**Variation in parasitoidism of *Protocalliphora azurea*
(Diptera: Calliphoridae) by *Nasonia vitripennis*
(Hymenoptera: Pteromalidae) in Spain**

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Abstract

Parasitoid wasps may act as hyperparasites and sometimes regulate the populations of their hosts by a top-down dynamic. *Nasonia vitripennis* (Walker, 1836) is a generalist gregarious parasitoid that parasitizes several host flies, including the blowfly *Protocalliphora* Hough, 1899 (Diptera: Calliphoridae), which in turn parasitizes bird nestlings. Nonetheless, the ecological factors underlying *N. vitripennis* prevalence and parasitoidism intensity on its hosts in natural populations are poorly understood. We have studied the prevalence of *N. vitripennis* in *Protocalliphora azurea* (Fallén, 1817) puparia parasitizing wild populations of pied flycatcher (*Ficedula hypoleuca*) and blue tit (*Cyanistes caeruleus*) birds in two Mediterranean areas in central and southern Spain. We found some evidence that the prevalence of *N. vitripennis* was higher in moist habitats in southern Spain. A host-dependent effect was found, since the greater the number of *P. azurea* puparia, the greater the probability and rate of parasitoidism by the wasp. Our results also suggest that *N. vitripennis* parasitizes more *P. azurea* puparia in blue tit nests than in pied flycatcher nests as a consequence of a higher load of these flies in the former. Based on the high prevalence of *N. vitripennis* in *P. azurea* puparia in nature, we propose that this wasp may regulate blowfly populations, with possible positive effects on the reproduction of both bird species.

Keywords: Blowfly, Parasitoid, *Nasonia vitripennis*, *Protocalliphora azurea*, *Ficedula hypoleuca*, *Cyanistes caeruleus*

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1. Introduction

Hyperparasitism is a special form of parasitism in which a parasite is infested by another parasite or parasitoid (Hochberg and Ives 2000; Sullivan 2009), thus establishing a multitrophic ecological system with at least three levels: the host, the parasite, and the parasitoid (Sullivan and Völkl 1999). Hyperparasites are usually parasitoid wasps, insects of the order Hymenoptera whose larvae parasitize several life stages of other arthropods, eventually killing them (Quicke 1997). The population dynamics of host species can influence the ecology of parasitoid wasps and vice versa; hence both the bottom-up and the top-down effects might regulate the host-parasitoid systems (Hawkins 1992). Forest and agricultural ecosystems where biological control is performed using parasitoid top-down dynamics are well documented (De Lange et al. 2018; Footitt and Adler 2017; Rivers 2004). Furthermore, parasitoid top-down dynamics in poultry and cattle farms can strongly affect host populations (Morgan 1980; Rutz and Axtell 1981; Skovgård and Nachman 2004). The success or failure of parasitoid top-down regulation may depend on optimal temperatures and moisture for the parasitoid (Skovgård and Nachman 2004), host microhabitat (Frederickx et al. 2014), host distribution, parasitoid searching behaviour (May 1978), or host density (Aung et al. 2011; May et al. 1981).

Nasonia vitripennis (Walker, 1836) (Hymenoptera: Pteromalidae: Pteromalinae), a generalist gregarious parasitoid wasp found in the Holarctic region, parasitizes several calyptrate flies (Desjardins et al. 2010; Peters and Abraham 2010; Werren and Loehlin 2009). Among the hosts of *N. vitripennis* is *Protocalliphora* Hough, 1899 (Diptera: Calliphoridae), a group of blowflies whose larvae are obligate hematophagous ectoparasites of nestling birds (Whitworth and Bennett 1992). Several studies have shown that blood-feeding *Protocalliphora* larvae have negative effects on the

physiology and survival of developing nestlings in different bird species (Hannam 2006; Merino and Potti 1995; Puchala 2004; Simon et al. 2004, 2005; Streby et al. 2009). Other studies have failed to find negative effects of blowflies on nestlings (Miller and Fair 1997; Thomas and Shutler 2001), perhaps, because short-term detrimental effects may be difficult to detect if parental compensation occurs through increased feeding rates to heavily parasitized nestlings (Johnson and Albrecht 1993).

Nasonia species have been widely used as model organisms (Cook et al. 2018; Godfray 2010; Lynch et al. 2006; Niehuis et al. 2010; Oliveira et al. 2008), but little is known on its ecology and prevalence of parasitoidism in its natural habitats. Most studies on the relative abundance, prevalence, and intensity of parasitoidism of this wasp have been conducted in urban areas or poultry and cattle farms, where the insect's prevalence is very low (less than 5%) (Marchiori 2004; Marchiori et al. 2007; Oliva 2008; Rodrigues-Guimarães et al. 2006; Skovgård and Jespersen 1999, 2000), perhaps because all hosts parasitized by the wasp in the aforementioned studies were flies that develop on decaying matter or parasitize cattle. By contrast, birds' nests may be the primary habitat for *N. vitripennis*, which shows high intensity of parasitoidism when parasitizing *Protocalliphora* (Peters 2010). In fact, most studies on the parasitoidism of *Protocalliphora* and other blowflies puparia by *Nasonia* Ashmead, 1904 report that 30-90% of bird nests containing blowfly puparia are infested by the wasp, with 20-50% of puparia being parasitized (Bennett and Whitworth 1991; Daoust et al. 2012; Grillenberger et al. 2008, 2009).

Climatic conditions, habitat, host availability or even the host species parasitized by *Protocalliphora* could play a role in the parasitoidism by this wasp and help explain the variability in its abundance. However, the ecological factors that determine parasitoid prevalence or intensity of *N. vitripennis* are still poorly understood. The present study

documents patterns of parasitoidism of *N. vitripennis* on puparia of *Protocalliphora azurea* (Fallén, 1817) in wild breeding populations of pied flycatcher (*Ficedula hypoleuca*) and blue tit (*Cyanistes caeruleus*) in Spain, and examines biotic and abiotic causes of variation in the prevalence and abundance of this parasitoid.

2. Materials and methods

Nasonia vitripennis is a gregarious ectoparasitoid whose females lay their eggs in the puparia of different calyptrate flies. After emergence of eggs, larvae feed, moult and metamorphose into adults at 14 days of age (at 25°C), which leave the puparium through a self-made exit holes to mate and reproduce (Whiting 1967). One female can carry hundreds of eggs (reviewed in Whiting 1967), but the number of parasitoids emerging from a puparium varies with the host species, ranging from 35-50 in flesh flies (Rivers and Denlinger 1995) to 15-25 in *Protocalliphora* hosts (Gold and Dahlsten 1989; Draber-Monko 1995; Peters 2010). *Protocalliphora azurea* is a blowfly whose larvae parasitizes cavity-nesting birds. Females of *Protocalliphora* lay a mean of 15-75 eggs directly in nests when nestlings hatch (Gold and Dahlsten 1989; Bennett and Whitworth 1991), but the number of eggs laid per nest varies according to bird species. Both insects are univoltine. *Nasonia vitripennis* larvae and pupae may sometimes overwinter inside the puparia of *P. azurea* in a diapause state until the emergence of adults the following spring (Gold and Dahlsten 1989), although in our study areas the adult typically emerges during the first spring. This species shows a relatively high level of overwintering survival among pteromaline wasps (Floate and Skovgård 2004). Both sexes of *P. azurea*, however, overwinter as adults and reproduce in spring near the nesting areas of their bird hosts (Bennett and Whitworth 1991).

We studied the prevalence of the wasp *N. vitripennis* on the blowfly *P. azurea* in wild populations of the pied flycatcher and the blue tit breeding in nest boxes in two different mountainous Mediterranean areas in central and southern Spain. In each area, we sampled two well-differentiated habitats and only nests infested by the blowfly were considered for *N. vitripennis* analyses. In 2009, we sampled pied flycatcher nests in two habitats from central Spain, an afforested Scots pine (*Pinus sylvestris*) area (n = 20 nests) and a nearby deciduous Pyrenean oak (*Quercus pyrenaica*) forest (n = 33 nests), both located in the Sierra de Ayllón (41°4'N; 3°27'W) at 1200–1400 m a.s.l. For a detailed description of this study area, see Camacho et al. (2015). Some blue tit nests were also sampled (n = 7 nests) in this area. In 2016 and 2017, we sampled blue tit nests in a mixed forest in Sierra Nevada (southern Spain; 36°57'N; 3°24'W), at 1700–1800 m a.s.l. This forest may be divided into two areas, a dry zone composed by holm oaks (*Quercus ilex*) and Pyrenean oaks (n = 29 nests), and a moist zone irrigated by the Chico river and the Almiar stream, composed by a mixed forest of Scots pines, holm oaks and Pyrenean oaks (n = 53 nests). In the two study areas, nest boxes were of the same type (ICONA C model; detailed descriptions in Potti and Montalvo 1990; Moreno-Rueda 2003) and were cleaned at the end of each breeding season.

Once all nestlings had fledged, nests were dismantled and their material was examined to collect blowfly puparia. In central Spain, puparia were included in plastic containers and placed in an incubator under a constant temperature of 25°C ($\pm 1^\circ\text{C}$) until emergence. After emergence, we counted the puparia from which *N. vitripennis* emerged and the puparia from which the blowfly emerged. In southern Spain, blowfly puparia were collected in labelled vials, preserved in 70% ethanol and taken to laboratory for examination. The presence or absence of *N. vitripennis* in puparia was determined using a stereo microscope by cutting the puparia in half and searching for *N.*

vitripennis larvae or pupae inside, or, in empty puparia (found in 6 nests), by confirming the occurrence of minute exit holes on the pupa exuviae, this being indicative that puparia had been parasitized by the wasp. In both cases, the identification of adult wasps and blowflies allowed to emerge confirmed that the only parasitoid and host species found in bird nests were *N. vitripennis* and *P. azurea*, respectively. Although not all *N. vitripennis* pupae collected in southern Spain were allowed to emerge, we are rather confident that parasitoids were *N. vitripennis*, as no other pteromaline species was found parasitizing *P. azurea* puparia in this study area.

For each nest examined, we counted the number of blowfly puparia. Based on the sampling units (nest and puparia), we defined two types of prevalence of *N. vitripennis*: (1) Prevalence by nests, calculated as the percentage of nests infested by the blowfly in which at least one puparium was parasitized by *N. vitripennis*; (2) Prevalence by puparia, calculated as the percentage of blowfly puparia per nest that were parasitized by *N. vitripennis*, only nests with *N. vitripennis* being considered. Aborted blowfly puparia, defined as those in which no adults developed, were excluded from *N. vitripennis* prevalence analyses.

To test for differences in prevalence by nests in relation to habitats and years, given that these are frequencies, we used the Chi-squared test (Rózsa et al. 2000). To test for differences in prevalence by puparia or number of parasitized puparia per nest in relation to habitat and year, given that they are mean values, we used Student's t-test (Rózsa et al. 2000). We also used the t-test to check for differences in the number of blowfly puparia in nests with or without *N. vitripennis*. We used a Generalized Linear Model (GLM), linked to a binomial distribution (infested or not), to assess the probability that a nest was infested by the wasp according to number of blowfly puparia. We checked the variables to evaluate whether they satisfied the premises of parametric

statistics (following Zuur et al. 2010). When the premises were not satisfied, variables were log-transformed (total number of puparia and number of parasitized puparia per nest) or non-parametric tests were used, employing the Mann-Whitney U-test instead of the t-test. Means are given with the raw data, even though all analyses regarding the number of total puparia and parasitized puparia per nest were performed with log-transformed variables. Mean values are given with the standard error. All tests were run in Statistica 8.0 (StatSoft 2007).

3. Results

3.1. Parasitoidism in central Spain

Overall, 86.8% (46 of 53) of pied flycatcher nests infested by the blowfly *P. azurea* were parasitized by the wasp *N. vitripennis*. In nests infested by *N. vitripennis*, an average of 78.2% of puparia per nest were infested by the wasp (in total, 252 of 312). Five of the seven blue tit nests in this study area were infested by *N. vitripennis* (prevalence per nest of 71.4%), with an average of 7.8 blowfly puparia parasitized by the wasp per nest (60.9% of puparia; Table 1). For pied flycatcher nests, no differences were found in wasp prevalence per nest between the oak (29 of 33) and pine (17 of 20) forests ($\chi^2 = 0.09$, $p = 0.76$; Table 1). Habitats did not differ in the number of blowfly puparia parasitized by the wasp per nest (5.52 ± 0.95 in oak forest and 5.41 ± 0.96 in pine forest; $t_{44} = 0.64$, $p = 0.52$), or in the prevalence per puparia (oak vs. pine: 79.0% and 77.7%; $t_{44} = 0.15$, $p = 0.88$; Table 1). Consequently, in subsequent analyses, the data from both forests were pooled together. Nests infested by *N. vitripennis* harboured significantly more blowfly puparia than did non-infested nests (infested: 6.78 ± 0.83 puparia; non-infested: 3.00 ± 1.21 puparia; $t_{51} = 2.59$, $p = 0.01$). The probability that some blowfly puparia were parasitized by *N. vitripennis* increased with the number of

total puparia in the nest (GLM, $\chi^2 = 7.02$, $p = 0.008$). The number of parasitized blowfly puparia per nest rose with the total number of blowfly puparia in the nest ($r = 0.87$, $p < 0.001$). However, the percentage of blowfly puparia parasitized did not correlate with the number of puparia in the nest ($r = -0.01$, $p = 0.95$).

3.2. Parasitoidism in southern Spain

During 2016 and 2017, 65.9% (54 of 82) of blue tit nests infested by the blowfly showed parasitoidism by *N. vitripennis*. In infested nests, 53.9% of the blowfly puparia were parasitized. In total, in both infested and non-infested nests, 434 of 977 (44.4%) blowfly puparia were parasitized by *N. vitripennis*. Significant differences were found between years in the prevalence per nest of *N. vitripennis*, this being higher in 2017 (40 of 53) than in 2016 (14 of 29; $\chi^2 = 6.17$, $p = 0.013$). Wasp prevalence per nest also differed between habitats, being higher in the moist zone (41 of 53) than in the dry zone (13 of 29; $\chi^2 = 8.82$, $p = 0.003$; Table 1). Similar to pied flycatcher nests, blue tit nests with wasps had a higher number of blowfly puparia (14.81 ± 1.14 puparia, $n = 54$) than did the nests without the wasp (6.32 ± 1.06 puparia, $n = 28$; U-Mann-Whitney test, $z = 4.68$, $p < 0.001$). The number of blowfly puparia in nests infested by *N. vitripennis* did not differ between habitats (moist: 6.77 ± 1.01 puparia, $n = 41$; dry: 8.44 ± 1.07 puparia, $n = 13$; $z = -0.27$, $p = 0.78$) or between years (2016: 9.21 ± 1.80 puparia, $n = 14$; 2017: 7.63 ± 0.96 puparia, $n = 40$; $z = 0.49$, $p = 0.96$). The prevalence of *N. vitripennis* per *P. azurea* puparia in infested nests did not vary between habitats (moist vs. dry: 56.0% and 47.3%, $t_{52} = 1.03$, $p = 0.31$; Table 1), nor did the number of puparia parasitized by the wasp (moist: 8.44 ± 1.07 , dry: 6.77 ± 1.00 , $t_{52} = 0.16$, $p = 0.88$). Although the number of parasitized puparia increased with the number of puparia in the nest ($r = 0.74$, $p < 0.001$), the percentage of parasitized puparia did not covary with the number of puparia ($r = -0.04$, $p = 0.79$).

4. Discussion

Parasitoidism drastically reduces host fitness, in most cases to zero, mainly due to the death of the host before producing offspring (Poulin 2011). Hence, parasitoids most often occur at low intensities and prevalence, mainly due to their extremely high virulence and impact on host survival (Poulin and Randhawa 2015). Nevertheless, unlike most parasitoid wasps, *Nasonia* registers high parasitoidism intensity and prevalence (Godfray 2010). We found that the percentage of nests with parasitoidism by the wasp *N. vitripennis* was 86.8% in pied flycatcher nests and 65.9% in blue tit nests. Furthermore, the percentage of the blowfly puparia parasitized was very high: 78.2% in pied flycatcher nests and 53.9% in blue tit nests. Few studies have been conducted on *Nasonia* hyperparasitism in wild populations, all suggesting high rates of parasitoidism on parasitic blowflies in bird nests, in contrast to the low rates of parasitoidism reported for other flies in non-natural environments (see Introduction). In line with our results, the findings by Daoust et al. (2012) indicated that *N. vitripennis* appeared in 85.3% and 67.2% of tree swallow (*Tachycineta bicolor*) nests containing *Protocalliphora* puparia in two consecutive years, with 50.4% and 39.3% of the puparia being parasitized by two *Nasonia* species. Grillenberger et al. (2008) reported that 60% of Central European bird nests contained fly puparia parasitized by *Nasonia*, with 46.8% of these puparia being parasitized. In another study in North America, Grillenberger et al. (2009) reported that 91% of the bird nests contained *Nasonia* and that a mean of 48% of puparia per nest were parasitized by these wasps. By contrast, Bennett and Whitworth (1991) recorded lower values: 29.5% of nests of several bird species contained parasitized puparia of *Protocalliphora* and only 16.7% of all the puparia were parasitized by *N. vitripennis*. A similar low prevalence of *N. vitripennis* in both blowflies and flesh flies hosts was reported by Werren (1983). These results show that the prevalence of *Nasonia* is

generally high in nature, implying a high mortality rate of *Protocalliphora* puparia due to this wasp. Therefore, *Nasonia* can be considered an important selective agent on *Protocalliphora* that likely regulates its natural populations.

This raises the question as to why the prevalence of *Nasonia* species in the puparia of *Protocalliphora* blowflies is so high. Although *N. vitripennis* is a generalist parasitoid, its parasitoidism rate decreases when it competes with other parasitoid wasps and attacks fly hosts that do not parasitize birds (Kaufman et al. 2001; Rutz and Scoles 1989; Skovgård and Jespersen 1999, 2000). The high level of parasitoidism of *N. vitripennis* on *Protocalliphora* in North America, when occurring in mixed infestations with other *Nasonia* species (e.g. *N. giraulti*) is slightly lower than the rate found in Europe, where *N. vitripennis* is the only *Nasonia* species found (Bennett and Whitworth 1991; Daoust et al. 2012; Grillenberger et al. 2008, 2009; our study). Moreover, the host range of this wasp includes puparia which are large, lack appendages and have flat surfaces (Peters 2010), and these attributes all match *Protocalliphora*. The above results suggest that *Protocalliphora* constitutes one of the most suitable hosts of *N. vitripennis*.

The high rates of parasitoidism reported by literature may also be due to the artificial nature of nests (i.e. nest boxes). However, nest material was removed each year once the breeding season of birds ended, thus reducing ectoparasite loads (Møller 1989), including *N. vitripennis* wasps that can overwinter inside blowfly puparia (Bennett and Whitworth 1991). Nevertheless, nesting in cavities seems to increase the probability of parasitization by *N. vitripennis* (72% vs. 22-32% of cavity and non-cavity nests, respectively; Bennett and Whitworth 1991), perhaps because the cavity-nest microclimate is more benign for wasps. If this is so, the microclimate created by nest boxes could enhance the establishment of *N. vitripennis*.

We found that *N. vitripennis* infested bird nests with a high host puparia load (on average, 6.78 puparia in flycatcher nests and 14.81 in blue tit nests). Both the probability of infestation of the blowfly puparia by *N. vitripennis* and the number of parasitized puparia increased with the number of puparia in bird nests, suggesting that the more puparia in the nest, the easier they are detected by wasps, possibly by chemoreception (Peters 2011). Some studies have shown a positive correlation between parasitoidism intensity of *N. vitripennis* and host pupal size (Rivers and Denlinger 1995; Wylie 1967), but the correlation between host density and the parasitoidism rate is less clear. Indeed, only 25% of host-parasitoid systems appear to work in a host density-dependent manner. In the superfamily Chalcidoidea, to which *Nasonia* belongs, a host density-dependent increase in the parasitoidism rate has been reported for less than 30% of parasitoids (Stiling 1987). However, we noted a clear density-dependent effect of the blowfly on the incidence of wasp parasitoidism, with higher rates of puparia being parasitized as their number increased. A plausible explanation for the discrepancy in parasitoidism rates is the spatial scale of the studies. Most studies examining density-dependent effects are conducted at small scales, where parasitoids show a negative density-dependence pattern. However, this may change at larger scales, where parasitoids need to search different—and sometimes distant—habitat patches to find their hosts, resulting in massive parasitoidism after a profitable habitat (i.e. holding numerous hosts) is found (Heads and Lawton 1983). In fact, in southern Spain, we found a trend for parasitoidism rate to be higher in the moist zone than in the dry zone, given that the blowfly prevalence was higher in the moist (89.7%) than in the dry (38.6%) zone. This finding supports the idea that the higher host density in a plot, the higher the parasitoidism rate (but see below for alternative explanations for the difference between habitats). However, in infested nests, the percentage of parasitized

puparia did not covary with the number of puparia available. A possible explanation for this result is that wasps may parasitize a limited number of puparia within the nest.

The prevalence of *N. vitripennis* was higher in central than in southern Spain (see Table 1). These differences might be due to different environmental factors between the two study areas or to variation between years, given that the two areas were sampled in different years. Alternatively, these differences could be due to the different bird species infested by the blowfly –pied flycatchers in central Spain and blue tits in southern Spain–, a possibility suggested by the fact that the rate of wasp parasitoidism in blue tit nests in central Spain was the most similar to that found in the moist forest of southern Spain (see Table 1). The intensity of blowfly parasitism was higher in blue tit nests (mean 14.7 blowflies per nest) than in pied flycatcher nests (mean 6.95 blowflies per nest), probably due to larger brood sizes in blue tit nests. Hence, it is not surprising that the wasp parasitized more puparia in blue tit nests (6-8 per nest) than in pied flycatcher nests (approximately 5 puparia per nest), simply because the former nests harboured more puparia.

In southern Spain, the probability that a nest or a puparium was parasitized by *N. vitripennis* tended to be greater in the moist zone than in the dry zone, but similar habitat-dependent variation was not found between the two habitats (pine vs. oak forests) in central Spain. One explanation is that climatic conditions in the moist zone were more suitable for adult survival of the wasp. In Canada, *N. vitripennis* adults under laboratory conditions do not prefer damp environments (Wylie 1958), but in Mediterranean environments dry conditions are probably more limiting, with moist, riverside zones being more benign for these wasps. Indeed, in Mediterranean ecosystems, moisture can impact on the survival and abundance of parasitoids, moderate to high relative humidity being favourable for some parasitoids (Sorribas et al.

2010). In central Spain, by contrast, the moisture was very similar between the two habitats (pers. obs.), which could explain the absence of habitat-specific differences in this region. On the other hand, the prevalence of the blowfly *P. azurea* was higher in the moist zone than in the dry zone. Therefore, as explained above, a higher host density in the moist zone may have provided a higher probability of parasitoidism by *N. vitripennis*. The moist zone also has more trees, shrubs, and herbaceous cover than the dry zone, so other factors such as food availability or shelter against predators could also account for the observed habitat differences and indirectly increase host parasitization by *N. vitripennis* (Edwards 1954; Wylie 1958).

As explained in the Introduction, some parasitoid wasps may regulate the populations of their hosts by a top-down dynamic and, hence, have a pervasive influence on the ecosystem. The high prevalence of *N. vitripennis* in the two Mediterranean mountain ecosystems studied here suggests that it might regulate the blowfly population by a top-down effect. Our study reports rates of parasitoidism of 44-88% of bird nests, in which 47-79% of blowfly puparia were parasitized. These rates imply that 21-69% of blowfly puparia would die as a consequence of *N. vitripennis* parasitoidism. This represents an important reduction of blowfly success and, in this way, the incidence of parasitism by blowflies in bird populations could be reduced. Blowflies have reportedly detrimental effects on the condition and survival of nestling birds (see Introduction), decreasing the host's body condition, growth rate, and survival both in blue tits (Hurtrez-Boussès et al. 1997) and in pied flycatcher nestlings (Merino and Potti 1995), and may even have long-term implications for future reproduction (Potti 2008). Therefore, a depressed blowfly population by wasp parasitoidism could boost the breeding success of the two bird species in these habitats.

In conclusion, our study shows a high prevalence of parasitoidism of *N. vitripennis* on *P. azurea* in four well-differentiated forest habitats from two geographically widely separated populations. These results, in addition to those reported in the literature, suggest that *N. vitripennis* has a major impact on *P. azurea*. The parasitoid appears to have more success in blue tit nests, which harbour higher quantities of blowfly puparia. A trend for higher wasp prevalence in moist environments was also found, suggesting that, in Mediterranean ecosystems, moisture enhances the wasp abundance.

5. References

- Aung KSD, Takagi M, Myint YY, et al (2011) Effect of host density on the progeny production of the egg parasitoids *Ooencyrtus nezarae* (Ishii) (Hymenoptera: Encyrtidae). *J Fac Agric Kyushu Univ* 56:71–74
- Bennett GF, Whitworth TL (1991) Studies on the life history of some species of *Protocalliphora* (Diptera: Calliphoridae). *Can J Zool* 69:2048–2058. doi: 10.1139/z91-286
- Camacho C, Canal D, Potti J (2015) Testing the matching habitat choice hypothesis in nature: phenotype-environment correlation and fitness in a songbird population. *Evol Ecol* 29:873–886. doi: 10.1007/s10682-015-9793-4
- Cook N, Parker DJ, Turner F, et al (2018) Genome-wide disruption of DNA methylation by 5-aza-2'-deoxycytidine in the parasitoid wasp. *bioRxiv* 437202. doi: 10.1101/437202
- Daoust SP, Savage J, Whitworth TL, et al (2012) Diversity and abundance of ectoparasitic blow flies *Protocalliphora* (Diptera: Calliphoridae) and their *Nasonia* (Hymenoptera: Pteromalidae) parasitoids in tree swallow nests within agricultural lands of Southern Québec, Canada. *Ann Entomol Soc Am* 105:471–478. doi: 10.1603/AN11155
- De Lange ES, Farnier K, Degen T, et al (2018) Parasitic wasps can reduce mortality of teosinte plants infested with fall armyworm: support for a defensive function of herbivore-induced plant volatiles. *Front Ecol Evol* 6:55. doi: 10.3389/fevo.2018.00055
- Desjardins CA, Perfectti F, Bartos JD, et al (2010) The genetic basis of interspecies host preference differences in the model parasitoid *Nasonia*. *Heredity* 104:270–277. doi: 10.1038/hdy.2009.145
- Draber-Monko A (1995) *Protocalliphora azurea* (Fall.) (Diptera, Calliphoridae) and other insects found in nests of sparrows, *Passer domesticus* (L.) and *Passer montanus* (L.) in the vicinity of Warsaw. *Int Stud Sparrows* 22:3–10
- Edwards RL (1954) The effect of diet on egg maturation and resorption in *Mormoniella vitripennis* (Hymenoptera, Pteromalidae). *Q J Microsc Sci* 95:459–468
- Floate KD, Skovgård H (2004) Winter survival of nuisance fly parasitoids (Hymenoptera: Pteromalidae) in Canada and Denmark. *Bull Entomol Res* 94:331–340. doi: 10.1079/BER2003308

- 397 Footitt R, Adler PH (eds) (2017) Insect biodiversity: science and society, Second edition. John
398 Wiley & Sons, Inc, Hoboken, New Jersey
- 399 Frederickx C, Dekeirsschieter J, Verheggen FJ, Haubruge E (2014) Depth and type of substrate
400 influence the ability of *Nasonia vitripennis* to locate a host. *J Insect Sci* 14:58. doi:
401 10.1093/jis/14.1.58
- 402 Godfray HCJ (2010) *Nasonia*: a jewel among wasps. *Heredity* 104:235–236. doi:
403 10.1038/hdy.2010.3
- 404 Gold CS, Dahlsten DL (1989) Prevalence, habitat selection and biology of *Protocalliphora*
405 (Diptera: Calliphoridae) found in nests of mountain and chestnut-backed chickadees in
406 California. *Hilgardia* 57:1–19. doi: 10.3733/hilg.v57n02p019
- 407 Grillenberger BK, Koevoets T, Burton-Chellew MN, et al (2008) Genetic structure of natural
408 *Nasonia vitripennis* populations: validating assumptions of sex-ratio theory. *Mol Ecol*
409 17:2854–2864. doi: 10.1111/j.1365-294X.2008.03800.x
- 410 Grillenberger BK, Van De Zande L, Bijlsma R, et al (2009) Reproductive strategies under
411 multiparasitism in natural populations of the parasitoid wasp *Nasonia* (Hymenoptera). *J*
412 *Evol Biol* 22:460–470. doi: 10.1111/j.1420-9101.2008.01677.x
- 413 Hannam K (2006) Ectoparasitic blow flies (*Protocalliphora* sp.) and nestling Eastern bluebirds
414 (*Sialia sialis*): direct effects and compensatory strategies. *Can J Zool* 84:921–930. doi:
415 10.1139/z06-079
- 416 Hawkins BA (1992) Parasitoid-host food webs and donor control. *Oikos* 65:159–162. doi:
417 10.2307/3544898
- 418 Heads PA, Lawton JH (1983) Studies on the natural enemy complex of the holly leaf-miner: the
419 effects of scale on the detection of aggregative responses and the implications for
420 biological control. *Oikos* 40:267. doi: 10.2307/3544591
- 421 Hochberg ME, Ives AR (eds) (2000) Parasitoid population biology. Princeton University Press,
422 Princeton, New Jersey
- 423 Hurtrez-Boussès S, Perret P, Renaud F, Blondel J (1997) High blowfly parasitic loads affect
424 breeding success in a Mediterranean population of blue tits. *Oecologia* 112:514–517.
425 doi: 10.1007/s004420050339
- 426 Johnson LS, Albrecht DJ (1993) Effects of haematophagous ectoparasites on nestling house
427 wrens, *Troglodytes aedon*: who pays the cost of parasitism? *Oikos* 66:255. doi:
428 10.2307/3544812
- 429 Kaufman PE, Long SJ, Rutz DA, Waldron JK (2001) Parasitism rates of *Muscidifurax*
430 *raptorellus* and *Nasonia vitripennis* (Hymenoptera: Pteromalidae) after individual and
431 paired releases in New York poultry facilities. *J Econ Entomol* 94:593–598. doi:
432 10.1603/0022-0493-94.2.593
- 433 Lynch JA, Brent AE, Leaf DS, et al (2006) Localized maternal orthodenticle patterns anterior
434 and posterior in the long germ wasp *Nasonia*. *Nature* 439:728–732. doi:
435 10.1038/nature04445
- 436 Marchiori CH (2004) *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) parasitóide de
437 dípteros muscóides coletado em Itumbiara, Goiás. *Arq Bras Med Veterinária E Zootec*
438 56:422–424. doi: 10.1590/S0102-09352004000300024

- 439 Marchiori CH, Leles AS, Carvalho SAD, Rodrigues RF (2007) Parasitóides de dípteros
440 muscóides coletados no matadouro Alvorada em Itumbiara, sul de Goiás, Brasil. Rev
441 Bras Parasitol Veterinária 16:235–237. doi: 10.1590/S1984-29612007000400010
- 442 May RM (1978) Host-Parasitoid systems in patchy environments: a phenomenological model. J
443 Anim Ecol 47:833–844
- 444 May RM, Hassell MP, Anderson RM, Tonkyn DW (1981) Density dependence in Host-
445 Parasitoid models. J Anim Ecol 50:855–865. doi: 10.2307/4142
- 446 Merino S, Potti J (1995) Mites and blowflies decrease growth and survival in nestling pied
447 flycatchers. Oikos 73:95. doi: 10.2307/3545730
- 448 Miller CK, Fair JM (1997) Effects of blow fly (*Protocalliphora spatulata*: Diptera:
449 Calliphoridae) parasitism on the growth of nestling savannah sparrows in Alaska. Can J
450 Zool 75:641–644. doi: 10.1139/z97-080
- 451 Møller AP (1989) Parasites, predators and nest boxes: facts and artefacts in nest box studies of
452 birds? Oikos 56:421–423. doi: 10.2307/3565628
- 453 Moreno-Rueda G (2003) Selección de cajas-nido por aves insectívoras en Sierra Nevada. Zool
454 Baetica 13/14:131–138
- 455 Morgan PB (1980) Sustained releases of *Spalangia endius walker* (Hymenoptera: Pteromalidae)
456 for the control of *Musca domestica* (L.) and *Stomoxys calcitrans* (L.) (Diptera:
457 Muscidae). J Kans Entomol Soc 53:367–372
- 458 Niehuis O, Gibson JD, Rosenberg MS, et al (2010) Recombination and its impact on the
459 genome of the haplodiploid parasitoid wasp *Nasonia*. PLoS ONE 5:e8597. doi:
460 10.1371/journal.pone.0008597
- 461 Oliva A (2008) Parasitoid wasps (Hymenoptera) from puparia of sarcosaprophagous flies
462 (Diptera: Calliphoridae; Sarcophagidae) in Buenos Aires, Argentina. Rev Soc
463 Entomológica Argent 67:139–141
- 464 Oliveira DCSG, Raychoudhury R, Lavrov DV, Werren JH (2008) Rapidly evolving
465 mitochondrial genome and directional selection in mitochondrial genes in the parasitic
466 wasp *Nasonia* (Hymenoptera: Pteromalidae). Mol Biol Evol 25:2167–2180. doi:
467 10.1093/molbev/msn159
- 468 Peters RS (2010) Host range and offspring quantities in natural populations of *Nasonia*
469 *vitripennis* (Walker, 1836) (Hymenoptera: Chalcidoidea: Pteromalidae). J Hymenopt
470 Res 19:128–138
- 471 Peters RS (2011) Two ways of finding a host: a specialist and a generalist parasitoid species
472 (Hymenoptera: Chalcidoidea: Pteromalidae). Eur J Entomol 108:565–573. doi:
473 10.14411/eje.2011.073
- 474 Peters RS, Abraham R (2010) The food web of parasitoid wasps and their non-phytophagous fly
475 hosts in birds' nests (Hymenoptera: Chalcidoidea, and Diptera: Cyclorrhapha). J Nat
476 Hist 44:625–638. doi: 10.1080/00222930903437317
- 477 Potti J (2008) Blowfly infestation at the nestling stage affects egg size in the pied flycatcher
478 *Ficedula hypoleuca*. Acta Ornithol 43:76–82. doi: 10.3161/000164508X345356

479 Potti J, Montalvo S (1990) Ocupación de áreas con nidales por el papamoscas cerrojillo
480 (*Ficedula hypoleuca*). *Ardeola* 37:75–84

481 Poulin R (2011) The many roads to parasitism. In: *Advances in Parasitology*, 1st ed. Elsevier,
482 London, pp 1–40

483 Poulin R, Randhawa HS (2015) Evolution of parasitism along convergent lines: from ecology to
484 genomics. *Parasitology* 142:S6–S15. doi: 10.1017/S0031182013001674

485 Puchala P (2004) Detrimental effects of larval blow flies (*Protocalliphora azurea*) on nestlings
486 and breeding success of tree sparrows (*Passer montanus*). *Can J Zool* 82:1285–1290.
487 doi: 10.1139/z04-111

488 Quicke DLJ (1997) *Parasitic wasps*, 1st ed. Chapman & Hall, London, New York

489 Rivers DB (2004) Evaluation of host responses to envenomation as a means to assess
490 ectoparasitic pteromalid wasp's potential for controlling manure-breeding flies. *Biol*
491 *Control* 30:181–192. doi: 10.1016/j.biocontrol.2004.01.004

492 Rivers DB, Denlinger DL (1995) Fecundity and development of the ectoparasitic wasp *Nasonia*
493 *vitripennis* are dependent on host quality. *Entomol Exp Appl* 76:15–24. doi:
494 10.1111/j.1570-7458.1995.tb01942.x

495 Rodrigues-Guimarães R, Guimarães RR, Carvalho RW de, et al (2006) Registro de *Aphaereta*
496 *laeviuscula* (Spinola) (Hymenoptera: Braconidae) e *Nasonia vitripennis* (Walker)
497 (Hymenoptera: Pteromalidae) como parasitóide de *Cochliomyia hominivorax*
498 (Coquerel) (Diptera: Calliphoridae), no estado do Rio de Janeiro. *Neotrop Entomol*
499 35:402–407. doi: 10.1590/S1519-566X2006000300017

500 Rózsa L, Reiczigel J, Majoros G (2000) Quantifying parasites in samples of hosts. *J Parasitol*
501 86:228–232. doi: 10.1645/0022-3395(2000)086[0228:QPISOH]2.0.CO;2

502 Rutz DA, Axtell RC (1981) House fly (*Musca domestica*) control in broiler-breeder poultry
503 houses by pupal parasites (Hymenoptera: Pteromalidae): indigenous parasite species
504 and releases of *Muscidifurax* raptor. *Environ Entomol* 10:343–345. doi:
505 10.1093/ee/10.3.343

506 Rutz DA, Scoles GA (1989) Occurrence and seasonal abundance of parasitoids attacking
507 muscoid flies (Diptera: Muscidae) in caged-layer poultry facilities in New York.
508 *Environ Entomol* 18:51–55. doi: 10.1093/ee/18.1.51

509 Simon A, Thomas D, Blondel J, et al (2004) Physiological ecology of Mediterranean blue tits
510 (*Parus caeruleus* L.): effects of ectoparasites (*Protocalliphora* spp.) and food abundance
511 on metabolic capacity of nestlings. *Physiol Biochem Zool* 77:492–501. doi:
512 10.1086/383512

513 Simon A, Thomas DW, Speakman JR, et al (2005) Impact of ectoparasitic blowfly larvae
514 (*Protocalliphora* spp.) on the behavior and energetics of nestling blue tits. *J Field*
515 *Ornithol* 76:402–410. doi: 10.1648/0273-8570-76.4.402

516 Skovgård H, Jespersen JB (1999) Activity and relative abundance of hymenopterous parasitoids
517 that attack puparia of *Musca domestica* and *Stomoxys calcitrans* (Diptera: Muscidae) on
518 confined pig and cattle farms in Denmark. *Bull Entomol Res* 89:263–269. doi:
519 10.1017/S0007485399000383

- 520 Skovgård H, Jespersen JB (2000) Seasonal and spatial activity of hymenopterous pupal
521 parasitoids (Pteromalidae and Ichneumonidae) of the house fly (Diptera: Muscidae) on
522 Danish pig and cattle farms. *Environ Entomol* 29:630–637. doi: 10.1603/0046-225X-
523 29.3.630
- 524 Skovgård H, Nachman G (2004) Biological control of house flies *Musca domestica* and stable
525 flies *Stomoxys calcitrans* (Diptera: Muscidae) by means of inundative releases of
526 *Spalangia cameroni* (Hymenoptera: Pteromalidae). *Bull Entomol Res* 94:555–567
- 527 Sorribas J, Rodríguez R, Garcia-Mari F (2010) Parasitoid competitive displacement and
528 coexistence in citrus agroecosystems: linking species distribution with climate. *Ecol*
529 *Appl* 20:1101–1113. doi: 10.1890/09-1662.1
- 530 StatSoft Inc (2007) STATISTICA (data analysis software system). StatSoft, Inc.
- 531 Stiling PD (1987) The frequency of density dependence in insect host-parasitoid systems.
532 *Ecology* 68:844–856
- 533 Streby HM, Peterson SM, Kapfer PM (2009) Fledging success is a poor indicator of the effects
534 of bird blow flies on ovenbird survival. *The Condor* 111:193–197. doi:
535 10.1525/cond.2009.080095
- 536 Sullivan DJ (2009) Hyperparasitism. In: *Encyclopedia of Insects*, 2nd edition. Academic Press /
537 Elsevier Science, pp 486–488
- 538 Sullivan DJ, Völkl W (1999) Hyperparasitism: multitrophic ecology and behavior. *Annu Rev*
539 *Entomol* 44:291–315. doi: 10.1146/annurev.ento.44.1.291
- 540 Thomas K, Shutler D (2001) Ectoparasites, nestling growth, parental feeding rates, and begging
541 intensity of tree swallows. *Can J Zool* 79:346–353. doi: 10.1139/z00-206
- 542 Werren JH (1983) Sex ratio evolution under local mate competition in a parasitic wasp.
543 *Evolution* 37:116. doi: 10.2307/2408180
- 544 Werren JH, Loehlin DW (2009) The parasitoid wasp *Nasonia*: an emerging model system with
545 haploid male genetics. *Cold Spring Harb Protoc* 2009:pdb.emo134-pdb.emo134. doi:
546 10.1101/pdb.emo134
- 547 Whiting AR (1967) The biology of the parasitic wasp *Mormoniella vitripennis* [= *Nasonia*
548 *brevicornis*] (Walker). *Q Rev Biol* 42:333–406. doi:
549 <https://www.jstor.org/stable/2818420>
- 550 Whitworth TL, Bennett GF (1992) Pathogenicity of larval Protocalliphora (Diptera:
551 Calliphoridae) parasitizing nestling birds. *Can J Zool* 70:2184–2191. doi: 10.1139/z92-
552 295
- 553 Wylie HG (1967) Some effects of host size on *Nasonia vitripennis* and *Muscidifurax raptor*
554 (Hymenoptera: Pteromalidae). *Can Entomol* 99:742–748. doi: 10.4039/Ent99742-7
- 555 Wylie HG (1958) Factors that affect host finding by *Nasonia vitripennis* (Walk.) (Hymenoptera:
556 Pteromalidae). *Can Entomol* 90:597–608. doi: <https://doi.org/10.4039/Ent90597-10>
- 557 Zuur AF, Ieno EN, Elphick CS (2010) A protocol for data exploration to avoid common
558 statistical problems: data exploration. *Methods Ecol Evol* 1:3–14. doi: 10.1111/j.2041-
559 210X.2009.00001.x

Tables

Table 1 Prevalence (in %) of *Nasonia vitripennis* per bird nest and per *Protocalliphora azurea* puparium (only in infested nests) in pied flycatcher and blue tit nests in two different habitats of two geographic regions of Spain. Sample size is indicated in brackets.

Geographic zone	Bird species	Prevalence per nest		Prevalence per puparium	
		Pine forest	Oak forest	Pine forest	Oak forest
Central Spain	Pied flycatcher	85.00 (N=20)	87.88 (N=33)	77.70 (N=19)	79.00 (N=29)
		71.40 (N=7)		60.90 (N=5)	
	Blue tit				
Southern Spain	Blue tit	Moist forest	Dry forest	Moist forest	Dry forest
		77.36 (N=53)	44.83 (N=29)	56.00 (N=41)	47.30 (N=13)