Interannual variation in *Protocalliphora* blowfly (Diptera: Calliphoridae) parasitism of Tree Swallows (*Tachycineta bicolor*) breeding in southern Québec's farmlands: 16 years of data on species composition, morphometry and hyperparasitism by *Nasonia* wasps (Hymenoptera: Pteromalidae)

Authors :

Coroller-Chouraki, Simon[1,2,4] ; Savage, Jade[3] ; Pelletier, Fanie[1] ; Garant, Dany[1] ; Bélisle, Marc[1,2]

Authors Affiliations :

[1]Département de biologie, Université de Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke (Québec), Canada

[2]Centre d'étude de la forêt, Pavillon des Sciences biologiques, Montréal (Québec), Canada

[3]Department of Biology and Biochemistry, Bishop's University, Sherbrooke (Québec), Canada

[4]Simon.Coroller@USherbrooke.ca

ABSTRACT

Although adult blowflies of the genus *Protocalliphora* are rarely observed in the wild, their larvae are among the most prevalent ectoparasites of altricial birds across the Holarctic. Yet, the spatial and temporal dynamics of infestations, species composition, and interactions with natural parasitoids remain poorly understood. Here, we present a long-term, multi-site studies of *Protocalliphora* infestations in 2,673 Tree Swallow (*Tachycineta bicolor*) nests, based on 16 years of data (2004-2019) collected across a 10,200-km² gradient of agricultural intensity in southern Québec, Canada. We found that *Protocalliphora* infestation prevalence and load (number of puparia) in Tree Swallow nests, varied markedly across space and time, with cyclical patterns emerging at approximately 75% of sites. This suggests the influence of both regional and local processes. Probability of hyperparasitism of *Protocalliphora* puparia by *Nasonia* parasitoid wasps was high but variable (min ~48%; max ~90%; mean ~70%), likely contributing to the observed temporal oscillations in *Protocalliphora* prevalence and load. We also documented substantial interannual shifts in the relative abundance of three *Protocalliphora* species (*P. bennetti*, *P. metallica*, *P. sialia*), emphasizing the importance of species-level resolution in ecological studies of parasitism. Morphometric analyses revealed considerable overlap in puparia size among species, challenging the utility of traditional diagnostic traits for species identification. Finally, we report the first field estimates of *Nasonia* dormancy and/or mortality within hosts, which were observed in 3% to 16% of *Protocalliphora* puparia depending on year. These findings highlight the importance of long-term, multi-trophic, and spatially explicit monitoring to unravel the complex ecological drivers of host-parasite-parasitoid dynamics in increasingly human-modified environments such as agroecosystems.

INTRODUCTION

Avian ectoparasites encompass an exceptionally diverse array of invertebrate taxa, ranging from leeches (Hirudinea) and ticks (Ixodida) to insects from various orders, including lice (Phthiraptera), true bugs (Hemiptera), fleas (Siphonaptera), and flies (Diptera; Rothschild & Clay 1952; Clayton et al. 2010). Many of these ectoparasites are hematophagous, a feeding mode that has evolved independently multiple times within Diptera and which occurs in many families of both the Nematocera and Brachycera sub-orders (e.g., Simuliidae, Ceratopogonidae, Hippoboscidae, Nycteribiidae). However, significant differences exist among dipteran ectoparasites in terms of host selection and specificity, whether they are obligatory blood-feeders or not, and regarding diet differentiation across life stages. For instance, whereas the larvae of *Protocalliphora* blowflies (Calliphoridae) are obligatory blood-feeding parasites of nestling belonging to a wide diversity of altricial bird species, the adults appear to feed on the pollen of herbaceous plants (Sabrosky et al. 1989; Bennett & Whithworth 1991). While the biology and ecology of hematophagous flies parasitizing humans and domestic animals are well known (e.g., Marquardt et al. 2004), flies parasitizing wild animals have received much less attention or have mainly been studied through the costs they can inflict on their hosts. *Protocalliphora* blowflies exemplify this case as most previous studies focused on measuring the impacts that their larvae may have on the nestlings and reproductive success of altricial birds (Simon et al. 2004; Maziarz et al. 2022). In recent years, however, the environmental factors that drive the prevalence and intensity of bird nest infestations by *Protocalliphora* have been increasingly addressed (Mennerat et al. 2021; Merino et al. 2024). Yet only a few studies have so far formally investigated the determinants of bird nest infestations by *Protocalliphora* in multiple years and sites (Mennerat et al. 2021; Maziarz et al. 2022; Merino et al. 2024). Moreover, these studies, which mainly focused

on the effects of weather or climatic factors, were conducted in a small number of forested sites, and thus, on a limited range of habitat heterogeneity. Thus, we have limited information on the spatial and temporal variability of infestations, and thereby on the influence of abiotic and biotic factors on the host-parasite dynamics involving *Protocalliphora*.

The lack of long-term monitoring data on bird nest infestations by *Protocalliphora* also concerns the species of blowflies involved in these interactions. At least 40 species of *Protocalliphora* blowflies have been described in the Holarctic region (Sabrosky et al. 1989), many of which have overlapping distribution ranges, resulting in occasional mixed infestations within a bird nest (Daoust et al. 2012). Moreover, the morphological identification of *Protocalliphora* species is challenging, at least to the non-specialists, and has been considered to be reliable only in adults (Heeb et al. 2000). Yet, locating and sampling adult blowflies in the field is no easy task (Stiner 1969; Bennett & Whitworth 1991), thus larvae and puparia often remain the primary evidence of blowfly parasitism and parasitic load (Jánošková et al. 2010). Despite significant progress regarding the taxonomy and identification of *Protocalliphora*, notably through the work of Sabrosky et al. (1989) and Whitworth (2002, 2003, 2019), identifying *Protocalliphora* species based on puparia morphology is complicated by the fact that key criteria, such as distance between specific spines, prothoracic fringe diameter, and distance between posterior spiracles can vary widely within species and overlap between species (Jánošková et al. 2010; Jamriška & Modrý 2013). Furthermore, identification based on DNA barcoding using the Folmer region of the common COI gene fails to discriminate between most described *Protocalliphora* species (Whitworth et al. 2007). These identification challenges, especially considering the high parasite load found in some nests, have understandably led ecologists to neglect *Protocalliphora* species composition and typically lump all individuals under "*Protocalliphora spp.*" (e.g., Heeb et al.

2000; Bańbura et al. 2004; Hannam 2006; Musgrave et al. 2019). Using *Protocalliphora* as a homogeneous entity can, however, bias species abundance estimates, and hinder the study of host-parasite dynamics by neglecting the potential competition that may occur among *Protocalliphora* species as well as their interactions with their hyperparasites within host nests.

Other overlooked aspects that may impact host-parasite dynamics involving *Protocalliphora* include within and between-species variation in body size that may partly reflect the quality of the growth conditions they experienced as larvae and ultimately their metamorphosis and emergence success, notably through their susceptibility to hyperparasitism by gregarious *Nasonia* "jewel wasps" (Hymenoptera: Pteromalidae; Werren & Loehlin 2009). These generalist wasps, which parasitize *Protocalliphora* at the pupal stage, are known for their effective control of insect host populations, including agricultural pests, and play a substantial role in the top-down regulation of calyptrate flies. Most research on *Nasonia*, including the well-known *N. vitripennis*, and *N. giraulti*, which attack *Protocalliphora* (Daoust et al. 2012; Garrido-Bautista et al. 2019), has however largely focused on their genetics and behavioral ecology under laboratory conditions (e.g., Werren & Loehlin 2009; Deveux et al. 2023). Past studies hence provide limited insights into the ecological interactions between *Nasonia* and *Protocalliphora* in natural habitats, and how their top-down regulation potential against blowflies may fluctuate under different conditions in either space or time. These gaps in our understanding of *Protocalliphora* extend beyond taxonomy, morphometry, and distribution. Long-term studies focusing on the prevalence and abundance of blowfly larvae within nests have noted important annual fluctuations, after accounting for some environmental conditions (Wesolowski, 2001; Daoust et al. 2012; Musgrave et al. 2019; Merino et al. 2024). However, only Merino et al. (2024) have described cyclical trends in abundance and

to our knowledge, no details on temporal variation in species composition and hyperparasitic pressures have been documented.

In this study, we quantify the spatial and temporal variability in the prevalence, intensity, and species composition of Tree Swallow (*Tachycineta bicolor*) nest infestations by *Protocalliphora* over a 16-year period within a 10,200-km² gradient of agricultural intensity in southern Québec, Canada. We also report species-specific size distributions for the puparia of three species of *Protocalliphora,* temporal variation in hyperparasitism rates by *Nasonia* wasps as well as the proportion of this hyperparasite dormancy within its host's puparia.

METHODS

Study system and field sampling

We collected *Protocalliphora* puparia between 2004 and 2019 within a network of 400 nest boxes distributed equally among 40 farms located along a 10,200-km² gradient of agricultural intensity in Southern Québec, Canada (Ghilain & Bélisle 2008). Nest boxes were placed ~1.5 m above ground and spaced by ~50 m along a linear transect that followed field edges. While the primary objective of the above network initiated in 2004 was to quantify the impact of land use on Tree Swallow breeding ecology, it has evolved into a comprehensive platform for diverse investigations, including the effects of *Protocalliphora* parasitic load on the swallows' breeding success (Daoust et al. 2012; Pigeon et al. 2012; Sigouin et al. 2021). At the end of each breeding season, we collected and bagged the entire content of nest boxes exclusively occupied by Tree Swallows, a single-brooded species. Collected nests were stored at 4°C prior to processing, which included 2 days at -80°C to kill any remaining living organisms before sorting under a ventilated hood. *Protocalliphora* puparia found within nest material were counted and stored in vials filled

with 75% ethanol and referenced according to farm and nestbox IDs and year of sampling. Note that *Protocalliphora* puparia from 2008 to 2009 were collected under two protocols by Daoust et al. (2012) to raise some pupae in the lab and collect emerging flies or *Nasonia* wasps. As a result, some specimens were no longer available for further examination in this study (i.e., for hyperparasitism, species composition, and morphometry). Nevertheless, our dataset allowed us to investigate nest infestation and parasitic load using nests from all nest boxes occupied by Tree Swallows with a single clutch event leading to ≥ 1 nestling on a given year between, 2004 and 2019 (including 2008 and 2009), for a total of 2,673 nests (Table 1).

Specimen examination

Due to the considerable number of *Protocalliphora* puparia and the significant time commitment required for individual specimen examination, a subsample of 263 infested nests was selected for hyperparasitism assessment, species identification, and morphometric measurements of puparia found therein (Table 1). Infested nests were subsampled as follows: two infested nests (when available) were randomly sampled from each of 10 farms randomly selected to represent the gradient of agricultural intensity on each year (excluding 2008 and 2009). When selected farms had nests with no or very low levels of infestation by *Protocalliphora* (i.e., < 100 puparia in total for all 10 nest boxes of a farm in a given year), additional nests, or farms if needed, were randomly selected until a minimum of 100 puparia could be processed for a given year. All *Protocalliphora* specimens from the resulting subsample of 263 nests (n = 3,994 puparia) were examined and only those with adequate structural integrity for the determination or measurement of a given attribute were considered in data analyses regarding hyperparasitism, species composition, and morphometry (Table 1).

Table 1. Number of Tree Swallow nests and *Protocalliphora* puparia collected and analysed between 2004 and 2019 in southern Québec, Canada. No puparia from 2008-2009 could be included in the hyperparasitism, species composition, and morphometry datasets as they were used in a previous by Daoust et al. (2012) and were no longer suitable for further processing.

	Datasets			
Samples	All data	Hyperparasitism	Species composition	Morphometry
# of nests examined	2,673	263	263	263
# of nests infested by *Protocalliphora* with > 1 puparium suitable for processing	1,227	259	216	174
# of nests infested by *Nasonia*	-	218	-	-
# of puparia examined	22,630	3,994	3,994	3,994
# of puparia excluded (damaged)		386	985	2,012
# of puparia without *Nasonia*	-	1,125	-	-
# of puparia with *Nasonia*	-	2,483	-	-
# of puparia with non-emerged *Nasonia*	-	318	-	-
# of puparia of *P. sialia*	-	-	2,426	1,569
# of puparia of *P. bennetti*	-	-	474	347
# of puparia of *P. metallica*	-	-	109	66

Hyperparasitism of Protocalliphora by Nasonia

Species-level identification of *Nasonia* wasps was not conducted in the present work due to fall sample collection when most hyperparasites had already emerged and those that did not were killed before completing their development. However, early subsampling and rearing of *Protocalliphora* pupae by Daoust et al. (2012) within the same study system allowed the post-emergence identification of two *Nasonia* species, *N. vitripennis* and *N. giraulti*, the first accounting for > 90% of *Protocalliphora* infestation events.

The occurrence of *Nasonia* hyperparasitism on a *Protocalliphora* puparium is typically diagnosed by the presence of one or more exit holes of 0.5-1 mm in diameter. However, *Nasonia* can also die or overwinter as pupae inside their host instead of emerging at the end of the active season (Garrido-Bautista 2019). Because puparia containing a dead *Protocalliphora* pupa and those containing unemerged *Nasonia* will look identical, we systematically dissected *Protocalliphora* puparia showing no sign of either fly or wasp emergence to verify the occurrence of *Nasonia* hyperparasites to discriminate pupation failures linked to hyperparasitism from those resulting from other causes. All 3,608 *Protocalliphora* puparia assessed for *Nasonia* infestation were hence classified into one of the following categories: no evidence of *Nasonia* hyperparasitism, presence of *Nasonia* emergence holes, and presence of unemerged *Nasonia* pupae without exit holes.

Protocalliphora species composition

We identified 3,009 well-preserved *Protocalliphora* puparia based on morphology following Sabrosky et al. (1989), and Whitworth (2002, 2003, 2019), with additional feedback from T.L. Whitworth (pers. comm.). Consistently with Daoust et al. (2012), who conducted research within

the same study system, only three species were identified: *Protocalliphora sialia* Shannon & Dobroscky, *Protocalliphora bennetti* (Whitworth), and *Protocalliphora metallica* (Townsend).

Protocalliphora morphometry

To investigate inter and intra-specific variability in maximum length and width of *Protocalliphora* puparia, each puparium was photographed using a Leica M165FC stereomicroscope equipped with a 0.63X planapo objective, a Leica MC170HD color camera, and the Leica LAS X image-capture software (v 3.0.3). All measurements were performed with Fiji image analysis software (Schindelin et al. 2012). This allowed us to model any photographed puparium as an ellipsoidal-shaped object and to calculate its theoretical maximum length and width. This approach minimized measurement biases as puparia from which a *Protocalliphora* had successfully emerged would otherwise appear shorter.

Data analysis

Interannual variability in Tree Swallow nest infestation by *Protocalliphora* between 2004 and 2019 was first evaluated by calculating, for each sampling year, the proportion of infested nests as well as the average number of *Protocalliphora* puparia per infested nest (±SD). We then assessed how the annual mean nest infestation rate across farms varied in time between 2004 and 2019, and to what level each farm followed that global trend, if any, using a Generalized Linear Additive Mixed Model (GAMM) fitted with the mgcv package (v.1.9-1; Wood 2023) in R (v.4.3.1, R Core Team, 2023). More specifically, we modeled the proportion of infested nests using a global smooth term for Year and group-level smooth terms allowing for farm-specific temporal trends that may differ in shape and wiggliness from the global trend (i.e., as per Model *GI* in Pedersen et al. 2019).

The GAMM was fitted using a binomial distribution and logit link function. Temporal autocorrelation in the residuals was assessed using the acf function in R (Simpson 2018). We then performed the same exploratory analyses for the average number of *Protocalliphora* puparia per infested nest with a GAMM that used a tweedie distribution and a log link function due to substantial overdispersion with respect to a (truncated) Poisson distribution. Detailed codes, model diagnostics and results for theses analyses are provided in Supplementary Material 1.

Interannual variability in *Nasonia* hyperparasitism of *Protocalliphora* puparia was evaluated by contrasting the yearly proportion of non-infested to infested puparia and the proportion of infested puparia from which *Nasonia* wasps had emerged against those still containing *Nasonia* pupae or adults. Interannual variability in *Protocalliphora* species composition was assessed by calculating the yearly relative proportion of each species for all specimens that could be unambiguously identified. Hyperparasitism rates and species composition from 2008-2009 were obtained from Daoust et al. (2012).

Intra- and between-species variability in the maximum length and width of puparia of *Protocalliphora sialia*, *Protocalliphora bennetti*, and *Protocalliphora metallica* were first assessed using density plots and boxplots based on the raw data. We then assessed inter-specific differences in mean puparium maximum length and width using Generalized Linear Mixed Models (GLMMs) fitted with the glmmTMB R package (Brooks et al. 2023). In this analysis *Protocalliphora bennetti*, the intermediate-size species (Sabrosky et al. 1989; Whitworth 2002), was designated as the reference category. Farm ID and Year were included as random effects to account for environmental variation and to control for potential confounding effects. Nestbox ID has been included as a random effect, but this led to convergence issues due to the low number of repeated measures within each nest. Therefore, this variable was omitted from the analysis. The

proportion of variance explained by Farm ID and Year random effects were estimated following Nakagawa and Schielzeth (2013). Species-specific maximum length and width being approximately normally distributed, GLMMs were fitted using a Gaussian distribution and identity link.

RESULTS

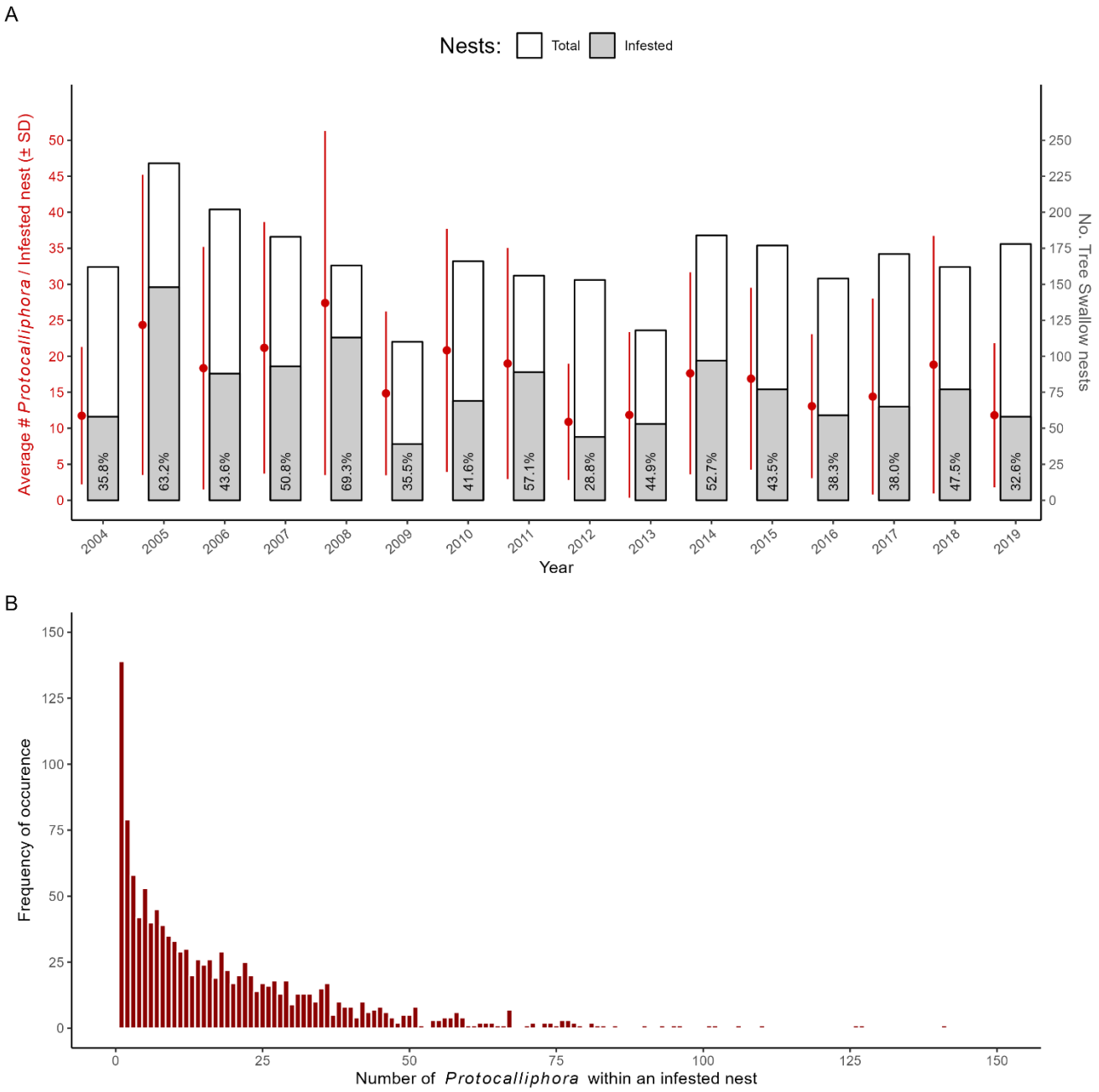
Spatiotemporal variation in Protocalliphora prevalence and load

Protocalliphora nest infestation occurred widely throughout the study area. All 40 monitored farms harbored ≥ 1 infested Tree Swallow nest at some point between 2004 and 2019 and between 67.65% and 92.31% of farms had ≥ 1 infested nest depending on year. Overall, 45.90% (1,227) of the 2,673 Tree Swallow nests with a single clutch event leading to ≥ 1 nestling were infested by ≥ 1 *Protocalliphora* larva based on counts of puparia. The proportion of infested nests varied substantially both among years (range: 28.76% in 2012 to 69.33% in 2008; Fig.1A) and farms (range: 13.37% to 79.49%). While most farms showed yearly fluctuations in nest infestation rate that followed the global, nonlinear trend observed across the study area, about 30% of farms showed mild to strong departures from that global trend (n = 587; GAMM R^2= 48.8%; Supplementary Material 1). Temporal autocorrelation in the GAMM residuals at time lags of 1, 2, 3 and 5 years supported the presence of a cyclical nest infestation dynamics with a ~3-year period at the scale of the study area (Supplementary Material 1).

A total of 22,630 puparia were counted among infested nests, resulting in an overall estimated average load (± SD) of 18.44 ± 19.16 *Protocalliphora* per infested nest. The average number of *Protocalliphora* per infested nest exhibited variability both within and across years, fluctuating between 10.89 ± 8.08 (2012) and 27.40 ± 23.90 (2008) with a yearly coefficient of variation ranging between 74.21 and 97.11% (Fig. 1A). The overall frequency distribution of the number of

Protocalliphora per infested nest was, however, highly skewed to the right with a median of 12 puparia (range: 1-141 puparia; Fig. 1B). Interestingly, most farms showed yearly fluctuations in average *Protocalliphora* loads per infested nest that followed a global, nonlinear trend, which peaks and troughs coincided with those observed for the proportion of nests infested by *Protocalliphora* (n = 1,227; GAMM R^2= 12.2%; Supplementary Material 1). However, the farms that deviated from the global load trend (i.e., 22.5% of farms) were not the same as for the proportion of infested nests and the GAMM residuals showed no temporal autocorrelation pattern.

Fig. 1. A) Patterns of Tree Swallow nest infestation by *Protocalliphora* observed between 2004 and 2019 in southern Québec, Canada. Left y-axis and corresponding red dots (± error bars) show the yearly average (± SD) number of *Protocalliphora* puparia per infested nest. Right y-axis and corresponding bars show the yearly number of infested nests (grey) over the yearly total number of examined nests (i.e., nests that harboured a



single clutch that experienced at least one hatching event; white). **B**) Frequency distribution of the number of *Protocalliphora* puparia found within infested nest (*n* = 1,227).

Hyperparasitism of Protocalliphora by Nasonia

Nasonia likely occurred throughout the study area as they were detected at least once in 30 of the 31 farms from which *Protocalliphora* puparia were subsampled to estimate hyperparasitism rate. Overall, *Nasonia* hyperparasitized ≥ 1 puparium in 85.08% of the *Protocalliphora* infested nests and 68.82% of the 3,608 subsampled puparia that could be examined (Table.1). Importantly, 12.81% of these hyperparasitized *Protocalliphora* puparia contained non-emerged *Nasonia* individuals. These non-emerged individuals occurred nearly exclusively at the pupal stage, suggesting that they either failed to produce adults or entered diapause for the coming winter. Hyperparasitism by *Nasonia* remained high across years, ranging between 48.25% (2006) and 90.48% (2012) of *Protocalliphora* puparia (Fig.2). Note that Daoust et al. (2012) estimated hyperparasitism rates in 2008 and 2009 based only on the presence of exit holes made by *Nasonia* to exit puparia and reported among the lowest hyperparasitism rates of the study period (i.e., 50.35% and 39.29%, respectively). The proportions of hyperparasitized *Protocalliphora* puparia from which *Nasonia* did not emerge before winter varied substantially, ranging from 3.07% in 2006 to 16.36% in 2019 (Fig.2).

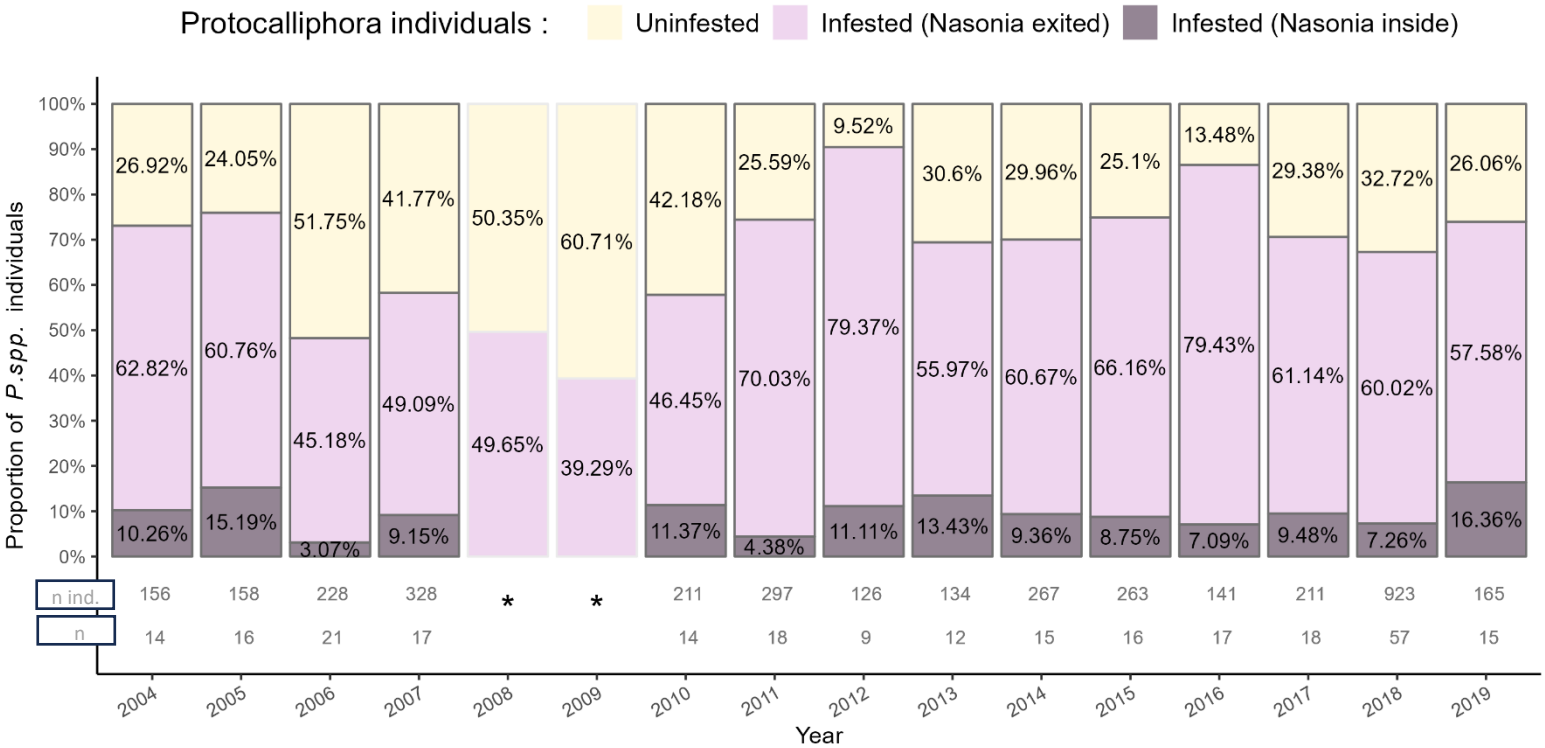


Fig. 2. Interannual rates of *Protocalliphora* puparia hyperparasitism by *Nasonia* within Tree Swallow nests collected in southern Québec, Canada, between 2004 and 2019. *Protocalliphora* puparia were classified as uninfested when they showed no signs of hyperparasitism, and as infested, if *Nasonia* exit holes were observed on the puparia or *Nasonia* first instars were found when *Protocalliphora* puparia were dissected. (*) Proportions for 2008 and 2009 were derived from Daoust et al. (2012; Table 3) based only on the presence of holes made by *Nasonia* to exit puparia.

Protocalliphora species composition

Of the 3,009 *Protocalliphora* puparia suitable for species identification, 82.62% belonged to *P. sialia*, 15.75% to *P. bennetti* (15.75%) and 3.62% to *P. metallica* (Table 1). *Protocalliphora sialia* was consistently the most abundant species in each year, ranging from of 61.72% in 2011 to 98.41% the following year (Fig. 3). In contrast, *P. bennetti* exhibited more pronounced fluctuations in relative abundance, ranging from quasi absence (0.79%) in 2012 to 32.01% in 2011. *Protocalliphora metallica*, the least abundant species in our Tree Swallow study system, also showed important variation in its relative abundance. It was either completely absent from our subsamples in 2005 and 2016 or equally to more abundant than *P. bennetti* in 2004, 2012 and 2017, but never exceeded 9.27% (2017) of *Protocalliphora* puparia in any given year (Fig.3). Data extrapolated from Daoust et al. (2012) for 2008 and 2009 also showed a strong dominance of *P. sialia*, with proportions of 96.59% and 90.74%, respectively. Overall, all three species were found over most of the study area (Supplementary Material 2)

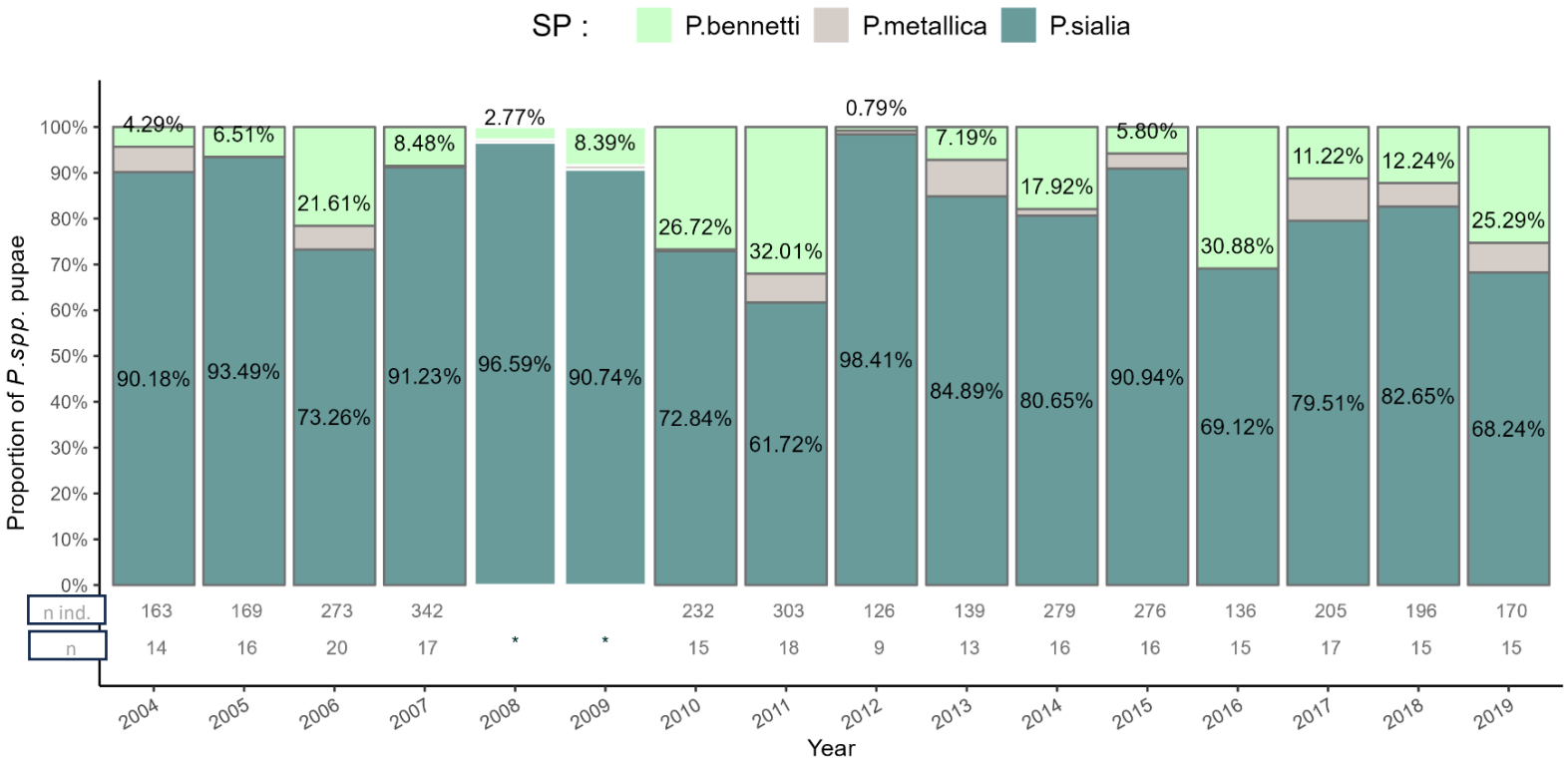


Fig. 3. Interannual *Protocalliphora* species composition within Tree Swallow nests collected in southern Québec, Canada, between 2004 and 2019. Three species were identified (*P. bennetti*, light green; *P. metallica*, grey; *P. sialia*, dark green). (*) Proportions for 2008 and 2009 were derived from Daoust et al. (2012; Table 1).

Protocalliphora morphometry

The maximum puparium length and width of the three *Protocalliphora* species were obtained from a subsample of 1,982 individuals (Table 1). The distribution of maximum length and width of the three species were unimodal and relatively symmetrical, though slightly skewed to the left in *P. metallica* (Fig. 4). Despite substantial intraspecific variation leading to distributional overlap, the GLMMs showed species differed in their average morphometry. *P. bennetti* exhibited a mean puparium length (± SD) of 8.18 ± 0.67 mm (range: 5.24-9.93 mm), while *P. metallica* showed a significantly shorter mean length (7.34 ± 0.71 mm; range: 5.82-9.29 mm; $P < 0.001$), and *P. sialia* a longer one (8.74 ± 0.58 mm; range: 5.70-10.27 mm; $P < 0.001$). Regarding width, *P. metallica* had a narrower puparium (3.36 ± 0.41 mm, range: 2.25-4.16 mm; $P < 0.001$) and *P. sialia* a wider one (3.89 ± 0.32 mm, range: 1.89-5.50 mm; $P < 0.001$) compared to *P. bennetti* (3.50 ± 0.39 mm, range: 1.84-4.41 mm), respectively. The proportion of variance in length and width explained by both the random (i.e., Farm ID and Year) and fixed (i.e., species) effects of the linear mixed models was 36.45% and 30.57% for the maximum puparium length and width, respectively. Random effects alone accounted for 51.58% and 44.16% of these proportions, respectively. These results suggest that morphometric variation of *Protocalliphora* puparia is strongly caused by agroenvironmental variables that have not been taken into account in the analysis.

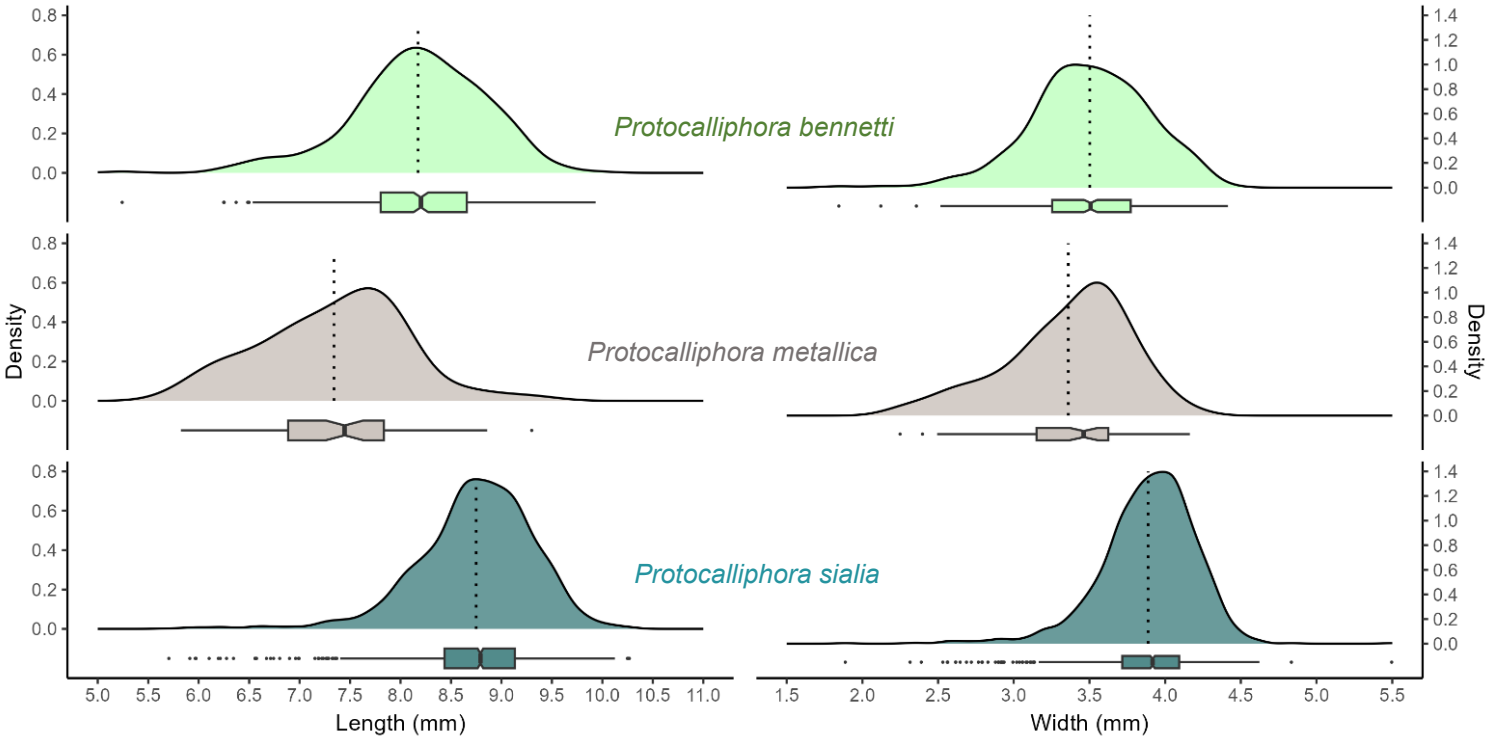


Fig. 4. Density distribution of morphometric measurements (length and width) of *Protocalliphora* puparia for *P. bennetti* (light green, n = 347), *P. metallica* (grey, n = 66), and *P. sialia* (dark green, n = 1,569) collected in southern Québec, Canada, between 2004 and 2019. Mean (dotted line) and quartiles (via boxplot) are shown.

DISCUSSION

Our study presents a rare time series on the prevalence of bird nest infestation by *Protocalliphora* blowflies and their abundance within infested nests. We found that infestation rates and loads of *Protocalliphora* within Tree Swallow nests were high over a 16-year period (2004-2019), yet widely variable in both space and time along a 10,200-km^2 gradient of agricultural intensity in southern Québec, Canada. Moreover, we found that these large temporal fluctuations were indicative of a cyclical dynamics that was shared by ~75% of the sampled locations (i.e., farms) and thus likely driven by both large-scale and local conditions or processes. Our study also shows that although *Protocalliphora* pupae were subjected to hyperparasitic pressures by *Nasonia* wasps that can vary as much as double among years, these were high enough to kill more than half of the *Protocalliphora* pupae produced in most years. Such an impact suggests that *Protocalliphora* and *Nasonia* may be part of a host-parasitoid dynamics responsible for the oscillations in *Protocalliphora* infestation rates and loads that we report. Consistent with the previous 2-year investigation in our study system by Daoust et al. (2012), we found both mono- and multispecific infestations in nests, involving three species of *Protocalliphora*, with *P. sialia* clearly dominating (>80% of puparia).. However, we found higher proportions of *P. bennetti* (~2x) and *P. metallica* (~4x) than reported by Daoust et al. (2012), emphasizing the value of multi-year studies in revealing the complete range of species composition variability in these flies. Lastly, we report highly variable puparium length and width with overlapping frequency distributions among *Protocalliphora* species. This finding suggests that these morphometric traits are not reliable as discrete diagnostic characters and not to be used in isolation for identifying *Protocalliphora* species (Whitworth 2002). It also raises important questions about the ecological factors driving

this variability and the potential consequences of size variation for emergence success and host selection by parasitoids.

Spatiotemporal variation in Protocalliphora prevalence and load

The prevalence and intensity of bird nest infestations by *Protocalliphora* have generally been found to vary widely in both space and time. For example, Bennett and Whitworth's (1992) comprehensive examination across 73 bird species and 4,668 sampled nests revealed infestation rates of 52% within the Rocky Mountain region (Utah, USA) versus 24% in the Great Lakes region (Ontario, Canada). The mechanisms underlying such variation likely involve multiple dependent and interactive factors and few studies have attempted to quantify the importance and spatiotemporal scales at which they operate. While large-scale geographic variation may result from differences in climate, landscape habitat composition, bird host community (species composition and abundance), and hyperparasitic pressure (Sabrosky et al. 1989; Bennett and Whitworth 1992; Daoust et al., 2012a, 2012b; Garrido-Bautista et al. 2019), local variation may origin from factors as specific as bird host brood size and hatching date (Williams 2017; Wolfe-Merritt et al. 2022), and nest type and material affecting the nest microclimate (Dawson et al. 2011; Griebel et al. 2020). With respect to Tree Swallows breeding within nest boxes, previous research reported *Protocalliphora* infestation rates ranging from 89% to 100% in most study sites across Canada (Rendell & Verbeek 1996; Dawson et al. 2005; Gentes et al. 2007; Griebel et al. 2020). Infestation rates are generally much higher than those we found either across years or farms within our study area (overall rate = 45.90%). In contrast to other study sites, many of which were located near wetlands, ours were located on farms distributed along a gradient of agricultural intensity with very low, if any, wetland cover (Garrett et al. 2022). Hence, we hypothesize that the much

lower infestation rates we observed is linked to intensive agricultural practices that may impact *Protocalliphora* through multiple pathways, including directly through wetland drainage or pesticide use, or indirectly by reducing the density of their bird hosts (e.g., Robert et al. 2019; Wagner et al. 2021; Powell et al. 2024). Evidence for such negative impacts has also been reported in our study area by Daoust et al. (2012b) who found that lower loads of *Protocalliphora* in Tree Swallow nests located occurred within agro-intensive landscape during a 2-year period (2008-2009).

Annual infestation rates of Tree Swallow nests by *Protocalliphora* varied between 29% and 63% across the 16 years that spanned our study. This level of interannual variation, which was observed at the scale of the entire study area (i.e., 10,200-km^2), was comparable to what we observed among nestboxes from a single farm (i.e., < 1 km^2 ; Supplementary Material 3). Wesolowski (2001) also reported highly variable annual nest infestation rates by *Protocalliphora* in Marsh Tits (*Parus palustris*) that ranged from 27% to 88% in four 0.5-km^2 plots separated by ~1-2 km during an 8-year period in eastern Poland. Similarly, Musgrave et al. (2019), found annual infestation rates between 30% and 91% in Western Bluebird (*Sialia mexicana*) nests monitored over 17 years in a ~100-km^2 area of northern New Mexico (USA). Despite potential confounding effects, the above comparisons suggest that nest infestation rates by *Protocalliphora* typically vary substantially among years across a range of spatial scales. Given the lack of reported time series of infestation rates (and loads; but see Merino et al. 2024) in the literature, the level of temporal autocorrelation among years and how it varies across spatial scales are largely unknown and prevent the investigation of the ecological mechanisms underlying such fluctuations. Our observation that yearly fluctuations in both *Protocalliphora* nest infestation rates and loads were to some degree cyclical and synchronous among ~75% of the farms sampled over 10,200 km^2 suggest that both

regional (e.g., climatic) and local (e.g., crop rotations), as well as some density-dependent (e.g., hyperparasitic pressure by *Nasonia* wasps) processes may be at play.

The observed loads of *Protocalliphora* within infested nests exhibited a right-skewed distribution, a phenomenon commonly observed in the field in parasitology (Wilson and Grenfell 1997). Furthermore, a high degree of variability between nests, between sites, and across years were reported (Griebel and Dawson 2019; Remeš and Krist, 2005). Part of the interannual variation is often associated with weather (Merino & Potti 1996; Dawson et al. 2005; Mennerat et al. 2021; Castaño-Vazquez et al. 2021; Merino et al. 2024) and spatial variation is typically linked to micro-habitat and nest-specific factors, such as the amount of feather lining or the species and number of nestling hosts (Eeva et al. 1994; Merino & Potti 2001; Tomás et al. 2007; Dawson et al. 2011; Williams 2017; Wolfe-Merritt et al. 2022). In our study system, part of the between-farm variation likely results from the diversified agricultural contexts in which Tree Swallows breed. Daoust et al. (2012b) found fewer *Protocalliphora* in Tree Swallow nests located in more agrointensive areas, particularly in maize and soybean monocultures. This pattern could result from several direct and indirect effects associated with intensive agriculture, such as reduced shelter and food resources for adult flies, lower avian host availability at both the landscape and brood scale and increased toxicological effects from pesticide exposure either directly or through their avian hosts (Daoust et al. 2012). Given the rarity of studies that examined the effects of agricultural intensity on ectoparasite of wild birds, more extensive and long-term research is needed to determine the mechanisms by which agriculture generates spatiotemporal variation and ultimately shapes host-parasite dynamics.

Hyperparasitism of Protocalliphora by Nasonia

Interannual variation in the proportions of *Protocalliphora* puparia hyperparasitized by *Nasonia* wasps showed no clear patterns while ranging between 48.25% and 90.48% among years, with an overall rate of 68.82%. These proportions are generally higher from those of Jamriška & Lučeničová (2019), who observed an average hyperparasitism rate of 37.91% over four years in Slovakia, and from those of Bennett & Whitworth (1991), who reported an overall rate of ~17% from two regions of North America, namely Utah and Ontario. In contrast, Grillenberger et al. (2009) reported a rate of ~44% in New York state (USA), while Garrido-Bautista et al. (2020) reported rates from 47% to 56% for southern Spain, and from 61% to 79% for central Spain, figures much closer to our own. While more extensive longitudinal and ecological studies are needed, current findings suggest that geographical and environmental contexts may significantly influence the likelihood of *Nasonia* hyperparasitism, a primary determinant of *Protocalliphora* emergence success. However, it is crucial to recognize that, in addition to the substantial control exerted by *Nasonia* wasps on *Protocalliphora* communities, uninfested *Protocalliphora* individuals may also die during pupation due to other ecological factors, such as environmental contamination. The specific impacts of those factors on *Protocalliphora* emergence success have yet to be thoroughly investigated and quantified.

Nasonia parasitoid wasps have mostly served as model species in numerous laboratory studies addressing themes in genetics, developmental biology, and behavioral ecology (Werren and Loehlin 2009). The ecological factors driving host selection and hyperparasitism by *Nasonia* under natural conditions are thus poorly understood (Garrido-Bautista 2019; Peters 2010). This is particularly true given the wide diversity of hosts in which they can lay eggs and the potential for regional variations due to their widespread distribution. This leaves the causes of yearly

452 fluctuations in *Protocalliphora* puparia hyperparasitic events or of dormancy within the host
453 puparia unresolved. Future research on *Nasonia* ecology would likely benefit from considering
454 factors such as vegetation type and density, as these features may influence host hyperparasitism
455 by affecting the availability of food and shelter (Wylies 1958). Avian nest availability associated
456 with these environmental factors could also potentially induce host-dependent effects, contributing
457 to an increased incidence of hyperparasitism on *Protocalliphora*.

458 This study reports the first quantitative exploration of dormancy and/or mortality of *Nasonia*
459 within their host in the wild. We noted consistently low yearly proportions of *Protocalliphora*
460 puparia containing dormant or dead *Nasonia* (range: 3.07-16.36%), with no discernible temporal
461 trend. Several biotic and abiotic factors, such as weather, luminosity or host and parasitoid body
462 condition, may affect the production of diapausing *Nasonia* offspring (Saunders et al. 1969; Yoder
463 et al. 1994; Bertossa et al. 2010; Li et al. 2014; Paolucci et al. 2013). Mechanisms linked to
464 phenology could thereby impact the dormancy and survival of *Nasonia* larvae. For instance,
465 *Protocalliphora* pupation phenology, which in turn determines host availability and development
466 conditions for *Nasonia*, must partly depend on avian host breeding phenology. Moreover,
467 agricultural intensity may affect *Nasonia* development and emergence success as pesticide use can
468 alter hyperparasitoid wasps cognition and fitness (Schöfer et al. 2023, 2024; Sulg et al. 2023), as
469 well as the availability and quality of their hosts (Li et al. 2014; Daoust et al. 2012).

470

471 *Protocalliphora species composition and morphometry*

472 Bird nestlings from the Nearctic region are hosts to at least 28 *Protocalliphora* species (Sabrosky
473 et al. 1989; updated by Whitworth 2002). While some bird blowfly species appear to specialize on
474 specific hosts through behavioral adaptations, some parasitize a wide array of bird species (Bennett

& Whitworth 1992), and the occurrence of multiple *Protocalliphora* species within a single host nest is not a rare event (Daoust et al. 2012). Our longitudinal study, one of the very few that describe *Protocalliphora* species composition, reports that such compositions can vary substantially across years. Indeed, our results show that certain species (in this case, *P. metallica*) may be practically absent in some years, only to reappear in relatively high numbers the following year. Even the highly prevalent *P. sialia* fluctuated widely, representing 58.93% of the *Protocalliphora* specimens in 2011 to being almost the only species found in infested nests the following year (98.32%). It thus seems essential to conduct longitudinal studies considering variability in species composition before drawing any conclusions about host specialization to avian parasites and mixed infestations.

While descriptive statistics of some morphometric measurements of *Protocalliphora* puparia can be found in the literature, few, if any, studies report the actual frequency distributions of these features that may help discriminating among species of this cryptic group (Heeb et al. 2000; Whitworth 2002, 2003; Jamriška & Modry 2013). The mean, standard deviation, and range estimates of puparium length and width for *P. bennetti, P. metallica, and P. sialia* that we obtained via photogrammetry align well with published estimates (Whitworth et al. 1991; Whitworth 2002). Yet, the fact that density plots highlighted substantial intraspecific variation in both puparium length and width, as well as significant overlap among the three studied species, indicates that these metrics should not be used on their own for *Protocalliphora* identification, even within a small regional species pool. Nevertheless, the ecological factors contributing to this high intraspecific variation, with certain puparia being more than twice as large and long as others, is unclear. Sexual dimorphism (Sabrosky et al. 1989) or factors like host availability and quality, as well as developmental period and temperature, which are often interconnected, have been

hypothesized as causes of size variation in *Protocalliphora*. For instance, underfed larvae may conclude their development as 'runts' (Sabrosky et al. 1989; Bennett & Whitworth 1991). Alternatively, intensive agricultural practices, including pesticide use, have been reported as either direct or indirect drivers of phenotypic variation (Wisniewska & Prokopy 1997; Verheyen & Stoks 2019; Gérard et al. 2022; Ponce-Méndez et al. 2022) and pupation success (Heneberg et al. 2020; Mahdjoub et al. 2020) in non-targeted arthropod species.

All the ecological parameters we studied showed substantial variation in space, time, or among individuals, warranting that future studies addressing the mechanisms driving host-parasite dynamics involving *Protocalliphora* adopt a long-term monitoring and multi-trophic approach in contrasting environments. Investigating the tri-trophic relationships involving birds, *Protocalliphora*, and *Nasonia* across diverse agroecosystems presents a promising avenue for such research. Given the complex interplay among environmental conditions and multi-trophic interactions, we further suggest that such endeavor be tackled using a causal inference framework to limit biases, especially given that each trophic level may respond to different factors, at different spatiotemporal scales, and potentially nonlinearly or in different directions, which has the potential to amplify, attenuate, and even create or mask effects (Arif and MacNeil 2023; Grace 2024).

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