Coexistence, habitat associations and puparia description of three dipteran species of the Family Carnidae

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Abstract

Differentiation of niche by means of resource partitioning facilitates coexistence of species with similar requirements. Here we analyse the association between different habitats (i.e. nest types) and two Diptera species of the poorly known Family Carnidae that coexist during their larval and pupal stage in the nests of troglobydic bird species. We also describe for the first time the puparium of Hemeromyia anthracina and Hemeromyia longirostris and offer morphometric data of the puparia of these two species and of Carnus hemapterus. Both the smaller size and the occurrence of well-developed spiracles allow easy differentiation of the puparium of C. hemapterus. The puparia of both Hemeromyia species is very similar and only differ in the distance between the small spiracles. Hemeromyia anthracina and C. hemapterus coexisted in nest boxes but the former species did not occur in natural sandy cavities where, in turn, C. hemapterus was highly prevalent. Carnus hemapterus prevalence did not differ between nest boxes and natural cavities but its abundance was higher in the first type of nest. This study shows clear associations of the two dipteran species with specific types of nests. Yet, some conditions are seemingly acceptable for both species.

Introduction

The study of the factors that allow coexistence of closely related species has been an important subject in ecology for decades and various mechanisms (e.g. niche differentiation, temporal segregation) have been reported to prevent or reduce competitive exclusion (Tilman, 1982; Hairston et al. 1996; Leisnham et al. 2014). Specifically, niche differentiation is a process by which competing species use the environment differently, therefore, facilitating coexistence. Niche differentiation can be achieved in different ways. For instance, differences in microhabitat selection criteria (even within the same general habitat type) may result in some spatial segregation that reduces interspecific competition. Studies based on interspecific comparisons among closely related species occurring in sympatry (Dearn, 1977; Dingle, 1978; Tauber and Tauber, 1981) can contribute to a better understanding of habitat selection criteria and stable coexistence by means of resource partitioning (Tauber and Tauber, 1981, 1982). Such studies can nonetheless be hampered by the lack of information on basic aspects of the study species, for example, the proper description and identification of different life stages of each species.

The Family Carnidae (Diptera, Schizophora) is a poorly investigated group of flies that includes parasitic species (genus Carnus Nitzsch 1818) as well as non-parasites belonging to the genus Meoneura (Rondani 1856) and Hemeromyia (Coquillett 1902) (Grimaldi, 1997; Brake, 2011; Stuke, 2016). Very little is known about the different species of the genus Hemeromyia and the available information is restricted to reports on their geographic distribution and to some notes on their biology (e.g. Papp, 1984, 1998; Carles-Tolrá, 2002). Carnus hemapterus has been studied in more detail (see, e.g., Capelle and Whitworth 1973; Guiguern et al. 1983; Dawson and Bortolotti 1997; Roulin 1998, 1999; Valera et al. 2004, 2006a, b; Václav et al. 2008; Valera and Zídková 2012; Amat-Valero et al. 2012), but several important aspects of its natural history are still unknown. Moreover, our knowledge is skewed since the most information available refers to the adult phase, although the requirements of other phases (e.g. larval and pupal stages), and therefore their biology and habitat preferences, may be very different. In fact, basic information, such as the description of the various life stages and of the puparium of many of these species of the family, is missing.

The only study about ecological aspects of Hemeromyia species is that by Valera et al. (2006b). These authors studied the coexistence of pupae and emergence phenology of imagos of C. hemapterus and two species of Hemeromyia (H. longirostris and H. anthracina). Since all three species develop larval and pupal stages in birds’ nests and in all cases the larvae feed on the organic matter that accumulates at the bottom of the nests (Grimaldi, 1997; Papp 1998), it has been hypothesized that coexistence within the same nest could result in competition among different species. Valera et al. (2006b) found no evidence of interspecific competition during the larval phase but they did find interspecific differences in habitat selection criteria: C. hemapterus appeared to avoid nests lined with plant material. They pointed out that more information about the occurrence of Hemeromyia species was necessary before drawing any
conclusions about their habitat preferences, Valera et al. (2006d) also emphasised that knowledge of the natural history of these species is insufficient, what hinders addressing fundamental questions for this interesting study system (see, e.g., Soler et al. 1999, 2014; Martín-Vivaldi et al. 2006; Václav et al. 2008; Calero-Torrallo et al. 2013).

Here we intend to: (i) offer a complete description of the puparium of the above-mentioned species; (ii) study possible differences in habitat selection of two sympatric species of carnid flies, *C. hemapterus* and *H. anthracina*. Larvae of both species are saprophagous and they can be found in the same cavities, so that interspecific competition is likely. Our hypothesis is that competition during the larval stage may decrease if adults of the two carnid species prefer different types of avian nest substrates for egg laying. We predict that the prevalence and abundance of *C. hemapterus* and *H. anthracina*, calculated on the basis of puparium occurrence, in two different avian nest types (nest boxes and natural cavities in sandstone cliffs) will differ.

Materials and methods

**Study species**

*Carnus hemapterus* is a generalist ectoparasite about 2 mm in length, parasitizing nestlings of various species of birds (Grimaldi, 1997; Papp 1998; Brake, 2011). Its life cycle encompasses an adult (parasitic) phase, three larval stages and a pupal stage (Bequaert, 1942). Diapausing pupae are found in the nests of the host species. Imagoes, initially winged, emerge at the beginning of the spring and can remain in the nest where they emerged or disperse in search of hosts. Once these are located, adult flies lose their wings and feed on blood, epidermal cells and skin secretions. Mating occurs on the host and eggs are laid in the nest. After the larval stages, the pupa enters into diapause. A short diapause of a few weeks (Amat-Valero et al. 2012), a long diapause of some months (allowing it to hibernate in the nest, Guiguen et al. 1983) and a prolonged diapause of several years (Valera et al. 2006a) have been reported. The puparium of *C. hemapterus* has been described (Capelle and Whitworth, 1973; Sabrosky, 1987; Papp 1998) even though data on its morphometry is very scarce. Little is known about the dispersion of this parasite. It is considered a generalist ectoparasite about 2 mm in length, parasitizing nestlings of various species of birds (Grimaldi, 1997).

**Study area and collection of nest material**

The main study area (c. 50 km²) lies in the Desert of Tabernas (Almería, SE Spain, 37°05′N, 2°21′W). The climate in this area is semi-arid with high annual and seasonal rainfall variability (mean annual rainfall c. 218 mm), and strong thermal oscillations with interannual differences. Summers are long and hot and winters are usually mild.

Ten nest boxes and eight natural cavities used by birds were sampled in Almería on 4 February and 18 March 2016 with the aim of highlighting habitat associations for each carnid study species. Nest boxes were made of wood or cork and were prepared for rollers. Thus, they contained a layer of sand where birds laid their eggs. All nest boxes sampled were used the previous breeding season by rollers even though in some cases starlings and sparrows bred there before the arrival of rollers. The former bird species usually add vegetal matter in the nest box to build their nests and, once the rollers occupy the boxes, some of such vegetal matter remains there. Natural cavities sampled had been previously used by rollers (six cases) or little owls (two cases) and the substratum was entirely sandy. Five additional nest boxes were sampled in July 2017 in search of *C. hemapterus* pupae for morphometric studies.

A second study area lies in Cáceres province (Western Spain, 39°03′N, 5°14′W), where Valera et al. (2006d) reported the coexistence of the three carnid species. A sampling of detritus from nest boxes located in the area was carried out on 24 January 2016 (25 nest boxes) to find and describe the puparia of the three study species. Since only a single individual of *H. longirostris* emerged from such samples, we sampled 17 different nest boxes on 22–23 February 2017.

Sampling consisted of taking material (sand, detritus and organic matter – feces, insect remains, and vegetal material used for the elaboration of the nest –) from nests by hand or with the aid of a spoon tied to the end of a stick. In the nests from Extremadura most of the detritus was collected whereas in Almería only a fraction of the nests content was taken. The samples were placed in plastic bags and transferred to the Estación Experimental de Zonas Áridas (Almería).

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**Samples treatment**

The samples were processed shortly after they were collected (on 28 January 2016 and on 27 February 2017 for samples from Extremadura; on 10 February 2016 and 21 March 2016 and on 2 August 2017 for samples from Almería). First, they were allowed to air-dry and then were put through a sieve column to obtain a sediment between 1 and 4 mm, thus ensuring that the pupae of *C. hemapterus*, about 2 mm in size (Capelle and Whitworth, 1973), were collected. It was assumed that the pupae of other Carnidae flies would have similar or slightly larger sizes, given the small difference in size between the imagos of the species under consideration. The resulting material from each nest was weighed and stored in individualized and labelled transparent tubes.

The sieved samples collected from Extremadura in 2016 were observed periodically in order to detect the emergence of flies. Once emergence of the study species was detected (in just three samples), the whole material of such samples was examined with a Nikon SMZ645 binocular loupe to find any kind of pupae. From each of the Almería samples collected in 2016, subsamples of 8 g were selected at random and scrutinized in search of pupae. We also sought for pupae in two subsamples of 5 g from each of the samples collected in 2017 from Extremadura and Almería.
Identification of the pupae of *C. hemapterus* was carried out according to Capelle and Whitworth (1973) and Papp (1998). Both the identified pupae of *C. hemapterus* and the remaining unidentified pupae were individually stored in Eppendorf tubes. Periodic monitoring (at least every 3 days) was done to check the emergence of the imagoes and, thus, identify the species with the aid of the binocular loupe. Whereas just one individual of *H. longirostris* was obtained from samples taken in 2016, several dozens of *H. anthracina* and *H. longirostris* emerged from samples taken in 2017. Pupae of identified imagoes were collected and measured with a micrometer and the binocular loupe. Measurements were taken of the maximum length, maximum width and the distance between spiracles of 30 pupae of *C. hemapterus* in 2016 and of 30 pupae of *C. hemapterus* in 2017, 23 pupae of *H. anthracina* and 68 pupae of *H. longirostris* in 2017. All *C. hemapterus* pupae used for the morphometric study come from Almeria, whereas the ones of *Hemeromyia* spp. come from Extremadura.

Photographs of the pupae were taken with a Nikon SMZ1500 binocular loupe equipped with a digital viewfinder and the software NIS-Elements BR3.1.

For a more detailed description of the pupae, pictures were taken with the scanning electron microscope (SEM) of the University of Almeria. The pupae were mounted on aluminium stubs and attached to them using double-sided graphite tape. They were coated with gold by the ion sputtering method using a BAL-TEC sputter coater, model SCD 005. The coating has a thickness of approximately 20 nm. Samples were visualized in high vacuum by the secondary electron signal (SE) with a HITACHI SEM, model S-3500N.

**Statistical methods**

Prevalence (percentage of infected nests among all examined) and 95% confidence intervals were calculated for each species. Two thousand replications were used for the estimation of confidence intervals. Median abundance (median number of pupae found in infected nests) and median intervals. Median abundance (median number of pupae found in infected nests) and their respective quartiles were also calculated.

We used Fisher tests to compare prevalences, and median tests to compare medians of abundances and intensities. Statistical tests were performed with Quantitative Parasitology 3.0 (Reiczigel and Rózsa, 2005) and STATISTICA (Dell Inc., 2016).

We used linear mixed effect models (LME) to study: (i) interannual differences in the size of the puparium of *C. hemapterus*, and (ii) interspecific differences in the size of the puparium of *Hemeromyia* spp. The dependent variables were maximum length, maximum width and the distance between spiracles. Fixed factors were the year for the first aim and species for the second one. In both cases, we considered nest as a random effect and examined and accounted for the correlation structure among dependent samples. The interaction between the factors was not studied because: (i) in the case of *C. hemapterus*, the nests sampled were different each year; (ii) we did not find pupae of both *Hemeromyia* species in each nest and in some nests the number of pupae found for one or both species was low. Normality of residuals was met. In cases with heterocedasticity we used the varIdent function in nlme 3.1-131 package (Pinheiro et al. 2017). These tests were carried out with R software, version 3.4. (R Development Core Team, 2017).

**Results**

**Description of the puparia of the Family Carnidae**

The puparium of three species (*C. hemapterus*, *H. anthracina* and *H. longirostris*) was identified after the emergence of the corresponding imagoes (more than 100 individuals for each of the first two species and several dozens for the third one).

The puparium of *C. hemapterus* is reddish-brown in colour, cylindrical or barrel-shaped, and has annular ornamentations distributed over most of its length (Fig. 1). Its main feature is the presence of two very notorious divergent spiracles, each with three digitiform extensions, at the caudal end (Fig. 2).

The puparium of *H. anthracina* is light brown, with marked annular ornamentations, larger, and wider than the one of *C. hemapterus* (see below) (Fig. 3). It also has two spiracles, but unlike the ones of *C. hemapterus*, they are quite small and without prolongations (Fig. 4). There is some variability in the size of the spiracles: some pupae have small but perceptible spiracles with the binocular loupe, whereas the spiracles can hardly be seen with the loupe in other individuals. All the pupae observed show, at the caudal end, a depression bounded by marked rims (Fig. 4).

The puparium of *H. longirostris* is very similar to the one of *H. anthracina* (Fig. 5), with short, non-ornamented spiracles at the caudal end and a conspicuous depression close to the spiracle (Fig. 6).

The identification by external characters examined with a binocular loupe is possible for *C. hemapterus* but not for both *Hemeromyia* species (Figs 7 and 8).

**Morphometry of the puparia of the Family Carnidae**

None of the dimensions of the puparium of *C. hemapterus* varied between years (LME model, year: *P* > 0.20 in all cases; Estimate ±
S.E.: length 2016: 1·80 ± 0·04, length 2017: 1·77 ± 0·04; width 2016: 0·63 ± 0·01, width 2017: 0·60 ± 0·02; distance between spiracles 2016: 0·09 ± 0·003, distance between spiracles 2017: 0·09 ± 0·003, n = 30 for 2016 and 30 for 2017; Fig. 9).

The maximum length and width of the puparium of *H. anthracina* and *H. longirostris* did not differ (LME model, species: *P* > 0·05 in both cases; Estimate ± S.E.: length *H. anthracina*: 2·13 ± 0·05, length *H. longirostris*: 2·06 ± 0·04; width *H. anthracina*: 0·79 ± 0·03, width *H. longirostris*: 0·76 ± 0·01, n = 23 for *H. anthracina* and 68 for *H. longirostris*; Fig. 10). However, the distance between spiracles was significantly longer for *H. anthracina* (LME model, *F* = 20·3, *P* < 0·001, Estimate ± S.E.: *H. anthracina*: 0·12 ± 0·003, *H. longirostris*: 0·10 ± 0·002, n = 23 for *H. anthracina* and 68 for *H. longirostris*; Fig. 10).

**Habitat associations of two sympatric carnid flies, *Carnus hemapterus* and *Hemeromyia anthracina***

Only two species, *H. anthracina* and *C. hemapterus*, were found in Almeria.
Fig. 9. Interannual differences in (A) maximum length, (B) maximum width and (C) distance between spiracles (estimated values ± S.E.) of the puparium of *Carnus hemapterus* in 2016 and 2017.
Fig. 10. Differences in (A) maximum length, (B) maximum width and (C) distance between spiracles (estimated values ± s.e.) of the puparia of *Hemeromyia anthracina* and *H. longirostris*.
Table 1. Prevalence, median abundance and intensity of viable pupae of Carnus hemapterus and Hemeromyia anthracina in 8 g of detritus taken from nest boxes and nests in natural cavities sampled in Almería (southeast Spain)

|                | Carnus hemapterus | Hemeromyia anthracina |
|----------------|-------------------|-----------------------|
|                | Nest box (10)     | Natural cavity (8)    | Nest box (10) | Natural cavity (8) |
| Prevalence     | 80.0 [44.4–97.5]  | 75.0 [34.9–96.8]      | 50.0 [18.7–81.3] | 0.0 |
| Median abundance | 17.5 [3.0–35.0]  | 3.50 [0.5–7.0]       | 0.5 [0.0–3.0] | – |
| Median intensity | 21.5 [14.0–51.5] | 5.5 [3.0–7.0]       | 3.0 [2.0–17.0] | – |

Sample size (in brackets), 95% confidence intervals for prevalence and quartiles for median abundance and intensity (in square brackets) are shown.

The prevalence of viable pupae of *Carnus hemapterus* and *Hemeromyia anthracina* in nest boxes did not differ significantly (80.0 vs 50.0%, respectively; two-tailed Fisher’s test, *P* = 0.35, Table 1). Pupae of both species were found in 50% (five out of ten) of the nest boxes. The median abundance of *Carnus hemapterus* pupae in nest boxes was significantly higher than that of *Hemeromyia anthracina* (Median test, *P* = 0.02). The median intensity of *Carnus hemapterus* pupae in nest boxes also tended to be higher than that of *Hemeromyia anthracina* (Table 1), although the differences were not significant (Median test, *P* > 0.10).

In contrast, *Hemeromyia anthracina* pupae were not found in natural cavities, whereas viable pupae of *Carnus hemapterus* were found in 75% of the cavities sampled (two-tailed Fisher’s test, *P* = 0.009) (Table 1).

The prevalence of *Carnus hemapterus* did not differ between the two nest types (two-tailed Fisher’s test, *P* = 1.0). However, both the median pupae abundance and the median pupae intensity were significantly higher in the nest boxes than in the natural cavities (Median test, abundance: *P* < 0.05; intensity: *P* < 0.01).

**Discussion**

This paper provides a full description, including morphometrics, of the puparium of *Carnus hemapterus*, *Hemeromyia anthracina* and *H. longirostris* after unequivocally verifying the emergence of adults of the collected pupae, being the most complete report until the date for *Carnus hemapterus* and the first one for the two later species. We also describe patterns in habitat associations of *Carnus hemapterus* and *Hemeromyia anthracina* that can reflect partial niche segregation.

**Description of the pupae of the Family Carnidae**

The determination of key traits for the identification of various stages of closely related species is important because it enables further studies on significant processes occurring at these phases (e.g. diapauses during the pupal phase, see Amat-Valero et al. 2013 for *Carnus hemapterus*) that are frequently longer than the adult phase for many insect species. Moreover, the possibility of identifying sister taxa facilitates comparative studies on relevant topics such as coexistence, niche partitioning or the evolution of life histories (Tauber and Tauber, 1981, 1982).

Our results show that the puparium of *Carnus hemapterus* that we describe coincides with the description provided elsewhere (Capelle and Whitworth, 1973; Sabrosky, 1987; Papp 1998). Both the occurrence of two obvious spiracles and its morphometry (much smaller size than both *Hemeromyia* species, Fig. 7) distinguish the puparium of this parasite from that of *Hemeromyia anthracina* and *H. longirostris*. In contrast, distinguishing the puparium of the two *Hemeromyia* species is not straightforward. Pupae of both species obtained from the same location and year did not differ in length and width and we only found significant differences in the distance between spiracles (larger in *H. anthracina*). Even though significant, such differences are small and could depend on factors such as food availability or seasonality that are known to influence larval and pupal mass and size (Williams and Richardson, 1983; Tsuda and Takagi, 2001; Temeyer, 2009). We did not find interannual differences in puparium size of *Carnus hemapterus* but this can be different for *Hemeromyia* spp. or for other study years. Therefore, caution about the reliability of the distance between spiracles for distinguishing between both *Hemeromyia* spp. is necessary.

**Differences in habitat selection and coexistence**

This study is based on the absence/occurrence and abundance of pupae of two dipteran species in two different cavity types used by birds. We assume that the presence of larvae and pupae of a given species in a cavity reflects the choice of such cavity by the adult phase as an appropriate habitat for subsequent developmental stages. In contrast, the absence of pupae of a given species in a cavity is more difficult to interpret. It could be that our study area is not within the range of the species or that the cavity is not selected by the imago because it is considered unsuitable for larval or pupal development. Other factors, such as larval/pupal predation or fungal infection, could also account for the absence of pupae of a given species in a cavity.

Coexistence of the three studied species in the same cavities has been cited by Valera et al. (2006b) in western and south Spain (ca. 40 km far from our study area). *Hemeromyia longirostris* was not detected in this study and, contrary to the two other species, has not been found in the study area in spite of intensive sampling of imagoes in nest boxes in several years (personal observation). Our study area lays in the most arid region of continental Europe and is quite different from the areas where this species was found, so that it could be that *H. longirostris* is not distributed along the arid southeastern Spain. Alternatively, other reasons, like the ones mentioned above, may account for the absence of the species in the cavities sampled.

We recorded the occurrence of *Hemeromyia anthracina* in nest boxes but not in natural cavities. The latter could be explained by several non-mutually exclusive factors: (i) larvae/pupae may have been differentially preyed/infected in natural cavities. However, given the similarities in the biology and morphology of the larval and pupal stages of *Carnus hemapterus* and *Hemeromyia* it seems unlikely that differential predation or infection occurs in a given cavity type for a given species; (ii) emergence of *Hemeromyia anthracina* imagoes could pass unnoticed to us. Still, Valera et al. (2006b) described a similar emergence phenology for both species and we did not find open pupae of *Hemeromyia anthracina* in the material from natural cavities neither in this study nor in previous ones; (iii) this cavity type is not selected by *H. anthracina*. The substratum of natural cavities in sandstone cliffs is essentially sandy, whereas in nest boxes (where the species is found) it may include plant material (depending on the bird species using it). Valera et al. (2006b) did not find any of the *Hemeromyia* species in nests of birds
breeding in the sandy substratum. These results suggest that *H. anthracina* could avoid nests with such substratum.

Concerning *C. hemapterus*, it was highly prevalent in both cavity types. The prevalence and abundance of this parasite are known to depend on innate host features such as ontogeny or immune capacity (Valera et al. 2004; Václav et al. 2008) but also on the habitat used by its hosts (Guiguen et al. 1983). Similarly to Fargallo et al. (2001) and Calero-Torrallo et al. (2013) we found that nest boxes were more infected than natural cavities. Microclimatic differences among nest types (see Amat-Valero et al. 2014) could influence parasites’ choice and/or survival. Alternatively, differences in the cleaning efficiency of both nest types by adult birds can result in different amounts of detritus (and diapausing pupae) left in the cavities. Coexistence of *H. anthracina* and *C. hemapterus* was therefore restricted to nest boxes. We found co-occurrence of both species in 50% of the nest boxes, a value somewhat higher than the 35% found by Valera et al. (2006b). Being non-parasites, *Hemeromyia* species are probably less dependent on the innate characteristics of the bird species, but they can still depend on host-related attributes such as the type of nest material used by the bird. Nest boxes in our study area were prepared for rollers and, thus, had a sandy layer. Nonetheless, some of them also had some vegetal matters (twigs, leaves) introduced by other birds (e.g. Spotless starling *Sturnus unicolor*) in the nest before the rollers took ownership of it. Valera et al. (2006b) found that *C. hemapterus* avoided nests lined with vegetable matter but the combination of sand and vegetal material seemingly resulted in an acceptable habitat for *C. hemapterus* and for *H. anthracina*. Moreover, the high variability in the abundance of *C. hemapterus* observed in the nest boxes sampled could result from differences in the amount of vegetal matter in them. Thus, the relative abundance of each fly species may depend on host nesting behaviour and on the bird species occupying the nest box. This is important because slight interspecific differences in habitat selection criteria (even within the same general habitat type) by carniv species, may result in some spatial segregation and reduced interspecific competition.

The reasons why a sandy substrate or a vegetal one within a cavity could be unsuitable for each insect species and the particular stage(s) of the insects sensitive to the type of substratum remain to be investigated. Future studies should also quantify the abundance of *C. hemapterus* and *Hemeromyia* species for each type of substratum.

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