fledging may lead to decreased predation risk, by giving the fledgling more daylight to find a safe location before dark, when it may be riskier to move (Johnson et al. 2004; Radersma et al. 2015; Chiavacci et al. 2015). Nevertheless, it is possible that as bottomland swamp habitat specialists, the predation pressures on prothonotary warblers are different from other, grassland or forest-dwelling species previously studied, and like some other cavity-nesting species, these warblers are also more fully flighted at fledging and may be able to better avoid danger when leaving the nest (Jones and Ward 2020). In addition, nests over deeper water in bottomland forests are less prone to predation (Hoover 2006) making warbler chicks potentially safer in than out of the nest which may alter fledging dynamics as compared to other species and habitats.

The timing of fledging may also not be related to post-fledge mortality, but instead be dependent on the attainment of certain developmental thresholds (Santema et al. 2021). In agreement with Santema et al. (2021), we found that nestlings that fledged at older ages fledged earlier in the day, although we only found a marginal effect of nestling size during the mid-nestling stage on fledging time. This could be the result of shifting pressures of both sibling competition and parent–offspring conflict, as parent–offspring conflict is expected to increase later in the nestling stage (Trivers 1974). We also found only a marginal effect of fledging order on the time of fledging, likely because many broods fledged over multiple days.

Although we found that male warbler nestlings were larger than females, we did not find any differences in characteristics of fledging between the sexes. This size difference was largely driven by wing length, with male nestlings having significantly longer wings, which has been found to be important in predicting the age of fledging (Michaud and Leonard 2000; Radersma et al. 2011; Johnson et al. 2013) and the order of fledging (Johnson et al. 2004; Radersma et al. 2011). However, Radersma et al. (2011) also did not find effects of sex on fledging time, asynchrony, or order of fledging between male and female great tits, when males are heavier, although the two had similar wing lengths at 14 days old. It is possible that we did not find any sex differences in fledging characteristics because adult male warblers have longer wings than females (Petit 1999), and this difference is simply already detectable at 6 days old. Males and females could have different developmental thresholds already accounted for by this difference.

The time spent in the nest entrance before fledging may represent competitive ability, as these nestlings could attempt to intercept provisions brought by parents (Radersma et al. 2011; Johnson et al. 2013). Nestlings with longer wings and nestlings that fledged first within a brood have both been found to spend more time in the nest entrance (Radersma et al. 2011; Johnson et al. 2013). However, we found that smaller nestlings spent more time in the nest entrance (as approximated by the number of RFID reads) and nestlings that spent the most time in the nest entrance were last to fledge in their brood. It is possible that in this study, time in the nest entrance primarily represents fledging hesitancy rather than an attempt to monopolize parent provisions. The earliest a nestling was detected in the nest entrance was 7 days before fledging; however, 94% of the PIT-tag detections were in the 24 h leading up to fledging, 63% of the PIT-tag detections within the last hour leading up to fledging. Nestlings may wait for a parent to be in

the vicinity of the nestbox before fledging (Michaud and Leonard 2000), which could explain why last-fledged nestlings spend the most time in the nest entrance, as the parents would have their attention already split between many fledglings and not be near the nestbox as often. Further research on parental proximity and calling behaviors around the nest during the late nestling stage should help elucidate the proximate causes of fledging in these nestlings.

Understanding the determinants of life stage transitions is essential in the study of life histories. Fledging is a critical, fitness-limiting behavioral and ontogenetic transition in the avian reproductive cycle, and it is necessary to study the relationships between characteristics of the nestling stage and fledging phenology to determine the causes of fledging, and in turn, its effects on post-fledging life. Our study builds on what little is known about this transition in life stages by investigating multiple aspects of fledging and their interactions and adds to the even less well-known effects of both hetero- and conspecific brood parasitism. Future work should focus on manipulating other features of nestling competition and parental influence, as well as investigating the effects of brood parasitism-driven fledging dynamics on the fledglings' immediate survival, their overwinter recruitment, and the overall lifetime fitness to further investigate this understudied life stage phase transition.

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APPENDIX A: SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. Chapter 4 - AIC_c table of model outputs investigating the importance of cowbird parasitism with non-experimental factors including total brood size, brood mass, ordinal date, and nestling age on prothonotary warbler nestling baseline plasma corticosterone levels. Models are ranked by decreasing Akaike weight (W_i), and those within 2 ΔAIC_c (highlighted in grey) of the top model are considered competitive. The “k” value represents the number of parameters per model. Additive models are denoted by “+” and interactive models are denoted by “*”.

	k	AIC_c	ΔAIC_c	W_i
(a) BASELINE CORTICOSTERONE LEVELS				
ordinal date + nestling age	6	283	0	0.338
ordinal date	5	284.6	1.64	0.149
cowbird * ordinal date	7	286.1	3.1	0.072
nestling age	5	286.1	3.15	0.07
null	4	286.3	3.3	0.065
cowbird + ordinal date	6	286.4	3.46	0.06
brood mass	5	286.9	3.9	0.048
cowbird + nestling age	6	287.6	4.69	0.032
cowbird	5	287.9	4.96	0.028
brood size	5	288	5.04	0.027
cowbird * ordinal date + brood size	8	288.2	5.21	0.025
cowbird * ordinal date + brood mass	8	288.2	5.22	0.025
cowbird * brood mass	7	288.2	5.24	0.025
cowbird + brood mass	6	289	6.05	0.016
cowbird + brood size	6	289.9	6.94	0.01
cowbird * brood size	7	290.1	7.15	0.009

Supplementary Table 2. Chapter 4 - AIC_c table of model outputs investigating the importance of cowbird parasitism with non-experimental factors including total brood size, brood mass, ordinal date, and nestling age on prothonotary warbler nestling immune response as measured by wing-web swelling 24 hours after injection of PHA. Models are ranked by decreasing Akaike weight (W_i), and those within 2 ΔAIC_c (highlighted in grey) of the top model are considered competitive. The “k” value represents the number of parameters per model. Additive models are denoted by “+” and interactive models are denoted by “*”.

	k	AIC _c	ΔAIC_c	W_i
IMMUNE RESPONSE				
cowbird * ordinal date + brood size	8	-227	0	0.672
cowbird * ordinal date	7	-223.5	3.49	0.117
cowbird * ordinal date + brood mass	8	-223.1	3.87	0.097
brood mass	5	-222.4	4.53	0.07
cowbird + brood size	6	-220.5	6.44	0.027
cowbird * brood size	7	-219.5	7.47	0.016
brood mass	5	-211.3	15.67	0
cowbird + ordinal date	6	-210.6	16.39	0
ordinal date	5	-210.4	16.55	0
cowbird + brood mass	6	-209.7	17.31	0
cowbird * brood mass	7	-209.1	17.86	0
ordinal date + nestling age	6	-208.5	18.44	0
cowbird	5	-204.3	22.67	0
null	4	-202.5	24.43	0
cowbird + nestling age	6	-202.3	24.72	0
nestling age	5	-200.5	26.46	0

Supplementary Table 3. Chapter 4 - AIC_C table of model outputs investigating the importance of cowbird parasitism with non-experimental factors including total brood size, brood mass, ordinal date, and nestling age on prothonotary warbler condition calculated as the residuals of mass on tarsus. Models are ranked by decreasing Akaike weight (W_i), and those within 2 ΔAIC_C (highlighted in grey) of the top model are considered competitive. The “k” value represents the number of parameters per model. Additive models are denoted by “+” and interactive models are denoted by “*”.

	k	AIC _C	ΔAIC_C	W_i
CONDITION				
cowbird * brood mass	7	736.9	0	0.228
cowbird * brood size	7	738	1.1	0.132
brood size	5	738.1	1.19	0.126
cowbird + brood mass	6	739.3	2.36	0.07
null	4	739.3	2.41	0.069
cowbird + brood size	6	739.4	2.54	0.064
cowbird	5	739.5	2.64	0.061
cowbird * ordinal date + brood mass	8	739.8	2.91	0.053
ordinal date	5	740.5	3.57	0.038
brood mass	5	740.7	3.76	0.035
nestling age	5	740.9	4.01	0.031
cowbird + ordinal date	6	741.1	4.17	0.028
cowbird + nestling age	6	741.1	4.24	0.027
ordinal date + nestling age	6	742.2	5.31	0.016
cowbird * ordinal date	7	742.7	5.84	0.012
cowbird * ordinal date + brood size	8	743.4	6.5	0.009

Supplementary Table 4. Chapter 4 - AIC_c table of model outputs investigating the importance of cowbird parasitism with non-experimental ordinal date on the proportion of prothonotary warbler nestlings that died per nest. Models are ranked by decreasing Akaike weight (W_i), and those within 2 ΔAIC_c (highlighted in grey) of the top model are considered competitive. The “k” value represents the number of parameters per model. Additive models are denoted by “+” and interactive models are denoted by “*”.

	k	AIC_c	ΔAIC_c	W_i
NESTLING DEATH				
cowbird	5	-71.2	0	0.648
cowbird + ordinal date	6	-69.0	2.26	0.209
cowbird * ordinal date	7	-67.2	4.01	0.087
null	4	-65.6	5.60	0.040
ordinal date	5	-63.8	7.46	0.016

Supplementary Table 5. Chapter 4 - AIC_c table of models investigating the importance of cowbird parasitism with non-experimental factors including total brood size, brood mass, ordinal date, and nestling age on prothonotary warbler nestling immune response as measured by wing-web swelling 24 hours after injection of PHA, with data truncated at day 194 to exclude non-parasitized nests that were later in the breeding season than the last parasitized nest. Models are ranked by decreasing Akaike weight (W_i), and those within 2 ΔAIC_c (highlighted in grey) of the top model are considered competitive. The “k” value represents the number of parameters per model. Additive models are denoted by “+” and interactive models are denoted by “*”.

	k	AIC _c	ΔAIC_c	W_i
IMMUNE RESPONSE				
cowbird * ordinal date + brood size	8	-169.9	0	0.399
brood size	5	-169.6	0.29	0.345
cowbird + brood size	6	-167.6	2.25	0.13
cowbird * ordinal date + brood mass	8	-165.8	4.07	0.052
cowbird * brood size	7	-165.6	4.3	0.047
cowbird * ordinal date	7	-164.3	5.58	0.025
brood mass	5	-158.7	11.16	0.002
cowbird + brood mass	6	-156.8	13.11	0.001
cowbird * brood mass	7	-155	14.89	0
cowbird + ordinal date	6	-152.9	17	0
ordinal date	5	-152.1	17.74	0
ordinal date + nestling age	6	-150.2	19.67	0
cowbird	5	-149.5	20.35	0
null	4	-149	20.93	0
cowbird + nestling age	6	-147.7	22.14	0
nestling age	5	-146.9	23	0

Supplementary Table 6. Chapter 4 - 95% confidence intervals and model averaged coefficients for models summing to a cumulative AIC_c weight of 0.95 investigating the importance of cowbird parasitism with non-experimental factors including total brood size, brood mass, ordinal date, and nestling age on prothonotary warbler nestling immune response as measured by wing-web swelling 24 hours after injection of PHA, with data truncated at day 194 to exclude non-parasitized nests that were later in the breeding season than the last parasitized nest.

	95% Confidence Interval	β
IMMUNE RESPONSE		
cowbird	-0.103, 0.012	-0.045
ordinal date	-0.038, 0.027	-0.006
brood size	-0.119, -0.018	-0.069
cowbird * ordinal date	0.012, 0.096	0.054
brood mass	-0.075, 0.001	-0.037

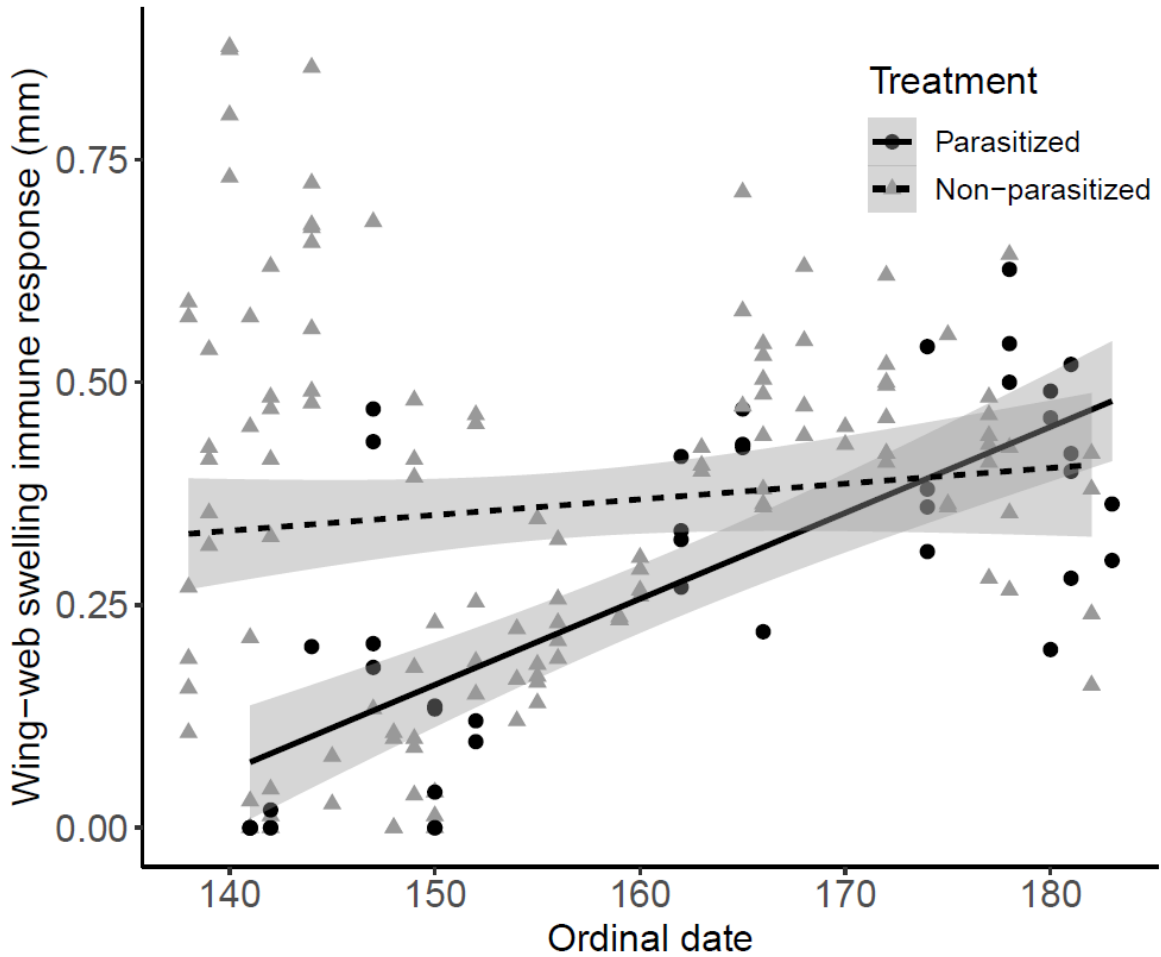
Supplementary Table 7. Chapter 5 - Effect sizes, degrees of freedom, t-values, and *P*-values associated with (a) sex effects on fledging characteristics and (b) extra-pair status effects on fledging characteristics. For sex, male is used as the reference category. For extra-pair status, extra-pair young are used as the reference category.

(a) Nestling sex effects:

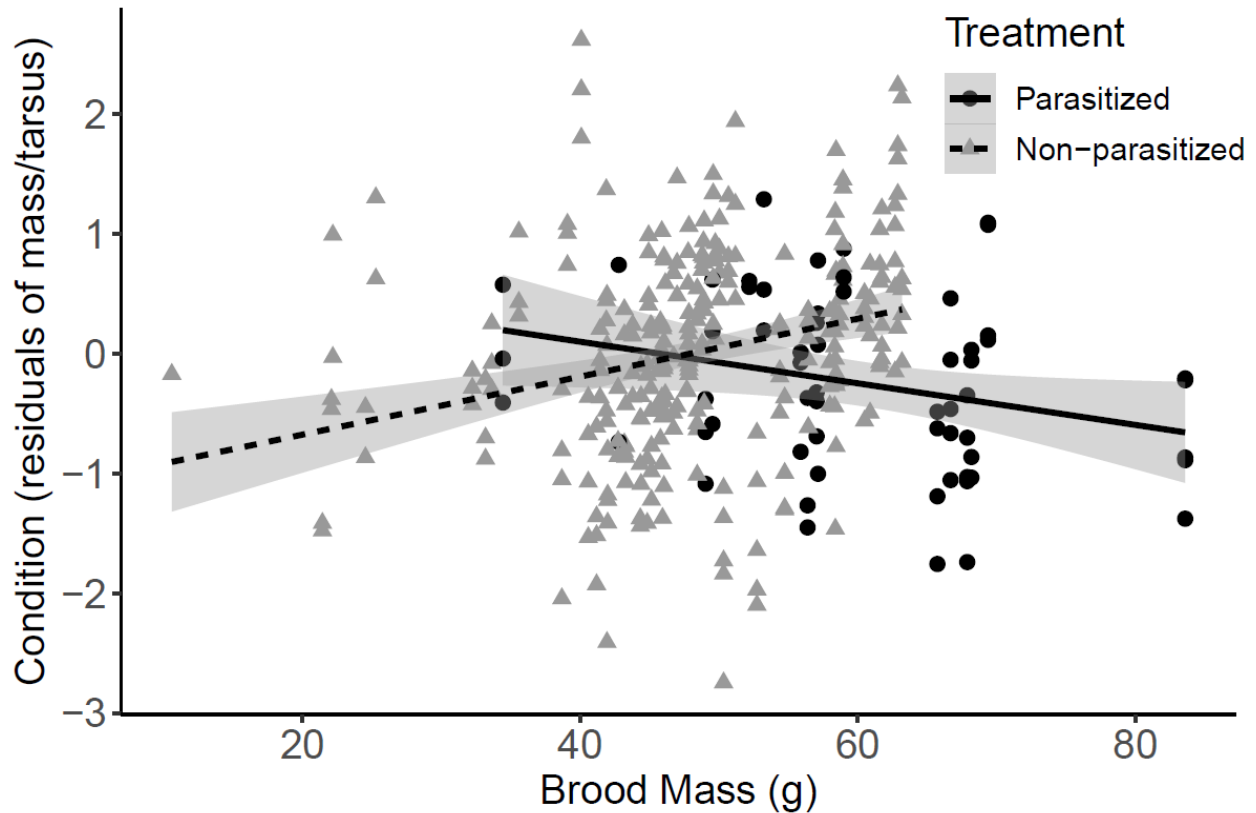
Response variable	β	DF	T-value	<i>P</i> -value
Fledging latency between PROW	0.738	175	0.644	0.521
Fledging age	-0.042	175	-0.673	0.502
Fledging time (hours after sunrise)	0.502	175	1.202	0.231
Fledging order (0-1)	0.047	175	0.953	0.342
Log ₁₀ number of RFID logs	-0.076	175	-0.417	0.677

(b) Nestling extra-pair status effects:

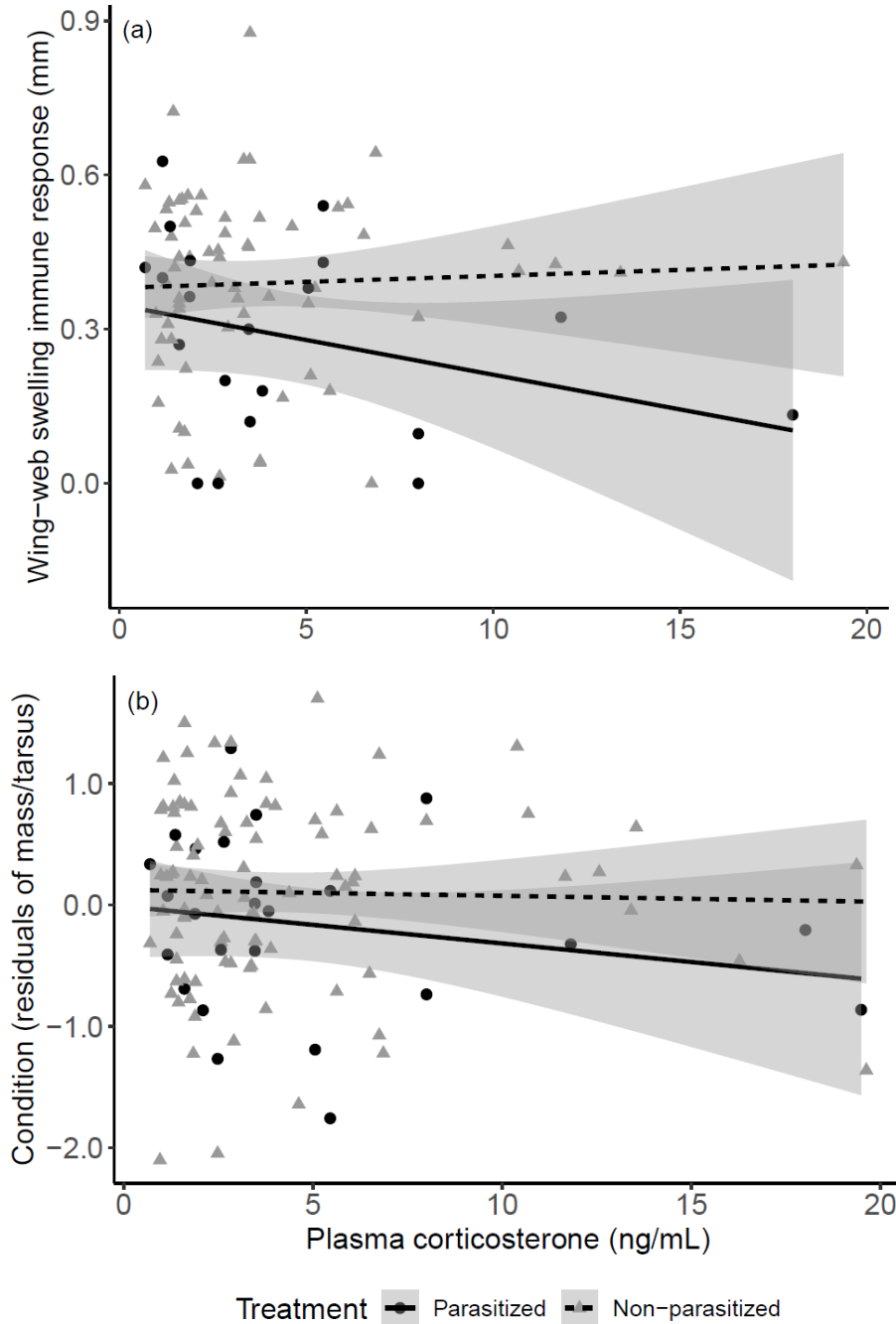
Response variable	β	DF	T-value	<i>P</i> -value
Fledging latency between PROW	1.135	172	0.490	0.625
Fledging age	0.083	172	0.585	0.560
Fledging time (hours after sunrise)	-1.502	172	-1.774	0.078
Fledging order (0-1)	-0.006	172	-0.075	0.940
Log ₁₀ number of RFID logs	-0.539	172	-1.496	0.137



Supplementary Figure 1. Chapter 4 - The effect of parasitism treatment on T-cell mediated immune response ($\beta = -0.057$, 95% CI = -0.092, -0.021) and its interaction with ordinal date ($\beta = 0.055$, 95% CI = 0.020, 0.090). Each point represents an individual nestling. Significant effects and interactions are indicated by the solid and dashed lines. The shaded area represents the 95% confidence intervals of the fitted lines.



Supplementary Figure 2. Chapter 4 - The effect of parasitism treatment on nestling condition, calculated as the residuals of mass on tarsus ($\beta = 0.022$, 95% CI = -0.207, 0.251) and its interaction with brood mass ($\beta = -0.143$, 95% CI = -0.275, -0.010). Each point represents an individual nestling. Significant effects and interactions are indicated by the solid and dashed lines. The shaded area represents the 95% confidence intervals of the fitted lines.



Supplementary Figure 3. Chapter 4 - Plot showing the relationship between raw plasma corticosterone levels of prothonotary warbler nestlings and (a) their immune response as measured by wing-web swelling in response to PHA injection in parasitized and non-parasitized broods ($F_{1,36} = 0.013$, $P = 0.908$), and (b) nestling condition, calculated as the residuals of mass on tarsus, in parasitized and non-parasitized broods ($F_{1,54} = 0.121$, $P = 0.730$). Each point represents an individual nestling. The shaded area represents the 95% confidence intervals of the fitted lines.