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Avoidance, tolerance, and resistance to ectoparasites in nestling and adult tree swallows

Joely G. DeSimone*, Ethan D. Clotfelter*, Elizabeth C. Black and Sarah A. Knutie

J. G. DeSimone, E. D. Clotfelter (http://orcid.org/0000-0002-6711-3873) (edclotfelter@amherst.edu) and E. C. Black, Dept of Biology, Amherst College, Amherst, MA, USA. JGD present address: Program in Organismal Biology, Ecology, and Evolution, Division of Biological Sciences, Univ. of Montana, Missoula, MT, USA. – S. A. Knutie (http://orcid.org/0000-0001-6423-9561), Dept of Ecology and Evolutionary Biology, Univ. of Connecticut, Storrs, CT, USA.

We examined avoidance, tolerance, and resistance strategies of nestling and adult tree swallows *Tachycineta bicolor* in response to ectoparasitic blowflies *Protocalliphora sialia*. Tree swallows avoided settling in north-facing nest boxes early in the breeding season. These boxes were more likely to be parasitized later in the season, suggesting that box selection may facilitate blowfly avoidance. After experimentally manipulating blowfly intensity, we found that nestlings were generally tolerant of parasitism. Parasites significantly reduced nestling blood hemoglobin but had no effect on nestling body mass, primary feather growth, age at fledging, or fledging success. Parents of parasitized nestlings did not increase their food provisioning rate to promote nestling tolerance. Adult female tree swallows demonstrated both tolerance and resistance: blowfly parasitism had no effect on adult hemoglobin and body mass, and those with higher *P. sialia*-binding antibody levels had fewer blowfly larvae in their nests. Nestling antibodies were unrelated to blowfly intensity. Despite considerable variation among years, our results suggest that the costs of blowfly parasitism to nestling and adult tree swallows are modest, and limited to blood loss in nestlings. Future work should examine the effects of reduced blood hemoglobin on fledgling survival and the importance of parasite-specific antibodies.

Keywords: host-parasite dynamics, breeding biology

Introduction

Hosts defend themselves against parasites through processes of avoidance, tolerance, and resistance (Miller et al. 2006, Råberg et al. 2007, Read et al. 2008, Medzhitov et al. 2012, Sears et al. 2013, Knutie et al. 2017a). Avoidance is comprised primarily of behavioral mechanisms that reduce exposure to parasitized prey, conspecifics, or nesting sites, including the removal of parasites before infection occurs (Wisenden et al. 2009, de Roode and Lefèvre 2012). Tolerance, which decreases damage to the host without affecting parasite fitness, includes tissue repair or the recuperation of resources lost to parasites. Tolerance can be represented by the slope of the relationship between host fitness and parasite burden, with high tolerance indicated by a shallow or flat slope and low tolerance indicated by a steeper negative slope (Simms 2000). Host resistance,

which is typically the result of immunological responses, reduces parasite fitness and thus damage to the host. Hosts exhibit genetic variation and plasticity in avoidance, tolerance, and resistance that are important in host–parasite coevolution (Koskela et al. 2002, Råberg et al. 2007), but field studies of these processes are relatively rare (Blanchet et al. 2010).

Parasite pressure can change across organisms' life stages and the relative importance of particular host defenses can fluctuate accordingly (Hamstra and Badyaev 2009, Koprivnikar et al. 2014). For example, hosts may be unable to avoid infections during early developmental stages when they are less mobile. Host tolerance of parasites can improve with age or developmental stage, as a smaller organism may experience proportionally greater parasitic damage than a larger organism (Rohr et al. 2010). Similarly, host resistance via adaptive immunity can become increasingly effective as animals progress from juvenile to adult stages (Thomas and Rudolf 2010, Killpack and Karasov 2012, Killpack et al. 2013).

Interactions across life-history stages (e.g. parents and offspring) can influence avoidance, tolerance, and resistance mechanisms. Among bird species, parents can facilitate their offspring's parasite avoidance by selecting uninfected nest sites, grooming their young (Stanback and Dervan 2001, Cantarero et al. 2013), or by adding green plant material or feathers to the nest (Winkler 1993, Mainwaring et al. 2014). Host resistance can be heritable (Råberg et al. 2009, Mazé-Guilmo et al. 2014) or enhanced via maternal transfer of antibodies (Hasselquist and Nilsson 2009). Offspring tolerance can be improved if parents increase their food provisioning rate in response to parasitism (Christe et al. 1996, Tripet and Richner 1997, Hurtrez-Boussès et al. 1998, Knutie et al. 2016). However, if adults themselves are severely parasitized, compensatory provisioning, and therefore nestling tolerance, may decline (Gallizzi et al. 2008).

Birds are hosts to many species of hematophagous arthropod parasites, including blowfly larvae in the order Diptera (Bennett and Whitworth 1992). Altricial nestlings, unable to leave the nest, often cannot avoid ectoparasites prior to fledging. Although larval blowflies (e.g. *Protocalliphora* spp.) consume enough blood to induce anemia (Johnson and Albrecht 1993, Hannam 2006), the literature provides equivocal evidence that blowflies negatively impact host nestling growth or survival (Roby et al. 1992, Saino et al. 1998, Puchala 2004, Simon et al. 2004, Hannam 2006, Huber 2008). Biotic or abiotic factors that create favorable conditions for hosts to defend themselves may contribute to the ambiguity of studies of blowfly parasitism and nestling fitness (Merino and Potti 1996, Heeb et al. 2000). Additionally, parents may offset the costs of parasitism for their offspring with tolerance mechanisms. Furthermore, parents can suffer from the costs of blowfly parasitism themselves. Numerous studies have demonstrated reduced survival and declines in future reproductive success among parasitized adult birds (Brown et al. 1995, Richner and Tripet 1999, Bize et al. 2004, Tomás et al. 2007).

Our study investigates the effects of ectoparasitic blowfly larvae *Protocalliphora sialia* on tree swallows *Tachycineta bicolor* and the various defense mechanisms employed by the hosts. Tree swallows construct nests in secondary tree cavities and are frequently parasitized by blowflies, including *P. sialia* (Winkler 1993, Proctor and Owens 2000, Dawson et al. 2005). Female blowflies oviposit directly in host nests concurrent with the hatching of the eggs (Gold and Dahlsten 1989). During their larval stage, blowflies spend most of their time within the nesting material, emerging to feed on the blood and other fluids of nestlings and attending adults. Parasite intensity can be experimentally manipulated by treating nests with a pyrethroid insecticide that has little effect on nestling growth or development (Tomás et al. 2007, Koop et al. 2013, Knutie et al. 2014, 2017b).

The goal of our study was to investigate avoidance, tolerance, and resistance mechanisms employed by nestling and adult tree swallows parasitized by hematophagous blowflies *P. sialia*. We predicted that avoidance mechanisms might include timing of breeding, nest box orientation, and nest construction, particularly the feathering of nests. Based on previous studies, we predicted that earlier nests, north-facing nests, and less-feathered nests would have fewer larval blowflies (Brown and Brown 1999, Dawson et al. 2005, 2011). We predicted that nestlings would tolerate blowflies – with body mass, skeletal growth, and fledging age similar to those of unparasitized nestlings – despite blood lost to parasites (Johnson and Albrecht 1993, Carleton 2008), and that this tolerance might be facilitated by compensatory parental provisioning of parasitized nestlings (Christe et al. 1996). Finally, we predicted that adults tending to parasitized nests would resist parasitism by a *P. sialia*-specific antibody response (Koop et al. 2013); this response is likely more robust in adults than in nestlings due to their developed adaptive immune system (Grindstaff et al. 2006).

Methods

We conducted fieldwork in the Amherst College Wildlife Sanctuary in western Massachusetts, USA (42°22′N, 72°31′W) during May–July 2014–2016. Temperature and precipitation data (collected at a weather station approximately 1 km from the study site) are included in Supplementary material Appendix 1 Table A1. We mounted 119 wooden boxes (floor = 7 × 11 cm, back = 20 cm) on metal poles 1.5 m off the ground, oriented their openings in haphazard directions, and spaced them approximately 30 m apart (Ardia et al. 2006). Tree swallows nested and were monitored in 57 boxes in 2014, 77 boxes in 2015, and 83 boxes in 2016. Nests were checked daily to determine clutch initiation date (CID) and clutch size, and then checked daily beginning 10–12 d later to determine hatching date (nestling day 0). Nests were characterized as either 'early' or 'late' in the season. Early nests were those with CIDs occurring before the median CID of that year (2014: Julian date 136; 2015: 132; 2016: 137); late nests were initiated on or after the median

CID of that year. Brood size ranged from 1 to 7 nestlings (mean ± SE = 4.55 ± 0.078 nestlings; median = 5 nestlings).

Experimental removal of blowflies

We removed blowflies from some nests to test for effects of parasitism on nestling and adult tree swallows, and to minimize the effect of confounding factors such as seasonality and brood size. In 2014, we ran a pilot version of this experiment. Twelve pairs of nests were matched for CID and – to the extent possible – brood size and location within the study area. One nest of each pair (randomly determined) was fumigated with permethrin, a synthetic pyrethroid insecticide (Permectrin II™, diluted with distilled water to 1%) at nestling days 0 and 4 (Koop et al. 2013, Knutie et al. 2016, 2017b). The other nest in each pair was sham-fumigated with distilled water on the same days. In both treatment groups, nestlings were removed from nests before spraying, and were returned to their nests once the nest material had dried, approximately 10 min later. The remaining nests in 2014 (33 of 57) were left unmanipulated. We expanded the experiment in subsequent years: there were 38 fumigated and 39 sham-fumigated nests in 2015, and 40 fumigated and 43 sham-fumigated nests in 2016 (Table 1). Tree swallow parents never abandoned their nests due to treatment and no permethrin-induced mortality was observed. Not all adults were banded, so we cannot state with certainty whether birds returned to the study area in multiple years. Tree swallow annual adult survival ranges from 13–51% (Winkler et al. 2011). We assigned treatments randomly both temporally and geographically to minimize the chance that the same bird was assigned to the same treatment more than once.

Nestling measurements

We weighed nestlings (± 0.1 g) using a portable balance and measured the second primary feather on the right wing (± 0.1 mm) using dial calipers on nestling day 13. Nestling day 13 was chosen because it is the last day we can reliably measure nestling tree swallows without causing premature fledging (Winkler 1993, Winkler et al. 2011). Skeletal measures such as tarsus and head-bill length were also taken, but yielded results similar to body mass and thus are not reported here.

Parental provisioning

We quantified parental provisioning behavior (number of feeding visits per nestling per hour of observation) on nestling day 8. We chose nestling day 8 because it is during the period of peak parental provisioning (Bortolotti et al. 2011) and to avoid conflict with other experimental objectives on days 10 and 13. A feeding visit was defined as any instance in which an adult tree swallow entered the nest box or inserted its head inside the entrance. In 2014 and 2015, human observers recorded the total feeding visits during a 30-min observation period between 09:00–12:00 EDT. In 2016, we video-recorded feeding visits for at least 30 min (37 ± 1 min) between 09:00–12:00 EDT. Thirty-minute observations have been shown to be adequate and reliable indices of adult tree swallow feeding rates when compared to 90-min observations (Bortolotti et al. 2011). We collected daily aerial insect samples using a suction trap at a nearby location to estimate and control for food availability. This suction trap (30 cm diameter × 90 cm tall), smaller but similar in design to a Rothamsted trap (Macaulay et al. 1988), used a fan to draw air through a mesh funnel inside a metal cylinder, which collected daily insect samples in a 125 ml bottle of 70% EtOH (Supplementary material Appendix 1 Fig. A1). These samples were dried overnight to calculate insect dry mass per day (Supplementary material Appendix 1 Table A1).

Nestling blood hemoglobin

We measured hemoglobin as an indicator of blood loss (Carleton 2008). Hemoglobin is a robust indicator of physiological condition in birds, more so than hematocrit (reviewed by Minias 2015). On nestling day 10, we collected 50–100 µl of blood from the brachial vein of nestlings using 26-gauge needles and heparinized microcapillary tubes (Owen 2011). Hemoglobin concentration (g dl^{-1}) was measured with a HemoCue™ Hb 201+ Analyzer. The remaining blood was placed on ice before returning to the lab, where it was centrifuged for 10 min at 10 000 RPM at 4°C. Plasma and red blood cells were each stored at –80°C; plasma was later used for antibody analysis.

Nestling and adult immunity

Enzyme-linked immunosorbent assays (ELISA) were used to detect the presence of *P. sialia*-binding antibodies (IgY) in

Table 1. Blowfly *Protocalliphora sialia* abundance in tree swallow nests for each treatment group per year and in all years of the study.

Year	Treatment group	Mean	Median	Range	SE	n
2014	Sham-fumigated	24.50	16.5	0–79	7.69	12
	Fumigated	0.67	0	0–8	0.67	12
2015	Sham-fumigated	14.49	7	0–102	3.31	39
	Fumigated	0.03	0	0–1	0.03	38
2016	Sham-fumigated	23.05	23	0–130	3.79	40
	Fumigated	0.03	0	0–1	0.03	38
All years	Sham-fumigated	19.57	14	0–130	2.43	91
	Fumigated	0.11	0	0–8	0.09	88

the plasma of nestling and adult female tree swallows, with a modification of the protocol in Knutie et al. (2016). In tree swallows, females perform most of the brooding of nestlings (Winkler et al. 2011), and thus males are unlikely to be exposed to blowflies (Koop et al. 2013). Nestling plasma samples were collected from nestlings in 2014 and 2015 (n = 179) and from adult females in 2015 (n = 80 captured early in the nestling period; n = 31 captured later in the nestling period), and were later analyzed for *P. sialia*-binding antibodies.

Ninety-six well plates were coated with 100 µl/well of *P. sialia* protein extract (capture antigen) diluted in carbonate coating buffer (0.05 M, pH 9.6). Plates were incubated overnight at 4°C, then washed and coated with 200 µl/well of bovine serum albumin (BSA) blocking buffer and incubated for 30 min at room temperature on an orbital table. Between each of the following steps, plates were washed three times with a Tris-buffered saline wash solution, loaded as described, and incubated for one hour on an orbital table at room temperature. Wells were loaded with 100 µl/well of individual host plasma (diluted 1:100 in sample buffer) in triplicate. Plates were then loaded with 100 µl/well of Goat-αBird-IgG-Heavy and Light Chain HRP (diluted 1:50 000) (Bethyl Laboratories; A140-110P). Finally, plates were loaded with 100 µl/well of peroxidase substrate (tetramethylbenzidine, TMB: Bethyl Laboratories) and incubated for exactly 20 min. The reaction was halted using 100 µl/well of stop solution (Bethyl Laboratories). Optical density (OD) was measured with a spectrophotometer (BioTek, PowerWave HT, 450 nm filter).

On each plate, a positive control of pooled plasma from naturally parasitized adult female swallows was used in triplicate to correct for inter-plate variation. In addition, each plate contained a non-specific binding (NSB) sample in which capture antigen and detection antibody were added, but plasma was excluded. Finally, each plate included a blank sample in which only the detection antibody was added, but plasma and capture antigen were excluded. NSB absorbance values were subtracted from the mean OD value of each sample to account for background binding of the detection antibody to the capture antigen.

Adult female body mass and blood hemoglobin

In 2015 and 2016, we opportunistically caught nesting females in their nest boxes twice during the nesting period, approximately one week apart. The first capture occurred between nestling days 0 and 7 (mean = 2.2 d after hatching, n = 124 females) and the second capture occurred between nestling days 9 and 14 (mean = 10.7 d after hatching, n = 94 females). If males were caught (sex determined by plumage, body size, presence/absence of cloacal protuberance and presence/absence of brood patch), we released them. We recorded the mass (± 0.5 g) of each nesting female; tarsus (± 0.1 mm) and wing chord (± 0.1 mm) were also recorded but not presented here. As with nestlings, we collected blood samples from the brachial vein to quantify hemoglobin and

separated and stored the plasma and red bloods cells for later analysis.

Fledging and nest collection

Nests were checked daily beginning on nestling day 18 to determine fledging date. Once nestlings had fledged, we collected nests and all loose nesting material in plastic bags. In the laboratory, we dissected nests and defined total blowfly abundance as the sum of the *P. sialia* larvae, pupae, and eclosed pupal cases within the nesting material. We weighed the total nest mass as well as the mass of the feathers lining the nest cup (± 0.001 g), after removing any feces or dirt from them.

Statistical analysis

All statistical tests were performed using the mosaic, dplyr, lme4, and circular packages in RStudio (ver. 1.0.136) and data were visualized using the ggplot2 package. A chi-squared test was used to compare the blowfly prevalence of fumigated and sham-fumigated nests. A Watson–Williams test for the homogeneity of means was used to compare the box orientations of parasitized and unparasitized untreated and sham-fumigated nests and, in an analysis of all nests, the box orientations of nests with eggs laid before or after the median CID of each year. Generalized linear models (GLM) were used in analyses of blowfly intensity between treatment groups; the relationship between blowfly intensity and CID; the relationship between blowfly intensity and nest feathers; food provisioning rates among treatment groups and study years; and adult age, hemoglobin, mass, and antibody levels between treatment groups. Generalized linear mixed models (GLMM) with Wald's chi-squared test were used to analyze the effect of blowfly parasitism on nestling hemoglobin, body size, and antibody levels, with nest box included as a random effect to control for non-independence of nestlings from the same brood. Brood size, CID, and study year were included in GLMs and GLMMs if they were significant. Means are presented ± standard error, and an alpha level of 0.05 was used to determine significance.

Data deposition

Data available from the Dryad Digital Repository: < http://dx.doi.org/10.5061/dryad.9bb60 > (DeSimone et al. 2017).

Results

Nest treatment and blowfly prevalence

Permethrin significantly reduced both the prevalence and intensity of blowflies in tree swallow nests (Table 1). Only 3 of 88 (3.4%) fumigated nests were parasitized compared to 70 of 91 (77%) sham-fumigated nests ($\chi^2 = 100.11$, p < 0.001). The intensity of blowfly parasitism was significantly greater

in sham-fumigated nests (19.57 ± 2.43 parasites per nest) than in fumigated nests (0.11 ± 0.09 parasites per nest; $F_{2,176} = 36.5$, $p < 0.001$; covariate: CID; Fig. 1). Intensity of blowfly parasitism did not differ significantly among years in sham-fumigated nests ($F_{2,87} = 0.38$, $p = 0.68$; covariate: CID). There was no relationship between brood size and number of blowflies in sham-fumigated nests ($F_{2,88} = 4.85$, $p = 0.13$; covariate: CID). In 2014, when some nests were left unmanipulated, 18 of 33 (54.5%) of these unmanipulated nests contained 12.85 ± 3.22 larvae. Blowfly abundance was significantly different between unmanipulated (12.85 ± 3.22) and sham-fumigated (24.50 ± 7.69) nests that year ($F_{3,40} = 11.58$, $p = 0.04$; covariate: CID).

Timing of nesting, nest site selection, and nest construction

We found that the intensity of blowfly parasitism increased with advancing clutch initiation date (CID) among sham-fumigated nests, when all three study years were combined ($F_{1,121} = 17.02$, $p < 0.001$; Fig. 2). However, this relationship was statistically significant only in 2014 ($F_{1,42} = 25.11$, $p < 0.001$).

When we pooled data from sham-fumigated and unmanipulated nests across all three years, we found that nests parasitized by blowflies differed in directional orientation from unparasitized nests ($F_{1,122} = 11.69$, $p < 0.01$; Fig. 3). The average orientation of unparasitized nests was 270.1° while that of parasitized nests was 343.3°. There was also a significant difference in the directional orientation of nest boxes selected earlier in the season than those selected later in the season ($F_{1,214} = 16.34$, $p < 0.001$). The mean orientation of early nests was 191.7°, whereas the mean orientation of late nests was 332.8°.

There was no significant relationship between the mass of feathers lining the nest and the intensity of blowfly parasitism in the sham-fumigated and unmanipulated nests of all years
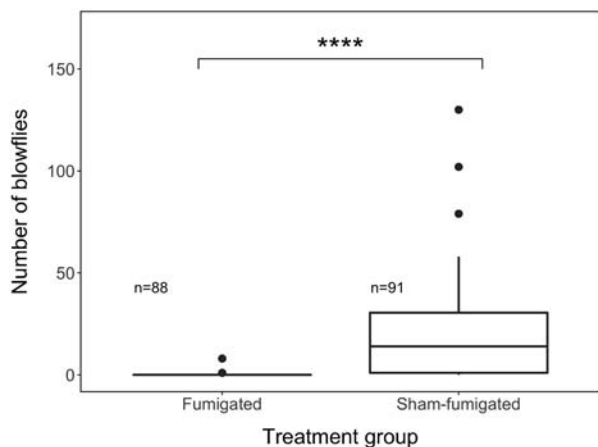

Figure 1. Tree swallow nests fumigated with permethrin had significantly fewer blowflies than sham-fumigated nests. Boxplot includes lines for first quartile, median and third quartile. p-value < 0.001.
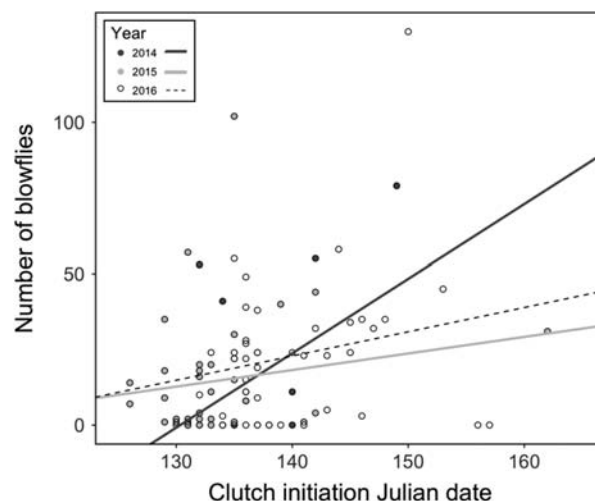

Figure 2. Seasonal changes in blowfly intensity in tree swallow nest boxes (2014–2016). Data shown include both unmanipulated and sham-fumigated nests; nests fumigated with permethrin were excluded. When all nests are included together, blowfly intensity increased significantly with clutch initiation date, though most of this effect was driven by data from 2014.

($F_{1,113} = 6.93$, $p = 0.31$; covariate: CID). There was a positive, but statistically nonsignificant, relationship between nest mass and blowfly intensity ($F_{4,115} = 83.74$, $p = 0.055$; covariates: brood size and study year), suggesting that larger nests can support more blowfly larvae.

Nestling hemoglobin, body size and fledging success

Tree swallow nestlings exposed to blowflies in sham-fumigated nests had significantly lower hemoglobin levels (11.09 ± 1.4 g dl^{-1}; n = 241 individuals from 74 nests) than nestlings in fumigated nests from which blowflies were removed (12.29 ± 0.09 g dl^{-1}; n = 212 individuals from 64 nests) ($\chi^2 = 24.66$, $p < 0.001$; covariate: CID). Results were similar when sham-fumigated nests without blowflies and fumigated nests with blowflies were excluded (Supplementary material Appendix 1 Table A2). When we analyzed hemoglobin as a linear function of blowfly intensity, we found a significant negative relationship ($\chi^2 = 88.41$, $p < 0.001$; covariate: study year; Fig. 4a).

Treatment group did not predict nestling body mass or primary feather length. Nestlings weighed 19.67 ± 0.19 g (n = 341) in sham-fumigated nests and 19.45 ± 0.19 g (n = 287) in fumigated nests ($\chi^2 = 1.24$, $p = 0.27$; covariates: brood size and study year). Primary feather length was 37.06 ± 0.39 mm (n = 349) in sham-fumigated nests and 37.18 ± 0.46 mm (n = 289) in fumigated nests ($\chi^2 = 0.27$, $p = 0.60$; covariates: brood size and study year). Results were similar when sham-fumigated nests without blowflies and fumigated nests with blowflies were excluded (Supplementary material Appendix 1 Table A2). In 2015, sham-fumigated nestlings' primary feathers were significantly longer than those of fumigated nestlings ($\chi^2 = 4.03$, $p = 0.04$). When analyzed
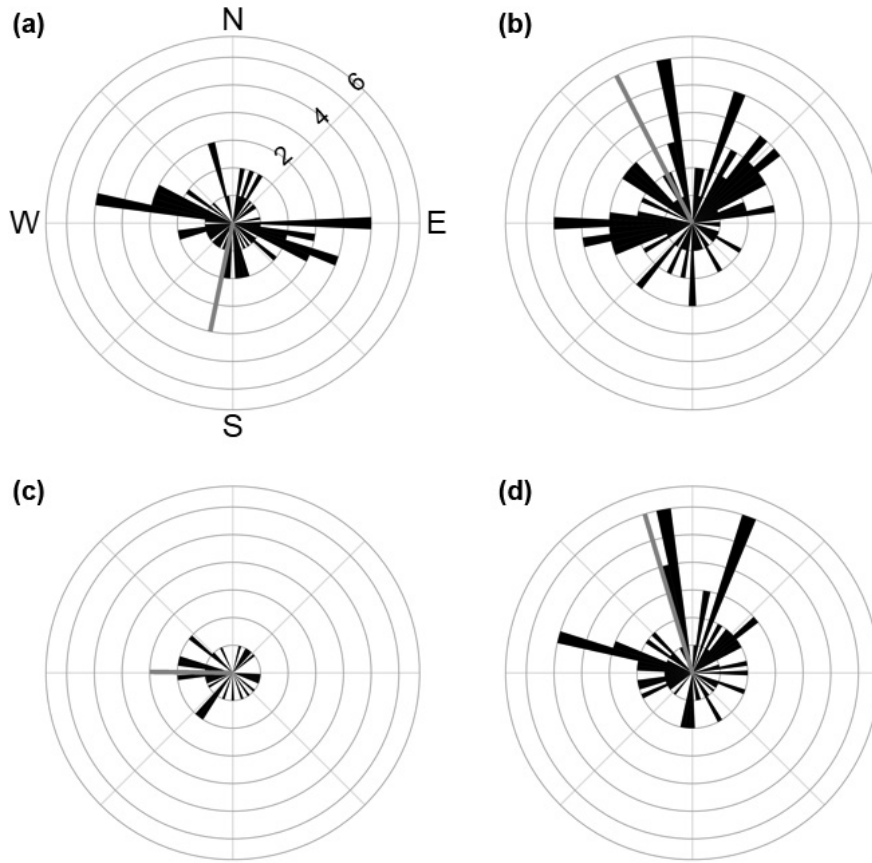
Figure 3. Directional orientation of nest boxes settled by (a) early-breeding and (b) late-breeding tree swallows. Orientation of the entrances of nest boxes that were (c) not parasitized by blowflies and (d) nests that were parasitized. Data shown in (a) and (b) include all boxes; (c) and (d) show only unmanipulated and sham-fumigated nests; fumigated nests are excluded. Average box orientation is indicated with a gray line.

across the range of blowfly intensities, we found similar results (Fig. 4b, c). Body mass (Fig. 4b) was not affected by blowfly intensity ($\chi^2 = 0.08$, p = 0.77; covariates: CID and study year), but was negatively related to brood size ($\chi^2 = 78.44$, p < 0.001; covariate: study year) and significantly higher in 2014 than either other year ($\chi^2 = 38.91$, p < 0.001; covariate: brood size). Similarly, primary feather length (Fig. 4c) was not affected by blowfly intensity ($\chi^2 = 0.31$, p = 0.58; covariates: brood size and study year). Nestling primary feathers were longer in smaller broods ($\chi^2 = 23.57$, p < 0.001; covariate: study year) and longer in 2014 compared to 2015 and 2016 ($\chi^2 = 59.95$, p < 0.001; covariate: brood size).

The age at which tree swallows fledged from their nests was unaffected by treatment group ($F_{5,140} = 18.1$, p = 0.78; covariates: CID, brood size, and study year). Sham-fumigated nestlings fledged after 21.84 ± 0.19 d; fumigated nestlings fledged after 21.94 ± 0.24 d. On average, nestlings in 2015 and 2016 fledged two days later than nestlings in 2014 (2014: 20.17 ± 0.23 d; 2015: 22.10 ± 0.21 d; 2016: 22.31 ± 0.24 d), a difference that was highly significant (p < 0.001) for both years. Nestlings in larger broods fledged later than those in smaller broods ($F_{4,141} = 22.75$, p < 0.001; covariates: CID and study year).

Finally, there was no effect of treatment group on fledging success (proportion of nestling fledged). Sham-fumigated and fumigated nests fledged 0.71 ± 0.04 and 0.69 ± 0.04 of all nestlings, respectively ($F_{4,174} = 3.52$, p = 0.67; covariates: CID and study year). Reproductive success did not vary significantly with blowfly intensity ($F_{4,173} = 3.61$, p = 0.44; covariates: CID and study year; Fig. 4d), but study year and CID were significant factors (higher survival rate in 2014 than 2016: $F_{1,101} = 5.80$, p = 0.02; higher survival rate in later clutches: $F_{3,175} = 4.65$, p = 0.006; covariate: study year).

Parental provisioning

There was no significant difference in feeding rate between treatment groups ($F_{4,142} = 4.62$, p = 0.74; covariates: study year and insect availability). Parents made 4.67 ± 0.29 feeding visits per nestling per hour to sham-fumigated nests on nestling day 8, compared to 4.64 ± 0.30 visits to fumigated nests. Provisioning rates to sham-fumigated nests were higher in 2014 than the other years in our study (2015: $F_{1,39} = 10.94$, p = 0.002, covariates: none; 2016: $F_{1,48} = 13.69$, p < 0.001; covariates: none).
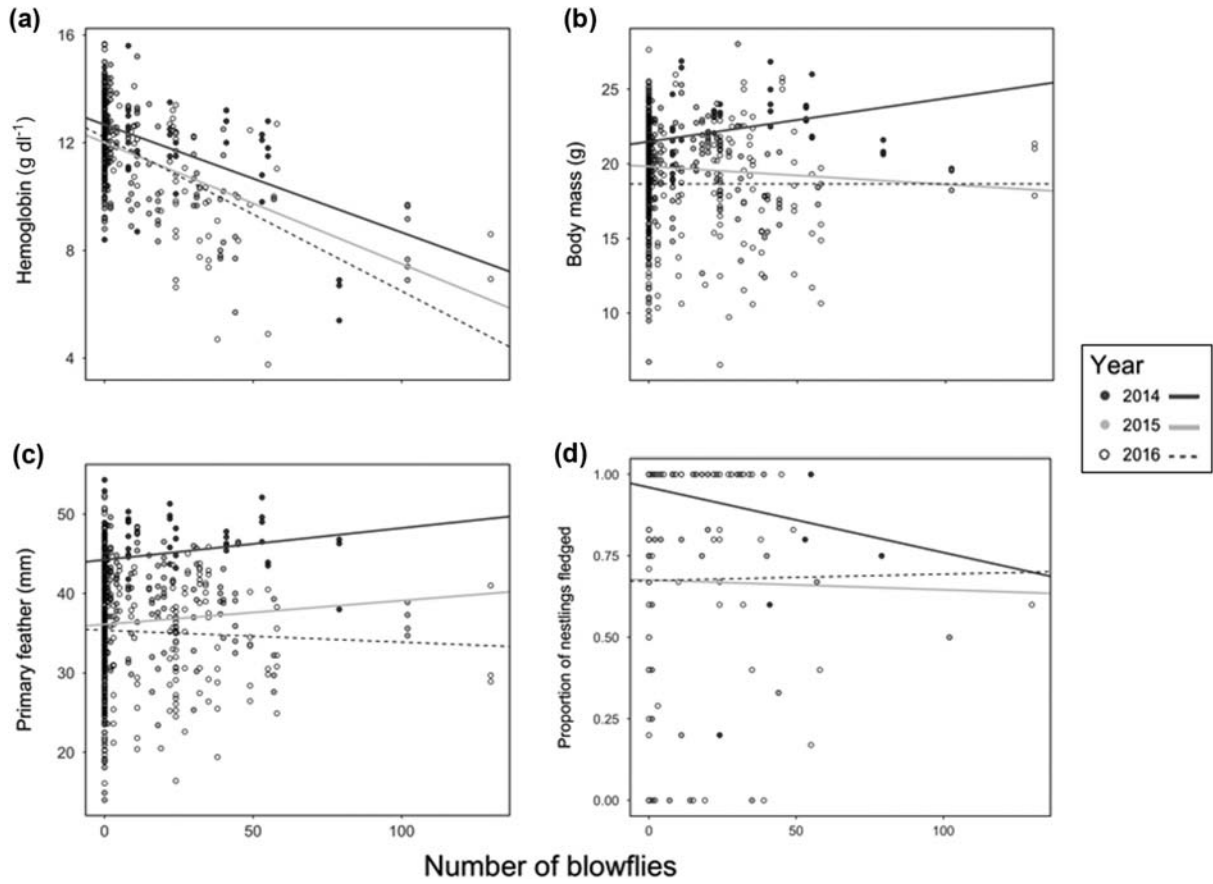
Figure 4. The effects of blowfly intensity on tree swallow nestling (a) hemoglobin, (b) body mass, (c) primary feather length, and (d) nestling survival in 2014–2016. These data include all experimental (fumigated and sham-fumigated) nests. In panels (a)–(c), data points represent individual nestlings (with nest box included as a random effect in the analysis), and in panel (d) data points represent whole nests.

Adult age, hemoglobin, and body mass

Treatment group did not significantly affect adult female hemoglobin levels ($F_{1,118}$= 0.02, p=0.90; covariates: none). Females with later CIDs had significantly lower hemoglobin levels than early-breeding females when hemoglobin was measured during the peak of nestling feeding (nestling days 9–14; $F_{1,85}$= 7.10, p=0.01; covariates: none). When captured and weighed early in the nesting period (nestling days 0–7), females in sham-fumigated nests weighed significantly more than those in fumigated nests ($F_{3,130}$=13.2, p=0.04; covariates: CID and study year). However, there was no effect of treatment on female mass when they were captured and weighed later in the nesting period (days 9–14) ($F_{2,89}$=3.03, p=0.80; covariate: study year). Among the modest sample of females that we captured twice, the change in mass between first and second capture did not significantly differ between treatment groups ($F_{3,82}$=11.32, p=0.33; covariates: brood size and study year).

Nestling and adult immunity

Treatment did not significantly affect *P. sialia*-binding antibody levels in nestlings (χ^2=0.17, p=0.68; covariate:

study year), adults captured early in the nestling period ($F_{1,47}$=2.82, p=0.10), or adults captured late in the nestling period ($F_{1,29}$=1.28, p=0.27). Nestling antibody levels were not significantly related to parasite intensity among sham-fumigated nests in 2014 (χ^2=2.36, p=0.12) or 2015 (χ^2=0.72, p=0.40). However, in 2015, as the antibody levels of adult females of sham-fumigated nests increased, parasite intensity decreased ($F_{2,13}$=5.80, p=0.05; covariate: CID; Fig. 5). Among sham-fumigated nests in 2015, nestling antibody levels tended to correlate positively with their mothers' antibody responses (χ^2=3.25, p=0.07). Among all of the birds for which we had data, we compared the adaptive immune responses of nestlings to those of adult females (plasma samples only from second captures). We found that females (0.54 ± 0.07, n=31) had significantly higher antibody levels than nestlings (0.22 ± 0.02, n=226; χ^2=31.29, p < 0.001).

Discussion

Our study evaluates host avoidance, tolerance, and resistance in a wild host–parasite system. We found that timing of breeding and nest site selection by adult tree swallows
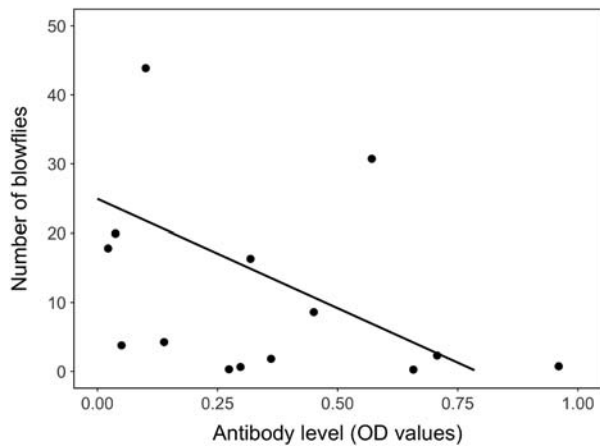
Figure 5. There was a negative relationship between *P. sialia*-binding antibody levels (OD) in adult female tree swallows and the number of blowflies in their nests. These data include sham-fumigated nests only.

Tachycineta bicolor could predict subsequent parasitism by hematophagous blowfly larvae *Protocalliphora sialia*, suggesting a potential mechanism of parasite avoidance. Nestling tree swallows were intolerant of parasitism in terms of blood loss: parasitized nestlings had significantly lower hemoglobin concentrations than unparasitized nestlings. However, there was no effect of treatment group or parasite intensity on nestling body mass, feather growth, age at fledging, or fledging success. Parental compensatory feeding did not explain the nestling tolerance we observed. Finally, while nestling antibody responses showed no significant relationship with parasite intensity, adult females with more *P. sialia*-binding antibodies had fewer blowflies in their nests, suggesting the possibility of resistance via adaptive immunity.

We investigated three mechanisms by which adult tree swallows may avoid blowfly ectoparasitism: timing of breeding, nest site selection, and nest construction. Timing of breeding is one of the most significant predictors of reproductive success in tree swallows, with late-laying birds experiencing decreased reproductive success (Winkler and Allen 1996). While this can be explained by correlates of clutch initiation date, such as food availability or variation in parents' phenotypic quality or reproductive effort (Moreno 1998, Verhulst and Nilsson 2008), ectoparasitism may also play a role. We found that blowfly intensity increases as the breeding season progresses, which is consistent with previous studies of tree swallows and other species (Roby et al. 1992, Merino and Potti 1995, Brown and Brown 1999). Thus, in addition to its other benefits (Winkler and Allen 1996, Moreno 1998, Brown and Brown 1999), early breeding may facilitate ectoparasite avoidance.

When nest cavities are not limited, nest site selection may provide a means of blowfly parasite avoidance. Orientation of the nest entrance is an important characteristic of cavity nests and may influence nest site selection. In a meta-analysis of woodpecker populations, Landler et al. (2014) found that populations in the Northern Hemisphere tended to have more southerly nest entrance orientations, presumably for

increased solar exposure. A previous study at our site showed that tree swallow boxes oriented SE were warmer, particularly early in the breeding season (Ardia et al. 2006). In the current study, the box orientations of early-settled nests significantly differed from those of later nests, suggesting a preference for factors associated with box orientation. Early nests were more likely to face SE and W and unparasitized nests faced S and W. Later-settled, and thus less-preferred, nests faced NW and N, as did parasitized nests. Thus, birds may select SE-facing boxes for thermal benefits (Ardia et al. 2006) and indirectly benefit by avoiding blowfly parasitism, though this was not the case in a study of nest-site selection in great tits *Parus major* (Goodenough et al. 2011). Avoidance of north-facing boxes may also affect parasite resistance mechanisms, as nestling tree swallows in experimentally cooled boxes have impaired innate immunity (Ardia et al. 2010). However, the apparent avoidance by tree swallows of blowfly parasitism via box selection may instead be an artifact of blowfly phenology. Blowflies may appear to preferentially parasitize N-faced boxes because these are the only boxes left late in the breeding season, when blowfly abundance has increased. Determining the causal relationship between host avoidance and box orientation would require an experimental manipulation of box orientation after birds have settled and established nests.

Nest construction is another potential mechanism of parasite avoidance; host birds may add materials to their nests to protect themselves and their nestlings from ectoparasites (reviewed by Mainwaring et al. 2014). Tree swallows are well-known for lavishly (and quite variably) feathering their nests, and it has been proposed that feathers may serve as a physical barrier between ectoparasites in the nesting material and the nestlings above (Winkler 1993). However, feathering has been shown to correlate positively with parasite load because blowflies prefer warm nests (Dawson et al. 2011) and feathered nests tend to be warmer than unfeathered nests (Hilton et al. 2004, Windsor et al. 2013). In our study, however, we found no relationship between nest feathering and blowfly intensity, thus providing no support for either alternative hypothesis. Total nest mass was nearly significantly and positively related to blowfly intensity even when brood size was statistically controlled. This finding suggests that other aspects of nest architecture that have heretofore been unstudied (Mainwaring et al. 2014) may be important predictors of ectoparasite burden in cavity-nesting birds.

Nestlings in sham-fumigated nests had significantly lower hemoglobin levels than fumigated nestlings, suggesting blood lost to ectoparasites (Johnson and Albrecht 1993, Słomczyński et al. 2006). Despite blood loss, we did not observe significant differences in mass, primary feather length, age at fledging, or nestling survival between the treatment groups in any of the three years of our study. This is similar to the findings of Carleton (2008): nestling eastern bluebirds *Sialia sialis* experienced a 25% decline in hemoglobin from ectoparasitic mites (*Dermanyssus prognephilus*), yet suffered no apparent decline in body mass or fledging success. However, our findings contrast with many studies recently reviewed by Minias (2015), who found that nestling

hemoglobin was frequently positively correlated with body mass and fat scores. Nestlings may be able to maintain normal somatic growth in spite of reduced hemoglobin if food availability is high. Simon et al. (2004) found that natural variation in caterpillar abundance was positively related to metabolic capacity in blue tit *Cyanistes caeruleus* nestlings, which promoted tolerance of *Protocalliphora* spp. parasitism.

Another possible explanation for the apparent tolerance we observed is that permethrin fumigation of experimental nests in our study resulted in slower nestling growth (López-Arrabé et al. 2014), in which case we might have underestimated the true costs of blowfly parasitism. The potential confound of permethrin fumigation merits more attention in future studies.

However, we saw no effect of a range of blowfly intensities on nestling body mass, primary feather length, or survival (Fig. 4b–d), giving us confidence that tree swallow nestlings are indeed tolerant of parasitism. This is consistent with the findings of Roby et al. (1992), who found that tree swallows were less impacted by *Protocalliphora* parasitism than were neighboring eastern bluebirds. It is also consistent with data reviewed by Simon et al. (2004), showing widespread tolerance in terms of fledging success among many hosts of *Protocalliphora*.

It has been hypothesized that nestlings in parasitized nests devote more energy to primary feather growth, allowing them to fledge early and avoid further exposure to ectoparasites (Saino et al. 1998). In contrast, a more recent study (Pirrello et al. 2015) showed that ectoparasitism delays rather than accelerates the first prebasic molt in European starlings *Sturnus vulgaris*. We did not observe any differences in feather length or fledging age between fumigated and sham-fumigated treatment groups, which could be further evidence of nestling tolerance of blowfly parasitism or the consequence of constrained growth by low hemoglobin levels (Minias 2015).

Adults may increase their food provisioning rate to facilitate nestling tolerance of ectoparasites, likely in response to the increased begging rates of parasitized nestlings (Johnson and Albrecht 1993, Christe et al. 1996, Hurtrez-Boussés et al. 1999). In fact, increased begging by Galápagos mockingbird *Mimus parvulus* nestlings led to parental compensatory feeding. The mockingbirds tolerated parasitism by the parasitic nest fly *Philornis downsi* better than the medium ground finch *Geospiza fortis*, which showed no such behavioral adaptation for tolerance (Knutie et al. 2016). In our study, parasitized (sham-fumigated) nestlings were fed at similar rates to unparasitized (fumigated) nestlings, suggesting that parental compensation does not contribute to nestling tolerance in our study system.

Adaptive immunity is less well-developed in nestlings than in adult birds (Grindstaff et al. 2006, Koop et al. 2013). As predicted, we found significantly higher antibody concentrations in adult female tree swallows than in their nestlings. While there were no significant differences in nestling or adult antibody levels between the fumigated and sham-fumigated treatment groups, there were some interesting

trends within the sham-fumigated (parasitized) group. In the only year for which we have both maternal and nestling antibody data (2015), we found that mothers with higher antibody levels had significantly fewer blowfly larvae in their nests, and that their nestlings also tended to mount stronger antibody responses. These results indicate resistance via adaptive immunity; the antibody responses of mothers (and perhaps their nestlings) significantly reduced the survival of *P. sialia* larvae. Koop et al. (2013) similarly found that the intensity of another species of parasitic fly, *Philornis downsi*, decreased in medium ground finches' nests as maternal *P. downsi*-binding antibody levels rose. Such antibody responses may directly defend against ectoparasitism by inciting an inflammatory response that reduces parasite fitness and survival (Owen et al. 2009, 2010). The link between adult and offspring antibody levels may indicate heritability of the antibody response, maternal transfer of antibodies via the egg yolk, or a combination of the two (Råberg et al. 2003, Boulinier and Staszewski 2008).

In summary, we found that nestling and adult tree swallow hosts employ avoidance, tolerance, and resistance mechanisms to varying degrees to defend against ectoparasitic blowfly larvae. Adults may be able to help their offspring avoid parasitism by breeding earlier in the summer and by selecting south-facing nest boxes. Despite significant blood loss to blowflies among sham-fumigated nestlings, we did not observe an effect of parasitism on body mass, primary feather length, or nestling survival. Adult compensatory feeding does not appear to be an important mechanism underlying nestling tolerance in this system. Resistance may be important, as females with higher antibody responses had nests with fewer parasites and offspring with slightly higher antibody levels.

Future studies should focus on the post-fledging effects of hematophagous ectoparasites such as blowflies, particularly on the effects that decreased oxygen-carrying capacity due to reduced hemoglobin might have on flight performance in tree swallows and other aerial specialist hosts (Cornell et al. 2017). Future work should also emphasize the importance of specific acquired immunity in bird-ectoparasite dynamics, and the degree to which heritability and maternal transfer of antibody response can influence these dynamics (Garnier and Graham 2014). Finally, the significant inter-annual variation we observed, with differences in nearly every parameter between 2014 and 2015–2016, emphasizes the importance of multi-year studies of host–parasite interactions.

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