Determinants of the host-parasite relationship in a system formed by a cavity-nesting bird and its ectoparasites in an arid ecosystem

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Abstract

Parasites are strongly influenced by numerous abiotic and biotic factors operating at different temporal and spatial scales. To understand parasites dynamics, host-parasite interactions and the underlying mechanisms of such interactions it is necessary to study how those factors influence parasites and their relationships with the hosts at the various scales.

Here we study the effect of off-host and host-related factors on the relationship between a cavity-nesting bird species, the European roller (*Coracias garrulus*) and its ectoparasites, mainly the haematophagous fly *Carnus hemapterus*. The main factors considered in this study involve habitat characteristics at the mesoscale (the host nest and the immediate surroundings) like nest-site type (a major source of habitat heterogeneity in our study area) as well as host and parasite density, breeding phenology of the host (i.e. seasonal effects) and host total brood mass (as a surrogate of cues for the parasites). We study the effect of these factors on the colonization success and distribution (prevalence and abundance) of the most common ectoparasite (*Carnus hemapterus*). However, since a given host species usually harbours several parasite species, the approach "one host-one parasite" overlooks the effect of the interactions among parasites that mainly occur at the infracommunity level. Therefore, we also explore the effect of the above-mentioned factors on the ectoparasite infracommunity of the European roller.

Our study is performed in a semi-arid environment, therefore contributing to our knowledge of host-parasite interactions in such habitats that are underrepresented in the scientific literature.

Both to fill the gaps on the basic biology of the study species and to build up our study on solid foundations, we also explore basic aspects of the natural history of *Carnus hemapterus* such as the characteristics of the pupal stage of the ectoparasite or its life span during the dispersal stage (closely related to colonization success).

We found that carnid flies have a very short life span during the dispersal stage (less than four days), in spite of which they are able to colonize ca. 100% of the host nests. Its longevity during this period increases with body size, environmental humidity and with access to food (flowers). Nest-site type influences colonization success of carnid flies, so that it is higher in nest boxes on sandstone cliffs-farmhouses than in nest boxes on trees. Colonization success also increased with host density and host brood mass but decreased as the season progressed. Interestingly, parasite density had no effect on host colonization success. Nest-site type also explained differences among nests in composition of the ectoparasite infracommunity and in abundance of various ectoparasite species. These variables were not affected by the spatial structure, nor by brood mass. However, host breeding phenology affects the ectoparasite infracommuity differences in abundance, acting mainly over *Carnus hemapterus*.

Our results also reveal that it is necessary to consider the non-infective stages of parasites when analysing host-parasite relationships since they are important for understanding habitat selection criteria, host-range and processes regulating coexistence with other species. In our study system, the observed patterns of prevalence and abundance of imagoes and pupae of three supposedly generalist ectoparasites (*Carnus hemapterus* and the louse flies *Pseudolynchia canariensis* and *Ornithophila metallica*) suggest that, for some parasite species, the requirements of non-infective stages may be more restrictive than the ones of the parasitic stages.

Since some of the ectoparasites here studied are vectors of haemoparasites and since the identification of associations host-vector-pathogens is critical for understanding the ecology of diseases, we studied the likely vectorial role of biting midges (Fam. *Ceratopogonidae*). We found that *Culicoides paolae* and *C. circumscriptus* were common visitors in nests of various species of troglodyte birds. These midges feed on various bird species and harbour four lineages of *Haemoproteus*. Thus, they can play an important role in the transmission of *Haemoproteus* in the study area.

Finally, this study reveals that the infracommunity of ectoparasites of a cavity-nesting bird species in a semi-arid environment is rich (made up of at least species of 8 different families, including a recently introduced species, *C. paolae*), with some species being particularly abundant.

We conclude that socioenvironmental characteristics at small scale are major determinants of the spatial distribution of nest-based ectoparasites in an arid environment.

Resumen

Los parásitos están marcadamente afectados por numerosos factores abióticos y bióticos que operan a diferentes escalas temporales y espaciales. Para comprender la dinámica de los parásitos, las interacciones hospedador-parásito y los mecanismos subyacentes a tales interacciones, es necesario estudiar cómo aquellos factores influyen en los parásitos y en sus relaciones con los hospedadores a diversas escalas.

Esta tesis estudia el efecto de factores ajenos al hospedador y otros relacionados con el mismo sobre la relación entre una especie de ave troglodita, la Carraca europea (*Coracias garrulus*) y sus ectoparásitos, principalmente la mosca hematófaga *Carnus hemapterus*. Los principales factores considerados en este estudio incluyen características del hábitat a mesoescala (el nido del hospedador y los alrededores inmediatos) como es el tipo de sitio de nidificación (una fuente importante de heterogeneidad del hábitat en nuestra área de estudio), así como la densidad del hospedador y del parásito, la fenología reproductiva del hospedador (es decir, estacionalidad) y la masa total de su pollada (indicativo de posibles pistas usadas por los parásitos). Estudiamos el efecto de estos factores sobre el éxito de colonización y la distribución espacial (prevalencia y abundancia) del ectoparásito más común (*Carnus hemapterus*). Sin embargo, dado que una especie hospedadora generalmente alberga varias especies de parásitos, el enfoque "un parásito – un hospedador" pasa por alto el efecto de las interacciones entre parásitos, que ocurren principalmente a nivel de infracomunidad. Por lo tanto, también exploramos el efecto de los factores mencionados anteriormente en la infracomunidad de ectoparásitos de la Carraca europea.

Nuestro estudio se ha desarrollado en un entorno semiárido, lo que contribuye a aumentar nuestro conocimiento de las interacciones hospedador-parásito en tales hábitats, que están subrepresentados en la literatura científica.

Tanto para conocer mejor la biología básica de las especies de estudio como para cimentar nuestro trabajo sobre bases sólidas, también exploramos aspectos básicos de la historia natural de *Carnus hemapterus*, como las características de la etapa pupal del ectoparásito o su esperanza de vida durante la etapa de dispersión (estrechamente relacionada con el éxito de la colonización).

Descubrimos que *Carnus hempaterus* tiene una vida muy corta durante la etapa de dispersión (menos de cuatro días), a pesar de lo cual puede colonizar casi el 100% de los nidos de su hospedador. La longevidad del parásito durante este período aumenta con el tamaño corporal, la humedad ambiental y con el acceso a alimento (flores). El tipo de sitio de nidificación influye en el éxito de colonización de hospedadores por parte de *Carnus hemapterus*, de forma que es mayor en las cajas nido colocadas en taludes y cortijos que en las cajas nido en árboles. El éxito de la colonización también aumentó con la densidad de hospedadores y con la masa total de pollada, pero disminuyó a medida que avanzaba la temporada. Inesperadamente, la densidad de parásitos no tuvo efecto en el éxito de colonización del hospedador. El tipo de sitio de nidificación también explicó las diferencias entre nidos en la composición de la infracomunidad de ectoparásitos y en la abundancia de varias especies de ectoparásitos. Estas variables no se vieron afectadas por la estructura espacial de los nidos o por la masa total de la pollada. Sin embargo, la fenología de cría del hospedador sí que afectó a las diferencias en abundancia de la infracomunidad de ectoparásitos, afectando principalmente a *Carnus hemapterus*.

Nuestros resultados también revelan que es necesario considerar las etapas no infecciosas de los parásitos al analizar las relaciones hospedador - parásito, ya que son importantes para comprender los criterios de selección de hábitat, el rango de hospedadores y los procesos que regulan la coexistencia con otras especies. En nuestro sistema de estudio, los patrones observados de prevalencia y abundancia de imagos y pupas de tres ectoparásitos supuestamente generalistas (*Carnus hemapterus*, *Pseudolynchia canariensis* y

Ornithophila metallica) sugieren que, para algunas especies de parásitos, los requisitos de las etapas no infecciosas pueden ser más restrictivos que los de las fases parasitarias.

Dado que algunos de los ectoparásitos aquí estudiados son vectores de hemoparásitos y puesto que la identificación de asociaciones patógeno-vector-hospedador es crítica para comprender la ecología de las enfermedades, estudiamos el probable papel vectorial de los jejenes (Fam. *Ceratopogonidae*). Descubrimos que *Culicoides paolae* y *C. circumscriptus* son visitantes comunes en nidos de Carraca europea. Estos dípteros se alimentan de varias especies de aves y albergan cuatro linajes de *Haemoproteus*. Por lo tanto, pueden desempeñar un papel importante en la transmisión de este hemoparásito en el área de estudio.

Finalmente, esta tesis revela que la infracomunidad de ectoparásitos de una especie de ave troglodita en un medio semiárido es rica (constituida al menos por especies de 8 familias diferentes, incluida una especie recientemente introducida, *C. paolae*), siendo algunos parásitos particularmente abundantes.

Concluimos que las características socioambientales a pequeña escala son las principales determinantes de la distribución espacial de ectoparásitos nidícolas en un hábitat semiárido.

Introduction

Parasitism is likely the most widespread life-history strategy (Price 1980), with major consequences on communities and ecosystems (Gómez and Nichols 2013). Parasites are a ubiquitous component of biological networks with strong consequences on them (Dunne et al. 2013) and the study of parasites and their relationships with their hosts is key to advance our knowledge on ecology and evolution.

It is well known that parasitism does not occur randomly across the space (Ostfeld et al. 2005, 2008, McCallum 2008) but we still ignore much about the rules and mechanisms explaining this evidence. Understanding the causes that regulate parasitism is essential to make predictions about its variability.

Parasites are strongly influenced by numerous factors such as climate, habitat characteristics, community context, host species identity and parasite species identity (see Duffy et al. 2010 for references). Moreover, these factors operate differently at different temporal and spatial scales. For instance, location and distance are major drivers of the ectoparasite community at large scales (Krasnov et al. 2006, Gómez-Díaz et al. 2008, Krasnov et al. 2008) whereas at smaller spatial levels factors such as microclimate, habitat characteristics or host-related features are also relevant (Poulin 2004, Kleindorfer and Dudaniec 2009, Krasnov et al. 2015, Kleindorfer et al. 2016, Dube et al. 2018). Hence, to understand parasites dynamics, host-parasite interactions and the underlying mechanisms of such interactions it is necessary to study how biotic and abiotic factors influence parasites and their relationships with the hosts at different scales.

Environmental conditions could influence parasitism through different overlapping routes: i) through the variation on the quality of habitats for hosts, parasites and the pathogens they transmit (e.g. Merino and Potti 1996, Cumming 2002); ii) through its influence on the structure and composition of the community of hosts and parasites (Keesing et al. 2010); iii) through the influence on the transfer of infectious individuals or stages of the parasite (Stapp et al. 2004). But, our knowledge of the relative importance of each route and factor is scarce. Similarly, host-related factors such as the abundance of potential hosts, gregariousness, breeding phenology or immunocompetence may influence parasitism via the likelihood of transmission, survival and fecundity of parasites or the ability of the latter to track the most suitable period to find the host (Kleindorfer and Dudaniec 2009, Owen et al. 2010, Calero-Torralbo et al. 2013).

The influence of both environmental and host-related factors on parasites is particularly evident during the process of habitat selection by the latter and when analysing the consequences of such choice.

Parasites' habitat selection

Habitat selection is the disproportionate use of available conditions and resources, and involves responses in space and time to perceived risks and rewards (Mayor et al. 2009). Habitat selection implies choice, and is commonly measured as use relative to availability or as use versus non-use. Understanding habitat selection is crucial both for basic and applied ecology.

Habitat selection is a scale-sensitive process. Detecting habitat selection depends on the scales of measurement and analysis, so that choosing the most informative scale of analysis is critical to understanding habitat selection (Dayton and Tegner 1984). However, identifying the right scale is difficult. What criteria can be used to estimate the relative importance among scales of habitat selection? As pointed out by Mayor et al. (2009) habitat selection depends, naturally, on "habitat". Frequently, habitat is deemed as little more than topography and vegetation. However, in its full sense, habitat encompasses the biotic and abiotic resources and conditions that govern the survival, reproduction, and presence of a population (Caughley and Gunn 1996).

For a parasite, the host itself is, in fact, the most important habitat (Bush et al. 1997), so that host identity is a critical determinant of parasite communities differences (Krasnov et al. 2008) and host-related factors such as age, gender, host body condition, immunocompetence… are known to influence parasite's host choice and fitness (McCurdy et al. 1998, Christe et al. 2003, Roulin et al. 2003, Valera et al. 2004, Hawlena et al. 2005). However, off-host characteristics are also important, even for endoparasites. Habitat characteristics are major rulers of the structure and diversity of the parasite community. Their effects have been assessed at different scales. For instance, the effects of locality on the structure of ectoparasite assemblages have mainly been found out on large spatial scales, such as across localities that differ substantially in environmental conditions (e.g. across distinct geographic regions) (Krasnov et al. 2006, 2008, Gómez-Díaz et al. 2008). At smaller scales, other factors such as vegetation cover, microclimate or proximity to watercourses can be also important. Lareschi and Krasnov (2010) found a significant effect of locality at small scale on the species composition of ectoparasite assemblages and suggested that subtle differences in the environment could influence some ectoparasites. They stated that it is unclear whether the patterns found on a large spatial scale also occur on a small scale and stressed the need of studies at this scale. As a result, host-parasite interactions will depend on the habitat where the host dwells. For instance, differences on parasite-host relationships based on host-habitat variation have been found in fleas (Krasnov et al. 1998).

The relative importance of the habitat and the host characteristics on the parasite will depend on the intimacy of the association between both members. The relationship between endoparasites and their hosts is more intimate than the one found for ectoparasites, which are more exposed to the environment. Intimacy with the host also varies among ectoparasites. Whereas some ectoparasites develop their whole life cycle on the host (i.e. lice), some others have just sporadic contact with it (i.e. mosquitoes).

An additional observation concerning the definition of habitat refers to specific cases such as nest-based ectoparasites, for which the immediate surroundings of the host, namely the nest or burrow where the host spends a significant part of its life, is of major importance. These nests usually offer relatively stable ambient conditions as well as protection against a hostile habitat (Cantarero et al. 2013). This is particularly evident for cavity-nesting bird species and their ectoparasites. Birds are known to host a wide array of ectoparasites (Clayton et al. 2010), and cavity-nesting bird species harbour important loads of ectoparasites that, in fact, become one main factor implicated in the evolution of cavity-nesting behaviour (Cantarero 2015). Thus, for a nest-based ectoparasite, habitat could be envisaged as the nest, its occupants and the immediate surroundings, whose dimensions are difficult to establish and are species-specific (based on aspects like parasite mobility, host detection abilities or thermal requirements).

Much work has been done on the relationships between cavity-nesting bird species and different ectoparasites, and the effect of host-related factors (e.g. body condition, gender, immunocompetence, see Tschirren et al. 2007, Václav and Valera 2018), of nest features (microclimate, nest composition, see Heeb et al. 2000, Malan et al. 2002, Dawson et al. 2005, Gwinner and Berger 2005) or of socioenvironmental characteristics (gregariousness, host density, see Liker et al. 2001, Brown and Brown 2004, Hoi et al. 2010) on parasites have been investigated. However, our comprehension of the habitat choice criteria used by ectoparasites is still incomplete. Moreover, in many cases the studies have focused solely on the parasitic stages, whereas for many ectoparasites (e.g. parasitic flies, haematophagous mites), the host's nest is also the habitat of other life cycle stages and, thus, the characteristics of the nest on these stages will influence factors such as life cycle duration, survival or host-parasite synchronization (Marshall 1981, Danks 1992, Calero-Torralbo and Valera 2008). This is also relevant for the definition of the host-range of a parasite. Parasites are frequently classified as generalist or specialist (even if this classification is controversial, see Loxdale and Harvey 2016) based on the preferences of the infective stage. Nonetheless, it could be that other stages show different or more restrictive habitat requirements since the balance between rewards and risks may change with life stage (Mayor et al. 2009). Finally, hosts are frequently attacked by several parasite species that may interact with each other, so that the approach "one host – one parasite" is very likely insufficient to understand the criteria used by parasites to select a habitat (see below).

We therefore could summarise the topic saying that the habitat of a particular ectoparasite is a specific host in a particular habitat (Krasnov et al. 1998) and that understanding habitat selection by parasites requires considering the influence of habitat characteristics at the most appropriate scales on the whole life cycle of the parasite.

Dispersal, host colonization and spatial distribution of parasites

“Why there are no parasite species exploiting all the members of large taxa such as mammals or birds?” Timms and Read (1999) proposed two major explanations: limited dispersal and limited adaptation.

Dispersal is defined as the movement of individuals between the location where they were born or bred to a location where they breed (Clobert et al. 2001, Ronce 2007). Most organisms have to face temporal or spatial variation in their environment and dispersal plays a central role to cope with such variation.

It has long been known that dispersal may be either temporal or spatial, and that temporal dispersal via developmental mechanisms, particularly diapause, is functionally equivalent to spatial dispersal (Hairston 2000, Hairston and Kearns 2002). Both types of dispersal have specific properties. Temporal dispersal via mechanisms such as diapause is usually interpreted as an adaptation to unpredictable environments (Hopper 1999) and requires synchronization between the host-feeding life stages of the parasite and the time when hosts become appropriate food resources. Spatial dispersal may result in colonization of new habitats/hosts, and parasite traits such as flight ability or host detection mechanisms can be under selection. Spatial dispersal is one of the most dangerous part of parasite life cycles (Ward et al. 1998) and a determinant factor that modulates the number of parasites that could reach a host.

Dispersing parasites aim at colonizing a host. Colonization (i.e. establishment of a population where none was present at the time, Bush et al. 1997) is intimately related to dispersal and analyzing colonization success is crucial to understand the capacity of a parasite to reach and establish in novel habitats. Many of the adaptations and strategies that parasites have developed have the function of effectively detecting, exploiting and transmitting among the hosts they parasitize. The parasites need to overcome two types of barriers to be able to successfully infect their hosts: the so-called encounter filters (ability to find the right host and be able to match it in space and time) and compatibility filters (ability to overcome the host's defenses against exploitation and transmission, as well as the ability to exploit the best resources from a nutritional and metabolic optimization point of view for the parasite) (Timms and Read 1999, Combes 2001). The distribution and abundance of the parasite will depend on the factors influencing these barriers. Features like host detectability, distribution and abundance or host predictability and persistence in space and time are fundamental parameters that will influence the success of overcoming the barriers of encounter and compatibility (Combes 2001, Vázquez et al. 2005). Other factors, such as reproductive filters (i.e. a successful mate to establish a population, Dick and Patterson 2007) or density-dependent mechanisms (Allee effects, notably related to the difficulty in finding mates at low density, Kada et al. 2017) are likely to interact with stage-dependent dispersal to strongly affect colonization dynamics.

Dispersal behavior and colonization success are fundamental to understand parasites virulence, epidemiology and host specificity (Tripet et al. 2002) and have been proposed as main rulers of the spatial distribution (geographical range or prevalence and abundance at local level) of the parasites communities (Poulin et al. 2011). Yet, our knowledge on this topic is fragmentary, mostly due to the difficulty of measuring and following dispersing individuals under natural conditions (Kokko and López-Sepulcre 2006).

Widening the scope: from one parasite species to parasites community

As said above, much effort has been done on the study of the relationship between ectoparasites and their avian hosts, particularly in cavity-nesting species (see, for instance, Møller 1989, Merino and Potti 1995, 1996, Heeb et al. 2000, Potti 2008, Cantarero et al. 2013). However, many of these studies have focused on the host and at the scale of individuals, reporting the fitness costs of parasitism

(Møller 1989, Lehmann 1993, Brown et al. 1995, Tripet and Richner 1997, Fargallo et al. 2001), or have concentrated on specific parasite species (but see Heeb et al. 2000). Whereas these studies are essential to understand how different parasite species behave spatially and temporally and to detect the factors that determine their abundance and prevalence, they frequently neglect the fact that different parasites species are usually found in the same host, and the consequences of the interactions among the various parasites both for themselves and for the host.

There are two main concepts in community studies, infrapopulation and infracommunity (Bush et al. 1997). An infrapopulation is defined as all the members of a given parasite species within a single host individual. An infracommunity includes all infrapopulations within an individual host.

The parasite infracommunity found in a specific host depends on different filters acting on the global pool of parasites that a species can host, and which define the final species composition. Among these filters, historical life-traits act on the host global pool of parasites while environmental and dispersal filters act to define the infracommunity of a specific host (Fig. 1).

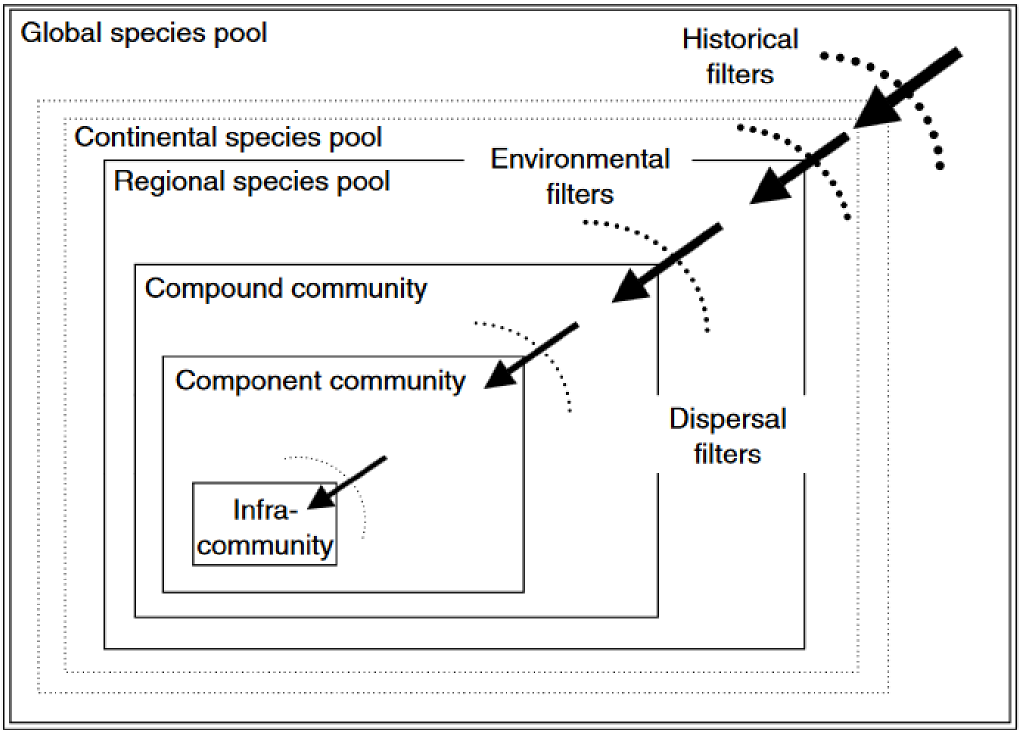

Fig. 1. Schematic illustration about the processes influencing parasite species diversity arranged into hierarchies in which different temporal and spatial factors may act. Extracted from Guégan et al*. (2005).*

Parasite community studies have revealed some general patterns (Guégan et al. 2005). For example, widespread hosts tend to have more parasites or increased host body size results in an increase of parasites. Morand et al. (2002) suggested that the pattern observed in parasite communities depends largely on spatial and demographic stochasticity and is the result of different colonization/extinction processes acting at the level of each ectoparasite. Thus, the recruitment of parasites from local host patches and the abilities of each parasite to colonize these host patches have important effects on the composition and richness of communities from local to global scale. Poulin et al. (2011) pointed out that the limited dispersal abilities of most parasites contribute to distance decay in community similarity. Furthermore, parasite communities show patterns of similarity in composition and richness depending on geographical distances and isolation (Poulin and Morand 1999).

In summary, parasites infracommunity is strongly affected by environmental and dispersal filters. Thus, to understand the infracommunity variation at a host population scale, habitat and host-related features together with geographical distances and parasites dispersal abilities should be studied in order to detect the relative importance of each factor.

Finally, an additional reason to study parasites at the community level is that it is within the infracommunity where the interactions between parasites occurs (Poulin 2007), which, in turn, can influence its structure (Heeb et al. 2000). Therefore, parasite communities should be studied as a whole to obtain deeper insights into the underlying mechanisms ruling their variation in composition and abundance.

Ectoparasites as vectors of pathogens

Many ectoparasites serve as vectors of hundreds of parasites. Different dipteran species (*Culicidae*, *Psychodidae*, *Hippoboscidae*, *Ceratopogonidae*, *Simuliidae*, see Levine 1988) are known vectors of parasites as human and avian malaria haemosporidians, West Nile, yellow fever and dengue viruses, or *Trypanosoma* and, thus, great emphasis has been put on studying this group of vectors in the context of emerging infectious diseases (Daszak et al. 2000, Dobson and Foufopoulos 2001, Harvell et al. 2002, Brooks and Hoberg 2007, Frick et al. 2010).

During the last decades, the ecology of emerging diseases has undergone a rapid progress in some areas, notably, pathogen and disease diagnostics (Jones et al. 2008). Yet, and even though parasite transmission has major consequences for the emergence and re-emergence of infectious diseases and their virulence, vectors behind many diseases remain still unidentified (e.g. Parola and Raoult 2001). While the specificity between specific hosts and parasites has been frequently revealed, that between parasites and their vectors is disproportionately less frequently established even for major host-vector-parasite systems (but see, for instance, Martínez-de la Puente et al. 2011). This has been suggested as a major limitation to deal with the current emerging infectious diseases crisis (Brooks and Ferrao 2005).

Avian haematozoa (genera *Leucocytozoon*, *Plasmodium*, *Fallisia* and *Haemoproteus*) are common vector-transmitted parasites of birds throughout the globe that have received considerable attention due to their taxonomic relation with human malaria and the potential detrimental effect on hosts (Peirce 1981, Merino et al. 2000, Waldenström et al. 2002, Fallon et al. 2003, Beadell et al. 2004, Scheuerlein and Ricklefs 2004, Valkiunas 2004, Szymanski and Lovette 2005). The degree of host-specificity may vary widely, ranging from those that only have been found in one bird species to those that apparently can complete their development in taxonomically very divergent bird species (Bensch et al. 2000, Ricklefs and Fallon 2002, Valkiunas and Ashford 2002, Waldenström et al. 2002, Scheuerlein and Ricklefs 2004, Hellgren et al. 2007). It is unclear if the distribution of parasites in host species is determined by the ability of a parasite to infect a certain host species or, alternatively, if the observed distribution patterns of the parasites among vertebrate hosts are affected by vector–host associations that limit transmissions between host species (e.g. Hellgren et al. 2008). For example, in the case of avian malaria-like parasites of the *Parahaemoproteus* subgenera which have been intensely investigated (a simple search of the word “*Haemoproteus*” in Web of Science returns 1765 documents), the vectors are almost unknown. Even when 150 species of *Haemoproteus* have been described (Iezhova et al. 2011), only 12 species of *Culicoides* have been proved to be true vectors, supporting the complete sporogony of 18 species of avian haemosporidian (Bernotienė et al. 2019, Bukauskaitė et al. 2019).

In conclusion, lack of ecological knowledge of vectors and vector-parasite associations prevents advances in our understanding of the factors affecting the occurrence and success of parasite transmission as well as practical results such as building precise prediction models of future disease distribution and prevalence or altering critical ecological pathways involved in parasite transmission.

A particular case study: ectoparasites of birds and aridity

Host-parasite interactions have been studied in depth in a variety of habitats and ecosystems but, apparently, the effort invested varies widely among habitats. For instance, searching “ectoparasites“ and “forests“ in Web of Science renders 1609 papers whereas a search of “ectoparasites“ and “deserts“ results in 308 papers, one order of magnitude lower. Is this due to the fact that there are less ectoparasites in arid areas?

Several studies have indicated that parasite diversity is higher at low latitudes, what is partly linked to latitudinal clines in climate (Møller et al. 2013). However, to our knowledge, general rules on the effect of specific factors such as aridity on the richness and abundance of ectoparasites are unknown. Cizauskas et al. (2017) pointed out that the geographical boundaries and ecology of ectoparasites can be affected by factors such as aridity or salt spray among others. In general, increased aridity should impair the transmission of parasites with stages that live in soil or of those that require water for larval development whereas low humidity could conceivably have little effect on blood-feeding ectoparasites, given the high water content of their diet (Moyer et al. 2002). Parasites whose life cycles include free-living stages (e.g. fleas) can also be vulnerable to arid environments (Amin 1966). Humidity has been reported to restrict the distribution and abundance of feather lice (*Phthiraptera: Ischnocera*) (Fabiyi 1996, Moyer et al. 2002, but see Carrillo et al. 2007). Other studies show that organisms are able to cope with climatic extremes given some conditions. For instance, Vial et al. (2018) found that *Ornithodoros* soft ticks could endure very arid conditions if the dry seasons were interrupted by small rain showers to maintain minimum moisture inside their habitat along the year. All in all, evidence gained so far reveal that the conditions prevailing in arid areas can exclude some parasites but it also suggests that many parasites are able to adapt to such conditions, so that the bias in our knowledge of parasites in arid areas is probably due to a lack of specific studies in such habitats.

Arid areas provide an interesting scenario to study host-parasite interactions. They are characterized by the lack of moisture. The soil is dry, the air is dry, and yearly precipitation is very low. Another characterisitic of arid and semi-arid lands is the high spatial environmental heterogeneity (Aguiar and Sala 1999, Bisigato et al. 2009). Vegetation heterogeneity at the landscape scale is usually controlled by geomorphological and edaphic factors in relation to their effects on water availability whereas biotic interactions usually influence fine scale (patch, intrapatch) heterogeneity (Bisigato et al. 2009). Human-related activities such as grazing or logging are additional sources of heterogeneity. How does such environmental heterogeneity affect parasites? And host-parasite interactions?

Concerning birds, an important feature of arid environments is its bidimensional physiognomy: arid and semi-arid areas lack trees what means that the guild of cavity-nesting bird species is restricted to breed in cavities in cliffs. However, installation of nest boxes in alternative habitats (e.g. trees) creates a streaking contrast since the environment (insolation, vegetation cover, microclimate and density of conspecifics and heterospecifics) close to nest boxes on sandstone cliffs and farmhouses differs widely from the one close to nest boxes on trees (see methodological section). Moreover, nest-site type also influences socioecological variables, since the density of breeding birds (i.e. potential hosts) in cliffs is usually higher than the one on trees. Nest box installation could be seen as a sort of experiment that enables the study of habitat selection by parasites and the effect of habitat type on host-parasite interactions.

Arid and semi-arid areas can be found in Spain, the most important one being in south-eastern Spain, the most arid region of the European continent. However, only a handful of papers on the ecology of parasites has been produced in these areas, therefore missing the opportunity that they offer to widen our knowledge on parasites and their interactions with the environment and their hosts.

General hypothesis and objectives

The general hypothesis of this study is that habitat-related and host-related factors influence the spatial variation in parasitism and that both types of factors interact to govern host-parasite interactions.

We assume that for small body size insects (most ectoparasites in our study area) nest-site type (the location of the nest box: trees, sandstone cliffs, farmhouses) is a major source of habitat heterogeneity in our scenario. Thus, we focus our study on habitat selection by parasites at this level, that could be named feeding site (even though it will be also a breeding site for some parasite species, e.g. the nidicolous ectoparasite *Carnus hemapterus* or the haematophagous mites). We hypothesise that nest-site type will be a major determinant of the colonization success and spatial distribution (prevalence and abundance) of parasites. Since nest-site type is associated to some socioecological factors (see above), we also consider these aspects, namely host density. Concurrently, host-related features could influence parasites' habitat selection. In fact, some of these factors (body condition, immunocompetence) have already been shown to influence host preferences of the most abundant ectoparasite in our area (Václav and Valera 2018). Here we focus on two main factors: i) host breeding phenology: rollers are transaharian migrants and, thus, late breeders in our latitudes. Ambient conditions vary widely along the season in arid environments, so that seasonality can set strong selective forces to organisms inhabiting these areas; ii) host brood mass: ectoparasites usually rely on host-related cues (temperature, odour, heat or carbon dioxide emission) to detect them. Host brood mass can be used as a surrogate of cues produced by the hosts that may serve for nest detection (Tomás et al. 2008).

The main objective of this study is to highlight the relative importance of habitat and host-related factors in determining the prevalence and abundance of ectoparasites of a cavity-nesting bird species, the European roller (*Coracias garrulus*), in a semiarid environment.

The achievement of this aim involves addressing other cross-cutting issues arising from various characteristics of our study system: i) the importance of non-infective stages of parasites for host-parasite interactions; ii) identification of host-vector-parasite associations; iii) host-parasite interactions in an arid environment.

Next, we will develop these objectives based on the research questions addressed rather than following the order of the chapters.

<u>Objective 1</u>: The effect of habitat characteristics on the colonization success, prevalence and abundance of the most abundant ectoparasite, and on the ectoparasites infracommunity.

This objective addresses several phases of the relationship between parasites and hosts (colonization and establishment) and two levels of organization: single parasite species and ectoparasite infracommunity.

Full understanding of the dynamics of host-parasite interactions requires elucidation of the principles governing host colonization, what in turn requires information about the dispersal ability of the parasites. Both processes determine the parasite pressure on hosts, but these processes are also influenced by socioecological factors (host density, habitat features) and by parasite-related factors. Logistic difficulties usually hamper studies on parasites dispersal but some aspects of our system provide some advantages for these investigations (see **Chapter 4**).

We studied the life span of the dispersal stage of the most abundant ectoparasite in our study area, *Carnus hemapterus*, as a first approach to evaluate the dispersal ability of this parasite (**Chapter 3**). We also analysed the effect of proximate factors (sex, body size), abiotic features (humidity) and biotic aspects (food, i.e. fueling) on the life span of the species. The latter factors are meaningful in the ecological context where the study has been performed since humidity and vegetal food can be seriously reduced in arid environments.

Then, we studied the influence of habitat characteristics (i.e. nest-site type) on the colonization success of *Carnus hemapterus* (**Chapter 4**) and on the variation in composition of the ectoparasite infracommunity and the abundance of the various ectoparasites (**Chapter 5**).

Objective 2: The effect of host- and parasite-related factors on the colonization success, prevalence and abundance of the most abundant ectoparasite, and on the ectoparasites infracommunity.

Similar to the previous objective, this one also addresses several phases of the relationship between parasites and hosts (colonization and establishment) and the two levels of organization mentioned above.

We analyse the effect of parasite and host density, of host brood mass and breeding phenology on the colonization success of *Carnus hemapterus* (**Chapter 4**) and of the latter two variables on the variation of the ectoparasite infracommunity at the host level (**Chapter 5**).

Objective 3: The importance of non-infective stages of the parasite for host-parasite interactions.

Studies on host-parasite interactions frequently focus on the infective stages of the parasite. This is logical since this phase allows us to study parasitism directly (on the spot and in the real time). Nonetheless, it should be considered that other stages of the parasite can also influence the relationship with the host. Studying non-infective phases of parasites can be frequently hampered by the lack of basic knowledge on the natural history of many species. Here we first establish a solid base to work with the pupae of *Carnus hemapterus*. This is important for further studies that require correct identification of this stage. Thus, we describe the pupa of *Carnus* in comparison to the ones of closely related and coexisting species (**Chapter 1**). We also study habitat preferences of this parasite based on the abundance of pupae and the processes that could facilitate coexistence with other dipterans (**Chapter 1**). In **Chapter 2** we explore the generally assumed idea that host selection by the infective phase of parasites is correlated with the suitability of the host and its environment for the development of the whole life cycle of the parasite. We examine in three allegedly generalist diptera ectoparasites the prediction that all stages of the parasites should perform better on the host where imagoes (the choosing stage) reach the higher prevalence and abundance (**Chapter 2**). Information from these chapters (e.g. suitability of the nests of some bird species for carnid pupae and, thus, the role of these nests as sources of parasites) are required to meet further aims (e.g. parasite density, **Chapter 4).**

Objective 4: Identification of host-vector-parasite associations

Since many insects are vectors of pathogens, preferences of some insect species for some habitats and/or host features could overexpose some individuals to specific pathogens. Here we intend to identify the role of two common ornithophilic species of biting midges (Fam. *Ceratopogonidae*) in the transmission of avian haemosporidians of the genus *Haemoproteus*. For this, we evaluate the abundance of the former in the nests of the focal host species, identify other host species they are feeding on and the lineages of the haemoparasite they harbour. In this way, we are able to identify the likely relations between hosts, vectors, and blood parasites in our study area (**Chapter 6**).

Objective 5: Host-parasite interactions in an arid environment

This is a cross-cutting objective that is attained as a result of the work done during the whole study period and reflected mainly in **Chapters 5 and 6**. Our knowledge of host-parasite interactions is biased since most work has been done on mesic areas. This study aims at contributing to complete such gaps (from a parasitological, entomological and ecological point of view) by studying the insects and arthropods associated to a cavity-nesting bird species in the most arid area of the European continent and their relationships with the avian host.

Material and methods

Study area

The study area is located at Campo de Tabernas and the plains before the villages of Sorbas and Uleila del Campo (37°05′N, 2°21′W, Almería province), a space bounded to the north by the Sierra de Los Filabres and to the south by Sierra Alhamilla (Fig. 2). It includes the municipal terms of Tabernas, Turrillas, Uleila del Campo, Tahal and Sorbas (Fig. 3). It is located in the area of influence of two spaces belonging to the Natura 2000 Network: the so-called Desert of Tabernas and Ramblas de Gérgal, Tabernas and South of Sierra Alhamilla.

Fig. 2. Tabernas district (Almeria province).

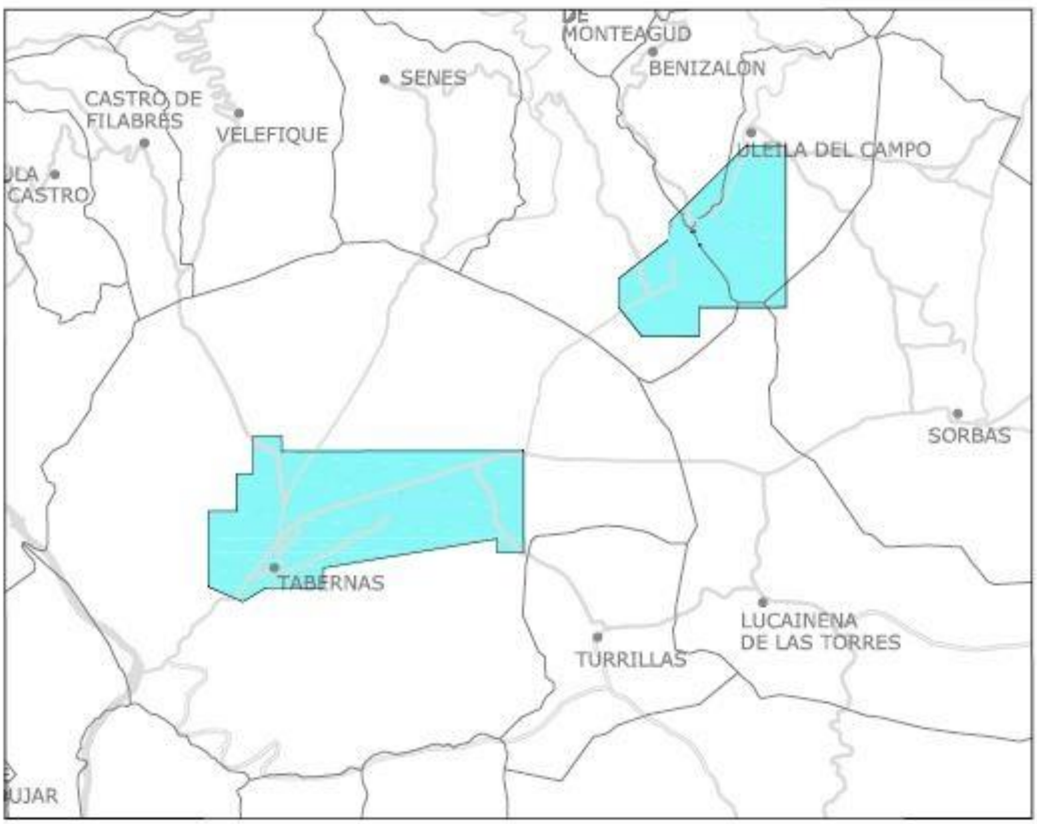

Fig. 3. Study area. The blue colour represents the main breeding areas of the European roller, even though some nests are also out of these areas.

Virtually lacking any tree vegetation, this area is, somewhat justifiably, viewed locally as ‘desert’, even if it does not belong to the category of Saharo-Sindhic deserts, not only in terms of rainfall (usually above 200 mm/year) but also of its flora (Charco 1999). Actually, this area displays features of other originally Mediterranean forest habitats, where human activity resulted in steppification and therophytization and, therefore, should be classified as anthropic pseudo-steppes or semi-deserts.

The area has a soft relief crossed by an intricate system of ramblas, with traditional olive and almond tree plantations and small plots dedicated to rainfed cereals. Recently, large areas of land have been dedicated to intensive olive groves. There are also plots of abandoned crops, as well as scrubland. This mosaic of habitats favours the presence of steppe birds, which select this type of poorly intensified environments with a variety of patches, among which hedges or natural discontinuities break the monotony and provide different resources for a variety of species. An added value is the occurrence of large farmhouses, and other traditional constructions, in many cases abandoned, which are of great interest since they provide breeding sites to various species of birds.

The high temperatures and the lack of rains condition the scarce herbaceous and shrub vegetation. Natural vegetation consists mainly of therophytic grasslands, chamaephyte *Labiatae* and *Fabaceae* (*Genista* sp., *Rosmarinus officinalis*, *Thymus* sp.), open halonitrophilous shrubland (*Salsola* sp., *Suaeda* sp., *Atriplex* sp., *Artemisa barrerieli*) and dense *Macrochloa tenacissima* and *Lygeum spartium*. Ramblas allow the development of more or less large spots of Oleander (*Nerium oleander*) and Tamarisk trees (*Tamarix* sp.). Nonetheless, it is interesting to note that in such relatively small area, the vegetation cover is heterogeneous due to a combination of geomorphological and edaphic factors, water availability, biotic interactions and human-related factors. In this regard, it is particularly noteworthy the occurrence of small *Eucalyptus* plots and isolated trees that increase the heterogeneity of the area.

The ramblas constitute authentic refuges for the fauna (Valera et al. 2011). They allow the reproduction of a variety of cavity-nesting bird species (Common kestrel *Falco tinnunculus*, Jackdaw *Corvus monedula*, European bee-eater *Merops apiaster*, European roller *Coracias garrulus*, Little owl *Athene noctua* and Rock pigeon *Columba livia*), that excavate burrows or colonize cavities and holes (Fig. 4).

Fig. 4. A long sandstone cliff in the study area where several cavity-nesting species coexist during the breeding season (Author: Jesús Veiga).

Close to the study area, there are upwellings of subsurface, saline water that allow the development of dense formations of halophilic vegetation (Fig. 5). These temporary watercourses facilitate the occurrence of a variety of vertebrate and insects, some of which are parasitic diptera (unpubl. information).

Fig. 5. A temporary influx of brackish water near the study area. (Author: Antonio Callejón).

Until about half of the last century, this area was mainly dedicated to dryland agriculture and the exploitation of sheep and goats. Both activities are currently in deep regression. In the 1970s and 1980s the low availability of water prevented land use change and agricultural intensification in many places. Recently, ploughing of large areas for cultivation of intensive olive groves has altered large extensions of semi-arid landscape and contributed to aridification of the area. Currently, a new threat to the area is the installation of massive photovoltaic plants that will alter the ecosystem definitely.

The climate is semi-arid with mild winters, long hot summers and low average annual rainfall (235 mm) with strong inter and intra-annual variation (Lázaro et al. 2001). The number of rainy days per year ranges between 25 and 55, although only 6% of the rainy episodes exceeds 20 mm. The average annual temperature is 17.9°C, the minimum average of the coldest month is between

3°C and 10°C. Maximum temperatures exceed 40°C in summer and they can reach 48ºC (Anonymous 2006). These conditions get milder to the eastern of our study area.

Study system

Our study system is mainly formed by a secondary cavity-nesting bird species, the European roller (*Coracias garrulus*) and the ectoparasitic fly *Carnus hemapterus*, even though other parasites and bird species have also been considered in our study (see below).

The European roller (hereafter roller) is a medium-sized (29-32 cm) bird species that breeds in warm steppe areas with hot summers. It is a secondary cavity-nesting bird species that depends on the occurrence of cavities dug by other birds (like the European bee-eater or woodpeckers) for breeding. In our study area rollers breed in abandoned bee-eater burrows, in natural cavities in sandstone cliffs and in holes and crevices in bridges and abandoned farmhouses. Rollers do not build a nest and the eggs are laid directly on the substrate (usually sand and detritus). They lay a single clutch of two to seven eggs. The incubation period lasts ca. 21 days in our population, and the nestlings hatch asynchronously. After hatching, juveniles fledge after 20-22 days (Václav et al. 2011). Its diet consists mainly of medium and large-sized terrestrial arthropods although it can include small mammals and reptiles. Rollers are transaharian migrants, arriving at our study area at the end of April, even though several waves can be distinguished, so that there is ample variation in the start or breeding within the population (unpubl. data).

A long-term nest box scheme starting in 2005 evidenced that the species in our study area was limited by the availability of nesting sites and nest box installation doubled the population (Václav et al. 2011). Currently, most of the rollers breed in nest boxes (Valera et al. 2019), that have been installed on trees (mainly *Eucalyptus*), sandstone cliffs and farmhouses. The location of the nest boxes has several important socioecological implications: i) the microhabitat around a nest box on a tree is very different from the one around a nest box on a cliff or farmhouse (e.g. insolation, vegetation, microclimate) (Fig. 6); ii) the occurrence of neighbouring breeding birds is more likely close to nest boxes on sandstone cliffs and farmhouses, given the presence of cavities and holes that are occupied by other bird species (House sparrows *Passer domesticus*, Spotless Starlings *Sturnus unicolor*, Kestrels, Little owls…). In contrast, rollers breeding in nest boxes on trees seldom have other birds breeding closely; iii) since *Eucalyptus* trees occur all over the study area (in ramblas, near farmhouses and bridges), the nearest nest box neighbour of a focal nest box is frequently in a different substrate (e.g. tree-farmhouse, tree-cliff, farmhouse-cliff).

Fig. 6. Two different nest-site types: nest box on a Eucalyptus tree and on a sandstone cliff.

A variety of parasites has been cited for rollers: endoparasites like *Lissonema coraciae* and *Diplotriaenia* sp. (Nematoda, López-Caballero et al. 1987, Cordero et al. 1994) and many ectoparasites that are favoured by the accumulation of detritus in the nests given that rollers do not expel their faeces from the nest cavity (Sosnowski and Chmielewski 1996, even though nest sanitation behaviour is common, pers. obs.). In our study area, feather lice, haematophagous mites, soft ticks and a variety of parasitic diptera have been recorded. The most abundant ectoparasite of the European roller (and other cavity-nesting bird species) in our study area is *Carnus hemapterus*.

Carnus hemapterus (Fam. *Carnidae*, Order Diptera) is a highly mobile, 2 mm-long, nest-dwelling haematophagous ectoparasite (Fig. 7). Within the genus *Carnus*, this is the only species widespread across Europe and the cold and temperate regions of Asia and North America (Grimaldi 1997). The adult phase feeds primarily on nestlings of a wide variety of bird species, for which is considered a generalist parasite (Brake 2011). Females lay eggs on the nestlings or in the detritus and larvae (3 stages) live within the nest and feed on organic debris. Pupae also overwinter there (Guiguen et al. 1983). The emergence of imagines, the dispersal stage, is synchronized to the hatch of host nestlings in the subsequent year (Calero-Torralbo et al. 2013).

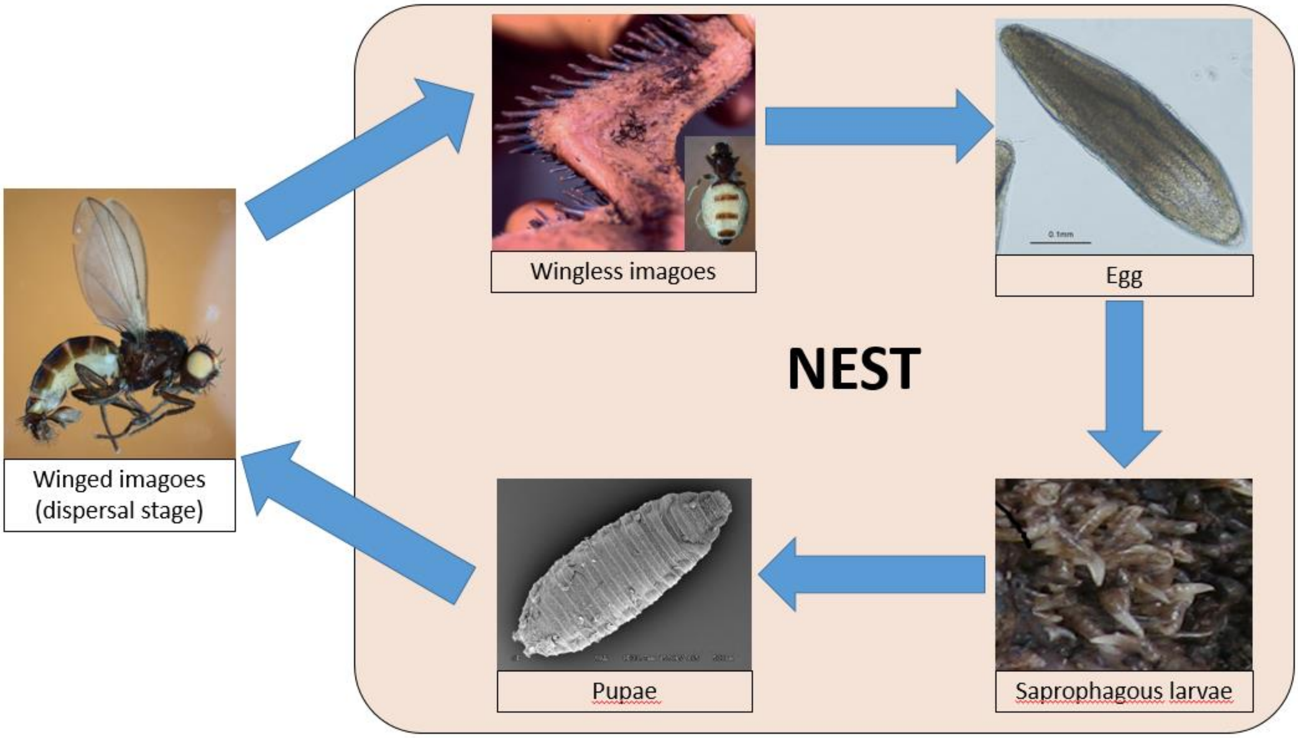

Fig. 7. Carnus hemapterus *life cycle. All the life stages occur in the host nest (pink square) except for the dispersal phase (winged imagoes).*

This ectoparasite is particularly adequate for studying some aspects of host-parasite interactions, even though lack of information on basic features of its biology hampers some studies. Previous work of our research team on this species has focused on the effect of abiotic factors on the emergence of the ectoparasite, host-parasite synchronization, life history strategies, diapause regulation, phylogeography and genetic structure (Calero-Torralbo 2011, Amat-Valero 2015). Other research teams have studied topics such as reproduction and fecundity (Roulin 1998, 1999), factors influencing parasite load (Liker et al. 2001, Hoi et al. 2010), the effect of the ectoparasite on the host (Hoi et al. 2018) and on the microbiota of host eggs (Tomás et al. 2018). More details about this species are given in the following chapters.

Other ectoparasites considered in this study are louse flies (*Hippoboscidae*), hematophagous mites, soft ticks, blackflies (*Simuliidae*), biting midges (*Culicoides*) and sandflies (*Phlebotominae*). Some of these parasites are known vectors of diseases: *Borrelia* and *Rickettsia* (ticks), *Lankesterella* (haematophagous mites), *Haemoproteus* (louse flies), *Parahaemoproteus* (biting midges), *Leucocytozoon* (blackflies) or *Leishmania* (sandflies).

Study period and methods

The study was performed during the breeding seasons 2016-2018. According to the precipitations observed before the breeding period during the last 15 years, our study period encompasses dry (2016), average (2018) and “humid” years (2017) (Fig. 8).

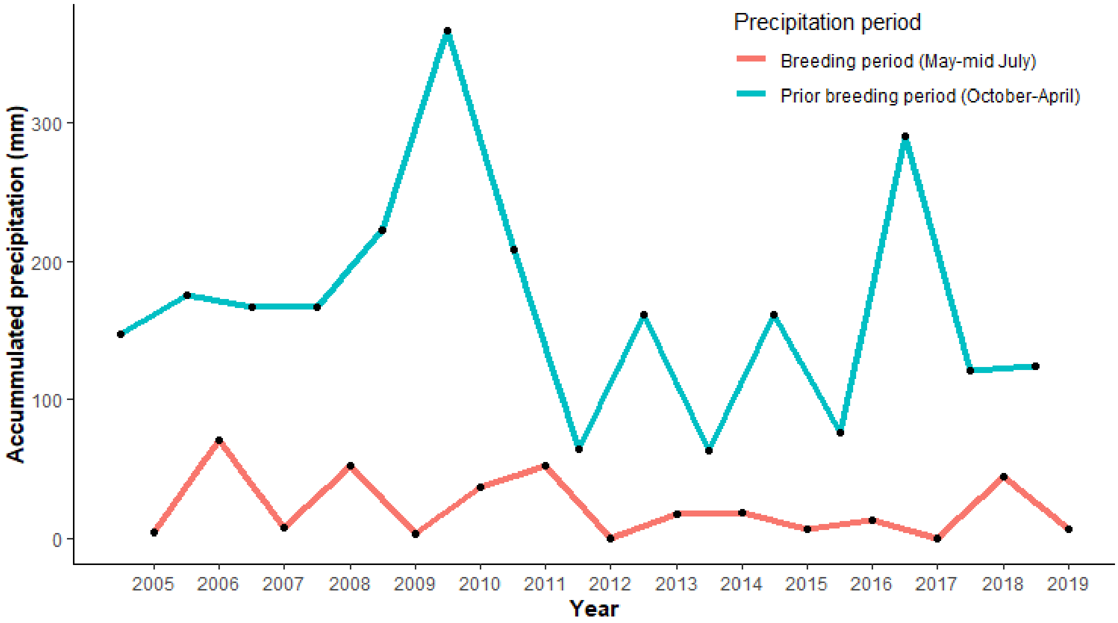

Fig. 8. Accumulated precipitation in the study area during the period 2005-2019 prior and during the breeding period of the European roller. A high variability for rainfall is evident. Source: Estaciones Agroclimáticas de Andalucía (https://www.juntadeandalucia.es/agriculturaypesca/ifapa/ria/servlet/FrontController?action=Init)

Precipitation has a profound impact on the vegetation (Fig. 9) and food availability for rollers and, thus, influences its breeding success.

Fig. 9. The study area during an exceptionally rainy year (2017) (above) and during a dry year (below).

During the three years, routine fieldwork included monitoring the breeding population of rollers and neighbouring bird species, periodic visits to the nest boxes to record basic breeding parameters (start of laying, clutch size, hatching date, fledgling success) and trapping, ringing and bleeding of adult and fledgling rollers.

The study of ectoparasites was performed during all years and following different techniques: visual estimation on the nestlings' body (mainly for carnid flies and louse flies), inspection of the nest box (for mites and ticks), sticky traps installed in the nest boxes (for blackflies, sandflies and biting midges) (Fig.10) and installation of Centers for Disease Control traps (CDC) (Fig. 11). The latter technique was performed during the years 2016-2018, and provided a huge amount of captures, that required identification of thousands of individuals to the Family level and then, for the families with hematophagous species, to species level.

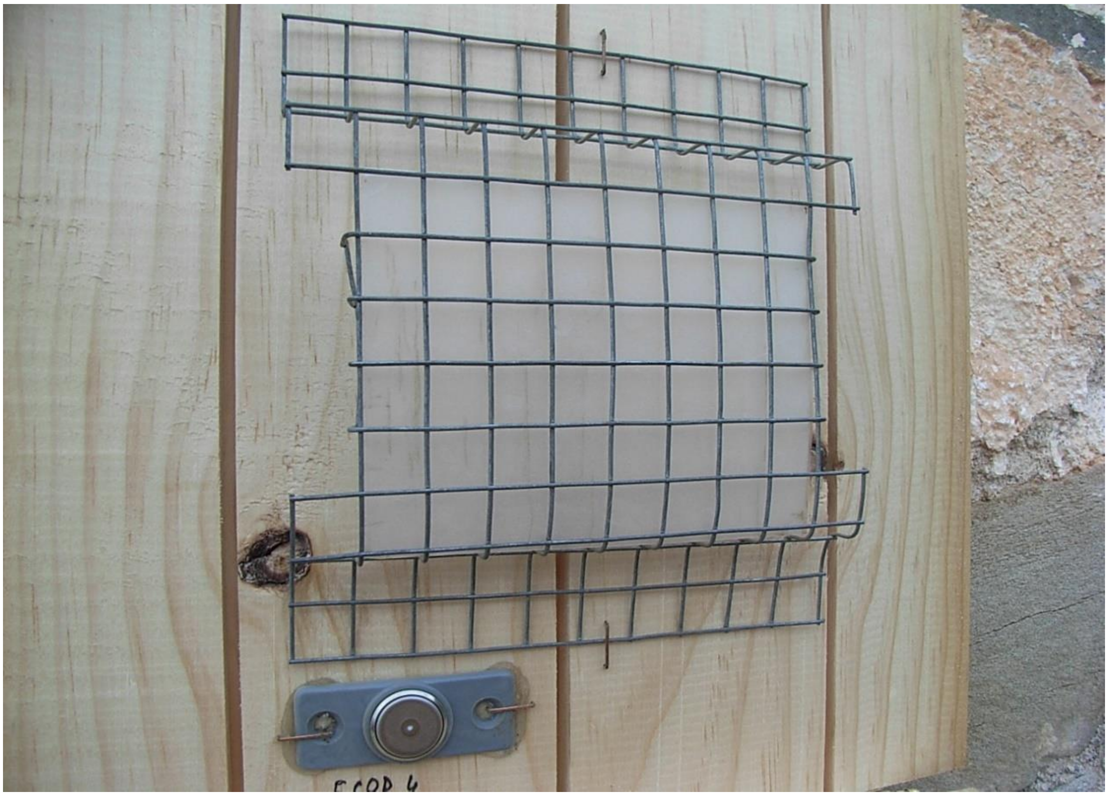

Fig. 10. Adhesive trap at the inner side of the lid of a nest box. A data-logger to register temperature and humidity is also shown.

Fig. 11. Centers for Disease Control (CDC) traps placed at a Eucalyptus tree, close to a nest box. Traps were baited with ultraviolet and incandescent lights as well as with a CO_2 source.

Nest boxes used during this period were cleaned prior to the start of each breeding season, so that colonization by parasites could be detected. Detritus of the nest boxes was collected after each breeding season and pupae of carnid flies and other diptera were collected and quantified.

For the hematophagous insects of the genus *Culicoides*, molecular techniques were used to confirm the species and to identify the hosts they had fed on and the pathogens they harboured.

Specific details about all these techniques are provided in the following chapters.

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Chapter 1: 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 troglodytic 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 discrimination 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* (Coquillet 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, Guiguen et al*.* 1983, Dawson and Bortolotti 1997, Roulin 1998, 1999, Valera et al*.* 2004, 2006*a*, 2006*b*, Václav et al*.* 2008, Valera and Zidková 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 species of this family, is missing.

The only study about ecological aspects of *Hemeromyia* species is that by Valera et al*.* (2006*b*). These authors studied the coexistence of pupae and emergence phenology of imagoes 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*.* (2006*b*) 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*.* (2006*b*) 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-Torralbo 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 pupae 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*.* 2006*a*) 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 that the flies are not transmitted by the host but colonize the nests actively during the winged phase of its life cycle (Grimaldi 1997).

Very little is known about the species of the genus *Hemeromyia* and even their basic requirements are undetermined, which explains that *H. anthracina* and *H. longirostris* have seldom been collected. Valera et al*.* (2006*b*) found both species and *C. hemapterus* in nest boxes in Western Spain. It can, therefore, be deduced that they develop various stages of their life cycle (egg, larva and pupa) in the nest of various species of birds, mainly troglodytic ones, such as the European roller *Coracias garrulus*, Common kestrel *Falco tinnunculus*, Spotless starling *Sturnus unicolor* and Little owl *Athene noctua*. These bird species nest in natural hollows in trees and sandy slopes, nest boxes and in human constructions. All of these birds are regular breeders in our study areas.

Hemeromyia anthracina and *H. longirostris* are flies about 2–3 mm in length and their adult phases, unlike *C. hemapterus*, are not parasitic. Adults are suspected of feeding on flower nectar (Carles-Tolrá 2002), while larvae appear to be saprophagous (Papp 1998). To our knowledge, the puparium of these species has never been described.

The main morphological difference between *C. hemapterus* and *H. anthracina* imagoes lies in alar venation (Papp 1998, Brake 2011), whereas *H. longirostris* is easily distinguished by the presence of an elongated rostrum (Carles-Tolrá 1992).

Study area and collection of nest material

The main study area (c. 50 km^2) 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 Almeria 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*.* (2006*b*) 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 Almeria 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).

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 imagoes 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 pupae

Apparently viable pupae (i.e. without external signs of breakage) were sorted in morphotypes according to size, presence/absence of spiracles and ornamentation of the latter.

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 Almería, 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 nests examined, whether or not they were parasitized) and median intensity (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).

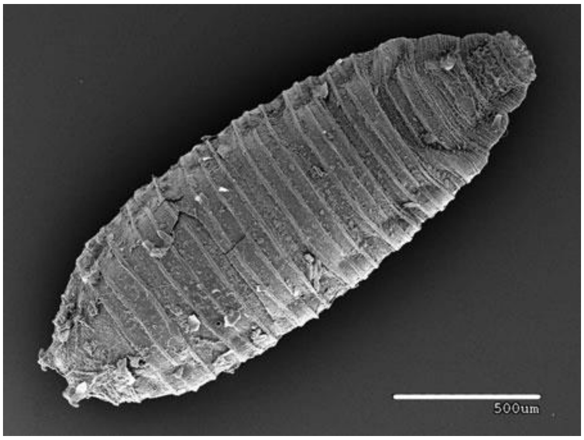

Fig. 1. Puparium of Carnus hemapterus*.*

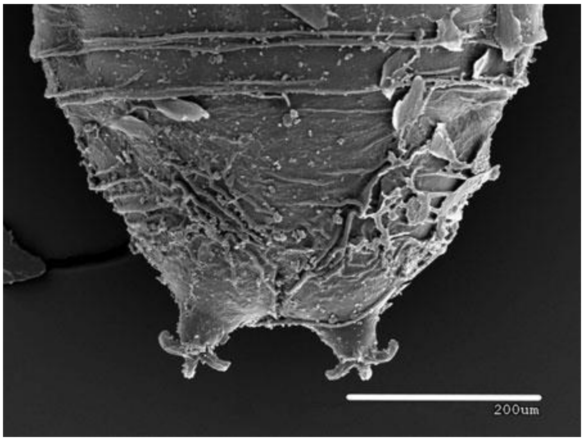

Fig. 2. Puparium of Carnus hemapterus*. Spiracles with short curved finger-like projections are evident.*

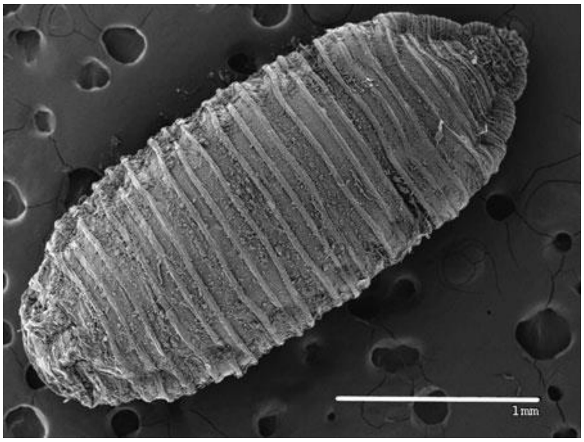

Fig. 3. Puparium of Hemeromyia anthracina.

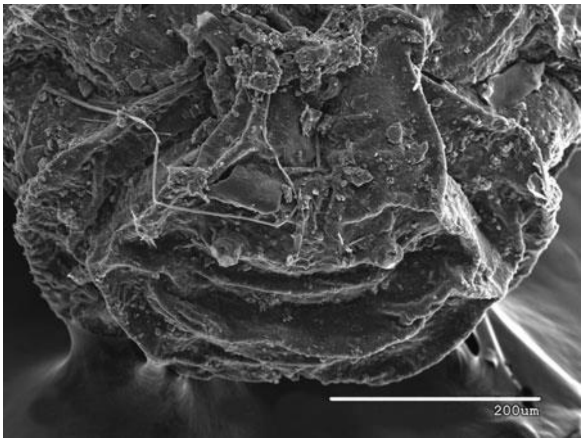

Fig. 4. Puparium of Hemeromyia anthracina *with small, non-ornamented spiracles and the depression bounded by marked rims.*

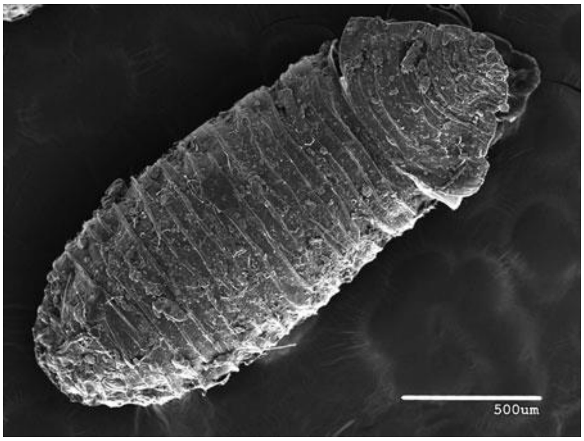

Fig. 5. Puparium of Hemeromyia longirostris.

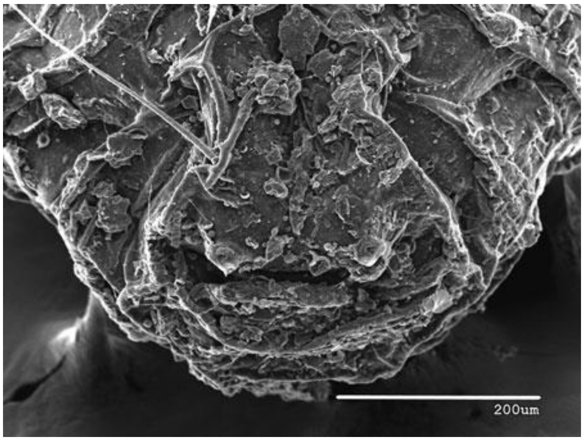

Fig. 6. Spiracles of the puparium of Hemeromyia longirostris *and the depression close to them.*

Fig. 7. Puparium of Carnus hemapterus (left), Hemeromyia longirostris *(middle) and* H. anthracina *(right).*

Fig. 8. Detail of the caudal end of the puparia of Carnus hemapterus (left), Hemeromyia longirostris *(middle) and* H. anthracina *(right).*

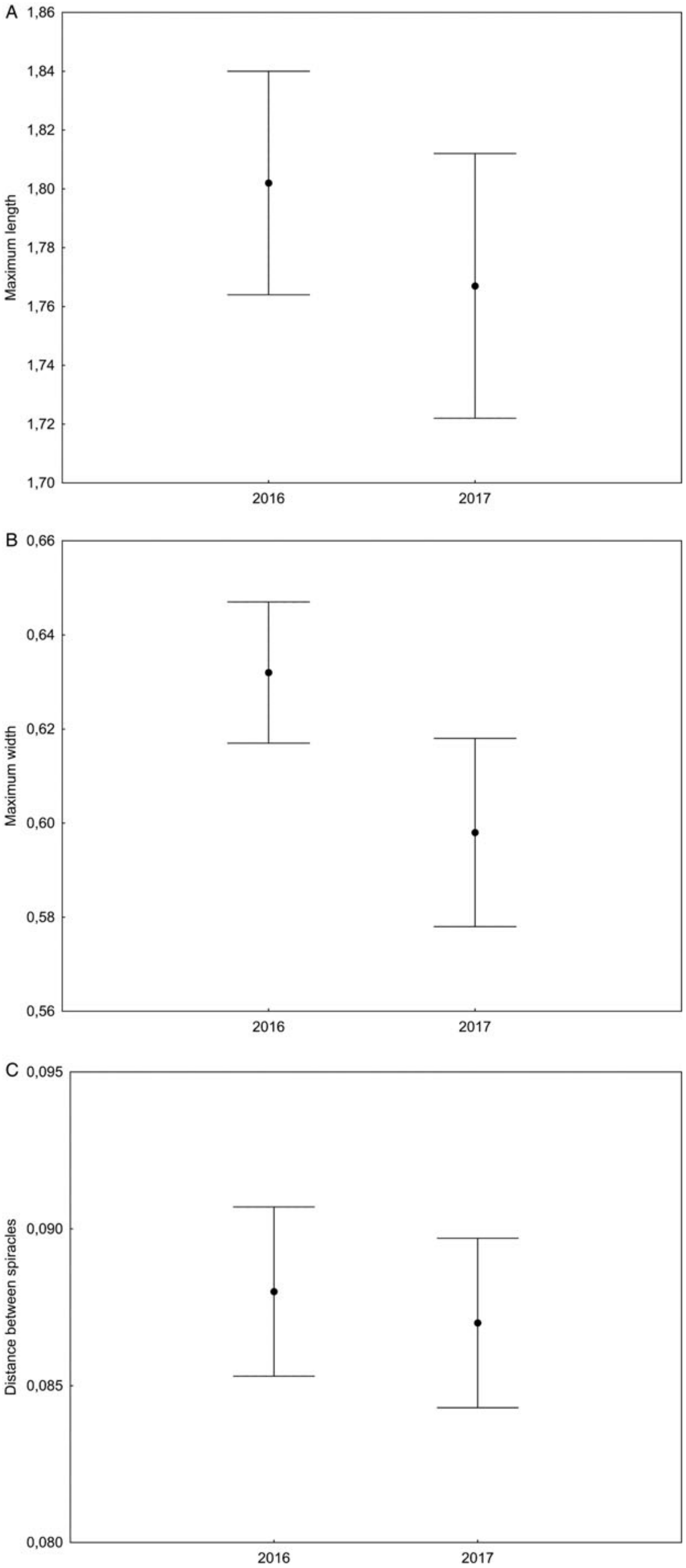

Fig. 9. Interannual differences in (A) maximum length, (B) maximum width and (C) distance between spiracles (estimated values ± S.E.) of the puparium of Carnus hemapterus *in 2016 and 2017.*

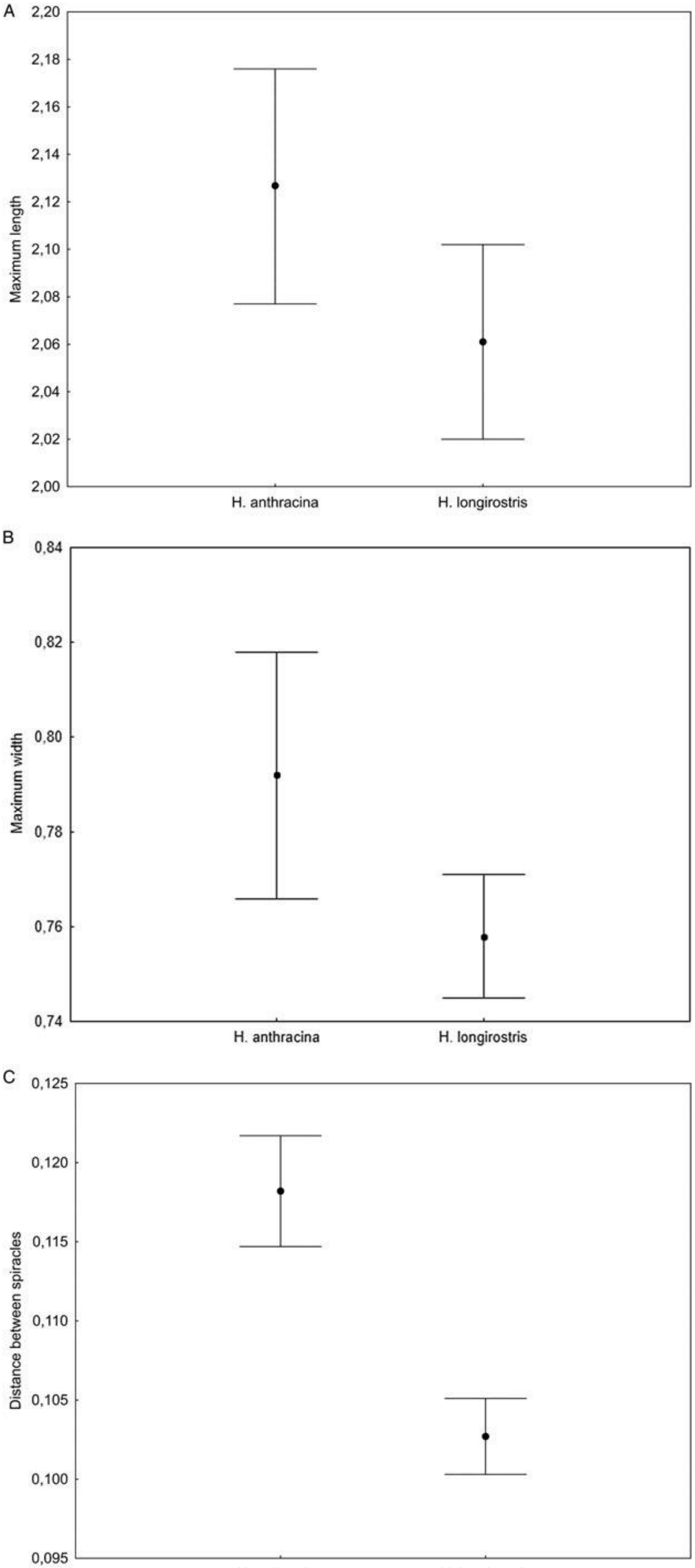

Fig. 10. Differences in (A) maximum length, (B) maximum width and (C) distance between spiracles (estimated values ± S.E.) of the puparia of Hemeromyia anthracina *and* H. longirostris.

Habitat associations of two sympatric carnid flies, C. hemapterus *and* H. anthracina

Only two species, *H. anthracina* and *C. hemapterus*, were found in Almería.

The prevalence of viable pupae of *C. hemapterus* and *H. 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 *C. hemapterus* pupae in nest boxes was significantly higher than that of *H. anthracina* (Median test, *P* = 0,02). The median intensity of *C. hemapterus* pupae in nest boxes also tended to be higher than that of *H. anthracina* (Table 1), although the differences were not significant (Median test, *P* > 0,10).

In contrast, *H. anthracina* pupae were not found in natural cavities, whereas viable pupae of *C. hemapterus* were found in 75% of the cavities sampled (two-tailed Fisher's test, *P* = 0,009) (Table 1).

The prevalence of *C. 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).

	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]	–

Table 1. Prevalence, median abundance and median 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). Sample size (in brackets), 95% confidence intervals for prevalence and quartiles for median abundance and intensity [in square brackets] are shown.*

Discussion

This paper provides a full description, including morphometrics, of the puparium of *C. hemapterus*, *H. anthracina* and *H. longirostris* after unequivocally verifying the emergence of adults of the collected pupae, being the most complete report until the date for *C. hemapterus* and the first one for the two later species. We also describe patterns in habitat associations of *C. hemapterus* and *H. 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. diapause during the pupal phase, see Amat-Valero et al*.* 2013 for *C. 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 *C. 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 *H. 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 *C. 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 south-eastern 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 *H. 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 *C. hemapterus* and *Hemeromyia* it seems unlikely that differential predation or infection occurs in a given cavity type for a given species; (ii) emergence of *H. anthracina* imagoes could pass unnoticed to us. Still, Valera et al*.* (2006*b*) described a similar emergence phenology for both species and we did not find open pupae of *H. 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*.* (2006*b*) 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-Torralbo 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*.* (2006*b*). 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 *S. unicolor*) in the nest before the rollers took ownership of it. Valera et al*.* (2006*b*) 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 carnid 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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Chapter 2: Defining host range: host–parasite compatibility during the non-infective phase of the parasite also matters

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Abstract

Host range and parasite specificity determine key epidemiological, ecological and evolutionary aspects of host–parasite interactions. Parasites are usually classified as generalists or specialists based on the number of hosts they feed on. Yet, the requirements of the various stages of a parasite may influence the suitability of a given host species. Here, we investigate the generalist nature of three common ectoparasites (the dipteran *Carnus hemapterus* and two species of louse flies, *Pseudolynchia canariensis* and *Ornithophila metallica*), exploiting two avian host species (the European roller *Coracias garrulus* and the Rock pigeon *Columba livia*), that frequently occupy the same breeding sites. We explore the prevalence and abundance of both the infective and the puparial stages of the ectoparasites in both host species. Strong preferences of *Pseudolynchia canariensis* for pigeons and of *Carnus hemapterus* for rollers were found. Moderate prevalence of *Ornithophila metallica* was found in rollers but this louse fly avoided pigeons. In some cases, the infestation patterns observed for imagoes and puparia were consistent whereas in other cases host preferences inferred from imagoes differed from the ones suggested by puparia. We propose that the adult stages of these ectoparasites are more specialist than reported and that the requirements of non-infective stages can restrict the effective host range of some parasites.

Introduction

Host range is a key element of parasites' ecology and evolution (Appelgren et al. 2016). According to host range, parasites (and other organisms such as herbivores or parasitoids) are usually classified as generalists or specialists (see, for instance, Barrett and Heil 2012, McCoy et al. 2013, Loxdale and Harvey 2016), even though such categories are vague and are currently under review (Jorge et al. 2014, Loxdale and Harvey 2016). Parasites success depends on both host profitability (in terms of resource acquisition) and the microenvironment provided by the host, which together define host–parasite compatibility and can differ between hosts (Lemoine et al. 2011). Therefore, the breadth of environments/hosts in which a parasite species can succeed is ultimately determined by the full pattern of its vital rates in each environment/host, including all the life stages (egg, larva, pupa and imago) (Caswell 1983).

Animals are expected to select resources according to their impact on fitness (Brodeur et al. 1998, Krasnov et al. 2004). However, an imperfect concordance between host selection and insect fitness has been frequently reported for phytophagous and parasitic insects (Thompson 1988, Courtney and Kibota 1990, Horner and Abrahamson 1992, Caron et al. 2010). This disagreement can arise from a variety of determinants. For instance, among parasites, host availability plays a key role, which depends on host densities but also on parasites' ability for finding a host (Kortet et al. 2010, McCoy et al. 2013). Increasing the range of hosts (e.g. by ecological fitting, Agosta et al. 2010, Araujo et al. 2015) could increase the chances of survival, but the new hosts could be suboptimal since the real host range will be determined by the fitness the parasite gets in each of the hosts (Ward et al. 1998). Another reason for an imperfect concordance between host selection and parasites' fitness is the inability of the latter to assess host suitability (reviewed by Fox and Lalonde 1993), that can occur, among other reasons, by the fact that different life cycle stages (e.g. larval, puparial or imaginal stages in insects) have different levels of specialization (Loxdale and Harvey 2016). Our knowledge on the requirements of the non-infective phases of many parasites has increased substantially. Yet, more research is needed since integration of the ecology of all life stages of parasites is necessary for a better understanding of the epidemiology of parasitic diseases (e.g. Pietrock and Marcogliese 2003, O'Connor et al. 2006).

Here, we examine host choice by three allegedly generalist avian, nest-based ectoparasites, the dipteran *Carnus hemapterus* and two species of louse flies (Family *Hippoboscidae*), exploiting two avian host species, the European roller *Coracias garrulus* and the Rock pigeon *Columba livia*. *Carnus hemapterus* is a widespread bird parasite in the Holarctic and Nearctic (Grimaldi 1997, Brake 2011). Hippoboscid flies (*Hippoboscidae*) are worldwide distributed, obligatory parasites attacking a wide variety of bird species (Boyd 1951, Maa 1969). Whereas the imagoes of both species feed on birds, the non-parasitic stages of their life cycle dwell in birds' nests. The European roller and the Rock pigeon are secondary hole-nesting birds whose nesting environments are ecologically similar but that, otherwise, differ in several key life-history traits (migration, breeding phenology, clutch size, composition of the nest material), that may impose divergent selective pressures on parasites.

We hypothesize that host selection by the infective phase of the parasites is correlated with the suitability of the host and its environment for the development of the whole life cycle of the parasite. We predict that all stages of the parasites should perform better on the host where imagoes (the choosing stage) reach the higher prevalence and abundance. If so, such estimates of parasitization will be good indicators of host-parasite compatibility and can be used for defining host range. Alternatively, prevalence and abundance of the imago in a given host will not correlate with prevalence and abundance of other stages of the parasite in the same host, so that imago's selection will not be a good indicator of host suitability and parasites' host range. To test this hypothesis, we evaluated during two years the parasitization of *Carnus hemapterus*, *Pseudolynchia canariensis* and *Ornithophila metallica* on two different avian hosts and estimated puparial abundance in the nests as a surrogate of host–parasite compatibility during the non-infective stage.

Materials and methods

Study species and study area

The study area (around 50 km^2) is located in the Desert of Tabernas (Almería, S.E. Spain, 37°05′N, 2°21'W). The landscape mostly consists of open shrubland with olive and almond groves interspersed among numerous dry riverbeds with steep sandstone banks – ramblas. The climate is temperate, semiarid Mediterranean with strong water deficit during the long, hot summer months. The mean annual rainfall is ca. 230 mm, with high inter-annual and intra-annual variability (Lázaro et al. 2001). The average temperature is 18 °C, with mild inter-annual oscillations of 3–4 °C and significant intra-annual fluctuations (Lázaro et al. 2004).

Carnus hemapterus (hereafter *Carnus*) is a 2-mm long, nidicolous ectoparasitic fly that colonizes nestling birds of several dozens of species (Grimaldi 1997, Brake 2011). Its life cycle comprises an adult phase, three larval stages and a puparial phase (Guiguen et al. 1983). The puparia are found in the detritus of the nests of the host species. The imagoes (the infective stage) emerge from the puparia after winter diapause and throughout the spring when nestling hosts are available (Valera et al. 2003, Calero-Torralbo et al. 2013). After their emergence, adults are initially winged but lose their wings as soon as they locate a suitable host (Roulin 1998). Once emerged, adult *Carnus* cannot survive a long time without feeding and its dispersal period is seemingly short (less than 4 days; Calero-Torralbo 2011, Veiga et al. 2018). Mating occurs on the host and eggs are laid in the nest. Larvae are saprophagous and perform two moults (Papp 1998). After the third larval stage, the pupa enters into diapause. In most cases, imagoes emerge the next breeding season. However, prolonged diapause has been recorded for this parasite, so that some pupae remain longer in diapause and adult flies emerge after two or more wintering seasons, therefore enabling *Carnus* to persist in the nest for several years (Valera et al. 2006). This haematophagous parasite (Kirkpatrick and Colvin 1989, Dawson and Bortolotti 1997) can have detrimental effects on nestling health (Whitworth 1976, Cannings 1986, Soler et al. 1999, but see Kirkpatrick and Colvin 1989, Dawson and Bortolotti 1997, Liker et al. 2001).

Hippoboscid flies are hematophagous ectoparasites. More than 200 species are recognized, most of them parasitize birds belonging to 18 orders (Maa 1969, Lloyd 2002, Lehane 2005). Imagoes spend most of their time on the body of the bird, where they feed on blood several times a day (Coatney 1931). Hippoboscids attack more juvenile than adult birds and imagoes die usually within two or three days when removed from the host (Boyd 1951). Larval development occurs almost entirely within the female. The pupa is formed almost immediately after laying, which occurs in the nest of birds. The insects apparently overwinter as puparia in the hosts' nests (Boyd 1951). With the exception of the larval and puparial phase, its dependence on the birds' nest is lower than in the case of *Carnus* since adult flies do not lose the wings and are capable of flying between hosts (Harbison et al. 2009, Harbison and Clayton 2011). Hippoboscids cause direct and indirect threats to the health and fitness of their hosts (Waite et al. 2012). In our study area, we have identified two species of hippoboscid parasites on birds (*Pseudolynchia canariensis* and *Ornithophila metallica*). *Pseudolynchia canariensis* (hereafter *Pseudolynchia*) parasitizes mainly pigeons, but it has a wider host range than closely related species and has been described attacking several dozens of bird species (Maa 1966, 1969). Adults copulate on the host. Eggs hatch in utero in the female fly, and then three stages of larvae feed from 'milk' glands in the female fly (Harwood and James 1979). The larvae pupate and female flies deposit puparia in the substrate in or around pigeon nests (Bishopp 1929). Pupal development is sensitive to temperature and can span 36–55 days (Klei and Degiusti 1975, Mandal 1989). The female produces on average eight pupae during its lifetime, which is on average 24 days under laboratory conditions (range 6–70 days) (Klei and Degiusti 1975). *Ornithophila metallica* (hereafter *Ornithophila*) is a poorly known species. It has been described parasitizing a variety of bird species, including several species of the families *Columbidae* and Coraciidae (Maa 1969).

The European roller (hereafter roller) is a secondary cavity-nesting bird. It is a trans-Saharan migrant that arrives into south Spain during April. In our study area, the nest is a slight depression at the sandy bottom of cavities in cliffs or in the nest boxes. They lay a single clutch of two to seven eggs. Nestlings are naked at hatching but, by the age of 13 days, their body is almost completely covered with closed feather sheaths (Cramp 1998). Juveniles fly from the nest about 20–22 days after hatching (Václav et al. 2008). Rollers do not expel their faeces from the nest cavity (Sosnowski and Chmielewski 1996), where detritus can accumulate after several breeding seasons, even though nest sanitation behaviour is common.

Rock pigeons (hereafter pigeons) also use natural cavities and human constructions to breed but not nest boxes. This resident species breeds at any time of the year, but peak times in our study area are spring and summer. The nest is a light platform of straw and sticks, laid under cover. Pigeons lay two eggs and incubation lasts around 18 days (Johnston and Janiga 1995). The newly hatched nestlings have pale yellow down and a flesh-coloured bill. For the first few days, the nestlings are fed exclusively on ‘crop milk’. The fledging period is about 30 days (Johnston and Janiga 1995). Droppings accumulate in the nest cavity that usually is filled becoming unsuitable for breeding after several nesting events.

The distribution of roller and pigeon nests along the study area is patchy and breeding patches can be defined according to distinct geomorphological units (Václav et al. 2011): (1) ramblas, with nest boxes for rollers and with natural cavities occupied by rollers, pigeons and other cavity-nesting bird species; (2) individual bridges with numerous, densely spaced cavities (ca. 2–3 m apart), suitable for rollers, pigeons and other birdspecies and (3) spatial aggregations of suitable nesting places – mostly trees with nest boxes, but also small sandstone banks with natural cavities and isolated country houses with cavities. Rollers, pigeons and other cavity-nesting species (mostly Common kestrel *Falco tinnunculus*, Little owl *Athene noctua*, Eurasian jackdaw *Corvus monedula*) co-occur more frequently along ramblas and bridges. Moreover, cavities in sandy cliffs and in bridges or abandoned country houses are frequently used successively (both within the season and among seasons) by these bird species.

Ectoparasite estimation in birds

Fieldwork was carried out in 2016 and 2017. Clean nest boxes provided with unsoiled sand were installed at the beginning of the 2016 and 2017 breeding season for roller reproduction. Cavities in sandy cliffs and in human constructions were examined in search of breeding pigeons. Occupied nest boxes and cavities were monitored along the breeding seasons.

The prevalence and abundance of *Carnus* imagoes in 251 nestling rollers (32 nests in 2016 and 38 in 2017) and 35 nestling pigeons (9 nests in 2016 and 10 nests in 2017) were determined by examining chicks at the mid-nestling stage (i.e. when they are covered by closed feather sheaths), when the peak of parasite infestation occurs (Václav et al. 2008). Roller and pigeon broods were carefully taken from the nest and placed in a cotton bag. Subsequently, each nestling was taken and the number of carnid flies on the body surface of each chick was counted twice and then we averaged both counts. This visual census method has been found to be reliable (Roulin 1998, Václav et al. 2008).

The prevalence and abundance of hippoboscid flies in 251 nestling rollers (32 nests in 2016 and 38 in 2017) and 42 nestling pigeons (10 nests in 2016 and 13 nests in 2017) were determined in each nest coinciding with the estimation of carnid flies following the same method (i.e. search of flies on body surface and between sheaths). Nonetheless, since the hippoboscid flies are much more mobile than *Carnus*, quickly leaving the bird when manipulated, underestimation of the actual parasite load is possible. Therefore, we took advantage of successive monitoring of the nests for other purposes and checked the abundance of louse flies on nestlings several times. We used the maximum number of flies observed in each nest for the calculation of prevalence and abundance of these parasites. Imagoes collected from both bird species as well as flies emerging from collected nest detritus (see below) were used for identification purposes.

Sampling nest detritus

During October–November 2016 and July 2017 nest boxes and cavities occupied by the study bird species during the previous breeding seasons were sampled (2016: roller: 32 nest boxes, pigeon: 26 cavities; 2017: roller: 38 nest boxes, pigeon: 10 cavities).

Nest material from rollers' nests (consisting essentially of sand, excrements and insect remnants) was collected by hand. Pigeons' nests, which consisted on sticks used to make the nest, and a compact mass of excrements that usually included organic remains like vegetable matter, shells and remains of dead nestlings, were completely removed.

The collected material was stored in plastic bags that were transferred to the Estación Experimental de Zonas Áridas where it was kept in a dark room with open windows to resemble natural conditions (i.e. ambient temperature moderated by partial enclosure and semi-darkness). The samples collected in 2016 were stored for 2–7 months until processing, whereas the ones collected in 2017 were stored for about 2 months.

Nest material treatment

Searching for Carnus hemapterus *puparia*

A sample of nest material of each roller and pigeon nests was sieved using a column of 4, 1 and 0.5 mm sieves. Material ⩽1 mm was collected and two subsamples of 5 g per nest were selected. During January–March 2017 (for samples from 2016) and July–August 2017 (for samples from 2017) such subsamples were visually examined with a binocular loupe Nikon SMZ645 in search of *Carnus* puparia, that were identified following Papp (1998) and Valera et al. (2018). We distinguished between apparently viable puparia (intact, closed puparia) and open puparia. Intact puparia were stored in transparent tubes.

For the calculation of the prevalence and abundance of *Carnus* puparia in pigeons and rollers nests, only viable puparia were considered. Counts of each subsample of 5 g per nest were averaged.

Searching for Hippoboscidae *puparia*

A second sample of nest material of each roller (except for 2017, for which only 14 nests were examined) and pigeon nests was sieved using a column of 4 and 1 mm sieves. Material collected in the second sieve was retained. In 2016, 100 g of such material was selected for rollers and pigeons, even though for this second species we could not get such amount in all nests (range 42– 100 g). In 2017, the amount of nest material scanned in search of Hippoboscidade pupae ranged 16–42 g for rollers and 42– 310 g for pigeons. During March–May 2017 (for samples from 2016) and August 2017 (for samples from 2017), the selected material was extended in a tray and puparia were sought visually. We distinguished between apparently viable puparia (intact, closed puparia) and open puparia. Only intact puparia (that were stored in transparent tubes) were used to calculate prevalence and abundance and values were extrapolated to 5 g in both years.

We found just one type of puparium that was identified as *Pseudolynchia canariensis* (following Hutson 1984) after the emergence of the corresponding imagoes from the puparia individually stored in plastic tubes.

In both years, nest detritus from rollers and pigeons not examined in search of pupae was also stored during the autumn–winter and scanned subsequently until next spring in search of emerged flies. In this way, we got some hippoboscid flies from pigeon detritus that were identified.

Statistical analyses

Prevalence (proportion of infected nests among all the nests examined) and mean intensity of imagoes and puparia of each parasite species (mean number of individuals found in the infected nests) and their respective 95% confidence intervals were calculated. Fisher's exact tests and bootstrap 2-sample *t*-tests were used for comparing prevalences and mean intensities, respectively; 2000 replications were used for estimation of confidence intervals and bootstrap *t*-tests. Unless otherwise noted, average values and standard errors are given and the tests performed are 2-tailed. Statistical significance was set at $P < 0.05$.

Statistical tests were done with the program Quantitative Parasitology 3.0 (Reiczigel and Rózsa 2005) and Statistica Academic 13 (Dell Inc. 2016).

Results

Occurrence of infective and non-infective phases of the parasites in two host species

Prevalence of imagoes and puparia of each parasite in each host species did not differ between years for any of the parasite species (Fisher tests, $P > 0.20$ in all cases), so that data from both years were pooled for each parasite. Similarly, no inter-annual differences in the mean intensity of imagoes and puparia per infected nest were found except for *Pseudolynchia* puparia in pigeon (see below). Thus, data for both years are shown separately only for this case.

Carnus hemapterus

The prevalence and intensity of imagoes of *Carnus* in rollers' nests are high. Correspondingly, the prevalence and intensity of puparia are also high (Table 1).

Pigeons seem to be less attractive than rollers for *Carnus*, given that both the prevalence and intensity of imagoes per infected nest are significantly lower (prevalence, Fisher test: $P < 0.001$, intensity: bootstrap 2-sample *t*-test: $t = 6.8$, $P < 0.001$, $n = 19, 70$). Importantly, the prevalence of carnid puparia in pigeon nests is more than nine times smaller than the prevalence of *Carnus* imagoes in nestling pigeons and a single puparium was found in samples of 36 nests (Table 1).

Ornithophila metallica

The prevalence of imagoes in rollers is ca. 17% and we found a mean intensity of one fly per infected nest. In contrast, we did not find a single puparium in samples from 46 roller nests (Table 1).

Pigeons were not infected by *Ornithophila metallica*: neither imagoes nor puparia were found in nestling pigeons and nests (Table 1).

Pseudolynchia canariensis

No imago or puparium were found in nestling rollers or nests (Table 1). In contrast, the prevalence of imagoes on nestling pigeons was high as it was the intensity of imagoes (mean 2.65 flies per nest, range 1–6). We also found that at least 36% of the nests harboured *Pseudolynchia* puparia. The intensity of puparia in pigeon nests varied significantly between years (bootstrap *t*-test: *t* = 2.6, *P* = 0.04, *n* = 10, 3) (Table 1).

		Carnus hemapterus		***Ornithophila metallica***		***Pseudolynchia canariensis***	
Host		**Imagoes/nest**	**Pupae/5 g detritus**	**Imagoes/nest**	**Pupae/5 g detritus**	**Imagoes/nest**	**Pupae/5 g detritus**
Roller	**Prevalence**	88.6% [62/70]	64.3% [45/70]	17.1% [12/70]	0.0% [0/46]	0.0% [0/70]	0.0% [0/46]
		78.7–94.9	51.9–75.3	9.1–28.0			
	Mean intensity	20.87	10.51	1.0	0.0	0	0
		16.45–27.19	8.02–14.0	0–0			
Pigeon	**Prevalence**	26.3% [5/19]	2.8% [1/36]	0.0% [0/23]	0.0% [0/36]	73.9% [17/23]	36.1% [13/36]
		9.1–51.2	0.07–14.5			51.6–89.8	20.8–53.7
	Mean intensity	2.20	1.0	0.0	0.0	2.65	2016: 0.19 (0.11–0.29)
		1.20–3.20	0–0			1.76–3.65	2017: 0.05 (0.03–0.07)

Table 1. Prevalence and mean intensity (with 95% CIs and number of nests sampled in square brackets) of imagoes and pupae of three ectoparasitic flies, Carnus hemapterus*,* Ornithophila metallica *and* Pseudolynchia canariensis*, on nests of two bird species, the European roller and the Rock Pigeon (data from 2016 and 2017 pooled except for the intensity of pupae of* P. canariensis *in pigeon nests)*

Discussion

Here, we provide information about the parasitization of three allegedly generalist ectoparasitic flies on two secondary hole-nesting bird species whose nesting environments are ecologically similar. Whereas these parasitic flies are widely distributed we were unable to find detailed information about their parasitic load on our study species or on other bird species (see below). Data on puparia in the nests are even scanter so that comparisons are done only when information was found. Thus, our data contribute to a better knowledge of the epidemiology of these common parasites. Moreover, we compared the parasitization pattern of these ectoparasitic flies considering both the prevalence and abundance of the infective, imaginal stage and the puparial stage on both bird species. In some cases (e.g. for *Carnus* in rollers, for *Pseudolynchia* in pigeons and rollers and for *Ornithophila* in pigeons), the pattern observed for imagoes and puparia was consistent whereas in other cases (e.g. *Carnus* in pigeons and *Ornithophila* in rollers) host preferences inferred from imagoes differed from the ones suggested by puparia.

All three parasite species have been frequently quoted as generalist ones. *Carnus* has been reported parasitizing 64 host species (including the roller and the pigeon) from 24 avian families from raptors to passerines (Grimaldi 1997, Brake 2011 and references therein). Similarly, although *Pseudolynchia canariensis* shows preference for Columbiformes, it has been described attacking many other bird species, including the genus *Coracias* (Maa 1966, 1969). Klei and Degiusti (1975) and references therein report lack of host specificity in laboratory colonies. *Ornithophila metallica* was classified by Maa (1969) in the group of louse flies with a very wide host range, citing this parasite species in 134 bird genera, including the genus *Coracias* and two *Columbidae*. In our study area, rollers and pigeons commonly breed interspersed, frequently at short distances from each other and even using successively the same cavities. So, detection of each parasite in both bird species would be expected. Yet, our results suggest strong host preferences and rejections. Considering the parasitic stage we found that: (i) *Carnus* prefers rollers over pigeons. The high prevalence and parasitic load of imagoes in nestling rollers found in this study agree with previous information (Václav et al. 2008, see also Soltész et al. 2018 for other species). We were unable to compare our results on pigeons since, to our knowledge, there is no published information; (ii) adult *Pseudolynchia* flies were frequently found on nestling pigeons but never on nestling rollers. Pigeons are known to be a preferred host of this louse fly, and the load of adult flies per nest in our study area is within the range reported for the species (Maa 1966, Adang et al. 2009, but see Amaral et al. 2013 for a higher load). Concerning rollers, we could find only a record of a *Coracias* sp. parasitized by *P. canariensis* (Maa 1966); (iii) adult *Ornithophila* flies were never recorded in pigeons but they were found parasitizing nestling rollers in ca. 20% of nests. Again, comparisons of our results are limited by the scant data available. These results therefore suggest that the low host specificity reported for these flies cannot be generalized.

In four out of six study cases (three parasites and two hosts) the information provided by the prevalence and abundance of puparia of each parasite in each host nest agrees with the one obtained from imagoes on nestling hosts: (i) parallel to imagoes, *Carnus* puparia are abundant in rollers nests (see also Valera et al. 2018); (ii) the occurrence of *Pseudolynchia* puparia in pigeon nests is compatible with the occurrence of imagoes in nestling pigeons; (iii) the absence of *Pseudolynchia* imagoes on nestling rollers agrees with the nil abundance of puparia in rollers nests; and (iv) similarly, the absence of *Ornithophila* imagoes on nestling pigeons matches with the absence of puparia in the nests. In these cases, clear and consistent preference/rejection criteria can be deduced.

In contrast, for two other systems, we found that host choice by the imago did not correspond with the occurrence of the puparial, non-parasitic stage in the host' nest. Carnid flies showed a moderate prevalence in pigeon nests (26%) whereas the occurrence (both prevalence and abundance) of puparia in the nests is very low. Pigeons often nest in cavities previously occupied by other birds, most commonly rollers that usually contain diapausing carnid puparia. Therefore, parasitization of the nestling pigeons by *Carnus* is very likely the result of the use of cavities infected with diapausing puparia (i.e. involuntary host shifting, see Calero-Torralbo and Valera 2008). Since the amount of puparia in rollers nests can be very high (e.g. here we found ca. 10 puparia/5 g and more than 0.5 kg of detritus can accumulate in a roller nest during a breeding season) and the mean intensity of adult flies in nestling pigeons is very low, we suspect that freshly emerged flies in pigeon nests migrate in search of other host species and that nestling pigeons are, in fact, rejected by *Carnus*. Similarly, *Ornithophila* flies were relatively common in roller nests and the parasitic load found (1 fly/nest) is probably underestimated (Maa 1969 reports that the highest density per infested bird was three flies). However, no puparium was found in any nest during two breeding seasons.

It could be argued that our sampling effort has not been intense enough to detect parasites in some cases. However, we think that our results are reliable because: (i) the number of sampled nests and nestlings of both species is appropriate and the results for both breeding seasons are consistent in nearly all cases; (ii) subsequent monitoring of the nestlings of both species for other purposes did not render different results; and (iii) we did not find *Carnus*, *Ornithophila* or *Pseudolynchia* imagoes emerging from non-monitored, stored detritus of pigeon and roller nests, respectively whereas we did record emergence of *Pseudolynchia* from stored pigeon nest detritus.

The cases where host suitability deduced from the occurrence of the infective and non-infective phases differs suggest that host compatibility filters occur at the later stage of the parasite. Pigeon nests does not seem a suitable environment for *Carnus* because, in contrast to rollers nests: (i) organic material (e.g. insect remains) is scarce in the nest so that food for the saprophagous larvae is probably scant, (ii) the nest substratum is probably adverse for *Carnus* eggs, larvae and puparia. Dung of nestling pigeons acts to cement the nesting material together into a sturdy adobe-like mound that has also been reported to inhibit the development of some ectoparasites (Johnston and Janiga 1995). Thus, the tiny eggs and larvae of *Carnus* can easily get embedded in the faeces of pigeons. In this case, adult *Carnus* flies are probably physiologically able to feed on nestling pigeons but parasite fitness is negatively affected given that the nest may jeopardize egg, larval and/or puparia survival. Rejection of pigeon as hosts by adult carnid flies (suggested by the very low load) is consistent with the unsuitability of this species for other life stages of the parasite. The misleading prevalence of adult flies on pigeons should be interpreted as an indirect consequence of other ecological pressures (nesting behaviour of pigeons when nest sites are limiting, Václav et al. 2008).

Concerning *Ornithophila*, we ignore the reasons why puparia are absent in roller nests. We do not think that the reasons given for *Carnus* in pigeon nests also hold for *Ornithophila* since louse flies lay their pupae in crevices and under layers of nest material (pers. obs. on *Pseudolynchia*, see also Waite et al. 2012).

Temperature is known to play an important role in puparial development of *Pseudolynchia canariensis* (Klei and Degiusti 1975, Mandal 1989) and it could also be the case for *Ornithophila*. Since the insulation ability of nest boxes is poor, with oscillations above 30 °C within one day occurring frequently in our study area (Amat-Valero et al. 2014), it could be that artificial breeding places such as nest boxes are unsuitable for development of louse flies. Interestingly, an exhaustive study of dipteran assemblages in nests boxes used by different bird species did not record hippoboscid flies (Soltész et al. 2018). Alternatively, predation could account for the absence of puparia in the nests. Kaunisto et al. (2016) found remarkable predation rates of deer ked (*Lipoptena cervi*) puparia presumably by lizards, spiders, harvestmen (Opiliones) and Formicinae-ants. This could also be the case for *Ornithophila* puparia since ants are frequently found in roller nests. More research is necessary to highlight the requirements of *Ornithophila* and the likely filters imposed by its host species and/or their close environment.

The current debate about the terms generalist and specialist warns about several flaws such as the ambiguous definition of the term or the problem raised by the abundance of cryptic species in many taxa (Loxdale and Harvey 2016). Our study suggests that the adult stages of these allegedly generalist parasites are more specialist than reported. We also suggest that the host range can differ among different phases of a parasite and that the requirements of some stages can be particularly restrictive (see also Dapporto and Dennis 2013). Thus, it is not only that simple species records are not enough to determine whether a parasite is a true host generalist (McCoy et al. 2013) but also that different phases of the parasite should be considered to define an organism selective environment.

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Chapter 3: Off-host longevity of the winged dispersal stage of *Carnus hemapterus* (Insecta: Diptera) modulated by gender, body size and food provisioning

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Abstract

Highlighting the dispersal ecology of parasites is important for understanding epidemiological, demographic and coevolutionary aspects of host–parasite interactions. Yet, critical aspects of the dispersal stage of parasites, such as longevity and the factors influencing it, are poorly known. Here we study the lifespan of the dispersal stage of an ectoparasitic dipteran, *Carnus hemapterus*, and the impact of gender, body size and food provisioning on longevity. We found that freshly emerged imagoes survive at most less than 4 days. Longevity increased with body size and, since this parasite exhibits sexual size dimorphism, the bigger females lived longer than males. However, controlling for body size suggests that males lived relatively longer than females. Furthermore, a humid environment and food provisioning (flowers) significantly increased individual life spans. We discuss the relative importance of spatial and temporal dispersal in relation to the infectious potential of this parasite.

Introduction

Dispersal is perhaps the most dangerous period in the life of most parasites (Ward et al. 1998) and a key process influencing the population dynamics and coevolution of host–parasite interactions (Hassell 2000, McCoy et al. 2003). Yet, little information is available on dispersal and on the factors that affect it. The lack of basic information has to do with the difficulties associated with the observation and experimental approaches during the highly mobile phase of parasites, which hampers further advance.

Opportunities for successful parasite dispersal are closely linked to host biology (e.g. foraging habits of the host – Harper et al. 1992 –, host density or colony size – Brown and Brown 1996, 2004, Kleindorfer and Dudaniec 2009 –) but they also depend on inherent characteristics of the parasite. The dispersal ability of arthropods is determined largely by morphology. Thus, for the insects that have evolved wings, the distance of dispersal is related to their flight ability. For instance, within the Diptera, the minute and delicate wings of midges and gnats make long-distance dispersal less likely than for strong fliers like horse flies or blowflies (Botzler and Brown 2014). The ability of the parasite to survive longer periods off the host also affects its dispersal opportunities. Hence, some arthropods, such as astigmatid mites, survive a short time off their hosts while others (e.g. ticks, fleas) are able to survive for long periods between meals (Botzler and Brown 2014). Size is also important for both survival and dispersal ability. Trade-offs between insect body size and life history parameters such as lifespan have been demonstrated (Roff 1993).

To know the lifespan of parasites during their dispersal stages as well as factors affecting longevity (e.g. food provisioning, temperature) during this phase is critical for evaluation of their dispersal ability. For instance, it is known that for many adult insects, including several mosquito species, carbohydrate-rich food is an important source of energy for longevity, fecundity and mobility (Clements 1955, Briegel and Horler 1993, Winkler et al. 2006). Moreover, whether insects feed or not during the adult phase is related to other critical traits such as size. In insects that do not feed as adults, a large size often contributes positively both to fecundity and longevity (Nilssen 1997). However, in species that feed as adults, there may be a selection towards small male body size due to energy constraints or as a response to host defences (Blanckenhorn et al. 1995, Clayton et al. 1999, and references therein).

Here we investigate the lifespan of the free-living stage of the nidicolous, non-contact-transmitted ectoparasitic fly, *Carnus hemapterus*. *Carnus* is a widespread bird parasite (Grimaldi 1997, Brake 2011) whose entire cycle, except for dispersal, takes place in its host's nest, where adult flies feed mainly on nestling birds. Pupae overwinter in the nests and after diapause nymphs emerge at the time after nest sites are reoccupied by birds, thus allowing the perpetuation of the parasite in the nest (unless the nest is not occupied by any host). Adult flies are initially winged but lose their wings as soon as they locate a suitable host (Roulin 1998), therefore limiting their dispersal ability from then onwards. Thus *Carnus hemapterus* flies are not transmitted by the host (Grimaldi 1997) but rather colonize host nests actively during the winged phase (Grimaldi 1997, Roulin 1998, 1999). To our knowledge, there is no information about the dispersal stage of this parasite, which is a critical episode to understand its infectious potential, nor about the factors that can affect its lifespan during this period.

In this work, our first aim is to analyse whether adult *Carnus hemapterus* is sexually dimorphic. If there are size differences between sexes, we would expect a positive relationship between size and longevity (Hasson et al. 1993, Sivinski 1993, Chen et al. 2005). Furthermore, we experimentally investigate whether *Carnus hemapterus* lifespan depends on some abiotic factors such as humidity and/or on the availability of feeding resources. It has been demonstrated that higher environmental humidity increases survival in some insects (e.g. Mellanby 1932, Tochen et al. 2015), or the other way round, drier environments may reduce longevity due to desiccation. Moreover, plants represent a source of food for many insects, mainly those looking for the high energy sugary substances. Floral volatiles have been argued to inform insects about the potential energy gain to be obtained from a flower (Wright and Schiestl 2009). In our experiments, we would thus expect adult *Carnus hemapterus* flies (i) living longer in a humid than in a drier environment, and (ii) live longer when also food is available. The results of our study provide insights into some of the factors influencing longevity in this parasite during the dispersal stage and contribute to the determination of its colonization abilities.

Material and methods

Study species

Carnus hemapterus (hereafter *Carnus*) is a 2-mm long, highly mobile ectoparasitic fly that colonizes nestling birds (Grimaldi 1997, Brake 2011). Its life cycle comprises an adult (parasitic) stage, three larval phases and a pupal phase (Guiguen et al. 1983). Diapausing pupae are found in the nests of the host species. After a diapause usually lasting several months (Guiguen et al. 1983) imagoes, initially winged, emerge when host nestlings are available. Emergence continues throughout the whole nestling period (Valera et al. 2003). Emerged flies can stay in the nest if occupied by a suitable host. Otherwise, flies are forced to disperse. Once a suitable host is located, adult flies lose their wings and feed on blood, epidermal cells and skin secretions of their host (Kirkpatrick and Colvin 1989, Papp 1998). This parasite prefers bird species nesting in sheltered areas or cavities and has never been found parasitizing ground or swamp nesters (Grimaldi 1997, Papp 1998).

Flies sampling and experimental design

Nest material (including diapausing pupae) from eight European roller (*Coracias garrulus*) nest boxes located at the Desert of Tabernas (Almería, SE Spain, 37°05′N, 2°21′W) was collected between 4th February 2016 and 14th February 2016. The samples were kept in transparent plastic bags and moved to the Estación Experimental de Zonas Áridas (EEZA, Almería, 36°50′N 02° 28′W) after collection. They were stored at room temperature until emergence started (mid-April). Then, plastic bags were opened and the material spread in a tray so that emerged carnid flies could fly away. After half an hour, we turned over the material to assure that no adults were left, put the nest material back into the plastic bag, closed it and waited for 1 h. After that, the emerged flies in the bag were collected and then assigned to the different experimental trials (see below). In this way, we were sure that the adult individuals we used in trials had emerged, at most, 1 h prior the experiment. This procedure was repeated several times over the experimental period (between 18th April and 2nd May 2016) for the collection of experimental flies.

To determine lifespan of adult *Carnus* flies during their free-living stage and the likely effect of both humidity and food provisioning on it, we performed an indoor experiment with the following three treatments:

(i) An untreated control. Adult flies were introduced in glass jars (one per jar; volume = 1000 cc) into which a dry sponge with a plastic flower (8 cm tall) simulating a wild plant was added. During the experiment, the jars were closed to avoid flies to fly off. This situation represents a dry environment with no food available (hereafter control treatment).

(ii) Experimental environment 1. The same experimental set up was used but, in this case, the sponge was maintained wet for the whole experiment. This represents a wet environment with no food available (hereafter moist treatment).

(iii) Experimental environment 2. The same device than in the experimental environment 1 was used, but an 8 cm flowered branch of *Cytisus scoparius* (Fabaceae) was used instead of the plastic flower. All branches used had two flowers. Flower blossoms were collected during the naturally flowering period of the species in our study area. Branches were inserted in the wet sponge and kept turgid during the whole experiment. Adult *Carnus* flies have been found on flowers of some plant species (Papp 1998) and adult individuals of the closely related *Hemeromyia anthracina* have been swept from flowering *Retama raetam* (Fabaceae) in Israel (Freidberg, pers. comm.). Thus, we assume that in this treatment food is available for adult flies, being labelled hereafter moist-food treatment.

Once one individual was introduced into the jar, it was monitored until it died. Previous observations (Calero-Torralbo 2011) and the results of a pilot study with the method described above showed that the life expectancy of carnid flies was at least 24 h. Therefore, the first control to check survival was 24 h after the start of each experiment. Then, each jar was revisited every 4 h (day and night) and we recorded whether the flies were dead or alive.

The experiments were done at room temperature (range: 17– 22 °C).

Overall, 55 flies were tested, 16 under the control treatment (seven males and nine females), 20 were assigned to the moist treatment (11 males and nine females) and 19 flies (nine males and 10 females) to the moist-food treatment. Flies entering the experiment were sexed according to their genitalia (Grimaldi 1997, Papp 1998) and measured after death to avoid that manipulation prior to the experiment could harm the individuals. We also sexed and measured nine flies not used for the experiment. As estimators of body size, maximum length and maximum width of the thorax of each fly was measured (Valera and Zídková 2012) with the aid of a stereo-microscope (Leica, MZ125) fitted with an ocular micrometer. Two measurements of each variable were taken in ten individuals for assessing the repeatability of our measures (Lessells and Boag 1987). Blind measurements of thorax length and width were highly repeatable ($F = 245.2$, $P < 0.001$, $R = 0.99$ and $F = 21.1$, $P < 0.001$, $R = 0.91$ respectively).

Estimating the lifespan

Lifespan was estimated as the number of hours flies were alive. Individuals entering the experiment were at most 1 h old (see above). We considered that death occurred at the midpoint between successive visits. Since the lapse between such visits was 4 h, the error of our estimate of lifespan is, at most, 3 h, thus yielding an accurate measure of longevity.

Statistical analyses

General linear models (GLMs), were used to analyse: (i) sexual size dimorphism: thorax length and width were the dependent variables, gender was a fixed factor and the nest of origin of the flies a random one; (ii) *Carnus* lifespan in relation to the experimental treatments. Lifespan (hours) was the dependent variable whereas treatment (control, moist, moist-food) and gender were fixed factors. To account for the effect of insect body size (covariate) maximum thorax width (or length) was included as a continuous predictor. Results obtained with thorax length and width are similar so that we report here the ones obtained with the latter variable. The nest of origin of the experimental flies was again included as a random factor. Residuals of the models were normally distributed.

Unless otherwise stated, values reported are means ± S.E. Analyses were done with STATISTICA version 13 (Dell Inc. 2016).

Results

Sexual size dimorphism

Carnus hemapterus is a sexually dimorphic species in body size, males being smaller than females. The thorax of male flies was significantly shorter (GLM: adjusted $R^2 = 0.29$, $F_{1,55} = 17.7$, $P < 0.001$; males: 0.52 ± 0.006 mm, $n = 32$; females: 0.58 ± 0.009 mm, $n = 32$) and thinner (GLM: adjusted $R^2 = 0.25$, $F_{1,55} = 15.0$, $P < 0.001$; males: 0.40 ± 0.004 mm, $n = 32$; females: 0.44 ± 0.006 mm, $n = 32$) than that of female flies.

Longevity of the dispersal stage of Carnus hemapterus *and its determinants*

Overall, flies lived on average 54.9 ± 1.8 h ($n = 55$ individuals). The earliest deaths were observed 26 h after emergence (two cases) whereas the two oldest flies lived 82 h. Only six out of 55 flies (10.9%) were alive after 3 days (three males and three females).

Lifespan was significantly explained by the experimental treatment (GLM: adjusted $R^2 = 0.49$, $F_{13,41} = 5.02$, $P < 0.001$; treatment: $F_{2,41} = 12.1$, $P < 0.001$) so that flies in the control group lived shorter than the ones in the two experimental groups (*post hoc* Tukey Honestly Significant Difference test, $P < 0.01$ in both cases, Fig. 1) and flies in the moist treatment lived significantly shorter than those in the moist-food treatment (*post hoc* Tukey Honestly Significant Difference test, $P = 0.024$). Lifespan was significantly and positively related to thorax width ($F_{1,41} = 19.7$, $P < 0.001$).

Gender had a significant effect on lifespan ($F_{1,41} = 4.2$, $P = 0.047$), so that, after controlling for body size, males lived longer than females (Fig. 1).

The interaction between gender and treatment was not significant ($P > 0.50$).

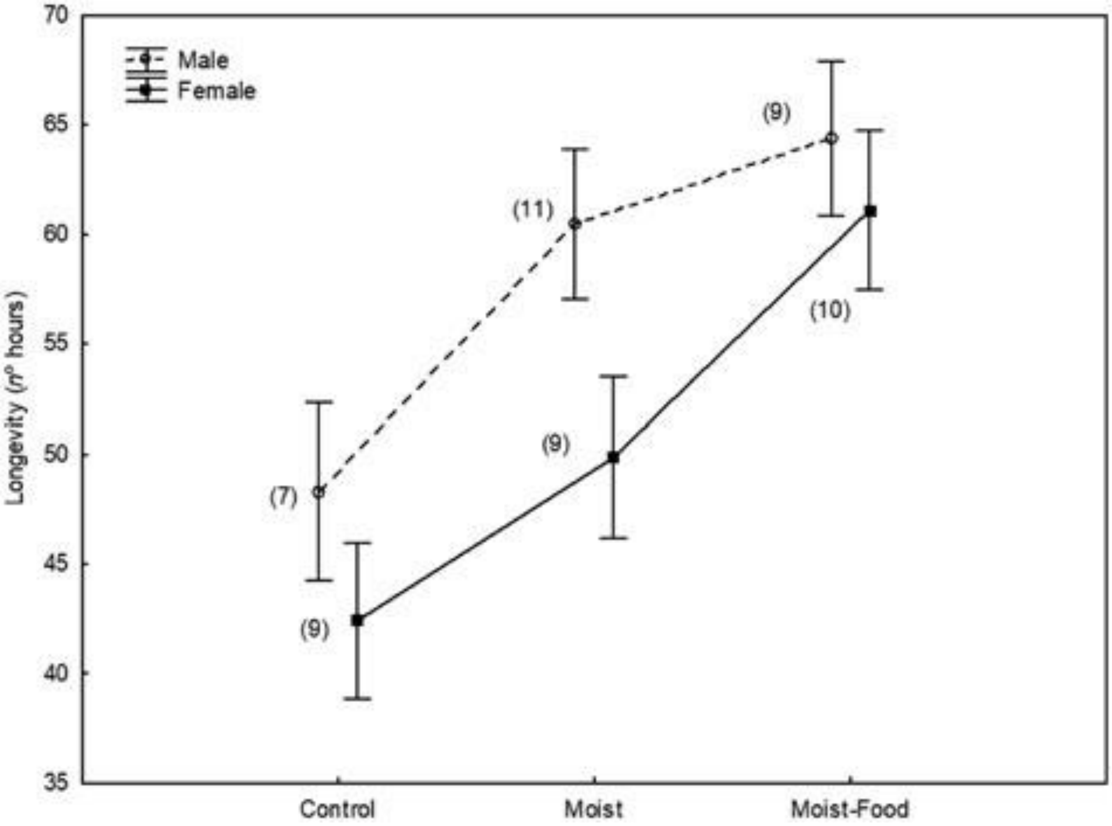

Fig. 1. Lifespan (n° hours) of male and female Carnus hemapterus *flies in three experimental setups (control, with moist environment, with moist environment and food) after controlling for body size (maximum thorax width, covariate means: 0.42). S.E. are shown. Figures in brackets are the number of flies used in each case.*

Discussion

Our study revealed that *Carnus hemapterus* is sexually dimorphic with females being larger than males, which is similar to many other insects (Fairbairn 1997). We also found that the length of the dispersal stage was short, lasting less than 4 days. We could also demonstrate that longevity was influenced by body size (see also Hasson et al. 1993, Sivinski 1993, Chen et al. 2005), so that larger flies namely females lived longer. Yet, statistically controlling for body size, males were longer-lived than females. In sexually dimorphic species, the fitness of the male is typically limited by the quantity of mates acquired while that of females depends more often on the quantity of other resources such as food (Darwin 1871). Thus, while selection may favour larger females due to a generally positive relationship between body size and fecundity in ectotherms (Roff 1993, see Valera and Zídková 2012, for *Carnus*), smaller males may be favoured because their lower energy requirements frees time to be invested in mating (Blanckenhorn et al. 1995). In *Carnus*, the necessity to actively disperse or not is unpredictable (Calero-Torralbo et al. 2013), as it depends on whether the nest is occupied during the following breeding season. If dispersal is not necessary, smaller males may have some advantages (Blanckenhorn et al. 1995, Clayton et al. 1999). In contrast, if the nest is not occupied and dispersal is necessary, larger males with longer lifespan might increase dispersal success. These opposing selection forces suggest that lifespan differences between males and females are not only the by-product of sexual and natural selection on body size and that other factors (e.g. metabolic differences or differences in patterns of resource allocation between males and females, see, for instance, Fox et al. 2003) probably account for sexual differences in lifespan.

Likewise, since small insects are particularly prone to water loss (Tochen et al. 2015, Bujan et al. 2016), thus an increase in longevity with humidity can be expected. We found that the lifespan of flies in the humid treatment increased 22.8% (for males) and 27.7% (for females) in comparison with the one observed for flies in a dry environment. The impact of humidity on the lifespan of adult *Carnus* flies is likely to vary along the broad range of the species, being probably higher in drier latitudes and lower in more mesic ones. Similarly, food provisioning has been described as an influential factor on the longevity of insects, including haematophagous ones (e.g. Foster 1995, Yu et al. 2016) and, as predicted, had a significant effect on the longevity of carnid flies: the lifespan of flies in the humid-food treatment increased between 11 and 19% (for males and females, respectively) in comparison with the lifespan observed in a humid environment without food.

One important question is to what extent our estimation of the longevity of *Carnus* under laboratory conditions resembles the lifespan of dispersing flies in the wild. On one side differences in influential abiotic factors (e.g. temperature, see Taylor 1981, Lessard and Boivin 2013) between field conditions and our experimental set up (with similar mean temperature but lower thermal oscillation) could have an effect on the calculated longevity. Some authors have reported a shorter lifespan of insects under alternating temperature regimes than under constant temperatures (e.g. Carroll and Quiring 1993, but see Cônsoli and Parra 1995). Ambient conditions during the experiment were not strenuous in the wild (maximum and minimum temperature during the period: 26.5 and 5.1 °C, respectively) nor they were at the experimental room so that we do not expect major differences. If so, the real lifespan of carnid flies would be even shorter in nature. This could be the case in parts of the range of the species given its broad distribution along the Holarctic. Thus, we would expect geographical differences in the infectious capacity of the parasite. Studies of the colonization ability of *Carnus* along its range could shed light on the effect of abiotic factors on the epidemiology of this parasite. On the other side, the food plant offered in our experiment could not be the most adequate one. Until now, it was ignored whether carnid flies could feed during their free-living phase. The significant increase in longevity of winged *Carnus* in presence of flowers suggests that this parasite can refuel during dispersal feeding on plant substrates. The plant species used in our experiment is a Fabaceae and species of this family have been reported to host *Hemeromyia anthracina*, a closely related species to *Carnus* (Freidberg, pers. comm.). It is known that the lifespan and dispersal ability of a broad spectrum of dipteran species depends on their diet during adulthood (Clements 1955, Briegel and Horler 1993, Foster 1995, Tochen et al. 2016). Moreover, previous studies have demonstrated that the plants chosen by mosquitoes for their sugar meals are those that maximize survival and fecundity (Manda et al. 2007, Yu et al. 2016). Thus, the lifespan of carnid flies during dispersal could be longer than our results suggest given that they very likely find more appropriate food sources in their environment.

Nonetheless, even if enlarged by fuelling during dispersal, the time carnid flies have for finding and colonizing a host is seemingly brief. Short free-living stages have been described for other parasites. For instance, trematode cercariae possess only limited energy reserves and have to invade their hosts within a short lifespan of a few hours to 3 days (Haas 2003). Adult fig wasps have never been kept alive for more than 48 h (Kjellberg et al. 1988) and most are likely to die the day they emerge unless they manage to find a fig quickly. The spatial dispersal of *Carnus* can also be hampered by its low flight ability. Given that controlling the direction of flight in moving air is problematical for small insects (Dudley 2000, Compton 2002), carnid flies probably cannot effectively control their own flight direction, particularly in windy weather, therefore decreasing the probabilities of successful dispersal.

Data available for this parasite suggest that it can reach high prevalences and loads. High infestations have been reported for colonial species (Hoi et al. 2010) but also for bird species breeding at low densities (Roulin 1998, Václav et al. 2008). Yet, this information can be misleading since carnid pupae can accumulate in the nest during successive breeding seasons (Roulin et al. 1998, Valera et al. 2006). To our knowledge, just two studies report the colonization of clean nests by *Carnus*. Liker et al. (2001) reported 94% of infected nests in a colony of Common starlings (*Sturnus vulgaris*) and Soltész et al. (2018) found that 76% of Red-footed falcon nests (*Falco vespertinus*) were infected by this parasite. Both studies suggest that the colonization ability of carnid flies is high. Whereas such a high colonization rate can be due to the high density of nest boxes (see Liker et al. 2001), colonization of new, isolated nest boxes has been observed frequently (Veiga et al. in prep.). Exceptional dispersal distances have also been reported for aphids and fig wasps, which share with *Carnus* the problems with aerial dispersal faced by small, delicate insects (Johnson 1969).

It is known that dispersal may be either temporal or spatial and that temporal dispersal *via* developmental mechanisms (especially diapause) is functionally equivalent to spatial dispersal (Hairston 2000, Hairston and Kearns 2002) and negatively correlated with migration (Hanski 1988, Bohonak and Jenkins 2003). Given *Carnus*' short lifespan of the dispersal stage, the seemingly low flight ability and frequently prolonged diapause (Valera et al. 2006), one could assume that spatial dispersal is not imperative and that temporal dispersal is more important for this species. Yet, the non-occupation of infected cavities, nest failure (i.e. loss of feeding resources) or asynchrony between the emergence of the parasite and the occurrence of the hosts (Calero-Torralbo et al. 2013) are important pressures forcing emerging flies to disperse spatially, what suggests that this species should be well-suited for detecting and reaching other hosts (i.e. occupied nests) in the surroundings. More studies on the colonization ability of *Carnus* on non-colonial or solitary host species could shed light on the dispersal ability and infectious potential of this parasite.

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Chapter 4: The effect of parasite density on host colonization success by a mobile avian ectoparasite

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Abstract

Full understanding of the dynamics of host-parasite interactions requires elucidation of the principles governing host colonization. With respect to mobile parasites, little is known about their dispersal behaviour and the factors affecting host colonization success. Here we experimentally explore the effect of parasite density manipulations on the colonization success of the carnid fly *Carnus hemapterus*, an avian ectoparasite. Most host nests were colonized within the same breeding season, but the abundance of flies colonizing the nests varied broadly both within and between years. Experimental increase in the density of carnid flies in the vicinity of host nests did not result in higher parasite abundance in these nests. Host colonization success in terms of parasite abundance was neither related to indices of parasite density around host nests. Parasite abundance in colonized host nests was positively related to host density and brood mass, and negatively related to date. Host nests in trees held less carnid flies than those on cliffs and farm houses. The dispersal ability of *Carnus hemapterus* is apt for rapid colonization of new host nests, but it is unable to explain the broad heterogeneity in parasite abundance between host nests.

Introduction

Dispersal is perhaps the most dangerous part of most parasites' life cycles (Ward et al*.* 1998), aiming to find an appropriate host and establish a viable population (Ran 2013). The factors influencing dispersal and the subsequent host colonization process have attracted attention because they can contribute to the understanding of the dynamics of host-parasite interactions (Boulinier et al*.* 2001), the evolution of local adaptation (Lively 1999), parasite virulence (Boots & Sasaki 1999, Wild et al*.* 2009) and the evolution of host-specificity (Timms & Read 1999). Yet, parasites' dispersal and colonization success is poorly known (but see Harbison et al. 2008, Pélisson et al*.* 2013, Clark & Clegg 2015), partly because the movement of parasites is difficult to measure under natural conditions (Brown & Brown 2004, Kokko & López-Sepulcre 2006).

Successful colonization of a host by a dispersing parasite depends on many factors, such as mobility or host detection mechanisms of the parasite, suitable environmental conditions, or the long-term presence of appropriate hosts. In particular, colonization of nests by nidicolous ectoparasites is known to depend on the host's foraging habits (Harper et al*.* 1992), host density or colony size (Brown & Brown 1996, 2004, Kleindorfer & Dudaniec 2009), host breeding seasonality (Merino & Potti 1995), nest microclimate (Dawson et al*.* 2005, Martínez-de la Puente et al*.* 2010), nest size and nests spatial dispersion (Tomás et al*.* 2008, Kleindorfer & Dudaniec 2009) or on the number of hosts in a nest (Dawson et al*.* 2005). Other relevant factors for host colonization such as the spatial distribution of parasite source sites (e.g. infested nests, see Brown & Brown 2004), have been seldom researched.

Here we study the colonization success of a nidicolous, mobile, directly transmitted ectoparasitic fly, *Carnus hemapterus* (Nitzsch, 1818) (hereafter *Carnus*). This is a widespread hematophagous bird parasite in the Holarctic and Nearctic, parasitizing 64 host species from 24 avian families (Grimaldi 1997, Brake 2011). Research on the factors determining the extent of individual host infestation with this parasite has revealed an effect of host size and age (Dawson

& Bortolotti 1997, Liker et al. 2001, Valera et al. 2004), increased prevalence with host density (Hoi et al. 2010, but see Liker et al. 2001), and preference for hosts in better body condition (Václav et al. 2008, Václav & Valera 2018). Yet, to our knowledge, little attention has been paid to the dispersal ability and colonization success of *Carnus* (but see Wiebe 2009), even if this is a particularly well-suited species for such studies for several reasons: (i) except for dispersal, its entire cycle takes place in the host nest, where it feeds mainly on nestlings, but also on incubating birds (López-Rull et al. 2007); (ii) pupae overwinter in the host nest and, after a diapause usually lasting several months (Guiguen et al. 1983), nymphs emerge the following spring, when nestling hosts are available, thus allowing the persistence of *Carnus* in the nest; (iii) adult flies lose their wings as soon as they find a suitable host nest (Roulin 1998), therefore limiting their dispersal ability after host nest colonization; (iv) *Carnus* does not need vectors for transmission, because flies colonize host nests actively during the winged phase of their life cycle (Grimaldi 1997; Roulin 1998, 1999).

In this paper, we examine the colonization success of *Carnus hemapterus*, both in terms of parasite prevalence in host nests and the abundance of colonizing flies in infested nests, by manipulating the density of parasites at various spatial scales. Given the short period of dispersal (at most 4 days, Veiga et al. 2019a), likely limiting the parasite's spreading potential, we predict that experimentally increased density of *Carnus* flies near the host nests should result in higher colonization success at these nests compared to control nests. Moreover, we explore if parasite density around host nests can explain the abundance of colonizing flies at these nests, while controlling for confounding factors such as host- and habitat-related features.

Material and methods

Study area and study species

The study area (around 50 km^2) lies in the Desert of Tabernas (Almería, south-eastern Spain, 37°05′N, 2°21′W). The landscape consists mainly of badlands and ramblas with olive and almond groves interspersed among numerous dry riverbeds. The climate is semiarid with long, hot summers and high annual and seasonal rainfall variability with a mean annual precipitation of c. 230 mm (Lázaro et al*.* 2001).

Carnus hemapterus' life cycle consists of three larval stages, a pupal period and an adult phase (Guiguen et al. 1983). The emergence of imagoes occurs throughout the whole host nestling period (Valera et al*.* 2003, Calero-Torralbo et al*.* 2013). Prolonged diapause has been recorded, so that *Carnus* can persist in the nest in the pupal stage for several years (Valera et al*.* 2006).

The European roller *Coracias garrulus* (hereafter roller) is the most abundant host species of *Carnus* in our study area. It is a trans-Saharan migrant that rears a single brood per year. Egg hatching is distinctly asynchronous with remarkable annual differences in hatching date and in clutch and brood size (Václav et al*.* 2008, 2011). Nestlings are naked at hatching, but their body covers with closed feather sheaths almost completely by the age of 13 days (Václav et al*.* 2008). Fledging occurs ca. 20–22 days after hatching (Václav et al. 2008).

Rollers are secondary cavity-nesting species. In our study area, rollers used to breed in burrows excavated by other birds in sandstone cliffs and in cavities in human constructions, namely bridges and farm houses (Václav et al*.* 2011, Valera et al*.* 2019). In 2005 a nest box installation program started and most of the roller population is currently breeding in nest boxes placed on trees, sandstone cliffs and farm houses (Valera et al*.* 2019). The nest boxes installed on trees are frequently farther from other species' nests than the ones installed on cliffs and on farms. The latter are usually located near natural cavities occupied by other *Carnus*' host species (Grimaldi 1997, Brake 2011): kestrels *Falco tinnunculus*, Jackdaws *Corvus monedula,* Rock pigeons *Columba livia* or Little owls *Athene noctua*. The nest boxes on cliffs and on farm houses share common features (devegetated surface of cliffs and farm houses vs dense tree canopy around nest boxes on trees, occurrence of other *Carnus* host species breeding on cliffs and farm houses vs absence of close neighbours in nest boxes on trees). As *Carnus* abundance did not differ between nest boxes on cliffs and on farm houses (see below), these two nest-site habitats were pooled and named “cliffs-houses” hereafter.

Field methods and experimental design

Fieldwork was carried out in 2010 and 2011 and from 2015 to 2018. We closely monitored rollers breeding in the study area all years. Nests were surveyed from the first observations of rollers in April and inspected during egg laying, incubation, hatching and during the nestling stage. Nests of other cavity-nesting bird species were also recorded in the study site.

Since carnid pupae overwinter in the nests, supply of new nest boxes and/or fumigation of previously used ones are a prerequisite for the study of *Carnus*’ colonization ability. Thus, nest boxes previously used by rollers were emptied, cleaned with soap and disinsected with a 10 ml/L solution of Arpon® (cipermetrine) prior to the breeding season. The solution was sprayed on the nest’s inner surface and walls. This insecticide has been proved to be highly efficient against *Carnus* (prevalence in treated boxes: 0%, Amat-Valero et al*.* 2012) and other insects. Consequently, all carnid flies detected in cleaned or new nest boxes can be classified as colonizers.

Exhaustive removal of nest material (containing diapausing pupae) and fumigation of used nest boxes was done at two different severity levels. In 2011, 42.4% (14/33) of nest boxes occupied by rollers were cleaned or newly supplemented prior to the breeding season and the remaining 19 occupied nest boxes were left uncleaned and soiled with nest material from other infested nests. In 2016-2018, most (2016: 96.9%, *n* = 31) or all (2017 and 2018, *n* = 37 and 36 respectively) nest boxes occupied were cleaned or newly supplemented prior to the breeding season. Consequently, we created conditions with two levels of background parasite density: higher background parasite density in 2011 and lower background parasite density in 2016-2018. In 2018 some nest boxes were involved in other experiments and were excluded from calculations of prevalence and abundance of carnid flies (Table 1).

During the breeding seasons of 2017 and 2018, we increased experimentally the density of carnid flies in the vicinity of occupied roller nest boxes to test whether increased parasite density results in increased colonization success at these nests. The density of parasites was experimentally increased near 9 nest boxes in 2017 and 8 nest boxes in 2018 by placing in their vicinity detritus of nests infested the previous year and stored at the Estación Experimental de Zonas Áridas (EEZA) during the corresponding winter seasons. A variable amount of detritus containing diapausing carnid pupae was placed in plastic trays (two in 2017 and one in 2018, mean weigh and range: 2017: $\bar{x}$ = 434 g, 345 - 535 g; 2018: $\bar{x}$ = 1038 g, 601 – 1235 g) and hanged by the hatching time of rollers' eggs in trees near the focal roller nest box (mean distance and range: 2017: $\bar{x}$ = 14.7 m, 5 - 30 m; 2018: $\bar{x}$ = 15.1 m, 5 - 30 m). A second subsample of detritus (mean and range: 2017: $\bar{x}$ = 282 g, 129 – 357 g; 2018: $\bar{x}$ = 491 g; 320 – 717 g) was kept at the EEZA and the number of emerging flies was counted every 2-4 days (mean and range of emerged flies: 2017: $\bar{x}$ = 26.4, n = 10, range: 1-108; 2018: $\bar{x}$ = 246.2, n = 8, range: 6-1357). This information allowed to estimate by extrapolation the number of potentially infesting flies emerging from the corresponding subsamples placed near the roller nests during the period from the hatching date of the focal nest until the date when *Carnus* abundance was estimated in the focal nest (2017: $\bar{x}$ = 102.1, range: 2.5-478.5, n = 9; 2018: $\bar{x}$ = 220.5, range: 10.4-880.6, n = 8).

This experiment was run only in isolated nest boxes on trees but not in nest boxes on cliffs or farm houses, where nests of several breeding pairs of various species readily occur and could act as sources of colonizing carnid flies. The prevalence and abundance of carnid flies in experimental nest boxes was compared with neighbouring occupied nest boxes located on trees but without parasite supplementation.

Colonization success of carnid flies

Rollers' nest colonization success by *Carnus* was recorded as the prevalence (proportion of infested nest boxes) and the abundance of flies in cleaned (i.e. new or fumigated) nests when nestling rollers are at the age with peaking *Carnus* abundance (13-14 days, then it declines rapidly, Václav et al*.* 2008). Specifically, we assessed *Carnus* abundance when nestlings age reached 8-13 days (mean = 10.31 days) in 2011, 11-17 days ($\bar{x}$ = 12.9 days) in 2016, 11-19 days ($\bar{x}$ = 13.57 days) in 2017, and 11-19 days ($\bar{x}$ = 13.83 days) in 2018. During *Carnus* abundance assessment, most carnid flies in the nest can be found on the nestlings and a few are located under them (see below). Variation in mean host age at parasite abundance assessment among years is unlikely to have had an effect on *Carnus* abundance in host nests. This is because of the marked hatching asynchrony of rollers and the wide range of host age and size for parasites within a nest. For the estimation of *Carnus* abundance in the nests, roller broods were carefully taken from the nest and placed in a cotton bag. Each nestling was then inspected for the number of carnid flies on the body surface. Carnid flies on each nestling were counted once in 2011 and twice in 2016, 2017 and 2018. This visual census method has been found to be reliable (Roulin 1998). The number of flies recorded in both counts for 393 nestlings from 2016, 2017 and 2018 was highly repeatable (R = 0.991, SE = 0.004, 95% CI = 0.981-0.997, P < 0.0001). In 2016, 2017 and 2018 we calculated means of parasite abundance from the two counts plus the number of carnid flies moving under the nestlings. In 2011 we did not count the number of flies under the nestlings. Still, the percentage of flies remaining in the nest after taking the nestlings is very low ($\bar{x}$ = 4.3%; SD = 6.97%, n = 103, data from 2016, 2017 and 2018). The date at *Carnus* abundance assessment was used to control for the seasonal effect of *Carnus* numbers (Calero-Torralbo et al*.* 2013).

Nest boxes on cliffs and farm houses were pooled because they share similar features and the abundance of carnid flies did not differ between them (GLMM, assuming the negative binomial error distribution, with nest identity as random effects and date (quadratic term), year, nest-site habitat and the interaction between the last two variables as independent variables – nest-site habitat: P = 0.70, year: P =0.17, and year × nest-site habitat: P = 0.80).

We also measured the mass of roller nestlings (with 0.1 g accuracy) during 2011 and 2016-2018 on the same day when *Carnus* abundance was estimated. We calculated host brood mass as a surrogate of the amount of heat and CO_2 that the brood produces and that may serve as a cue for nest detection (Tomás et al. 2008). Replacement of this variable by the number of nestlings gave similar results.

The procedure for bird trapping and handling used here was approved by the Dirección General de Gestión del Medio Natural, Consejería de Medio Ambiente, Junta de Andalucía.

Density of hosts and parasites

Breeding birds around rollers' nests could contribute to attracting dispersing carnid flies. We estimated the density of potential *Carnus* hosts in two ways: (i) the mean distance between the focal roller nest and the two nearest active nests of any cavity-nesting bird – the distances were classified as: 1 = mean distance < 100 m, and 2 = mean distance > 100 m, after establishing the distribution of distances between focal and neighbouring nests (Supplementary Fig. S1); (ii) the distance between the focal roller nest and the nearest active nest of any cavity-nesting bird.

Natural and semi-natural cavities used by cavity-nesting bird species are often infested with *Carnus*. As they usually contain pupae generated the previous season, they are potential sources of emigrant flies, especially if they are not occupied and parasites are therefore forced to disperse (see Brown & Brown 2004 for a similar system). Cavities used by pigeons and Jackdaws are an exception in that pupae in these nests are very scarce (Veiga et al. 2019b, Valera et al. in prep.). Thus, as a surrogate of *Carnus* density around the focal roller nests in year *t* we estimated the density of parasite sources on the basis of the number of nests occupied in year *t* -1 regardless of whether or not these nests were occupied in year *t*. We considered two indices of parasite density in year *t*: (i) the mean distance to the two nearest nests occupied in year *t* -1 (excluding pigeons and Jackdaws) – the distances were classified as 1 = mean distance <

100 m, and 2 = mean distance > 100 m, after establishing the distribution of distances between focal and infested nests (Supplementary Fig. S2); (ii) the distance between the focal roller nest and the nearest nest occupied in year *t* -1 (excluding pigeons and Jackdaws). Nests occupied in 2010 and non-fumigated nests in 2011 and the parasite supplementation trays used in 2017 were considered as "infested nests".

Statistical methods

A GLMM with negative binomial distribution was used to test whether increased parasite density near occupied nests resulted in differences in *Carnus* abundance between manipulated and non-manipulated nests. The two groups did not differ in host brood size or breeding phenology ($P > 0.10$), and the two variables were therefore not included in GLMMs. Year and experimental treatment were included as fixed factors and nest identity as a random factor. Prior to this analysis, we ran another GLMM to explore whether the number of carnid flies that emerged from the experimental trays was related to the abundance of carnid flies in the manipulated focal nests. We assumed negative binomial distribution and included nest identity as a random factor. Since we did not detect any significant relationship between the number of emerged flies from the trays and *Carnus* abundance on the nestlings (Supplementary Table S1), we do not control for the number of *Carnus* flies emerged from trays in the GLMM on the effect of experimental treatment.

The predictors of the abundance of colonizing *Carnus* in disinsected nests in 2011, 2016 and 2017 were examined with GLMM assuming the negative binomial error distribution. The independent variables used were: (i) host brood mass per nest (linear and quadratic term); (ii) nest-site habitat (trees vs cliffs-houses); (iii) date when *Carnus* abundance was checked in each colonized nests (linear and quadratic term); (iv) parasite density (see above); (v) host density (see above), and (vi) the interaction between the latter two variables. Nest identity and year were set as random factors. Separate GLMMs were conducted for categorical and continuous indices of host and parasite density (see above).

Although both sets of GLMMs yielded qualitatively comparable results, we report the results obtained by the statistically more robust GLMM involving categorical data on host and parasite density. Host brood mass and the date when *Carnus* abundance was assessed were scaled and centred before being included in the models to address collinearity and to obtain meaningful regression estimates involving main and interaction effects (Quinn & Keough 2007; Schielzeth 2010). A model averaging approach based on Akaike information criterion (AIC) was used to obtain weighted averages estimates for fixed parameters (Grueber et al*.* 2011). Model averaging was done on models with the cumulative sum of AICc weights > 0.95.

The test on the repeatability of the number of carnid flies recorded in two counts was calculated with 1000 permutations and 1000 bootstraps, assuming the Poisson distribution for the response variable.

All analyses and plotting were conducted with R software 3.5.2 (R Core Team 2018), using the packages rptR (Stoffel et al*.* 2017), lme4 1.1-18-1 (Bates et al*.* 2015), MuMIn 1.42.1 (Barton 2018), effects 4.0-3 (Fox 2019) and ggplot2 3.0.0 (Wickham 2016).

Results

Experimental increase in parasite density and colonization success

Prevalence of carnid flies in roller nests was close to 100% in all years (Table 1). Experimental increase in parasite density near focal clean roller nests did not result in a higher abundance of carnid flies in the focal nests compared to control clean nests (GLMM, Nest identity: *SD* = 0.63; Intercept: Estimate = 2.45, *SE* = 0.32, *z* value = 7.55, *P* < 0.001; experimental treatment: Estimate = 0.19, *SE* = 0.38, *z* value = 0.52, *P* = 0.60; Fig. 1). Year did not account for variation in *Carnus* abundance in roller nests (year: Estimate = 0.38, *SE* = 0.34, *z* value = 1.11, *P* = 0.26).

	2011	**2016**	**2017**	**2018**
N	14	31	37	18
Prevalence (95% CI)	0.93 (0.66-0.99)	0.90 (0.74-0.98)	0.89 (0.75-0.97)	0.94 (0.73-0.99)
Mean abundance ± SD (range)	55.14 ± 77.40 (0-270)	18.65 ± 20.62 (0-99.5)	20.62±24.54 (0-98.5)	29.81 ± 36.46 (0-139)

Table 1. Prevalence (and Clopper-Pearson 95% confidence intervals), mean abundance (± standard deviation) and range of carnid fly numbers in disinsected roller nests (2011, 2016 - 2018).

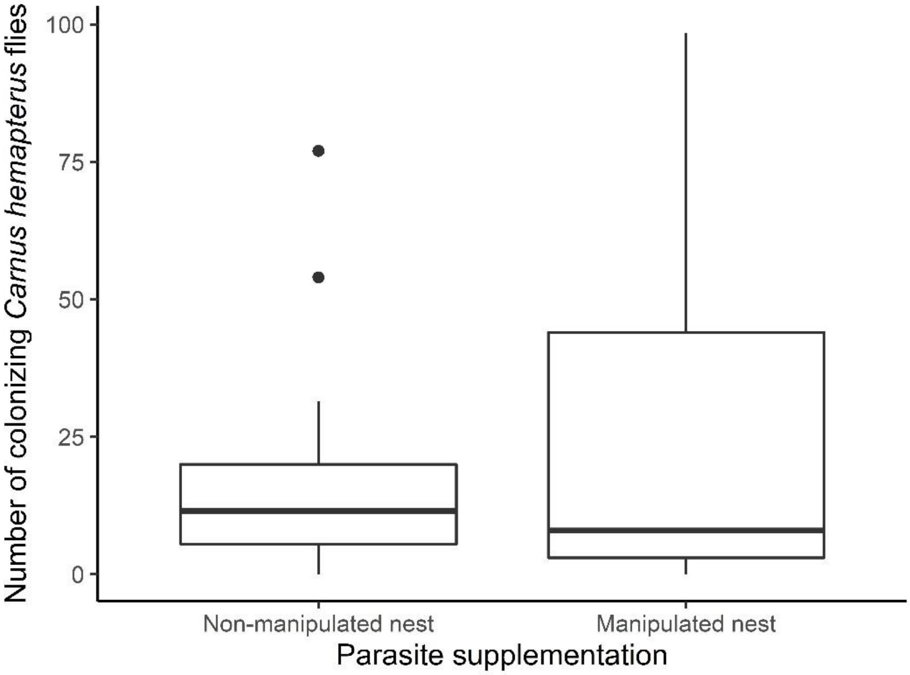

Fig. 1. Tukey boxplot of the number of colonizing Carnus hemapterus *flies in manipulated (with parasite supplementation near the nest) and non-manipulated (control) nest boxes. Median values, first and third quartiles, the largest and smallest values no further than 1.5*IQR (Interquartile range) from the hinge, and the outlying points are shown.*

Parasite density and parasite abundance in host nests

The most parsimonious models (ΔAICc < 2) on the abundance of colonizing *Carnus* flies in roller nests include nest-site habitat, date when *Carnus* abundance was estimated, host brood mass and host density around the focal nests (Table 2). Nest-site habitat and date are the most important variables (relative importance = 1.0, *P* < 0.001, in both cases, Table 3), with *Carnus* abundance being lower for nest boxes on trees compared to those on cliffs-houses (Fig. 2) and decreasing along the season (Fig. 3). Brood mass is the second most important variable (relative importance = 0.95; *P* = 0.001; Fig. 4) with *Carnus* abundance increasing with brood mass. Finally, *Carnus* abundance was lower at low compared to high host density (relative importance = 0.68; *P* = 0.04, Table 3, Fig. 5). Neither parasite density nor the interaction between parasite and host density were important in the most parsimonious models.

Model	**Total mass**	**Total mass^2**	**Date**	**Date^2**	**Nest-site habitat**	**Parasite density (P)**	**Host density (H)**	**P × H**	**AICc**	**ΔAICc**	**Akaike weight**
1	X		X		X		X		651.76	0.00	0.20
2	X	X	X		X		X		652.86	1.10	0.12
3	X		X		X				652.99	1.23	0.11

Table 2. Most parsimonious GLMM models with ΔAICc < 2 on the abundance of colonizing Carnus hemapterus *flies in roller nests cleaned prior to breeding season in 2011, 2016 and 2017. "X" means that the variable was included in the model.*

Parameter	Estimate	Adjusted-SE	Z-value	P-value	Relative importance
Intercept	3.53	0.20	17.37	<0.001	
Total mass	0.36	0.11	3.24	0.001	0.95
Total mass^2	-0.08	0.06	1.33	0.18	0.35
Date	-0.67	0.09	7.17	<0.001	1.00
Date^2	-0.04	0.06	0.64	0.52	0.24
Nest-site habitat	-0.96	0.22	4.35	<0.001	1.00
Host density (H)	-0.52	0.25	2.06	0.04	0.68
Parasite density (P)	-0.07	0.24	0.31	0.76	0.31
H × P	0.70	0.35	2.01	0.04	0.11

Table 3. Results of model-averaging on the abundance of colonizing Carnus hemapterus *flies in roller nests cleaned prior to breeding season in 2011, 2016 and 2017. Values reported are conditional averages after model averaging. Estimates for parasite and host densities are shown for the density category "> 100 m". Estimate for nest-site habitat is shown for the category "trees".*

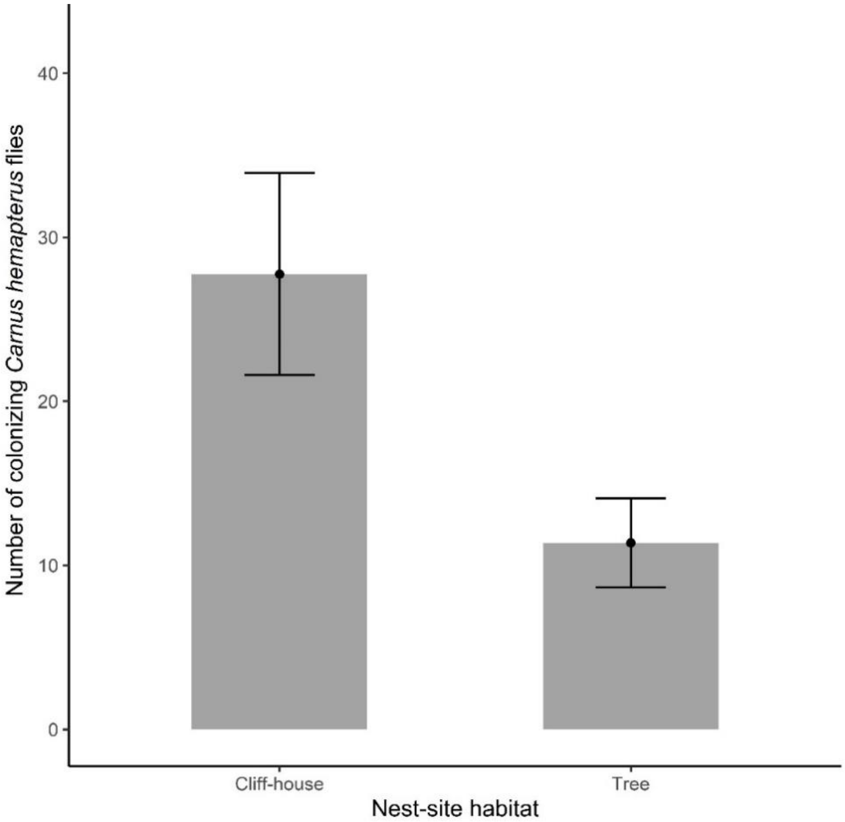

Fig. 2. The total number of colonizing carnid flies inside the nest with respect to two nest-site habitats. Means and standard errors are shown. Estimates were obtained from the model with the lowest AICc (model 1, Table 2).

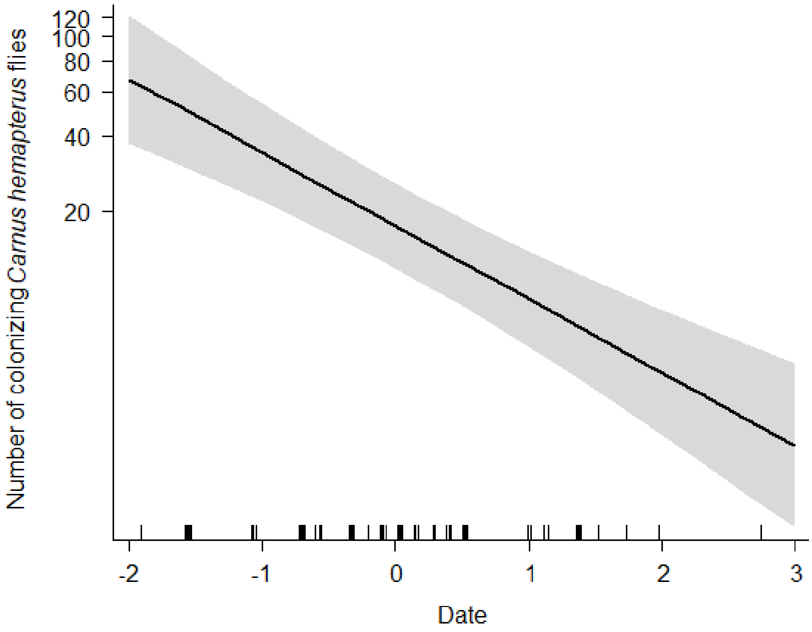

Fig. 3. The total number of colonizing carnid flies inside the nest as a function of time of season (date at Carnus abundance estimation). Shadow shows the 95% confidence interval; date was scaled and centred. Estimates were obtained from the model with the lowest AICc (model 1, Table 2).

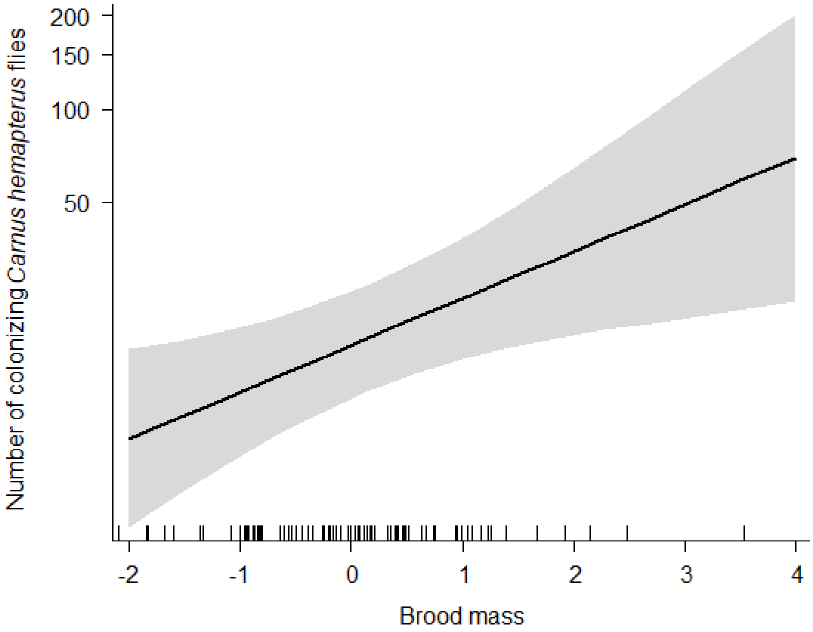

Fig. 4. The total number of colonizing carnid flies inside the nest as a function of host brood mass. Shadow shows the 95% confidence interval; host brood mass was scaled and centred. Estimates were obtained from the model with the lowest AICc (model 1, Table 2).

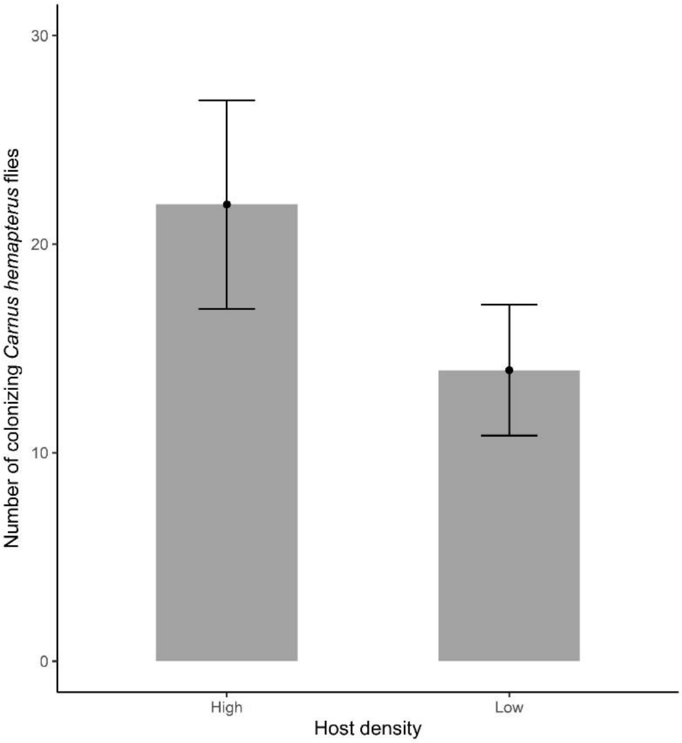

Fig. 5. The total number of colonizing carnid flies inside the nest with respect to two categories of host density. Means and standard errors are shown. Estimates were obtained from the model with the lowest AICc (model 1, Table 2)

Discussion

Identifying the mechanisms behind host colonization success requires an experimental approach (Kokko & López-Sepulcre 2006). This study on the colonization ability of a mobile, non-contact transmitted ectoparasite has been performed under natural circumstances and involves the manipulation of parasite density. The results reveal *Carnus*' highly effective spatial dispersal abilities: despite exhaustive removal of parasite sources by nest cleaning and fumigation, carnid flies colonized ca. 100% of the nests within single seasons (see also Dawson & Bortolotti 1997, Liker et al*.* 2001, Wiebe 2009, Soltész et al. 2018, that report prevalences ranging 76% - 100% for new nests). In contrast to *Carnus* prevalence, the abundance of colonizing flies in hosts' nests did vary both within and among years, decreasing substantially (46%-66%) in 2016-2018 compared with 2011. Several factors (e.g. weather-induced mortality) may have accounted for the decrease, but cleaning of all nest boxes of the most abundant *Carnus* host was likely the most important one. Nest box cleaning resulted in the elimination of thousands of infesting flies, which would otherwise be available in the subsequent host breeding period, and affected the spatial distribution of *Carnus* sources, increasing the distance among host nests and sources of emigrant *Carnus* flies. Thus, decreased *Carnus* density at a large scale may have affected the colonization probability of roller nests as well as parasite abundance in colonized nests.

Parasite density and colonization success

We predicted that increased density of a mobile parasite in its host-seeking stage should result in higher nest colonization success. However, experimentally increasing parasite density near nests (within 30 m) did not lead to higher carnid fly numbers in the focal nests. Moreover, our findings also suggest that parasite density around the focal nests has no effect on the number of colonizing flies in rollers' nests.

Several reasons may account for the lack of relationship between parasite density and the number of colonizing carnid flies: i) inter and/or intraspecific interference among parasites could limit the colonization rate of nests. Dawson & Bortolotti (1997) suggested that intraspecific competition among adult carnid flies may prevent immigration to certain nests, thus limiting parasite abundance within nests. Interspecific competition with other parasites (e.g. blackflies, biting midges) that have been found to be more abundant in some nests than in others (Veiga et al. in prep.) could also account for our results; ii) small insects usually undergo a form of stratified dispersal that combines wind-borne dispersal over long distances with short-range dispersal. In these cases, the distribution of host-containing patches, landscape traits and the prevailing wind directions may interactively influence the spread of the parasite (Colombari & Battisti 2016), probably limiting the role of parasite density; iii) other dispersal strategies not related to parasite density could also play some role. For instance, phoresis has been regarded as an efficient transmission route for various parasites (see Harbison et al. 2008 and references therein). We are not aware of any paper reporting *Carnus* phoresis, but we found three carnid flies on a hipoboscid fly (*Pseudolynchia canariensis*) captured in a pigeon nest. This may be an anecdotic observation, but also a widespread strategy. If the latter were true, it could explain the lack of relationship between parasite density and *Carnus* abundance in colonized nests.

Factors accounting for the variation in abundance of colonizing flies

Carnid flies colonized nearly all nests and reached high but variable abundances in nests ranging 1-270 individuals in our study (see also Kirkpatrick & Colvin 1989, Liker et al*.* 2001, Hoi et al*.* 2010). Although the effect of host-related factors on *Carnus* load has been addressed to some extent (e.g. Valera et al. 2004, Václav et al*.* 2008, Václav & Valera 2018), the factors influencing *Carnus* colonization success remain unknown. Our results show that, after controlling for the effect of year and nest identity, host density contributed to explaining the abundance of colonizing carnid flies in nests; the higher the breeding density of cavity-nesting birds around the focal roller nests, the higher the abundance of colonizing parasites in the focal nests. This finding runs counter the usual expectations that contact-transmitted ectoparasites spread more efficiently in group-living hosts (Poulin 1991, Brown & Brown 1996, 2004), whereas host colonization by mobile ectoparasites is predicted not to differ depending on host sociality (Poulin 1991). Thus, our results agree with those obtained for *Carnus* flies by Hoi et al*.* (2010) and Wiebe (2009), but see Liker et al*.* (2001), even though our results were obtained at a larger spatial scale for an inter-specific community of birds, not at a scale of intra-specific breeding colony. Similarly, Kleindorfer & Dudaniec (2009) found that the intensity of the mobile, non-contact-transmitted fly *Philornis downsi* was higher in aggregated nests of their avian hosts and suggested that higher density of hosts probably implies more host cues for emigrant parasites.

The sensory cues used by *Carnus* to find suitable new hosts are unknown, but stimuli such as odour, heat or carbon dioxide emission may be important

(Gatehouse & Lewis 1973, Gold & Dahlsten 1989, Martínez-de la Puente et al*.* 2010 for other mobile ectoparasites). These attractants are likely to increase with host brood size (e.g. see Andreasson et al*.* 2016 for temperature). Accordingly, we found that the abundance of colonizing carnid flies was positively related with host brood mass (see also Roulin 1998 for a similar effect of Barn owls *Tyto alba* brood size). However, Dawson & Bortolotti (1997), Liker et al*.* (2001), and Wiebe (2009) found no relationship between brood size and carnid fly abundance.

The most important predictors of the number of carnid flies colonizing rollers' nests in our study area are nest-site habitat and date. Habitat parameters such as vegetation structure and plant architecture are known to play an important role in host-parasite interactions (see, for instance, Meiners & Obermaier 2004). In our case, roller nest boxes on trees held less carnid flies than nest boxes on cliffs-houses. In our study site, both nest-site habitats are usually interspersed, so that the chance by *Carnus* flies to encounter nest boxes in these habitats should be similar. Our results, however, suggest that colonizing flies find host nests more easily on cliffs-houses and/or are more attracted by nests located in this habitat. This result implies that nest box placement with respect to habitat type can have important consequences for parasite loads (see also Kleindorfer & Dudaniec 2009). Concerning date, our results agree with the ones by Calero-Torralbo et al. (2013), who suggested that early emergence of carnid flies may be an effective strategy for exploiting late host breeders and may reduce the chance of parasite reproductive failure if host reproduction fails.

Our results show that carnid flies can colonize nearly all nests available even for a solitary host species. In fact, most host species of *Carnus* studied so far are solitary breeding species with considerable inter-nest distances (Hoi et al*.* 2010). Factors such as the vacancy of cavities infested the previous year, nest failure (with the subsequent loss of feeding resources) or asynchrony between the emergence of the parasite and host availability (Calero-Torralbo et al*.* 2013) may exert strong selection pressures on spatial dispersal abilities of this parasite.

Further research is necessary to deepen the knowledge of the importance of the parasite's natural history (e.g. flight ability, spread distance, host detection cues) and environmental factors (e.g. wind characteristics, the scale of host-containing patches in relation to the scale of movement of the organism, habitat type) on host colonization success, particularly through experimental approaches.

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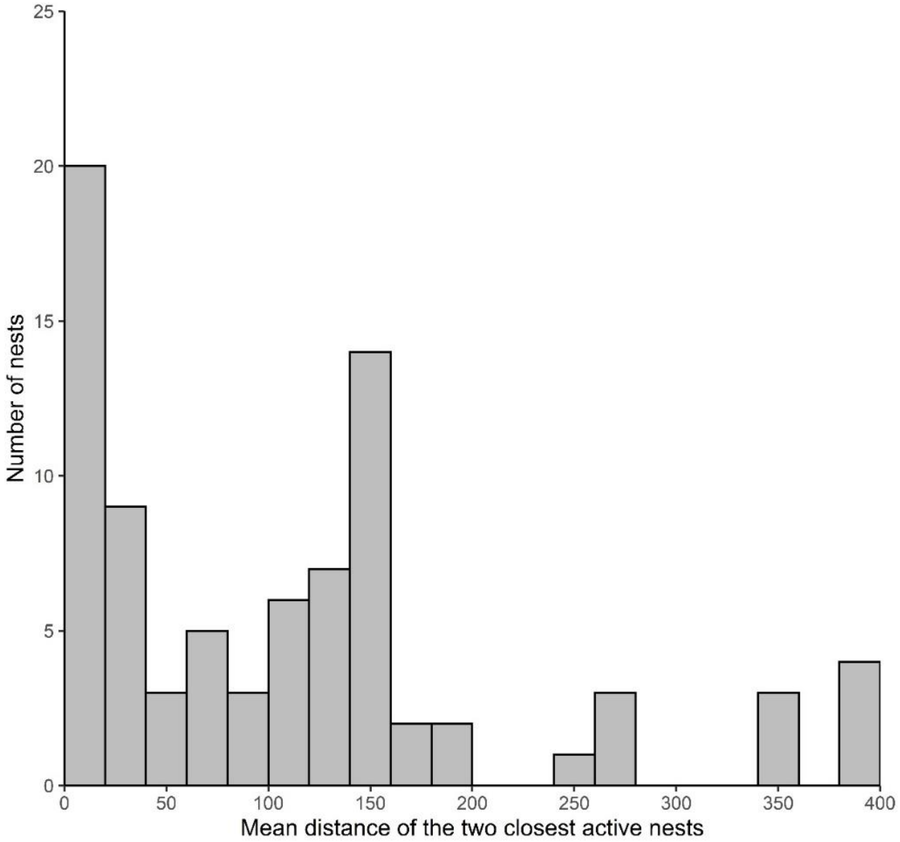
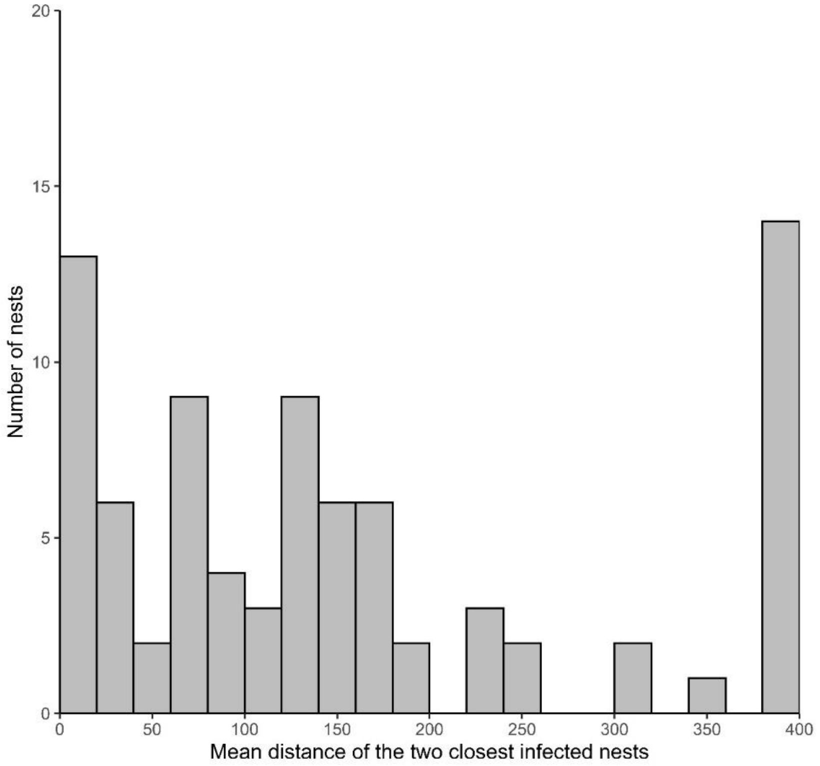
Supporting information

Parameter	Estimate	SE	Z-value	P-value	Std. Dev
Intercept	3.12	0.49	6.37	<0.001	
Emerged flies from trays	0.0005	0.001	0.39	0.69	
Nest ID					0.12

Supplementary Table S1: GLMM exploring the relationship between the number of Carnus flies emerged from trays and the total number of flies found in colonized host nests. Nest ID was included as a random factor.



Supplementary Fig. S1. Histogram of the distance between the focal roller nest and the two closest active cavity nests (host density). The last bar denotes distances > 400 m.



Supplementary Fig. S2. Histogram of the distance between the focal roller nest and the two closest infested nests (parasite density). The last bar denotes distances > 400 m.

Chapter 5: Nest-site type: a major determinant of the ectoparasite infracommunity of a cavity-nesting bird species in an arid environment

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Abstract

Birds host an impressive diversity of ectoparasites that may have detrimental effects on their fitness. Consequently, much work has been done on the factors regulating the occurrence and abundance of single parasite species. However, comprehensive studies focused on the parasitic community of a given host are scarce. Here we focus on the ectoparasite infracommunity of a secondary cavity-nesting bird species, the European roller, breeding in nest boxes in a semiarid environment. During three breeding seasons, we examined the composition and abundance of parasites at the nest level and explored their spatial structure and the effect of nest-site type, breeding phenology and host number (brood mass) on the variability of the infracommunity. Nest-site type (nest boxes on trees vs nests on cliffs and human constructions) contributed the most to explain differences in prevalence and abundance of the various ectoparasite species. Host breeding phenology affected the abundance of the most prevalent and abundant ectoparasite (*Carnus hemapterus*), but host brood mass had no significant influence on any ectoparasite. Neither the occurrence nor the abundance of the infracommunity of parasites had a significant spatial structure. This study, performed at the host population scale, reveals that the socioenvironmental characteristics resulting from the selection of nest-site microhabitat explain most of the variation of the ectoparasite infracommunity. Accordingly, nest boxes for vulnerable species should be placed with solid knowledge of the effect of such features.

Introduction

Ectoparasites are a taxonomically diverse group of organisms able to affect the hosts' fitness (Lehmann 1993) and provoke antiparasitic adaptations by their hosts (Clayton et al. 2010). Birds are known to host a wide array of ectoparasites (Clayton et al. 2010), many of which are vectors of pathogens (e.g. avian malaria and closely related parasites transmitted by *Culex* mosquitoes, biting midges and louse flies, Valkiūnas 2004) that also have detrimental effects on their hosts (Merino et al. 2000, Marzal et al. 2005, Valkiūnas et al. 2006, Tomás et al. 2007, Martínez-de la Puente et al. 2010, Asghar et al. 2015). In fact, the abundance of ectoparasites is a key epidemiological variable (Sol et al. 2000, Martínez-Abraín et al. 2004, Griffing et al. 2007, Martínez-de la Puente et al. 2013).

Much research has been dedicated to exploring the factors accounting for the prevalence and abundance of specific ectoparasitic species or families (for ticks see Oorebeek and Kleindorfer 2008, for biting midges and blackflies see Martínez-de la Puente et al. 2009a, for mites, fleas and blowflies see Merino and Pott, 1996 and Cantarero et al. 2013) on different bird species. However, a given host species or individual (adult, nestling, infested nest) usually hosts a variety of ectoparasitic species, the ectoparasite infracommunity (Bush et al. 1997). Considering this level of organization is important because, for instance, it is mainly within the framework of the infracommunity where interactions among different parasite species occur (Poulin 2007) and such interactions can influence the structure of the infracommunity (Heeb et al. 2000). Thus, analysing separately a given ectoparasite species just gives an incomplete picture of the factors influencing that ectoparasite and, in turn, the host.

Optimally, the ectoparasite community should be studied as a whole, what could give deeper insights into the underlying mechanisms ruling variations in composition and abundance. For instance, studies focused on some ectoparasite taxa have shown that factors such as distance at large scale (Gómez-Díaz et al. 2008 on birds) or locality features (Lareschi and Krasnov 2010, on mammals), host characteristics (Krasnov et al. 2008, Lareschi and Krasnov 2010, Sponchiado et al*.* 2017 on mammals), off-host environment such as temperature and precipitation (Krasnov et al. 2005, 2008 on mammals) or habitat characteristics (Manzoli et al. 2013) are partly responsible of the variation in the ectoparasite community. Yet, comprehensive studies on the ectoparasitic community of bird species are scarce (see Hamstra and Badyaev 2009, and Lareschi and Krasnov 2010, Sponchiado et al. 2017 for a similar approach on rodents and marsupials). Such scarcity is partly due to the fact that studying different ectoparasite species (each of them with different life cycles, requirements and adaptations) is methodological and logistically complicated. However, cavity-nesting birds offer an excellent opportunity to address this issue. They usually host a wide variety of ectoparasites (Marshall 1981), some of which are nidicolous whereas others just visit temporarily the nest to feed on the adults and/or the nestlings. Thus, studying the nests of hole breeding birds facilitates the investigation of the ectoparasitic community.

The European roller *Coracias garrulus* (hereafter roller) is a migratory, secondary cavity-nester species breeding in sandy cliffs, tree holes, human constructions and artificial nest boxes (Cramp 1998). Several ectoparasites have been described for this species: carnid flies (*Carnus hemapterus*, Calero-Torralbo et al*.* 2013; Václav et al. 2016), biting midges, blackflies and sandflies (Václav et al*.* 2016, Veiga et al. 2018), hematophagous mites (Václav et al. 2008, Roy et al. 2009), lice (Sosnowski and Chmielewski 1996), louse flies (Nartshuk and Matyukhin 2019, Veiga et al. 2019a) and ticks (Hoogstraal and Kaiser 1961, Tsapko 2017). Specific studies on such ectoparasites show that factors such as host body features (Clayton and Walther 2001, Valera et al. 2004, Martinez-de la Puente et al. 2009b, Václav and Valera 2018), breeding phenology (Martínez-de la Puente et al. 2009a, Calero-Torralbo et al. 2013) or habitat features (Černý et al. 2011, Manzoli et al. 2013) account for variation in their abundance and prevalence.

In this paper, we examine during three breeding seasons the ectoparasites of rollers breeding in nest boxes in a semi-arid landscape. We focus on the effect of nest-box location (i.e. nest-site type), breeding phenology (i.e. seasonality) and host number (brood mass) on the composition and abundance of ectoparasites at the infracommunity level (nest), its variation and spatial distribution. Furthermore, we searched for evidence of interactions among co-infecting parasite species by examining species co-occurrence patterns.

Materials and methods

Study area

The study area (of ca. 50 km^2) is located in the Desert of Tabernas (Almería, SE Spain, 37º05'N, 2º21'W). The landscape mostly consists of badlands and wadis with olive and almond groves interspersed among dry watercourses. The climate is semi-arid with mild winters, long hot summers and low average annual rainfall (235 mm) with strong inter and intra-annual variation (Lázaro et al. 2001). During this study rainfall varied broadly given the very rainy 2017 year (accumulated precipitation during the hydrologic year 1st October - 30th September: 2016 = 100.4 mm; 2017 = 334.8 mm; 2018 = 200.2 mm).

Study system

The European roller is a migratory bird species evaluated as "least concern", even if the population is thought to be declining (BirdLife International 2019). It is the most common cavity-nester in the study area, where it used to breed in burrows excavated by other birds in sandstone cliffs or in cavities in human constructions. Currently, due to a nest box supplementation program started on 2005 (Václav et al. 2011), most of the roller population in the study area breeds in nest boxes located on trees, sandstone cliffs and human constructions (Valera et al. 2019). Nest boxes on trees are usually more isolated from other breeding bird species, which mostly consist of open nesters including the Eurasian Collared Dove *Streptopelia decaocto* and Common Wood Pigeon *Columba palumbus*. In contrast, nest boxes located on sandstone cliffs are usually near other natural cavities occupied by cavity-nesting birds such as Common kestrels *Falco tinnunculus*, Jackdaws *Corvus monedula,* Rock Pigeons *Columba livia* and

Little Owls *Athene noctua*. Similarly, nest boxes on farmhouses are usually neighbours to bird species breeding in cavities and crevices such as Rock Pigeons, Common kestrels, Spotless Starlings *Sturnus unicolor* and House Sparrows *Passer domesticus*. Another common feature of nest boxes on sandstone cliffs and farmhouses is that they are located on devegetated surface, while nest boxes on trees are covered by dense tree canopy.

During the three breeding seasons under study (2016-2018) 61, 60 and 59 nest boxes were available for rollers. Some of them were occupied all years whereas others were never occupied. Nest boxes in our study area are frequently interspersed so that, averaging data from the ones occupied during the study period, in 61% (range: 48% - 62%) of the cases the nearest nest box neighbour of a focal nest box is in the same nest-site type (e.g. tree-tree, or cliff-cliff), the remaining 39% (range: 51% - 38%) being in a different nest-site substrate (e.g. tree-farmhouse, tree-cliff, farmhouse-cliff).

During 2018, a thermal pad was placed on the inner wall of 18 nest boxes during the nestlings phase (day 6 to day 21, day 1 = hatching date of the first egg) for a different study. Its effect on microclimate was not significant and subdued by factors such as nest orientation or nest-site type. Nevertheless, we tested the effect of the thermal pads on the occurrence and abundance of the ectoparasites via partial RDA analysis. We found that thermal pads had no significant effect in any model (p-value>0.3).

Rollers rear a single brood per year, and egg hatching is distinctly asynchronous with remarkable annual differences in hatching date and in clutch and brood size (Václav et al. 2008, 2011).

Rollers in our study area are parasitized at least by biting midges (Fam. *Ceratopogonoidae*), blackflies (Fam. *Simuliidae*), sandflies (Fam. *Psychodidae*), carnid flies (*Carnus hemapterus,* Fam. *Carnidae*), louse flies (Fam. *Hippoboscidae*), hematophagous mites (Fam. *Macronyssidae* and Fam. *Dermanyssidae*) and soft ticks (Fam. *Argasidae*). Since each group of ectoparasites has different habits, mobility and strategies of host exploitation (e.g.

nidicolous vs temporary parasites, diurnal vs nocturnal), different methods are required for estimating their respective abundances (see below).

Routine fieldwork

The reproduction of rollers and their ectoparasite community was studied in 31, 37 and 36 nest boxes occupied by rollers during the breeding seasons of 2016, 2017 and 2018 respectively (although the sample size decreased due to the occurrence of non-infested nests or nests where the abundance of some parasites was not estimated, see below). Occupied nest boxes were followed closely from occupation (end of April) until fledging and inspected periodically during egg laying, incubation, hatching and nestling development. At least three visits were done every year during the nestling phase: the first one to place a sticky trap (see below), the second one to remove the trap and the third one to ring the fledglings. The number of nestlings was recorded in each visit. We also measured the mass of roller nestlings (with 0.1 g accuracy) when the oldest nestling of each nest was ca. 13 days old (see below).

Prior to each breeding season, nests were emptied, cleaned with soap and disinsected with a solution of 10 ml/L of Arpon® (cipermetrine). The solution was sprayed on the nest’s inner surface and walls. This insecticide has been proved to be highly efficient against *Carnus hemapterus* (prevalence in treated boxes: 0%, Amat-Valero et al*.,* 2012) and other insects. Thus, infestation in a given year does not affect the next year. After fumigation, each nest was provided with fresh, clean sand.

Ectoparasite sampling

Winged diptera visiting the host temporarily (biting midges, blackflies and sandflies) were sampled by means of sticky traps placed under the upper lid of the nest boxes (Tomás et al. 2008). This method has been found reliable and effective for sampling small flying insects, which are captured while entering or leaving the nest. Sticky traps were placed when the oldest nestling of each nest was ca. 13 days old (2016: mean =12.9, range =11-17; 2017: mean =13.57, range =11-19; 2018: mean = 13.83, range =13-19). Sticky traps were maintained three days in 2016 (sticky size = 57,6 cm^2), and four days in 2017 (sticky size = 80 cm^2) and 2018 (sticky size = 330 cm^2). The number of ectoparasites captured by the sticky traps was then standardized to captures per day and 1 cm^2.

Carnus hemapterus (hereafter *Carnus*) is a nidicolous ectoparasite that parasitizes nestlings of many bird species but it may also attack adult birds during incubation (López-Rull et al. 2007). Carnid flies are winged when emerged from the pupa and during dispersal (Veiga et al. 2019b) but they lose the wings when a suitable host is found. Then, flies remain on the nestlings and in the nest debris. Therefore, sticky traps are not suitable to estimate the abundance of carnid flies. Instead, direct estimation of wingless carnid flies during the peak infestation phase (when nestlings have sheaths, see Václav et al. 2008, 2018) is a reliable method (see also Roulin, 1998, Roulin et al. 2001). *Carnus* estimation in each nest was done when the older nestling was ca. 13 days old (Václav et al. 2008). Roller nestlings were carefully taken from the nest and placed in a cotton bag. Subsequently, each nestling was taken and the number of carnid flies was counted twice. Both counts were averaged. The number of carnid flies that remains in the nest debris (even if small, mean = 4.3%, see Veiga et al. 2020) was also counted and added to the sum of the number of carnid flies in all nestlings to get the total number of *Carnus* flies inside the nest.

Louse flies are robust flying insects and they can probably escape from the sticky traps. Thus, they were estimated following the same method employed for carnid flies. However, since they are not nidicolous and highly mobile parasites, its detection during a single visit is unlikely and, thus, we screened all the nestlings in search of louse flies in every visit. Furthermore, in some cases we took advantage of adults captured brooding the nestlings to check the presence of hipoboscid flies too, exploring carefully between the feathers. This method probably gives accurate estimates of prevalence but not of abundance, that can be easily underestimated due to the high mobility and escape behavior of louse flies (Veiga et al. 2019a).

Regarding hematophagous mites, we have recorded the occurrence of *Pellonyssus reedi*, *Ornithonyssus sylviarum*, *Dermanyssus gallinae* and *Dermanyssus hirundinis* in nest boxes occupied by rollers. In general, hematophagous mites have short generation times. They are able to reach high numbers rapidly (Pacejka et al. 1996, Proctor and Owens 2000, Stoehr et al. 2000), so that they are easier to detect when nestlings are grown, but their abundance is highly variable. Similar to louse flies, we took advantage of successive monitoring of the nestlings along the breeding cycle to detect parasitic mites.

Argasidae ticks are usually found under the lid of nest boxes. They also become more abundant along the nestling phase (see, for instance, Dupraz et al. 2017). We recorded their presence during the routine checks of nestlings.

Louse flies, hematophagous mites and soft ticks have low prevalence in our study area, and a reliable estimation on their abundance requires specific and demanding surveys. Therefore, we just analyzed their prevalence.

After discarding the nests where no ectoparasites were found and those where the abundance of some ectoparasites was not estimated, the sample size for the three study years was respectively 30, 36 and 36 for presence-absence data and 29, 36 and 36 for abundance data.

Statistical analysis

The ectoparasite infracommunity was analyzed in terms of species prevalence (presence-absence of biting midges, blackflies, sandflies, carnid flies, louse flies, hematophagous flies and ticks) and abundance (of biting midges, blackflies, sandflies and carnid flies).

In order to test whether geographical location of nests accounts for variations in the ectoparasite infracommunity, Moran's eigenvector maps (MEM) were performed on both the presence-absence and abundance data in each year. MEM analyses are considered robust and suitable for discriminating between spatial and environmental effects on community composition (Griffith and Peres-

Neto 2006). This method computes the principal coordinates of a matrix of distances among geographic neighbours (i.e. geographic connectivity matrices among sampling sites) (Borcard et al. 2011a). MEM decomposes the spatial relationships into eigenvectors, which represent the variation at specific spatial scales. First, we explored for each year whether there was significant linear relationships between the infracomunity composition or abundance data and the geographical coordinates. Since no relationship was found we worked on non-detrended data (Borcard et al. 2004). Then, we selected the eigenvectors describing significant spatial autocorrelation (only positive eigenvalues, see Dray et al. 2006). CCA analyses (for presence-absence data) and RDA analyses (for abundance data) were run for each year which the corresponding set of eigenvectors selected. According to Borcard et al. (2004) forward selection of the MEM eigenvectors should follow in those ordination analyses (CCA or RDA) that proved significant (but see Results). Selection of eigenvectors in non-significant cases would lead to spurious models. The forward-selected eigenvectors should then be included in the final CCA and RDA to compare the effect of socioenvironmental and distance-related variables (see below).

Ordination analysis is an adequate method to examine complex ecological data sets (Legendre and Legendre 2012). In order to examine how environmental and host-related parameters contributed to explain the variation of the ectoparasite infracommunity Constrained Canonical Analysis CCA (for presence/absence of all parasite groups) and Partial Redundancy Analysis RDA (for abundance of carnid flies, blackflies, biting midges and sandflies) were used. To control for the effect of the different sampling methods, the abundance of a given parasite group in each nest was scaled by dividing it by the maximum value of that parasite group in all the nests. In CCA and partial RDA analyses, year was included as conditioning variable, and brood mass (sum of the mass of all nestlings in day 13), nest-site type (cliff, tree, farmhouse), breeding phenology (hatching date of the first egg of the population = 1, calculated separately for each year) and nest identity as constraining variables. Brood mass and breeding phenology were scaled and centred before being included in the analyses.

Forward selection was applied to the overall models to increase parsimony and reduce correlation between explanatory variables. ANOVA-like permutation tests to assess the significance of the models and constraining variables were employed after 999 permutations.

Even if our goal is to examine the effect of the explanatory variables, unconstrained ordination analysis of response variables should be included to explore their relationship without constrains (Borcard et al. 2011b). Therefore, unconstrained Canonical Analysis CA (for presence/absence) and Principal Component Analysis PCA (for abundance) were also run.

All analyses and plotting were conducted with R software 3.6.1 (R Core Team 2019), using the packages vegan (Oksanen et al. 2019) and MASS (Venables and Ripley 2002).

Results

Spatial analysis

CCA analyses (for presence-absence data) with the set of selected eigenvectors for each year were non-significant in all cases (2016: $F_{7,22} = 1.37$, $P = 0.11$; 2017: $F_{8,27} = 0.87$, $P = 0.69$; 2018: $F_{9,26} = 1.24$, $P = 0.18$), so that the procedure was stopped since no significant spatial structure was detected.

Concerning the abundance of ectoparasites, no spatial structure was found in any of the three study years either (RDA analyses: 2016: $F_{7,21} = 1.07$, $P = 0.39$; 2017: $F_{8,27} = 0.59$, $P = 0.95$; 2018: $F_{9,26} = 1.34$, $P = 0.17$).

Determinants of the variation in the composition of the ectoparasites infracommunity

Unconstrained canonical analysis on the occurrence of all ectoparasites groups showed a strong negative relation between the presence of sandflies and hipoboscid flies and the presence of biting midges and hematophagous mites (Supplementary Fig. S1 and Table S1). Furthermore, the occurrence of blackflies was negatively related with the presence of ticks (Supplementary Fig. S1 and Table S1).

The CCA on the presence-absence of the seven ectoparasitic groups rendered a significant overall model ($\chi^2 = 0.87$, $F_{45,54} = 1.56$, $p = 0.002$). Forward selection on the former only retained the variable nest-site type to explain the variation on the occurrence of ectoparasites. The selected model included two significant CCA axes (axis 1: $\chi^2 = 0.29$, $F_{1,97} = 23.08$; $p = 0.001$; axis 2: $\chi^2 = 0.029$, $F_{1,97} = 2.31$, $p = 0.041$). The constraining variable included in the model selected (nest-site type) accounted for 20% and the conditioning variable (year) for 3.5 % of the variation (Supplementary Table S2). Nest-site type contributed significantly to explain the variation in ectoparasite community composition ($\chi^2 = 0.32$, $F_{2,97} = 12.69$, $p = 0.001$), so that the occurrence of blackflies, biting midges and hematophagous mites was associated to nest boxes on trees, while ticks occurred preferentially in nest boxes on cliffs, and sandflies in nest boxes on cliffs and farmhouses. The presence of carnid flies and louse flies was not related with any constraining variable (Fig. 1).

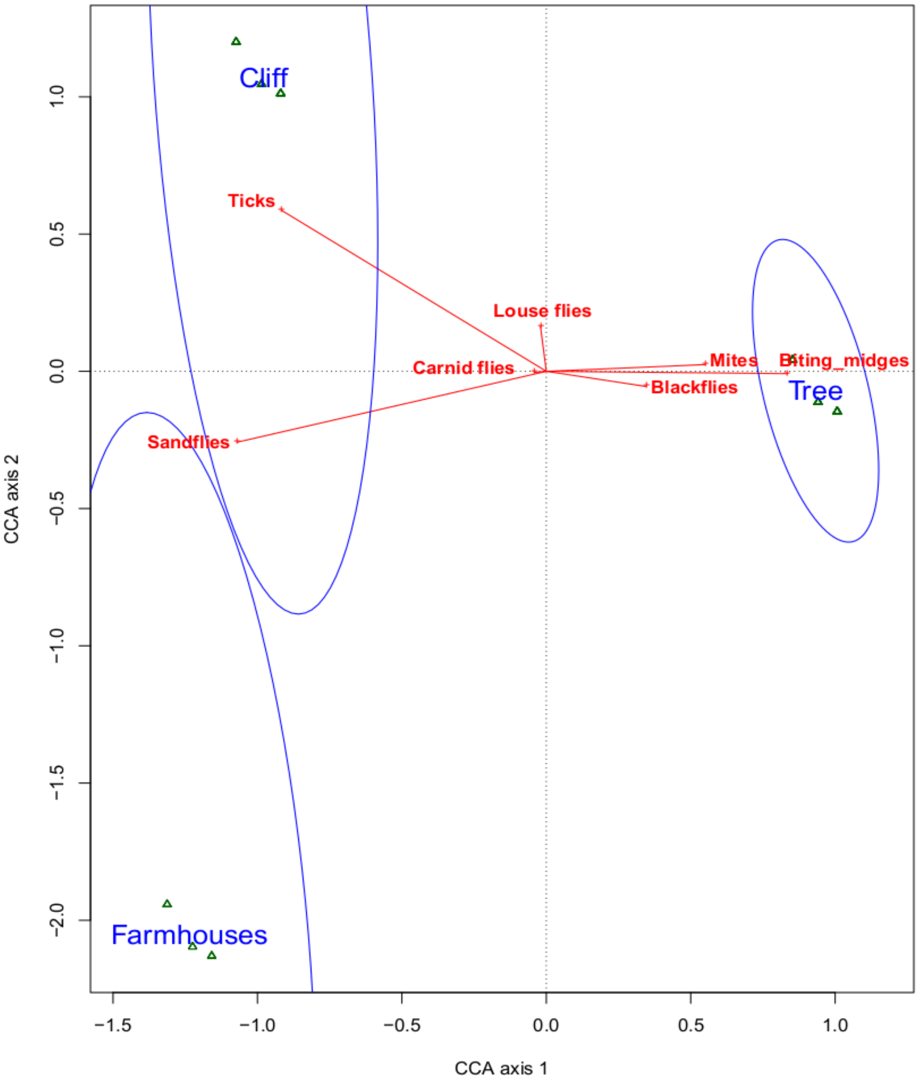

Fig. 1. Constrained Canonical Analysis on the effect of nest-site type on the composition of the ectoparasite infracommunity of breeding European rollers in south eastern Spain. The canonical correspondence correlation bitplot (scaling 2) shows the relation between the constraining (blue labels and ellipses) and the response variables (red labels and arrows). The blue labels of the three levels of the explanatory variable (nest-site type) represent the centroids of each level, and the blue ellipses depict 95% confidence limits for the SE of these centroids.

Determinants of the variation in the abundance of the ectoparasites infracommunity

The PCA on the associations among the abundance of carnid flies, biting midges, blackflies and sandflies revealed that carnid flies and blackflies are separated by the first PCA axis (that accounts for 45.2% of the variance) (Supplementary Fig. S2 and Table S3). In contrast, carnid flies and blackflies are segregated from sandflies and biting midges by the second PCA axis (accounting for 33.5% of the variance) (Supplementary Fig. S2 and Table S3).

Constraining the relationships among abundances of the ectoparasitic groups by their associations with environmental and host-related variables, partial RDA rendered a significant overall model (Variance = 0.07, $F_{45,53} = 1.84$, p = 0.003). Forward selection on the former retained the variables nest-site type and breeding phenology. The selected model was globally significant (Variance = 0.03, $F_{3,95} = 9.12$, p = 0.001) with two significant axes (axis 1: Variance = 0.02, $F_{1,95} = 19.75$, p = 0.001; axis 2: Variance = 0.006; $F_{1,95} = 6.67$; p = 0.001). The constraining variables (nest-site type and breeding phenology) and the conditioning variable (year) included on the model selected accounted for 21.3% and 4.8% of the variance respectively (Supplementary Table S4). Nest-site type contributed significantly to explain the variation on the ectoparasite infracommunity abundance (Variance = 0.017, $F_{2,95} = 9.79$, p = 0.001), so that nests on cliffs and farmhouses had higher abundance of carnid flies and sand flies, while nests on trees held higher abundances of biting midges and blackflies (Fig. 2). Breeding phenology also contributed significantly to explaining the variation on abundance of ectoparasites (Variance = 0.007, $F_{1,95} = 7.78$, p = 0.001) affecting negatively to the abundance of *Carnus hemapterus*.

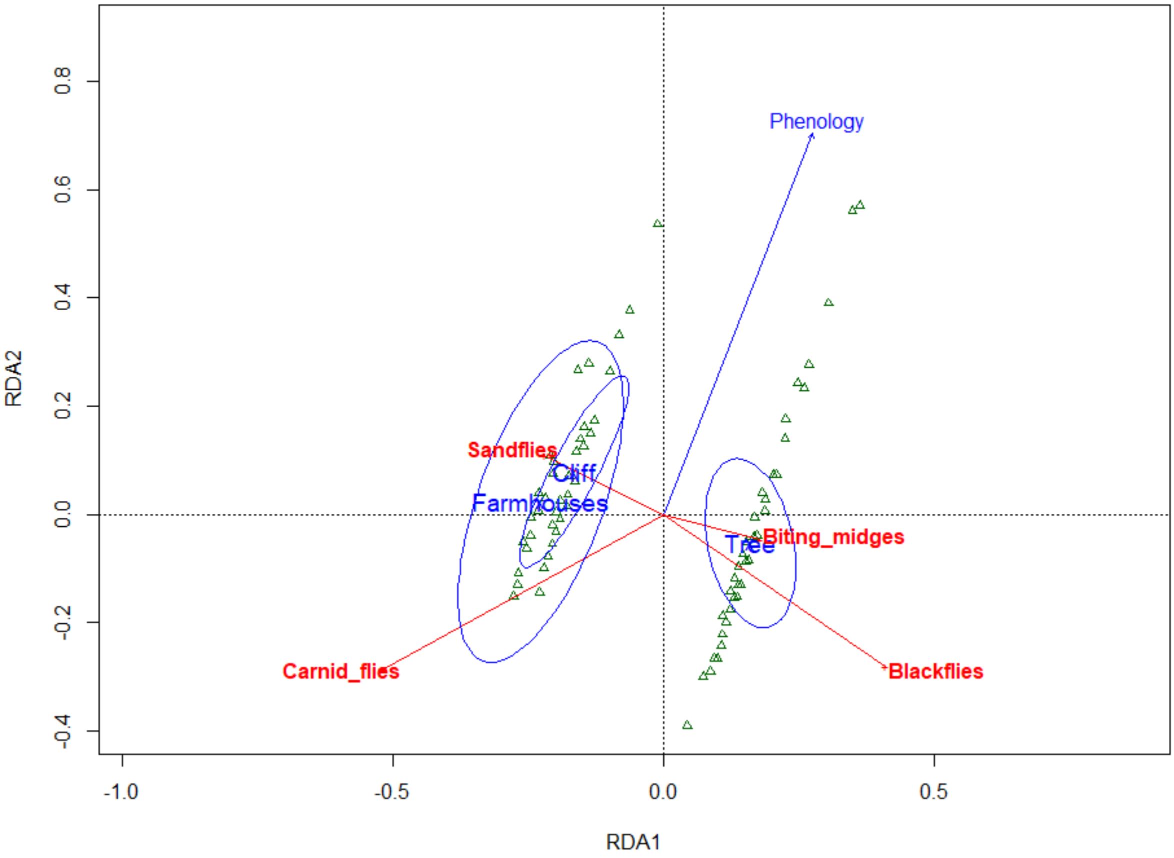

Fig. 2. Partial RDA on the effect of breeding phenology and nest-site type on the abundance of ectoparasites of breeding European rollers in south eastern Spain. Redundancy analysis correlation bitplot (scaling 2) shows the relation between the constraining (blue arrows, labels and ellipses) and response variables (red arrows and labels). The blue labels of the three levels of the explanatory variable (nest-site type) represent the centroids of the variable, and the blue ellipses denote 95% confidence limits for the SE of these centroids.

Discussion

Studies on ectoparasites of arid birds do not agree in a clear effect of aridity on the richness and abundances of the former (see, for instance, Moyer et al. 2002, Carrillo et al. 2007, Malenke et al. 2011), probably because of differences among species on the ability to cope with such harsh environment (Carrillo et al. 2007). Our study of the ectoparasite infracommunity of a cavity-nesting bird species in a semiarid environment during three years reveals a remarkable variety and abundance of ectoparasites. Among the most common avian nest-based ectoparasites only fleas and *Culex* mosquitoes are missing in our records. To our knowledge, fleas have not been cited parasitizing European rollers (see, for instance, Tripet and Richner 1997) and we have never found them in natural and artificial cavities occupied by various breeding bird species. *Culex* mosquitos are frequently found in CDC traps in our study area (unpubl. data). Their absence in our sampling could be due to the fact that sticky traps are not effective in capturing them. However, we have rarely observed *Culex* mosquitos in the nest boxes, which may be due to the exophylous nature of the species in the area. In contrast, the abundance of blackflies is remarkable since only scarce and temporary brackish watercourses occur in our study area.

Effect of spatial location and internest distance on the ectoparasite infracommunity

We did not find any spatial structure both in the occurrence and in the abundance of the infracommunity of ectoparasites. A strong effect of location and distance has been found at large scales (hundreds or thousands of kms) where environmental conditions differ substantially (Gómez-Díaz et al. 2008, Krasnov et al. 2008). However, at smaller spatial scales the effect of locality on ectoparasites richness and abundances has been reported (Lareschi and Krasnov 2010) together with factors such as microclimate, habitat characteristics at mesoscale or host-related features (Poulin 2004, Kleindorfer and Dudaniec 2009, Krasnov et al. 2015, Kleindorfer et al. 2016, Dube et al. 2018). Given that water availability and moist habitats determine the distribution and abundance of biting midges, mosquitoes and blackflies (Braverman et al. 1974, Ferraguti et al. 2016), we expected some geographical pattern associated to the proximity of temporary water courses or ponds to some nests. However, the spatial scale of our study and the high dispersal abilities of most of our study species (Crosskey 1990, Murray and Kirkland 1995, Veiga et al. 2020) may account for the lack of a clear spatial pattern and an effect of distance on the ectoparasite infracommunity.

Determinants of the spatial distribution of the ectoparasite infracommunity

Habitat characteristics close to the nests (nest-site type) are the most important variables defining the differences on ectoparasites occurrence and abundance. This is particularly interesting since nests in our study area are interspersed, so that nest boxes in a given habitat (e.g. trees) can be closer to another in a very different one (e.g. cliff). Since this could influence host detection and facilitate infestation, we would expect similar infracommunities in neighbouring nest. However, this is not always the case. Ticks occur almost exclusively in nest boxes on cliffs (78.6% of the infested nests, n= 14) whereas hematophagous mites select nest boxes on trees (81.8% of the infested nests, n= 18). Blackflies and biting midges are more frequently found and are more abundant in nest boxes on trees while sandflies are more prevalent and abundant in nest boxes on cliffs and farmhouses. The prevalence of other parasites (carnid flies and louse flies), does not seem to be affected by the habitat around the nest, although *Carnus* is more abundant in nest boxes on cliffs and farmhouses (see below).

Most Argasidae ticks are nest, burrow or roost parasites (Klompen et al. 1996), and *A. reflexus*, the most common species in our study area, is frequently found in pigeons (Dautel et al. 1999), but also in Little owls and Jackdaws (Murillo et al. 2013, personal observations). In our study area, these bird species usually breed in crevices and cavities on cliffs, so that the social environment of rollers breeding on sandcliffs could be responsible of the higher prevalence of ticks.

The higher prevalence of hematophagous mites (a contact-transmitted parasite) in nest boxes on trees could be explained by the preferential occupation of such locations by starlings (*Sturnus unicolor*), a common host of hematophagous mites (Błoszyk et al. 2016), prior to rollers arrival. No breeding attempt of starlings in nest boxes on cliffs has been registered in our study area for 14 years.

The preferences of blackflies and biting midges for nest boxes located on trees agree with previous studies reporting their selection of tree canopies for resting (Carpenter et al. 2008, Černý et al. 2011). Similarly, sandflies use to inhabit undergrowth, rock crevices, animal burrows and human dwellings as resting sites during daytime (Lane 1993). In our study area, these microhabitats are mostly found in cliffs and farmhouses.

The high prevalence of carnid flies (92%, n = 104) can be explained by their remarkable dispersal ability (Veiga et al. 2020). Its higher abundance in nests on cliffs and farmhouses than in nests on trees was also reported by these authors. Veiga et al. (2020) also found that *Carnus* abundance was positively related to the density of other potential neighbouring host species, which is higher in cliffs and farmhouses than on trees. Habitat-related differences in the predation pressure to which these parasites are subjected (in prep.) could also explain the differences in the abundance of carnid flies among nest site types.

Our analyses revealed that breeding phenology contributed to explaining the variation in ectoparasites abundances, but this effect affected almost entirely to carnid flies, that are less abundant in late nests (see also Calero-Torralbo et al. 2013). These authors suggested that early emergence of carnid flies enhanced the probability of host finding at the beginning of the breeding season. We did not find an effect of phenology on the abundance of the other ectoparasites, even though other studies found higher abundances of biting midges and blackflies late in the breeding season (Tomás et al. 2008, Martínez-de la Puente et al. 2009a). This can be explained by the different length of the breeding periods between species and locations. Nonetheless, studies at lower taxonomic levels could reveal seasonal effects (see Veiga et al. 2018 for two biting midge species).

Brood mass seemingly had no effect on the variation of the ectoparasite infracommunity. Previous studies did find a relation between brood mass and the abundance of some ectoparasites (see, for instance, Veiga et al. 2020 for *Carnus hemapterus*, and Martínez-de la Puente et al. 2009b for biting midges), probably due to an associated increase in host cues or food availability. Nonetheless, this variable could contribute similarly to all the ectoparasite taxa here studied, thus increasing similarly their abundances.

Interactions among parasites at the infracommunity level

Several kinds of interactions could occur within the infracommunity, from antagonistic to facilitating (Pedersen and Fenton 2007). Our observational approach suggests some positive (prevalences of biting midges with mites and of louse flies with sandflies, and abundances of biting midges with blackflies) and negative relationships (prevalences of blackflies with ticks, of biting midges and mites with louse flies and sandflies, and abundances of carnid flies with blackflies). Some of these correspondences may just reflect common or different habitat preferences (e.g. blackflies and biting midges preferring nests on trees, ticks and sandflies associated to cliffs and farmhouses), but others could reveal competition (carnid flies and blackflies preferring nestlings of the same age and rivalling on them). Evidencing interactions among parasites is out of the scope of our study, but our results do suggest some research lines that could contribute to fill gaps in our knowledge of multiple interactions occurring within parasites communities (Johnson et al. 2010).

In conclusion, studies on the ectoparasites of birds at the infracommunity level are scarce. However, they are required to gain a complete view of the relationships among parasites, hosts, vector-borne pathogens and socioenvironmental variables. This study reveals that habitat characteristics at small scale, more than host-related traits, determine the assemblage of parasites of a cavity-nesting bird species. Nest-site type accounted for the variation in prevalence and abundance of various ectoparasite species and this occurred apparently in two ways: i) via the habitat requirements and preferences of ectoparasites, and ii) determining the social environment of the host: the preferential occupation (prior to the arrival of the focal species) of some nest locations by other secondary cavity bird species and the identity and density of neighbouring breeding birds could favour or exclude some ectoparasite species. Since the location of the nest boxes may critically influence the ectoparasites (and also probably the vector-borne pathogens) the hosts will be exposed to, nest box schemes should consider the (direct and indirect) consequences of a given location in terms of exposure to (ecto)parasites, what seems unusual. Moreover, studying a single parasite species or a group of parasites will probably result in a misleading view of host-parasite interactions and their consequences on host health.

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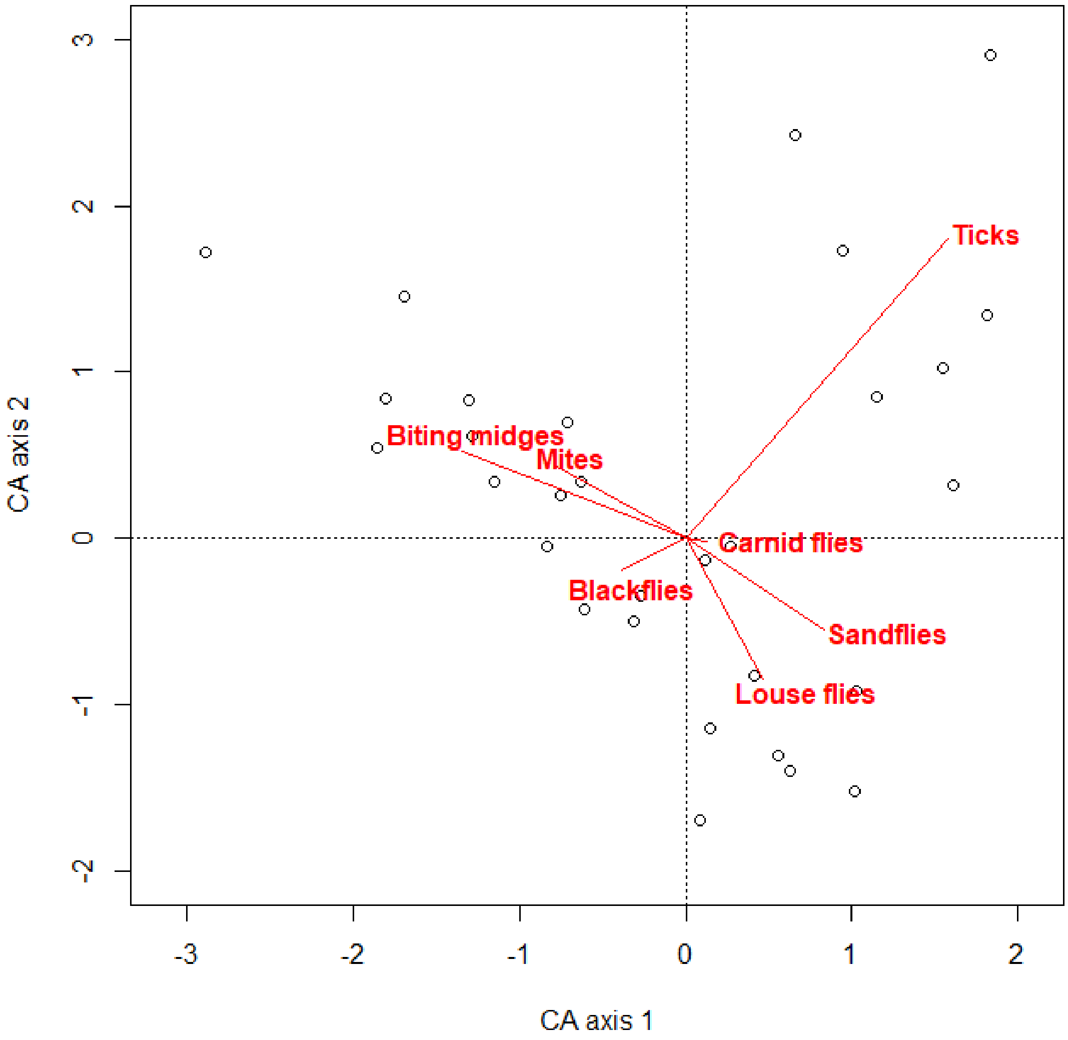
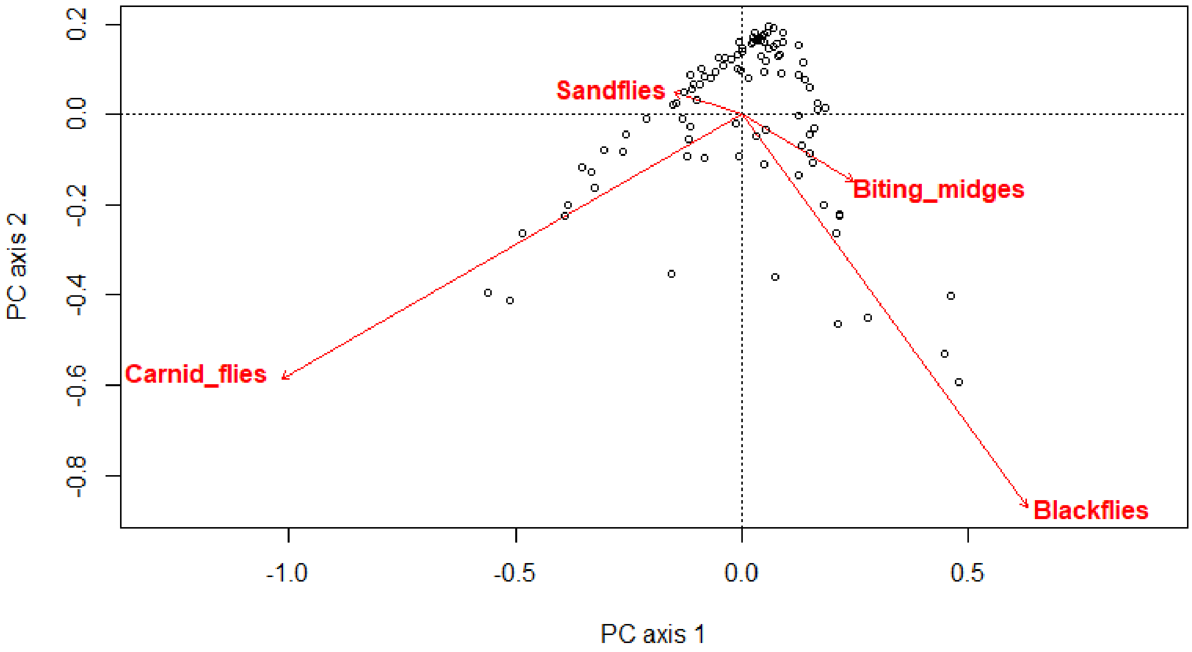
Supplementary material



Supplementary Fig. S1. Unconstrained Canonical Analysis biplot (scaling 2) on the relationships between the occurrence of different ectoparasite species of the European roller. The biplot shows the relationships among ectoparasites (red arrows and labels). Black circles denote individual nests. The first two axes explain 48.8% of the variation (axis 1 = 29.4%; axis 2 = 19.4%).



Supplementary Fig. S2. Principal Component Analysis correlation biplot (scaling 2) on the relationships between the abundance of different ectoparasites of the European roller. The biplot shows the relationships among ectoparasites abundances (red arrows and labels). Black circles denote individual nests. The first two axes explain 79% of the variation (axis 1 = 45.2%, axis 2 = 33.53%).

Partitioning of scaled Chi-square

	Inertia	Proportion
Total	1.586	1
Unconstrained	1.586	1

Importance of the two first components

	CA1	CA2
Eigenvalue	0.4662	0.3075
Proportion Explained	0.2939	0.1938
Cumulative Proportion	0.2939	0.4878

Species scores for the two first component

	CA1	CA2
Carnid_flies	0.1269	-0.01508
Mites	-0.7893	0.44626
Louse flies	0.4613	-0.84282
Ticks	1.5784	1.80311
Blackflies	-0.3855	-0.19594
Biting_midges	-1.3477	0.52908
Sandflies	0.8307	-0.54689

Supplementary Table S1. Results of CA analysis on the occurrence of ectoparasites of the European roller at the infracommunity level.

	Inertia	**Proportion**	**Rank**
Total	1.58628	1.00000	
Conditional	0.05490	0.03461	2
Constrained	0.31768	0.20027	2
Unconstrained	1.21370	0.76512	6

Eigenvalues for constrained axes

CCA1	**CCA2**
0.28882	0.02886

Permutation test for CCA under reduced model

	Df	**Chi Square**	**F**	**Pr(>F)**
CCA1	1	0.28882	23.0831	0.001
CCA2	1	0.02886	2.3065	0.041
Residual	97	1.21370		

Permutation test for CCA under reduced model. Marginal effects of terms

	Df	**Chi Square**	**F**	**Pr(>F)**
Nest site type	2	0.31768	12.695	0.001
Residual	97	1.21370		

Supplementary Table S2. Results of CCA analysis on the occurrence of ectoparasites of the European roller at the infracommunity level.

Partitioning of variance

	Inertia	**Proportion**
Total	0.1123	1
Unconstrained	0.1123	1

Importance of the two first components

	PC1	**PC2**
Eigenvalue	0.05078	0.04118
Proportion Explained	0.45205	0.33528
Cumulative Proportion	0.45205	0.78733

Species scores for the two first components

	PC1	**PC2**
Carnid flies	-1.0164	-0.58397
Blackflies	0.6314	-0.87105
Biting midges	0.2467	-0.14728
Sandflies	-0.1497	0.04811

Supplementary Table S3. Results of PCA analysis on the abundance of ectoparasites of the European roller.

	Inertia	Proportion	Rank
Total	0.112333	1.000000	
Conditional	0.005391	0.047992	2
Constrained	0.023901	0.212852	3
Unconstrained	0.083032	0.739156	4

Eigenvalues for constrained (RDA) and unconstrained (PC) axes

RDA1	RDA2	RDA3	PC1	PC2	PC3	PC4
0.017258	0.005829	0.000824	0.03183	0.03040	0.01124	0.00957

Permutation test for RDA under reduced model

	Df	Variance	F	Pr(>F)
RDA1	1	0.017258	19.7456	0.001
RDA2	1	0.005829	6.6688	0.003
RDA3	1	0.000824	0.9423	0.420
Residual	95	0.083032		

Permutation test for RDA under reduced model. Marginal effects of terms.

	Df	Variance	F	Pr(>F)
Nest site type	2	0.017113	9.7898	0.001
Phenology	1	0.006797	7.7771	0.001
Residual	95	0.083032		

Supplementary Table S4. Results of RDA analysis on the abundance of ectoparasites of the European roller.

Chapter 6: *Culicoides paolae* and *C. circumscriptus* as potential vectors of avian haemosporidians in an arid ecosystem

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Abstract

Haemosporidians are the most important vector-borne parasites due to their cosmopolitan distribution and their wide range of hosts, including humans. Identification of their vectors is critical to highlight ecologically and epidemiologically relevant features such as host specificity or transmission routes. Biting midges of the genus *Culicoides* are considered the main vectors of *Haemoproteus* spp., yet important information on aspects such as vector feeding preferences or vector-host specificity involving haemosporidian parasites is frequently missing. We assessed the abundance of *Culicoides circumscriptus* and *C. paolae* and blood sources of the latter at the nests of cavity-nesting bird species (mainly the European roller *Coracias garrulus*) and in their surroundings. We also explored the prevalence and genetic diversity of avian haemosporidians in parous females of both species. Both *C. circumscriptus* and *C. paolae* were abundant in the study area and common at European roller nests. *Culicoides paolae* had a diverse ornithophilic diet, feeding on at least seven bird species. Human DNA was also detected in the blood meal of some individuals. Four *Haemoproteus* lineages, including a new one reported here for the first time, were isolated from parous females of both biting midges. In conclusion, *Culicoides circumscriptus* and *C. paolae* can play a locally important role in the transmission dynamics of *Haemoproteus* parasites in a community of cavity-nesting bird species in an arid ecosystem.

Introduction

Biting midges of the genus *Culicoides* are small, cosmopolitan blood-sucking insects playing an important role as vectors of numerous viruses, filarial nematodes and protozoa affecting human, livestock and wildlife (Borkent 2005, Carpenter et al. 2013). *Culicoides* are regarded as the main vectors of *Haemoproteus* (class Aconoidasida, order Haemosporidia, subgenus *Parahaemoproteus*) (Atkinson and Van Riper 1991). Still, they are the least studied of the major dipteran vector groups and our knowledge of their vectorial role is biased. On one side, their participation in the transmission of livestock and human viruses has received much attention (e.g. for bluetongue virus see Mellor et al. 2000, for African horse sickness virus see Mellor and Boorman 1995, for Oropouche virus see Da Rosa et al. 2017). On the other side, much less is known about their role in the transmission of avian haemosporidians. In particular, malaria-like parasites of the genus *Haemoproteus* are highly prevalent avian haemoparasites (Valkiūnas 2005) with a relevant impact on the health status, longevity and fitness of their avian hosts (Merino et al. 2000, Marzal et al. 2005, Valkiūnas et al. 2006, Tomás et al. 2007, Martínez-de la Puente et al. 2010, Asghar et al. 2011, 2015). *Haemoproteus* presents a high diversity in host-parasite associations (Valkiūnas 2005) and it is unclear to what extent this diversity is due to host-parasite, host-vector or vector-parasite specificity (Hellgren et al. 2008, Ishtiaq et al. 2008, Kimura et al. 2010, Martínez-de la Puente et al. 2011, Njabo et al. 2011, Santiago-Alarcon et al. 2012a). Moreover, the vector identity and ecology of most *Haemoproteus* lineages is unknown (Garnham 1966, Valkiūnas 2005).

Tracing the feeding preferences (i.e. feeding patterns) of female *Culicoides* is critical to identify host-vector-parasite associations as well as ecologically and epidemiologically relevant features such as host specificity or transmission routes. Biting midges have a clear preference to feed mainly on either birds or mammals, with some species showing an opportunistic behaviour (Martínez-de la Puente et al. 2015). Traditionally, the feeding preferences of *Culicoides* have been assessed based on morphological characterization of the sensory structures (i.e. for palps and antennae see Jamnback 1965, Braverman and Hulley 1979, Augot et al. 2017a). Other methods, such as the precipitin test

(Weitz 1956, Braverman et al. 1971, Nevill and Anderson 1972), immunological assays (Blackwell and Mordue 1994, Blackwell et al. 1995), and more recently, MALDI-TOF (Niare et al. 2016) and molecular tools (Alcaide et al. 2009, Van der Saag et al. 2016), have been applied to specifically identify the blood meal sources of female *Culicoides* and other insect vectors. Methods like the immunological assays are useful when the suitable hosts are suspected, which is commonly the case for the *Culicoides* surveys done in relation with livestock (Goffredo et al. 2004, Lysyk 2006, Casati et al. 2009, Narladkar and Shivpuje 2014). Yet, biting midges trapped in the wild may have a broad range of potential hosts, supporting the necessity to use approaches allowing the identification of a wide range of vertebrate species (Martínez-de la Puente et al. 2015). Studies on *Culicoides* feeding preferences in natural areas are especially scarce, although they provide a more complete view of the circulation of the blood parasites in the wild.

Here we studied the role of two common ornithophilic species of *Culicoides* in the transmission of avian haemosporidians in the driest European area, the Desert of Tabernas (south-eastern Spain). In this area, the prevalence of infection by *Haemoproteus* spp. varies between avian species, with a total absence of parasites found in adult Trumpeter finches (*Bucanetes githagineus*) while all the adult European rollers (*Coracias garrulus*) sampled showed evidence of infection (Valera et al. 2003, Václav et al. 2016). The haemosporidian species described for rollers in this area (*Haemoproteus coraciae*) was clustered, based on phylogenetic analysis, with other *Haemoproteus* spp. vectored by *Culicoides* (Václav et al. 2016), although the dipteran species involved in its transmission remains unidentified.

At least 81 species of the genus *Culicoides* are present in Spain (Alarcón-Elbal and Lucientes 2012). In south-eastern Spain, different ornithophilic *Culicoides* species have been recorded including *Culicoides paolae*. This species, registered for first time in Spain in 2008 (Estrada et al. 2011), has been frequently associated with livestock farms (Boorman et al. 1996, Goffredo et al. 2004, Barceló and Miranda 2017). However, analysis of the sensory structures suggests an ornithophilic preference in this species (Meiswinkel et al. 2004), although the host sources of blood remain unidentified, and its role for the transmission of avian haemosporidians is completely unknown. This contrasts with other well-known, sympatric ornithophilic species such as *C. circumscriptus*, a common species in southern Spain which may be involved in the transmission of *Haemoproteus* parasites (Černý et al. 2011, Martínez-de la Puente et al. 2011, Ferraguti et al. 2013).

To assess the potential of *C. paolae* and *C. circumscriptus* as the vectors of blood parasites in a community of birds in south-eastern Spain we: (i) collected the specimens of the two species inside and in the surroundings of the nests of the European roller, one of the locally most abundant troglodytic species; (ii) identified the blood meal sources of engorged females; and (iii) studied the prevalence and genetic diversity of *Haemoproteus* parasites harboured by parous biting midge females.

Material and methods

Study area

This study was performed in an approximately 50 km^2 area located in the Desert of Tabernas (Almería, SE Spain, 37° 05'N, 2°21'W). The landscape mostly consists of open shrubland with olive and almond groves interspersed among numerous dry riverbeds (ramblas). Inhabited farms are scarce and scattered along the study area. The climate is temperate, semiarid Mediterranean with a strong water deficit during the long, hot summer months (June to September), when the absolute maximum monthly temperature is higher than 40 °C and the monthly average of the maximum daily temperatures remains above 30 °C (Lázaro et al. 2004). The average annual temperature is 18 °C, with mild inter-annual oscillations of 3–4 °C and significant intra-annual fluctuations (Lázaro et al. 2004). The mean annual rainfall is *c.*230 mm with high inter-annual and intra-annual variability (Lázaro et al. 2001).

The bird community comprises species that breed mainly in cavities in the study area (e.g. the Little owl *Athene noctua*, scops owl *Otus scops*, Eurasian jackdaw *Corvus monedula*, Common kestrel *Falco tinnunculus* and feral pigeon *Columba livia*), chiefly in natural holes in sandy cliffs but also in cavities in human constructions (Valera et al. 2018). The European roller (hereafter roller) is a common breeding species in the study area where it is distributed patchily according to distinct geomorphological units (Václav et al. 2011): (i) ramblas (dry stream channels with steep sandstone banks), which are linear, continuous geographical units separated from neighbouring ramblas by hills and human settlements; (ii) individual bridges with numerous, densely spaced cavities (*c.*2–3 m apart); and (iii) spatial aggregations of suitable nesting places, mostly trees with nest boxes but also small sandstone banks with natural cavities and isolated country houses with cavities. Wooden nest boxes have been placed in these habitat types and most rollers individuals are currently breeding in them (height × length × width: 310 × 232 × 230 mm, entrance diameter: 60 mm, with a removable upper lid to allow nest monitoring) installed on isolated eucalyptus trees, sandstone banks and isolated and deserted country houses (Václav et al. 2011, Valera et al. 2018). Rollers are migratory birds wintering in Africa and arriving at the breeding grounds in the study area when resident, secondary cavity-nesting birds are already settled. Eggs (mean clutch size = 4.23) (Avilés et al. 1999) are incubated by both sexes (Cramp 1985) during *c.*21 days. Rollers rear a single brood per year (Cramp 1985) with fledglings leaving the nest approximately 20–22 days after hatching in the studied population (Václav et al. 2011).

Culicoides trapping

Culicoides spp. specimens were trapped using two methods: sticky traps and CDC light traps. Sticky traps were placed in nest boxes occupied by rollers during the 2016 and 2017 breeding seasons (from 18 May to 4 July in 2016 and from 2 June to 18 July in 2017). Specifically, sticky traps were fixed under the upper lid of 69 nest boxes (32 in 2016 and 37 in 2017). In 2016 we took advantage of a pair of kestrels breeding in a next box close to a breeding pair of rollers, thus resembling natural nesting conditions with different cavity-nesting bird species breeding in close proximity (Václav et al. 2011). We followed the method described by Tomás et al. (2008) (i.e using Petri dishes smeared with body gel-oil as a non-attractant glue) but replacing Petri dishes by white vegetal papers that were fixed by thumbtacks on the inner side of the upper lid. In 2016, these sticky traps (size = 63.6 cm^2) were kept for three days in two periods of the breeding cycle: (i) at the end of the incubation phase (18–20 days after the first egg was laid); and (ii) during the nestling phase, when all chicks had already hatched (13–15 days after the first egg hatched). In 2017, sticky traps were only placed during the nestling stage, because most vectors were captured during this stage in 2016, and the trap size was increased (size = 175.5 cm^2). Thus, in 2017, a first trap was set 13 days after the first egg hatched and kept for four days. Then, it was replaced by a new trap that was kept for a second period of four days. Additionally, opportunistic catches of *Culicoides* at the nests were made by hand during routine visits.

Additionally, CDC traps were set throughout the study area during 2016 and 2017. We used traps with UV light as they are recommended to attract *Culicoides* (González et al. 2016). Moreover, since this study is part of a broader one aimed at studying the community of dipteran vectors, we also used incandescent light traps. Both trap types were put together and were also baited with CO_2 in order to use as many different stimuli as possible. Dry ice was used as source of CO_2 (1 kg of dry ice per night and pair of traps to ensure the continued emission of CO_2 until the collection of the traps at dawn). Thus, 20 pairs of CDC traps (each pair formed by one trap with incandescent light and one with UV light, *c.*50 cm apart from each other, both baited with CO_2) were set all over the study area and in the main breeding habitats of the roller, namely trees, ramblas and bridges (see above), so that eight traps were located on ramblas, eight on trees and four on bridges during 2016 and 2017. The traps were powered by a 6 V battery of 12 Ah. The trapping sessions were adjusted according to the breeding season of rollers and the moon calendar, so that traps were active on the days during or close to the period of the new moon (reducing the effect of ambient light [McDermott and Mullet 2018]), and avoiding windy nights. In 2016 we placed one group of 10 pairs of traps from 8 June to 10 June and a second group of 10 trap pairs from 7 July to 8 July. In 2017, all 20 trap pairs were set from 22 June to 1 July. Most traps (82.5%) were set before dusk or shortly after and were removed after sunrise. Captured insects were moved to the Estación Experimental de Zonas Áridas and frozen in 70% ethanol until identification.

Morphological identification

Biting midges were identified to the species level based on González & Goldarazena (2011) and Mathieu et al. (2012) taxonomic keys under a Zeiss

Discovery V8 stereomicroscope. *Culicoides circumscriptus* and *C. paolae* were the most abundant biting midges at the nests (see Results), and individuals of these species collected at the nests and with CDC light traps were analysed for blood meal origin (engorged females) or *Haemoproteus* detection (parous females). Engorged females were identified based on the presence of blood remains in the abdomen. The abdomen of each *C. paolae* engorged female was separated from the head-thorax using sterile tips on chilly Petri dishes and, subsequently, maintained in individual vials. Diet analyses were restricted to *C. paolae* as only two engorged *C. circumscriptus* females were captured. Parous females were identified based on the presence of burgundy-red pigmented abdomen that develops during the first gonotrophic cycle (Dyce 1969). As previous studies have reported a low prevalence of avian haemosporidians in *Culicoides* from southern Spain (Ferraguti et al. 2013, our unpublished observations), parous females were grouped in pools from 1 to 11 individuals according to species, date and site of capture.

DNA extraction and molecular analyses

Genomic DNA from the abdomen of each engorged *C. paolae* females and biting midge pools was extracted using the DNeasy Blood and Tissue® kit (Qiagen, Hilden, Germany) following company specifications. Negative controls (reagents without a template) were used to detect possible contaminations. DNA was stored at -20 °C until PCR amplification. To confirm the morphological identification of *Culicoides* species, we amplified a 658 base pair (bp) fragment of the mitochondrial cytochrome *c* oxidase 1 (*cox*1 gene, barcoding region) of four individuals following Gutiérrez-López et al. 2015. The vertebrate hosts of *Culicoides* females were identified by amplification of a fragment of 758 bp of the vertebrate *cox*1 gene following Alcaide et al. (2009). Finally, the presence and identity of *Haemoproteus* and *Plasmodium* spp. were assessed for the pools of parous female *Culicoides* specimens using the protocol by Hellgren et al. (2004). Parasite determination was conducted at least twice per sample to avoid false negative results (McClintock et al. 2010). The presence of amplicons was verified on 1.8% agarose gels. Positive amplifications were sequenced using the Macrogen laboratories sequencing service (Madrid, Spain) and sequences were edited using the software Sequencher™ v.4.9 (Gene Codes Corp, Ann Arbor, MI, USA).

The identity of *Culicoides* species and their vertebrate hosts were established by comparison with sequences deposited in GenBank DNA sequence database (National Center for Biotechnology Information BLAST) or the Barcode of Life Data Systems (BOLD). The molecular identification of two female *C. paolae* and two female *C. circumscriptus* confirmed the morphological identifications. Vertebrate species were confirmed if agreement was ≥ 98% with deposited sequences. Parasite lineages and morphospecies were identified by BLAST comparison with the sequences available in GenBank and MalAvi (Bensch et al. 2010).

Statistical analyses

The abundance of parous *C. paolae* and *C. circumscriptus* captured in CDC traps were analysed with a generalized linear mixed model (GLMM) with the negative binomial distribution of errors. Year (2016 and 2017) and biting midge species were included as independent variables. Scaled and centred date of sampling was included in a GLMM as a covariate. The number (log-transformed, scaled and centred) of blood-feeding parasitic dipterans captured per pair of traps was included as an offset variable to correct for their abundance in each sampling point. Trap location, identical during both years, was included as a random factor. The interaction between sampling date and *Culicoides* species was introduced to explore a seasonal effect in the capture of the two species. One outlier due to the capture of 94 parous *C. paoale* was detected and the analyses were run with and without this datum. Given that the results obtained were qualitatively comparable, we report the analysis including this datapoint.

The prevalence of *Haemoproteus* spp. in *Culicoides* pools was estimated considering variable pool sizes and 100% test specificity and sensitivity following Sergeant 2018. Statistical analyses were performed using the R envionment (R Core Team 2015) with the *lme4* and *effects* packages (Fox 2003, Bates et al. 2015).

Results

Abundance of Culicoides spp. *in avian nests*

Overall, 57 *Culicoides* spp. were collected in avian nest during both years (*n* = 42 in 2016 and *n* = 15 in 2017, Table 1). From the 57 captures, four individuals were collected opportunistically in the nest and 53 were collected by sticky traps. In addition, *Simuliidae* (*n* = 230) and *Phlebotominae* (*n* = 105) were other blood-feeding dipterans collected with the sticky traps at the nests.

The most abundant biting midges were *C. paolae* (57.9%, 33 out of 57) and *C. circumscriptus* (22.8%, 13 out of 57). Twenty-seven *C. paolae* specimens were parous and six were engorged, whereas 11 *C. circumscriptus* specimens were parous, two nulliparous (not included in Table 1) and no engorged individual was captured. Twenty-two out of the 32 *C. paolae* collected in 2016 were captured in a Common kestrel nest.

Abundance of Culicoides spp. *in CDC traps*

Overall, 7764 *Culicoides* spp. were captured using CDC traps (Table 1). Of them, 341 were parous females of *C. paolae* and *C. circumscriptus*, representing 4.4% of the total *Culicoides* spp. specimens captured (Table 1). The average number of parous females per pair of traps was 3.8 of *C. paolae* and 1.7 of *C. circumscriptus* in 2016 and 9 of *C. paolae* and 2.5 of *C. circumscriptus* in 2017. Furthermore, 30 engorged females of *C. paolae* and two engorged females of *C. circumscriptus* were also captured (Table 1).

	Inside nest		**Outside nest**	
	2016 (*n* = 33[a] nests)	2017 (*n* = 37 nests)	2016 (*n* = 20 trap pairs)	2017 (*n* = 20 trap pairs)
***Culicoides* spp.**	42 1.27 ± 4.38 (0–25)	15 0.41 ± 0.90 (0–4)	3585 179.25 ± 160.77 (0–423)	4179 208.95 ± 187.31 (2–380)
***C. paolae* parous**	26 0.79 ± 3.56 (0–20)	1 0.03 ± 0.164 (0–1)	77 3.85 ± 6.79 (0–27)	180 9 ± 20.59 (0–94)
***C. circumscriptus* parous**	3 0.09 ± 0.29 (0–1)	8 0.22 ± 0.75 (0–4)	34 1.70 ± 3.42 (0–15)	50 2.5 ± 3.01 (0–11)
***C. paolae* engorged**	6 0.18 ± 0.58 (0–2)	0	14 0.7 ± 0.92 (0–3)	16 0.8 ± 1.06 (0–3)
***C. circumscriptus* engorged**	0	0	0	2 0.1 ± 0.45 (0–2)

Table 1 Abundance, mean ± SD, and range (in parentheses) for Culicoides spp*. (overall data set) and for the subset of parous and engorged females of* C. paolae *and* C. circumscriptus *trapped in avian nests and their surroundings during 2016 and 2017.*

[a] *All nests corresponded to European roller nests with the exception of a single Common kestrel nest sampled in 2016*

The abundance of parous biting midges (*C. paolae* and *C. circumscriptus*) was greater in 2017 and decreased through the breeding season. *Culicoides paolae* was significantly more abundant than *C. circumscriptus* (Table 2). The interaction between *Culicoides* species and sampling date was also significant (Table 2), because parous *C. paolae* was more abundant late in the roller breeding season and parous *C. circumscriptus* was more abundant early in the season (Fig. 1).

Fixed effects	**Estimate**	**SE**	***z*-value**	***P***
Intercept	0.043	0.28	0.16	0.88
Species (*C. paolae*)	0.64	0.29	2.26	0.024
Date	-0.63	0.23	-2.71	0.007
Year (2017)	0.56	0.29	1.92	0.053
Species (*C. paolae*)*Date	1.10	0.31	3.57	0.0004

Table 2. Results of a generalised mixed model analysing the abundance of parous Culicoides paolae *and* C. circumscriptus *collected using CDC traps in relation to year (2016, 2017), date of capture, and the interaction between date of capture and the species of Culicoides biting midges*

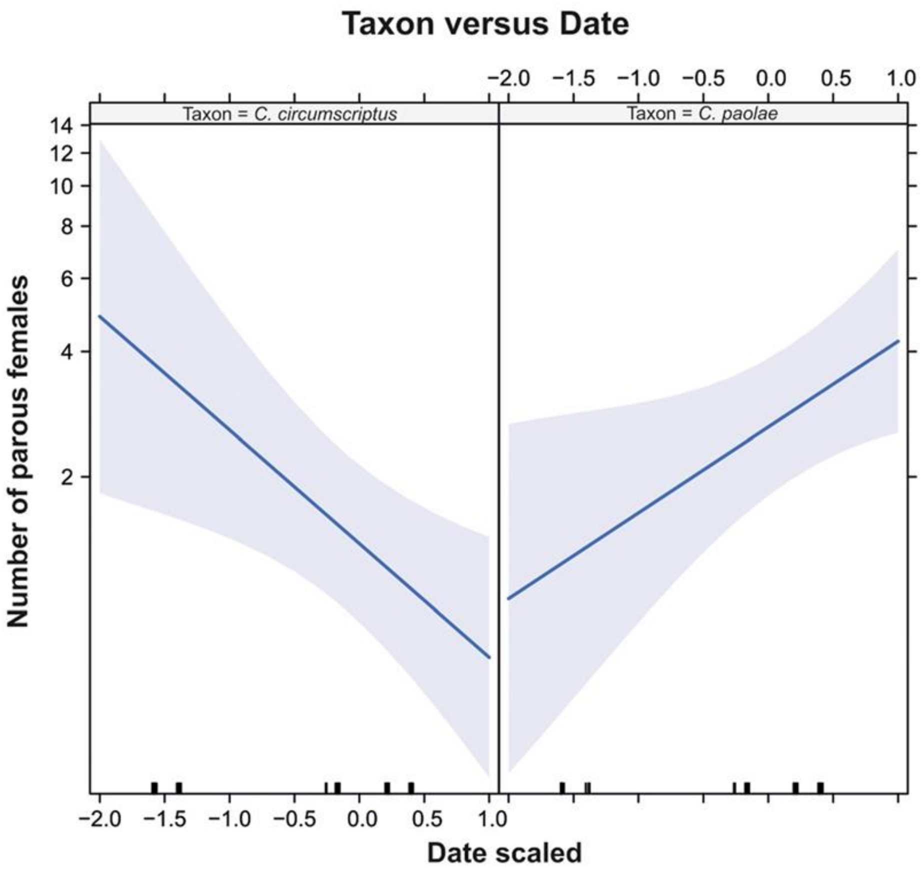

Fig. 1 Relationship between capture date and abundance of parous females of Culicoides circumscriptus *(estimate ± SE = -0.63 ± 0.23, P < 0.01) and* C. paolae *(estimate ± SE = 0.48 ± 0.21, P = 0.022) captured with CDC traps in south-eastern Spain during 2016 and 2017. Date of capture was scaled and centred. Lines represent fitted values with shaded regions showing areas delimited by 95% confidence intervals.*

Culicoides paolae *feeding patterns*

Blood meals of 21 (58.3%) out of the 36 engorged *C. paolae* were successfully identified. Of them, the six females captured in avian nests fed on the species breeding in these nest boxes (two on Common kestrels and four on rollers) (Table 3). *Culicoides paolae* females captured with CDC traps (*n* = 15) fed mainly on birds (66.7% of the identified blood meals) corresponding to five different species including cavity-nesting and open-nesting species. Finally, human DNA was found in five (33.3%) out of 15 *C. paolae* collected with CDC traps (Table 3).

	Host	Cavity/open nester	No. of successful amplifications
Inside nests	European roller (*Coracias garrulus*)[a]	Cavity-nester	4
	Common kestrel (*Falco tinnunculus*)[a]	Cavity-nester	2
CDC traps	Humans (*Homo sapiens*)	–	5
	House sparrow (*Passer domesticus*)	Cavity-nester	4
	Common blackbird (*Turdus merula*)	Open-nester	3
	Eurasian hoopoe (*Upupa epops*)	Cavity-nester	1
	Eurasian collared dove (*Streptopelia decaocto*)	Open-nester	1
	Common linnet (*Linaria cannabina*)	Open-nester	1

Table 3. Hosts of C. paolae based on the molecular identification of blood meal origin. The number of successfully identified blood meals is shown for each species.

[a] *The blood in the abdomen of the biting midge belonged to the avian species breeding at the nest where the biting midge was collected*

Prevalence and identification of haemosporidian parasites

The prevalence of *Haemoproteus* spp. was 4.4% (95% CI: 1.39–9.95%, *n* = 95 individuals) and 0.7% (95% CI: 0.12– 2.21%, *n* = 284 individuals) for *C. circumscriptus* and *C. paolae* pools, respectively. Overall, four *Haemoproteus* and one *Plasmodium* lineages were found. Of them, the three *Hemoproteus* lineages, TURDUS2, GAGLA03 (= GAGLA05, both sequences with equal coverage and identity) and AEFUN03, and the *Plasmodium* lineage SYAT05 (*Plasmodium vaughani*), were identified with 100% coverage and identity. The three *Haemoproteus* lineages, TURDUS2, GAGLA03 (=GAGLA05) and AEFUN03, were isolated from *C. circumscriptus* specimens (Table 4). The *Haemoproteus* lineage TURDUS2 and the *Plasmodium* lineage SYAT05 were isolated from *C. paolae* specimens (Table 4). In addition, a new lineage (CUPAO-01, GenBank: MH237967) was isolated from a *C. paolae* specimen. This lineage showed 93% overlap and 99% similarity with the *Haemoproteus coraciae* lineage

H1CG.1 (GenBank: KU297278) (Table 4). In fact, six nucleotide bases differed between both lineages.

Pool code	Host in this study	Closest lineages (morphospecies)	GenBank ID	Potential avian hosts and vectors described	Coverage/ identity (%)
N7c35 (Accession no.: MH237967)	*C. paolae*	H1CG.1 (*Haemoproteus coraciae*)	KU297278	*C. garrulus*	93/99
N26c1 NFc1	*C. circumscriptus* *C. paolae*	Turdus2 (*H. minutus*)	MF625183 KM361485 KJ488583 KC818452 JN819398 JN819388 JN819383 HQ398208 DQ630013 DQ060772	*E. rubecula* *G. glandarius* *M. striata* *T. merula* *T. assimilis* *T. icterocephala* *B. lineola*	100/100
NEc3 NEc4	*C. circumscriptus*	GAGLA05 GAGLA03 (*Haemoproteus* sp.)	KX831071 KJ488735 GU085197 MF594402 MF095639	*G. glandarius* *C. circumscriptus*	100/100
AGALM4	*C. circumscriptus*	AEFUN03 (*Haemoproteus* sp.)	KP715101	*A. funereus*	100/100
N31c2	*C. paolae*	SYAT05 (*Plasmodium vaughani*)	MF817773 MF347700 KJ488789 JF411406 AB477124 DQ847271	*C. caeruleus* *S. maurus* *S. unicolor* *S. atricapilla* *T. merula* *T. migratorius* *T. philomelos* *T. viscivorus* *C. pipiens*	100/100

Table 4. Molecular identification of haemosporidians in pools of parous C. circumscriptus *and* C. paolae *females trapped in avian nests and surroundings. Lineages and accession numbers from GenBank sequences showing the highest percentage of coverage and identity to those found in this study are shown. Previous information regarding these sequences is reported including the parasite morphospecies (when described), avian hosts and potential insect vectors (in bold) according to information of the reported sequences.*

Discussion

This study reveals that *C. circumscriptus* and *C. paolae* are common endophagous insects at the nests of cavity-nesting species, with *C. paolae* being identified for the first time, using identification of blood meals, as a potential vector of avian haemosporidians. This assertion is supported by detecting a high diversity of avian hosts including cavity-nesting and open-nesting species and the identification of avian haemosporidian parasites for *C. paolae*.

Whereas *C. circumscriptus* is common in Spain (Ortega et al. 1998, Ventura et al. 2005, Martínez- de la Puente et al. 2009, González and Goldarazena 2011, Del Río 2012, Estrada et al. 2013, Alarcón-Elbal et al. 2016), *C. paolae* was detected for the first time in 2008 (Estrada et al. 2011). It has been proposed that the latter species was introduced into Europe by Columbus's travels from America five centuries ago (Meiswinkel et al. 2004, Augot et al. 2017b). In spite of some morphological differences, *C. paolae* is very similar to the American *Culicoides jamaicensis* (Meiswinkel et al. 2004) and a recent phylogenetic study related the former species with *Culicoides* from the New World (Augot et al. 2017b). Nowadays, in addition to Spain, where *C. paolae* is currently expanding its distribution range (Estrada et al. 2011), this biting midge is the most widespread and abundant species of all *Culicoides* in Malta (Goffredo et al. 2004) and one of the most abundant species in central Tunisia (Slama et al. 2015) and Sardinia (Foxi et al. 2011), where its importance on the local transmission of avian vector-borne pathogens should be considered. *Culicoides paolae* is commonly found near livestock farms (Goffredo et al. 2004, Foxi et al. 2011, Slama et al. 2015), but according to our results, this species may also be widespread in the wild and, at least for the study period, it is even more abundant than *C. circumscriptus* (Tables 1, 2). Data from two breeding seasons suggest that the two species exhibit different phenologies, *C. paolae* being more abundant late in the roller breeding season whereas the opposite is true for *C. circumscriptus*. Furthermore, whereas the ability of some ornithophilic biting midges to feed inside enclosed places (endophagy) has been previously shown (Tomás et al. 2008, Martínez-de la Puente et al. 2009, Votýpka et al. 2009, Václav et al. 2016), to our knowledge this is the first time that endophagy has been recorded for *C. paolae*.

Culicoides paolae is defined as ornithophilic according to its sensory structures (Meiswinkel et al. 2004). Here we provide for the first time, unequivocal identification of its hosts, including seven different bird species within the study area. This broad spectrum of hosts has already been described for other ornithophilic *Culicoides* species (Petterson et al. 2013, Martínez-de la Puente et al. 2015).

Interestingly, some of the host species are not particularly abundant in the study area, suggesting a remarkable feeding range of this biting midge and excellent host-searching abilities. Nonetheless, a greater effort in sampling engorged females *C. paolae* together with an analysis of the bird community composition around the traps is still necessary for a better knowledge of host selection by this dipteran. Our results also suggest that *C. paolae* could feed on humans. Even though we tried to minimize the risk of contamination, we did not type the human-positive samples with DNA samples of the experimenters (e.g. Malmqvist et al. 2004). Thus, we cannot discard the possibility of sample contamination. Nonetheless, other ornithophilic species like *C. kibunensis* (Santiago-Alarcon et al. 2012b, 2013), *C. circumscriptus* (Lassen et al. 2012), or *C. pictipennis* (Bobeva et al. 2015), have previously been reported to feed on humans. The broad range of hosts could help biting midges to face environmental changes (Santiago-Alarcon et al. 2012b), and in our case, it could have facilitated the establishment of *C. paolae* in a new area.

DNA from four *Haemoproteus* lineages and one *Plasmodium* lineage was detected in parous *C. paolae* and *C. circumscriptus*. Even though multiple *Plasmodium* lineages have been molecularly detected in *Culicoides* (Martínez-de la Puente et al. 2011, Ferraguti et al. 2013), this does not imply vector competence (Valkiūnas 2011). *Plasmodium* is mainly transmitted by *Culex* mosquitoes (Valkiūnas 2005) and our result could simply reflect the presence of abortive stages of *P. vaughani* in *C. paolae* (Valkiūnas et al. 2013).

We isolated four different *Haemoproteus* lineages from six pools of *Culicoides* females. A lineage of *H. minutus* (TURDUS2) was detected both in *C. paolae* and *C. circumscriptus*. This is a geographically widespread lineage (northwest Africa, northwest Iberia, Transcaucasia and western Greater Caucasus) infecting different avian species, with *Turdus merula* probably playing a central role as reservoir (Drovetski et al. 2014). Additionally, GAGLA03 (=GAGLA05) was previously isolated in Bulgaria from *Garrulus glandarius* (Dimitrov et al. 2010), *C. circumscriptus* in Spain (Ferraguti et al. 2013, this study) and Turkey (GenBank: MF594402 and MF095639). The lineage AEFUN03 that had been only detected previously in *Aegolious funereus* (Synek et al. 2016) was found in *C. circumscriptus* in south-eastern Spain. This bird species is absent from the study area and probably this *Haemoproteus* lineage is infecting another locally abundant owl (e.g. Little owl *Athene noctua*). Finally, we also detected a new *Haemoproteus* lineage highly similar (99% similarity) to the one corresponding to the haplotype H1CG.1 (identified as *H. coraciae*), which was detected previously in the same roller breeding population by Václav et al. (2016). Microscopic examination of smears suggested that this lineage might correspond to the species *Haemoproteus coraciae* (Václav et al. 2016), a parasite identified in rollers in Bulgaria (Shurulinkov and Golemansky 2002) and Kazakhstan (Valkiūnas and Iezhova 1990). For the case of avian malaria parasites and related haemosporidians, different lineages are described with differences of a single nucleotide base in their sequences (Bensch et al. 2009). However, different lineages showing few differences may correspond to the same parasite morphospecies. Thus, it is likely that the new lineage reported here (H1CG.1) corresponds to the *H. coraciae* morphospecies*.* Further analyses are necessary to confirm this possibility. *Haemoproteus coraciae* were widely prevalent in adult rollers and also present in nestlings, suggesting the presence of a competent vector in the breeding area (Václav et al. 2016). Václav et al*.* (2016) pointed out that the detection of a *Haemoproteus* species only infecting adult rollers was intriguing because all the *Culicoides* species studied by Bobeva et al. (2015) were feeding on a wide range of avian host. Our results suggest that *C. paolae* may be a competent vector for *H. coraciae* probably playing a role on the transmission of locally circulating parasites that could be amplified by the migratory behaviour of rollers. Further analyses are necessary to confirm the vector competence of this *Culicoides* species for the transmission of the lineages isolated here (Valkiūnas 2011).

The prevalence of *Haemoproteus* in *C. circumscriptus* in the study area (4.4%) is slightly lower than the one observed in central Spain (16.7% in Martínez-de la Puente et al. 2011) and south-western Spain (10.3% in Ferraguti et al. 2013), yet it is higher than the prevalence found in the sympatric *C. paolae* (0.7 *vs* 4.4%). On the other hand, *C. paolae* is seemingly locally more abundant than *C. circumscriptus* both at the nests and in their surroundings. Therefore, both species could play an important role in the transmission dynamics of haemosporidian parasites in the study area. Nevertheless, other factors such as the efficiency of parasite transmission or seasonality in vector abundance should be considered. Concerning the latter, our study reveals that differential exposure of the hosts to individual biting midge species along the season is worth studying to fully understand the risk of haemosporidian transmission by each species.

In conclusion, vectors for most haemosporidians are unidentified (Cleaveland et al. 2001, Valkiūnas et al. 2005) and thus parasite-vector associations remain an enigmatic aspect of haemosporidian parasite ecology (Atkinson et al. 2008, Kimura et al. 2010, Njabo et al. 2011). Here, we provide valuable information about the *Haemoproteus* lineages potentially transmitted by two biting midges species. *Culicoides paolae* and *C. circumscriptus* were abundant both at the nests of cavity bird species and in their surroundings, with seasonal differences in abundance during the study period. We assessed the ornithophilic diet of *C. paolae* that fed on at least seven bird species and possibly also on humans. Both biting midge species harboured several *Haemoproteus* lineages. These findings provide an important first step towards the identification of *C. paolae* and *C. circumscriptus* as potential vectors of avian haemosporidian parasites.

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Discussion

This thesis has intended to add to the understanding of the factors affecting the spatial distribution of ectoparasites and, thus, to a better comprehension of host-parasite interactions. Much work has been done in this regard and some general rules can be drawn. Overall, it is known that community assemblage is determined by competition among species (MacArthur and Levins 1967), by environmental constraints (Ackerly and Cornwell 2007) and by dispersal limitations and population dynamics (Hubbell 1979). The relative contribution of these components in the organization of the parasite communities can differ across different scales (see, for instance, Krasnov et al. 2015): at large scales location and distance have strong effects whereas at smaller spatial scales other factors (such as microclimate, habitat characteristics at mesoscale or host-related features) become more important (Poulin 2004, Krasnov et al. 2015). However, we miss information on several aspects: i) many of the studies performed so far have focused on intestinal helminths whereas work on communities of ectoparasites have been less studied (but see, for instance, Krasnov et al. 2006a, b, 2008, Lareschi and Krasnov 2010); ii) reports focused on ectoparasites usually are restricted to specific species (see Clayton et al. 2010 for references) or to one ectoparasite taxon (e.g. fleas, see Krasnov et al. 2006b); iii) investigations into the effect of factors such as locality on the structure of ectoparasite assemblages have mainly been carried out at large spatial scales (Krasnov et al. 2006a, Gómez-Díaz et al. 2008, Krasnov et al. 2008) and it is unclear whether such patterns also occur on a small spatial scale. As a result, we ignore to what extent ectoparasite communities respond to subtle variations in the environment (but see, for instance, Lareschi and Krasnov 2010). We also ignore whether the particular characteristics of each environment (e.g. an arid environment, a marshland…) produce different results.

Considering these issues, we have focused on the following questions:

- To what extent does an ectoparasite respond to subtle changes in the environment at a local scale? And the ectoparasite community?
- At a local scale, are host-related factors important for ectoparasites in comparison to off-host variables?

- Given that many ectoparasites are vectors of pathogens, to what extent do off-host and host related factors influence the exposure of hosts to vector-borne pathogens?
- Is there enough information to address these questions? Or should be work on the basis of (more or less) educated guesses?
- What do we know about host-parasite interactions in arid and semi-arid environments?

We believe that this thesis offers some answers to the above questions. The most logical start should refer to the penultimate question: we frequently miss basic information on the study species and, then, the dilemma is whether effort should be devoted to fill these gaps or whether we can progress based on some educated assumptions. We have chosen the first way. Overall, we know little about the natural history and biology of many parasites. And this prevents, to our opinion, addressing some questions thoughtfully. For instance, we ignore much about the entomofauna dwelling in the detritus of birds' nests. Is then logical to assume that all flies emerging in a nest are the ubiquitous, cosmopolitan ectoparasite *Carnus hemapterus*? Some works report that *Carnus hemapteurs* can coexist with closely related and morphologically similar, non-parasitic species (e.g. *Hemeromyia anthracina*) that are pretty unknown for entomologists (Valera et al. 2006). We therefore studied the pupal stage of carnid flies and of *H. anthracina* to be able to distinguish both species and to study habitat selection of each of them. Our results showed that both species coexist in nest boxes in our study area even though the abundance of the latter species is low and probably determined by the occurrence of vegetal matter added to the nest boxes by some bird species. This helped us to prepare our working scenario in the best way. This scenario, based on the installation of nest boxes in different nest-site types, can be seen as an experimental approach that enables the study of habitat selection by parasites at a local scale and the effect of habitat type on host-parasite interactions. We offered clean nest boxes every year during the study period, what discards the occurrence of *H. anthracina* and allows the study of host colonization by ectoparasites.

Our study is not restricted to the nest boxes. We also considered the effect of the social environment (density of potential avian hosts and the associated density of parasites) around our focal study species on the colonization success of carnid flies. But, again, information about species-specific preferences of *Carnus hemapterus* is scarce. Instead of assuming that this parasite has no marked preferences for some of the bird species in our study area, we analysed its prevalence and abundance (both during the adult and the pupal stage) in other abundant bird species, the Rock pigeon and the Jackdaw (the results for the latter are currently in preparation), that are common neighbours of the European roller. Our work revealed the preferences of carnid flies (and of other parasitic diptera) for some avian hosts and that the infestation patterns observed for imagoes and puparia were not always consistent. These results allowed us to contribute to the concept of host range (see **Chapter 2**). Importantly, they also allowed us to estimate correctly parasite density around our focal study species and analyse its effect on host colonization success with solid foundations.

Dispersal ability of parasites is another little-known topic even though it is basic for the understanding of the factors affecting host colonization success (Boulinier et al. 2016). We found that carnid flies have a very short life span during their dispersal stage and that both environmental factors and parasite-related factors influence it. Yet, our results on several years show that they can colonize ca. 100% of the nests, what agrees with the results found elsewhere (see Liker et al. 2001, Soltész et al. 2018). Short free-living stages and exceptional dispersal abilities have been described for other parasites (Johnson 1969). This could be explained by fine-tuned host detection mechanisms and/or by high numbers of dispersing individuals, some of which can finally find a host. We tried to examine experimentally the cues that carnid flies could use to detect a host but, unfortunately, the flies did not respond to various stimuli (probably because the experimental set up was too artificial). Concerning the second explanation, interestingly, we found that experimental increase in the density of carnid flies near hosts' nests did not result in higher parasite abundance in these nests. This was an unexpected result. Among the various explanations that could account for this result (see **Chapter 4**), we would like to remark here two of them. The first one refers to the possibility that *C. hemapterus* uses other organisms to reach the hosts nests, since we found some carnid flies on a hipoboscid fly (*Pseudolynchia canariensis*). Transmission of feather lice by hitchhiking on parasitic louse flies has been reported (Keirans 1975). This type of transmission is risky for lice since they are usually host-specific and hipoboscid flies are reported to be generalist ectoparasites, so that they could carry the lice to an unsuitable host. One could argue that this is not our case, since *C. hemapterus* is a generalist ectoparasite. However, we argue that some louse flies can be more specialist than previously recognized. We did not find a single individual of *P. canariensis* on rollers, so that carnid flies hitchhiking on this louse species have high probabilities of landing on pigeons, which are suboptimal hosts for carnid flies (**Chapter 2**). Unfortunately, evaluating the importance of this type of transmission is quite difficult both observationally (a high sample size would be required) and experimentally. The second explanation refers to the fact that intra or interspecific interference among parasites could limit the colonization rate of nests by *C. hemapterus*. Our experimental manipulation of parasite density was done near nest boxes on trees. This habitat is preferred by blackflies and our results suggest a negative association between both ectoparasites (**Chapter 6**), so that this explanation is likely. Nonetheless, such association should be studied carefully. It would also be interesting to repeat the experiment around nest boxes on sandstone cliffs, after controlling for host density.

Concerning the first and second questions (effect of local changes in the environment and of host-related factors on ectoparasites) we found that nest-site type influenced colonization success of the most abundant ectoparasite, *Carnus hemapterus*, so that larger number of flies colonized nest boxes on sandstone cliffs-farmhouses than nest boxes on trees. Nest-site type also explained differences among nests in composition of the ectoparasite infracommunity and in abundance of other ectoparasite species. These results are, to our opinion, solid since they are not the consequence of a short-term sampling but have been obtained during several breeding seasons. The effect of locality on ectoparasites has been found elsewhere (Krasnov et al. 2005, Lareschi and Krasnov 2010). Lareschi and Krasnov (2010) revealed that there was a significant effect of locality for some species, but not for others. They explained such differences on the basis of differences among parasites in life history and the degree of dependence of the host, what influences ectoparasites sensitivity to spatial or temporal variations in the environment (Marshall 1981). Consequently, they expected that the relative effect of locality would be weaker in parasites that are closely associated with their hosts and stronger in parasites that spend most of their lives off-host (Lareschi and Krasnov 2010). In our case, the distances among localities are smaller than in the work by Lareschi and Krasnov (2010). In fact, nests in the various site types are frequently interspersed in our study area. However, we found that, in spite of the small spatial scale of this study, most ectoparasites responded to differences in the environment. We found clear preferences of mites, blackflies and biting midges for nest boxes on trees while ticks, carnid flies and sandflies showed preferences for nest boxes on cliffs and farmhouses. Louse flies were not affected by the habitat around the nest. Thus, our results suggest that, in a semi-arid environment, the differences between a nest under a vegetated cover and an exposed nest in a devegetated cliff are probably not so subtle and elicit clear responses by several parasites. Interestingly, our results do not agree with the above mentioned expectation: parasites closely associated to the host (or to its intimate environment) like mites and carnid flies showed clear responses to local differences. Parasites spending most of their lives off-host (biting midges, sandflies and blackflies) also showed clear responses to local differences (with the single exception of louse flies). Probably these preferences are not elicited only by environmental factors. We think that the distribution of mites can be associated to other hosts (starlings that occupy nest boxes on trees but not on cliffs-farmhouses). Socioenvironmental factors (occurrence of other host species or density of potential hosts) could also explain the distribution of ticks and carnid flies (see **Chapter 5**). Finally, some aspects of the life cycles of these parasites as well as interspecific interactions could account for some of our results. These possibilities remain to be investigated.

The host-related factors here considered are seemingly less influential for ectoparasites than the socioenvironmental ones. Specific studies on carnid flies revealed an effect of brood mass and breeding phenology of the host. Higher abundance of flies on larger broods could be due to higher detectability or to a mere size effect (host individuals can be seen as habitat islands for parasites and the larger an island is, the more parasites are expected to inhabit it, Valera et al. 2004). As said before, laboratory experiments on the sensory cues used by carnid flies to detect hosts did not render any result. It would be interesting to explore this issue in the field and, in fact, some studies on the effect of gas composition in the nest boxes on the abundance of *C. hemapterus* are in progress. However, the results of this study will be correlational so that additional effort should be done to get an appropriate experimental set up to study this issue. The seasonal decrease of the abundance of *C. hemapterus* has been regarded as a way to diminish the risk of losing suitable hosts (Calero-Torralbo et al. 2013). Carnid flies emerge continuously from the beginning of the season and bivoltinism has been also recorded (Amat-Valero et al. 2012). Consequently, early emerging flies have many hosts available and the selective forces for prompt emergence must be strong. An alternative explanation is that carnid flies also emerge late in the season, when some late roller pairs are still breeding, but that the colonization success of these flies is very low, given that humidity and flowering plants (i.e. food) (that decrease along the season) influence the life span of the dispersal stage (**Chapter 3**). Seasonal effect on other parasites were not detected in our study at the infracommunity level, even though they were expected. For instance, parasites with stages that require water for larval development (e.g. blackflies) should be affected by the drying of temporary watercourses as the season progresses. More detailed studies are probably required to detect such effect. In fact, we found that two species of biting midges exhibit different phenologies (**Chapter 6**). Similar cases are likely to occur in other taxa and are worth to be studied.

Could there be confounding factors accounting for our results? For instance, could it be that rollers in nest boxes on trees have nestlings with worse body condition or poorer immunocompetence (that is also affecting the abundance of carnid flies, Václav et al. 2008, Václav and Valera 2018)? Preliminar work has not found significant differences in these variables but deeper studies should be done.

Our results show that nest-site type determines the risk of exposure of adult and nestling rollers to different ectoparasites. Since many of them are vectors of pathogens, birds could also be exposed differently to such pathogens. For instance, rollers breeding in nest boxes on trees are attacked by biting midges that harbour up to four lineages of *Haemoproteus* (**Chapter 6**). However, rollers breeding in nest boxes on cliffs are attacked by soft ticks, in which we have found a high prevalence of *Rickettsia* (unpubl. information). These results have obvious management implications about the criteria that should be used to install nest boxes for endangered bird species that are readily discussed in **Chapter 5**. It would be important to know whether there is some kind of nest-type imprinting or nest-type fidelity that could enhance the differential exposure of some individuals to specific pathogens. Again, the data set available enables addressing such questions.

Finally, concerning the last question posed at the beginning of this section, we are afraid that we know little about host-parasite interactions in arid and semi-arid environments. Of course, some work has been done in our country and elsewhere but we feel there is much to do. One could wonder, for instance, whether the patterns found in other habitat types (see, for instance Lareschi and Krasnov 2010 in marshlands) hold in arid environments. The results of some studies also offer seemingly contradictory results. Valera et al. (2003) found a low prevalence of haematozoa in Trumpeter finches (*Bucanetes githagineus*) close to our study area and suggested that the lack of vectors could account for this result. However, nearly 100% of adult rollers in our population harbour *Haemoproteus* and vectors are seemingly abundant (see below). Furthermore, we found that the life span of the dispersal stage of *C. hemapterus* is influenced by humidity and vegetation, which is very variable in our study area (Introduction, Figs. 8 and 9). Yet, the colonization success is almost 100% every year (**Chapters 3 and 4**). Answering these questions and asking new, meaningful ones requires much basic information. To begin with, we ignore the parasite species in such areas. We have registered at least 12 species of biting midges, 4 species of sandflies, 3 species of louse flies of birds, and one species of blackfly. This is an exciting scenario that we should take advantage of.

Conclusions

1. When studying parasite-host relationships, it is necessary to consider the various phases of the life cycle of the parasite and not just the infective stage. This requires a basic knowledge of the natural history of parasites that is not always available. This work allows to differentiate the pupa of *Carnus hemapterus*, an abundant and cosmopolitan parasite, from non-parasitic sister species that coexist with it in birds' nests. This information allows studying various aspects of the parasite fundamental to understand its relationship with the host.
2. Habitat requirements of the non-infective phases of ectoparasites are important to determine the potential host range or basic processes such as the coexistence of sister species facilitated by spatial segregation. We found differences in habitat selection criteria of *Carnus hemapterus* and *Hemeromyia anthracina* that may facilitate their coexistence. In addition, the observed patterns of prevalence and abundance of imagoes and pupae of three purportedly generalist ectoparasites reveal that the requirements of non-infective stages may restrict the range of suitable hosts of some parasites.
3. *Carnus hemapterus* has a short life span (less than four days) during its dispersive stage. Its longevity during this period increases with body size, ambient humidity and with access to food (flowers). Despite this short period of dispersion, the parasite is able to colonize almost 100% of the nests of its main host in the study area.
4. The colonization success of nests of the European roller (*Coracias garrulus*) by *Carnus hemapterus* does not depend on the density of parasites. Colonization is favoured by host density and total brood mass and hindered by the late host breeding phenology. The nest-site type of the host affects the success of colonization, the abundance of colonizing parasites being greater in nests on sandstone cliffs and human constructions than in nests on trees.
5. The infra-community of ectoparasites of breeding European rollers in a semi-arid environment is varied and consists at least of species from 8 different families. Nest-site type largely explains the differences in prevalence and abundance of parasites between nests. On the contrary, neither the

geographical distribution nor the total brood mass have an effect on the variation of the composition of the infra-community and on the abundance of the studied parasites. The effect of breeding phenology is restricted to the abundance of *Carnus hemapterus*. Studying parasites at the community-level is essential for a better understanding of parasite-host relationships.

6. This study identifies associations between vectors and hemoparasites: *Culicoides paolae* and *C. circumscriptus* were common in nests of various species of troglodyte birds. We found that these biting midges feed on various bird species and that they harbour four *Haemoproteus* lineages. Both *Culicoides* species can play an important role in the dynamics of *Haemoproteus* transmission in a community of troglodyte birds in a semi-arid ecosystem.

Conclusiones

1. En el estudio de las relaciones parásito-hospedador es necesario tener en cuenta las diversas fases del ciclo vital del parásito y no sólo la fase infectiva. Esto requiere un conocimiento básico de la historia natural de los parásitos que no siempre se encuentra disponible. Este trabajo permite diferenciar la pupa de *Carnus hemapterus*, un parásito abundante y cosmopolita, de especies hermanas no parásitas que coexisten con él en los nidos de las aves. Esta información permite estudiar diversos aspectos del parásito fundamentales para entender su relación con el hospedador.
2. Los requerimientos de hábitat de las fases no infectivas de ectoparásitos son importantes para determinar el rango de hospedadores potenciales o procesos básicos como la coexistencia de especies hermanas facilitada por segregación espacial. Encontramos diferencias en los criterios de selección de hábitat de *Carnus hemapterus* y de *Hemeromyia anthracina* que pueden facilitar su coexistencia. Además, los patrones observados de prevalencia y abundancia de imagos y pupas de tres ectoparásitos pretendidamente generalistas revelan que los requerimientos de estadios no infectivos pueden restringir el rango de hospedadores adecuados de algunos parásitos.
3. *Carnus hemapterus* tiene un corto periodo de vida (menos de cuatro días) durante su fase dispersiva. Su longevidad durante este periodo aumenta con el tamaño corporal, la humedad ambiente y con el acceso a alimento (flores). A pesar de este corto periodo de dispersión, el parásito es capaz de colonizar casi el 100% de los nidos de su principal hospedador en la zona de estudio.
4. El éxito de colonización de nidos de Carraca (*Coracias garrulus*) por parte de *Carnus hemapterus* no depende de la densidad de parásitos. La colonización se ve favorecida por la densidad de hospedadores y la masa total de pollada y dificultada por la tardía fenología de cría del hospedador. El sitio de nidificación del hospedador afecta al éxito de colonización, siendo la abundancia de parásitos colonizadores mayor en nidos en taludes y construcciones humanas que en nidos en árboles.
5. La infracomunidad de ectoparásitos de la Carraca en un medio semi-árido es variada y está compuesta al menos por especies de 8 familias distintas. El tipo de sitio donde se encuentra el nido explica en buena medida las

diferencias en prevalencia y abundancia de los distintos parásitos entre nidos. Por el contrario, la distribución geográfica y la masa total de la pollada no tienen efecto en la variación de la composición de la infracomunidad ni en la abundancia de los distintos parásitos. La fenología de cría sólo afecta a la abundancia de *Carnus hemapterus.* El estudio de los parásitos a nivel de comunidad es fundamental para una mejor comprensión de las relaciones parásito-hospedador.

6. Este estudio identifica asociaciones entre vectores y hemoparásitos: *Culicoides paolae* y *C. circumscriptus* fueron comunes en nidos de diversas especies de aves trogloditas. Se ha comprobado que se alimentan de diversas especies de aves y que albergan cuatro linajes de *Haemoproteus*. Ambas especies de *Culicoides* pueden jugar un importante papel en la dinámica de transmisión de *Haemoproteus* en una comunidad de aves trogloditas en un ecosistema semiárido.

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