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Ectoparasitic mites and their *Drosophila* hosts

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RESUMEN

Los parásitos al ser ubicuos y tener un impacto negativo sobre la eficacia biológica del huésped, presentan un papel importante en la evolución y regulación de las poblaciones del mismo. Un factor clave en la extensión y abundancia del huésped es la especificidad parasitaria. Las especies de mosca del género *Drosophila*, son excelentes modelos biológicos para contestar preguntas ecológicas. Hasta el día de hoy, sólo dos interacciones con parásitos han sido descritas: moscas *Drosophila* micófagas parasitadas por nemátodos y la asociación entre ácaros parasíticos *Macrocheles subbadius* y la mosca *D. nigrospiracula*. Esta es la única asociación descrita a pesar de que otras especies de *Drosophila* también comparten su nicho ecológico con ácaros ectoparásitos de otros géneros. Los ácaros como parásitos, tienen severos efectos en la adecuación biológica de *D. nigrospiracula*, por lo tanto, al entender el grado de infestación así como la distribución de ácaros ectoparásitos entre especies del género *Drosophila*, nos dará información sobre la evolución de las interacciones huésped-parásito en general. Haciendo uso de claves morfológicas y marcadores moleculares, encontré 13 especies del género *Drosophila* infestadas con ácaros, además de la ya conocida *D. nigrospiracula*. Nueve de estas especies pertenecen al grupo *repleta* dentro del subgénero Drosophila, a pesar de haber encontrado más abundancia de moscas de otros subgéneros en las áreas de colecta. La mayoría de los ácaros parasíticos fueron identificados como *Macrocheles subbadius*, lo cual es relevante dada la gran diversidad de ácaros en el orden Mesostigmata. Sin embargo, se identificaron también dos ácaros pertenecientes a la familia Blasttisociidae, *Paragarmania bakeri* y *Lasioseius sp.*; el primero se encontró exclusivamente asociado a *D. hexastigma*. Experimentos de elección y no-elección revelaron que los ácaros tienen una preferencia por *D. hydei* sobre *D. simulans*. Los datos de distribución de este estudio no sólo demuestran que el grado de parasitismo de ácaros asociados a especies del género *Drosophila* es mayor a lo que estaba reportado anteriormente, sino que sugiere un sesgo en la distribución de ácaros ectoparasíticos mediada por la preferencia de un huésped y/o un mecanismo de resistencia exclusivo de algunos linajes de Drosophilidos.

Los parásitos reproductivos, *Wolbachia* y *Spiroplasma* han sido encontrados en un amplio rango de especies del género *Drosophila*. A pesar de la extensa evidencia de transmisión horizontal de estos endosimbiontes, el mecanismo por el cual sucede en la naturaleza no se ha determinado. En búsqueda de evidencia de una posible participación por parte de los ácaros en la trasmisión de endoparásitos, también se realizó un muestreo de bacterias endoparasíticas en *Drosophila* así como en sus ácaros asociados. Como se ha reportado previamente, se encontraron más especies de *Drosophila* infectadas por *Wolbachia* que por *Spiroplasma*. Aunque fue posible comparar las secuencias de *Wolbachia* aisladas de una mosca y su ectoparásito, no es suficiente evidencia para demostrar la existencia de transmisión horizontal.

ABSTRACT

Growing evidence on the ubiquity and negative impact of parasites on host fitness has lead to the recognition of the important role they play in the evolution and regulation of host populations. A key determinant of host range and abundance is host specificity. Understanding the basis of host specificity as well as the extent and distribution of parasitism in a species with a known biology will broaden our understanding of host-parasite interactions in general. *Drosophila* species, given their known ecological and genetic properties, are excellent models to increase our understanding of these ecological and evolutionary issues. Despite their potential to add to our understanding of these issues, only two interactions with parasites have been described to date: mycophagous *Drosophila* parasitized by allantonematid nematodes and the association of parasitic mite *Macrocheles subbadius* with Sonoran Desert endemic *Drosophila nigrospiracula*. Although several *Drosophila* species routinely encounter parasitic mites, the relationship between the mite *M. subbadius* and *D. nigrospiracula* is the only one described to date. Ectoparasitic mites exert profound effects on the fitness of adult *D. nigrospiracula;* therefore, understanding the extent and distribution of mite parasitism among other species of genus *Drosophila* could provide insights into the evolution of host-parasite interactions in general. I found 13 species of *Drosophila* in addition to *D. nigrospiracula* to have mite infestations. Nine of these species belong to the *repleta* species group of the subgenus Drosophila, despite numerous species from other subgenera being more abundant at the collecting sites. In all but two cases, the associated mites were identified as the generalist *M. subbadius,* which is surprising given the large number of parasitic mites from order Mesostigmata. *Drosophila hexastigma* was found to have not only *M. subbadius*, but a Mesostigmatid mite from family Blattisociidae, *Paragarmania bakeri,* as well. *Drosophila hydei* was also found to be associated with a *Lasioseius sp*. also from family Blattisociidae. Choice and no-choice experiments revealed that mites have a preference for *D. hydei* over *D. simulans*. Mite parasitism clearly is much broader than previously documented for *Drosophila* but also reflects a host bias mediated either by mite preference and/or some mechanism of resistance in particular Drosophilid lineages.

Reproductive parasites *Wolbachia* and *Spiroplasma* are known to infect a range of *Drosophila* species but, despite extensive evidence of horizontal transmission, a mechanism has never been identified in nature. Thus, I also surveyed endosymbiotic bacteria in *Drosophila* as well as in their associated mites, seeking evidence that mites might be involved in the transmission of endosymbionts in nature. As expected from previous studies, *Wolbachia* was found infecting more *Drosophila* species than *Spiroplasma*. This is the first time, however, that mites have been screened for either endosymbiont and both were found. Although a *Wolbachia* sequence from one mite and its host were identical, it is insufficient evidence for horizontal transmission.

INTRODUCTION

Parasites represent a large proportion of global biodiversity. Furthermore, parasitism is possibly more common than any other feeding strategy known to date (Korallo et al 2007). Parasites are ubiquitous; therefore they create a strong force on potential hosts to evolve resistance mechanisms (Combes 2001). Even ectoparasites, restricted to an area away from vital organs have pronounced impacts on host fitness. Parasites can play a key role in regulating the sizes of host populations and communities, restricting them to levels well below the carrying capacity set by resources (Jaenike and Pearlman 2002).

Host range and specificity is limited at a proximate level both by physiological and ecological factors (Poulin and Keeney 2008). Ultimately, the determinants of host range will reside in the evolutionary and biogeographical history of a parasite as well as of its potential hosts. Different levels of host specificity are displayed by different parasite taxa; some parasites are highly host-specific and thus restricted to a single host or group of hosts. On the contrary generalist parasites can exploit a wide range of alternative hosts. Dissecting the basis of host range is best accomplished in a group of organisms with defined ecological and genetic properties.

Species that can be studied in the wild as well as genetically in the laboratory offer an opportunity to broaden our understanding of a host-parasite interaction from both ecological and evolutionary perspectives. *Drosophila* species, because of our

extensive knowledge of their genetics, biology, and ecology (Markow 2015) have great potential to disentangle the contributions of both host and parasite to patterns of infestation and resistance.

In the wild, flies encounter predators both as larvae and adults, as well as parasites. The field ecology, species distribution, abundance, and diversity of *Drosophila* parasites has been insufficiently investigated (Fleury et al 2009). The only horizontally transmitted *Drosophila* parasites whose host ranges have been studied in an ecological and evolutionary context are obligate parasitic nematodes of the family Allantonematidae associated with mycophagous flies (Jaenike and Perlman 2002; Perlman and Jaenike 2003). These Drosophilids use a wide variety of mushroom species to feed and reproduce and their larvae are in the same substrate with the nematodes. An inseminated female nematode pierces the larva's cuticle, injecting larval nematodes that will grow and feed within the fly's hemocoel that will mature and reproduce several days after the adult fly has emerged. Juvenile nematodes will be passed from the fly's ovipositor or anus to the mushroom (Jaenike and Pearlman 2002). Studies on the survival of wild mycophagous *D. putrida* and *D. neotestacea* revealed a greater mortality rate in infested flies (Jaenike 1992; Perlman and Jaenike 2003). Furthermore, female fly fertility can be reduced or even eliminated by nematode parasitism. In males, fertility and mating success are affected in both wild-caught and laboratory-reared males of *D. neotestacea* (Jaenike and Pearlman 2002; Perlman and Jaenike 2003). Of the 10 nematode species (eight species of *Howardula* and two of

Parasitylenchus) parasitizing mycophagous *Drosophila,* the majority are generalists.

Less well studied are ectoparasitic mites. Several *Drosophila* species routinely encounter mites that are ectoparasites of adults and/or predators of eggs and larvae (Castrezana and Markow 2001). The association between an ectoparasitic mite and *Drosophila* initially was described in detail by Polak and Markow (1995) for the mite *Macrocheles subbadius* and the Sonoran Desert endemic cactophilic *D. nigrospiracula*. *Drosophila nigrospiracula* is a member of the *repleta* species group within subgenus *Drosophila*. This is the largest subgenus within genus *Drosophila* (Markow and O'Grady, 2005) and it has a wide distribution across the globe, with members occupying different ecological niches such as cacti and fungi (Morales-Hojas and Vieira 2012). There are over 100 species in the *repleta* species group and the majority are associated with cacti in arid and semiarid regions of the Western Hemisphere (Markow and O'Grady 2005). While some species of *repleta* use a broad variety of substrates as habitats, others are more specialized (Oliveira et al 2012). *Drosophila nigrospiracula* utilizes necrotic saguaro (*Carnegiea gigantea*) or cardón (*Pachycereus* pringlei) cactus as its feeding and breeding site (Heed 1978). When a cactus is injured, it is colonized by bacteria and yeast that create a rot pocket (Heed 1978). This releases volatiles that attract cactophilic species of insects and arthropods to the necrotic cactus tissue were they carry out their life cycles in semiarid or arid environments (Heed 1978; 1982). Different species of *Drosophila* are attracted to volatiles produced by distinct host cactus (Fogleman et al 1986). Adult flies find mates and oviposition sites at the

cactus necrosis where they feed. While both egg and pupa stages are sessile, larvae move within the decaying tissue were they undergo development (Markow 2015).

Necrotic tissue of large columnar cacti such as saguaro and cardón also serves as breeding sites for a large number of arthropods, including mites, among many other organisms (Breitmeyer and Markow 1997; Castrezana and Markow 2001). Due to their size and limited mobility, dispersal is vital for mites, especially those with restricted environmental tolerance (Mumcuoglu and Braverman 2010). They often depend on other animals, particularly arthropods, to transport them to a fresh substrate. Mites have been observed to arrive, transported by dispersing flies to new cactus necroses where they dismount from their hosts (Walter and Proctor 2013; Polak and Markow 1995). Mites eventually detach and reproduce along with the other arthropods (Polak 1996; Castrezana and Markow 2000). By monitoring cactus rots over a period of time, Polak and Markow (1995) demonstrated that intensity and prevalence of parasitism by mites increases rapidly and linearly as function of rot age. When the next necrosis finally dries out, mites attach to departing flies to colonize new cactus rots. The generalist mite *Macrocheles subbadius* also has been found on *D. mettleri*, which shares the same cactus host with *D. nigrospiracula.*

Dispersing *Macrocheles subbadius* are usually mated females, which attach to adult flies of both sexes by inserting the entire length of their chelicerae into the fly's tissue (Polak 1993) (Figure 1). These pincer-like mouthparts are the primary

organs of feeding (Walter and Proctor 2013). The association of *M. subbadius* with *D. nigrospiracula* was originally assumed to be exclusively passive. In this type of relationship, referred to as phoresy, at a certain life stage, a migrating animal actively seeks and attaches to the surface of another animal that will transport the phoreont to a more favorable environment (Mumcuoglu and Braverman 2010). Nonetheless, the mode of attachment of *M. subbadius* suggested that mites actually feed from their host hemolymph, as was confirmed by Polak (1996). Mites extract hemolymph from the flies, severely impacting the nutrient availability flies need to carry out activities like long-distance dispersal or combating disease (Polak 1998). Diversion of nutrients often coincides with reduction in egg output in females and reduction in testis size in males (Polak 1998). Besides affecting fecundity, body condition and longevity, mites interfere with mating, causing differential mating success and driving host sexual selection (Polak and Markow 1995; Polak 1996). The presence of mites increases wing load and introduces asymmetry depending on the attachment site affecting flight endurance (Luong et al 2015), which has important consequences for gene flow and metapopulation dynamics.

Figure 1. *Drosophila nigrospiracula* parasitized by *Macrocheles subaddius*.

Macrocheles subbadius belongs to the Mesostigmata, the most diverse and broadly distributed order of Parasitiformes with approximately 8,000 species of mites (WoRMS 2015). Half of these species are free-living predators occupying a variety of habitats from rotting wood, compost, herbivore dung, nests, or house dust and many are parasitic on other insects (Walter and Proctor 2013; WoRMS 2015). Most are fluid-feeders that exploit ephemeral resources, therefore tend to have rapid developmental rates.

Given the large number of Mesostigmatid mite species, it would be surprising if parasitism were limited to *M. subbadius-D. nigrospiracula* association of the Sonoran Desert. Little is known, however, about the host range of *M. subbadius* with respect to other Drosophilids. A parasite's host range is determined by the physiological, biochemical, and behavioral properties of a host (Perlman and Jaenike 2003). According to Mouillot et al (2006), selection of the most suitable host is the main external ecological attribute of parasite species and it involves several phases which may be under genetic and environmental influence, including habitat location, host location, host recognition, and host acceptance (Desjardins et al 2010). More specifically, a mite may be faced with choosing among a number of hosts inhabiting the same ecological niche, some of which may be 'better' than others (Walter and Proctor 2013). Some insects differ in their cuticular hydrocarbon composition (Francis et al 1995; Hunter and Rosario 1988; Fogleman and Danielson 2001), which could serve as a cue. In other systems where ectoparasitic mites are involved: *Macrocheles mycotrupes* and *M. peltotrupetes* locate their scarab hosts by means of olfactory stimulus (Krantz 1991). These Macrochelid

species even reacted to surface compounds extracted from their respective beetle hosts (Krantz 1991). Host recognition by mites for particular *Drosophila* species might also be determined by the hydrocarbon profile of their hosts, a character involved in mate choice recognition *in D. mojavensis* and other Drosophilid species (Fogleman and Danielson 2001). Furthermore, mites may avoid certain individuals or some potential hosts may escape parasitism through behavioral or morphological characteristics known as “front line” forms of defense. Observations in the lab show that when a mite approaches, flies exhibit reflex behavior in the form of sudden movements away from the mite. When touched by a mite, they rapidly burst into flight away from the substrate where they are standing on and when grabbed by the tarsus, flies perform vigorous grooming and tarsal flicking until they get rid of the mite (Polak 2003).

Regarding the host range of *M. subbadius* with respect to other Drosophilids, infestation has not been reported for other Sonoran Desert cactophilic *Drosophila* species, such as *D. mojavensis* and *D. pachea.* One explanation is that these other species have not been the target of studies measuring mite infestation in nature. Another could be the particular host resource of the flies. These other two *Drosophila* species utilize different columnar host cacti, senita (*Lophocereus schottii*) and organ pipe (*Stenocereus thurberi*) respectively, both of which are smaller and chemically different from saguaro or cardón (Kircher 1982; Breitmeyer and Markow 1998). Larger cacti produce necroses that last much longer than those of smaller cacti, providing a longer time for development of new generations of mites. Senita and organ pipe, for example, are smaller than saguaro and cardón

and the necroses do not last as long (Breitmeyer and Markow 1998). But mites have been found in the necrotic tissues of both of these other cactus species (Castrezana and Markow 2001), arguing against the effect of host resource on the apparent differential distribution of mites on *Drosophila* species. Furthermore, *M. subbadius* is a widespread generalist, associated with other Diptera such as houseflies (*Musca domestica*) and stable flies (*Stomoxys calcitrans)* on other continents (Mumcuoglu and Braverman 2010), where its immature stages are thought to develop in dung. The generalist ecology of *M. subbadius* thus predicts that multiple additional *Drosophila* species, such as members of large subgenera like Sophophora, which use substrates other than cacti (O'Grady and Kidwell 2002), would be parasitized. Given the fitness consequences of mite infestation for the flies (Polak 1996), understanding the extent and distribution of parasitism among Drosophilids should provide insights into the evolution of host-parasite interactions in general.

Development, ecology, and evolution of animals are strongly influenced by the associations they form with microorganisms, particularly bacteria. *Drosophila* species, in contrast to other insect groups, possess an apparently robust innate immune system that eliminates many bacterial groups (Mateos et al 2006). Only two heritable bacterial endosymbionts have been able to avoid recognition by the *Drosophila* immune system. *Spiroplasma* and *Wolbachia* are maternally transmitted reproductive parasites that have a range of phenotypic effects on their associated arthropods.

Wolbachia, a *Rickettsia*-like bacterium, is typically localized in the reproductive tissues of arthropods (Breeuwer 1997) and is estimated to be found in more than 65% of all insect species (Werren et al 2008). The effects this reproductive parasite has on its host include feminization of genetic males, induction of parthenogenesis, male-killing and cytoplasmic incompatibility. While the mechanism remains obscure, these alterations bias sex ratio toward female offspring (the transmitting sex) (Werren et al 1995; Zhou et al 1998; Werren et al 2008). Less well studied is *Spiroplasma*, a small Gram-positive bacterium associated with many host plants and arthropods (Haselkorn et al 2009; Xie et al 2010). The phenotype associated with *Spiroplasma* is the reproductive parasitism known as male-killing (Haselkorn et al 2009; Watts et al 2009; Xie et al 2010) although in some *Drosophila* species, *Spiroplasma* does not affect sex ratio (Watts et al 2009). All these modifications of reproduction caused by parasites may have implications to basic processes such as sex determination and sexual selection. Moreover, *Wolbachia* can interfere with the population dynamics of the host by increasing reproductive isolation between diverging populations that harbor different *Wolbachia* strains, which can eventually lead to speciation (Vavre et al 1999; Bordenstein and Werren 2007; Moran et al 2008). Both these heritable endosymbionts have been found in several *Drosophila* species. While infection rates vary among species and among populations of the same species, *Spiroplasma* has been reported in 16 species (Watts et al 2009). In an extensive survey of 225 species, 19 *Drosophila* species were found to have *Wolbachia* (Mateos et al 2006).

Spiroplasma and *Wolbachia* are vertically transmitted but many studies have demonstrated a lack of concordance between the phylogeny of the endosymbiont and the phylogeny of its host, indicative of horizontal transmission between host species (O'Neill et al 1992; Werren et al 1995; Vavre et al 1999; Werren et al 2008; Baldo et al 2008). Additionally, closely related strains of either *Spiroplasma* or *Wolbachia* in distantly related insect species suggest infection by horizontal transfer (Jaenike et al 2006; Sintupachee et al 2006). While some lineages of *Spiroplasma* are transmitted horizontally, usually via a plant host (Xie et al 2010), the mechanism of transmission of *Wolbachia* and most strains of *Spiroplasma* has not been identified. By placing an infected *D. nebulosa* in contact with mites, detaching the mites after 24 h and transferring them to a pipette tip containing an uninfected fly (either a conspecific *D. nebulosa* or *D. willistoni*), Jaenike et al (2007) demonstrated that in the lab, ectoparasitic mites could serve as a vector to transfer a strain of *Spiroplasma* from one *Drosophila* species to another. Whether this occurs for either endosymbiont in nature has never been studied.

HYPOTHESIS

Given that Drosophilids occupy the same ecological niche as generalist mites, multiple *Drosophila* species should be parasitized. Mites can be a vector of the endosymbiotic bacteria commonly found in some *Drosophila* species.

OBJECTIVES

General Objectives

The general aim of this study was to examine the extent and distribution of mite parasitism among Drosophilids in Mexico, in order to provide insights into the evolution of host-parasite interactions in general. A secondary aim was to seek evidence for mites acting as vectors of either *Spiroplasma* or *Wolbachia* in Drosophilids.

Specific Objectives

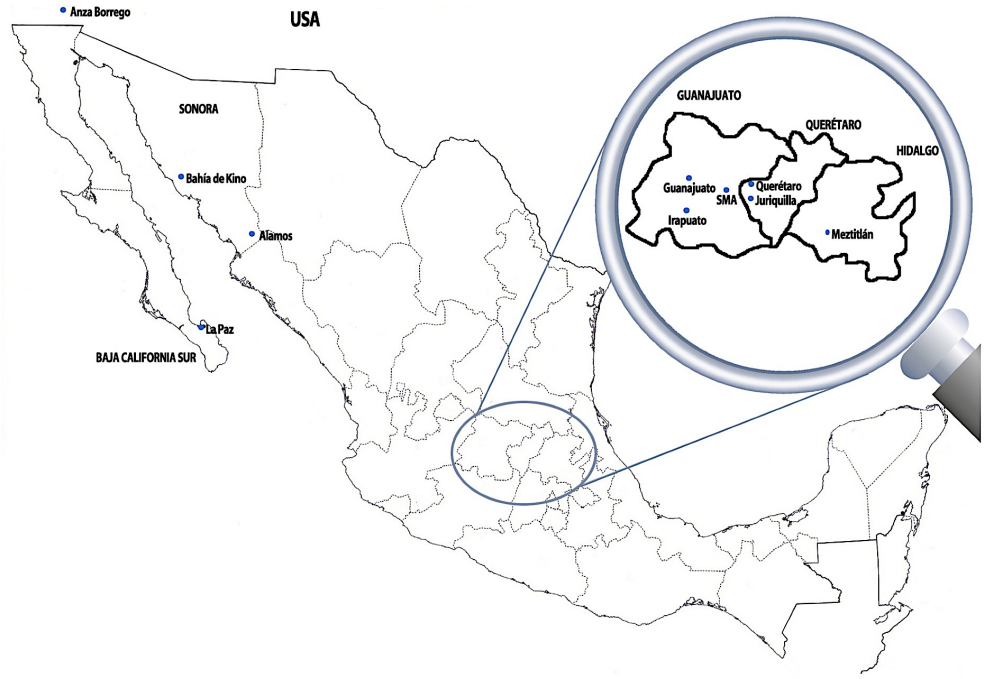
1. Determine the distribution of mite parasitism in species of the genus *Drosophila* in Mexico.
    a. Are all *Drosophila* species equally parasitized?
    b. How many species of mites are associated with *Drosophila*?
2. Evaluate the potential role of mite behavior in the infestation bias observed in nature.
3. Determine if mites contain the endosymbionts *Wolbachia* and *Spiroplasma*.
4. Determine if the association between mites, endosymbionts, and flies could explain horizontal transmission.

MATERIALS AND METHODS

Field collections of Drosophila

Flies were collected either directly from rotting fruit or from banana baits placed in habitats in the states of Guanajuato, Queretaro and Hidalgo that had either high densities of cactus or of domestic fruits such as citrus or berries. In 2014, I collected from September through December and in 2015 from April through October. I placed from one to five baits, prepared from banana and yeast (Markow and O'Grady, 2005) in each location. Flies were aspirated from the baits, preserved in 95% ethanol and taken directly to the laboratory where they were sorted by species and sex and scored as to whether they had a mite or mites attached. The prevalence of infection was calculated by dividing the total number of flies with mites by the total number of collected flies of each species groups. Comparisons were done by subgenus between cactus and fruit habitats using the Mann-Whitney U test. I also noted the location on a fly where a mite was attached. Mites were removed for morphological and molecular identification and the host flies were saved for species determination. Also included in this study were some samples of flies collected by Therese Markow in 2012 and 2014 from Anza Borrego Desert, California (USA), La Paz, Baja California Sur (Mexico), Bahia de Kino, Sonora and Alamos, Sonora (Mexico) that had been preserved until the present study. Collection localities are shown in Figure 2. Complete information on samples, collection localities and dates of collection available in Supplementary table S1.

Figure 2. Map of collection sites. In all collection sites, sampling was performed near cactus habitats. Fruit habitats also were sampled in Guanajuato and Irapuato, in the state of Guanajuato (San Miguel de Allende is abreviated SMA).



Identification of Drosophilid species

Flies that were found infested with mites were identified first by morphology to species group and if possible to species. Afterwards, using the barcode region of the mitochondrial cytochrome oxidase 1 (COI) gene (Table 1), all infested flies were identified to species.

Table 1. Primers used to identify *Drosophila* species (mitochondrial cytochrome oxidase 1) and mites (18s rDNA).

Primer pair	Sequence
LCO-1490 HCO-2198	5'-GGTCAACAAATCATAAAGATATTGG-3' 5'- TAAACTTCAGGGTGACCAAAAAATCA-3'
Fw1230 ConsR18S	5'- TGAAACTTAAAGGAATTGACG-3' 5'- ATTCAATCGGTAGTAGCGACG-3'

Identification of mites

Mites were identified using morphological and molecular methods. Dr. Gerald Krantz, Oregon State University, assisted me in the morphological identification of the mites, which I then verified by sequencing a 530-bp fragment 18s rDNA gene (Table 1). I chose this gene because it is the most commonly used molecular marker in studies of arthropod relationships (Klompen et al 2007; Dabert et al 2010); therefore, plenty of sequences were available in GenBank for identification. Furthermore, 18S has proven useful to identify distantly related lineages of Acari (Dabert et al 2010) and has been used with considerable success at generic and familial levels in Ixodida (ticks) (Klompen et al 2000).

PCR conditions and sequencing

Extractions of total genomic DNA from individual flies and mites were performed using the DNeasy Blood and Tissue kit (QIAGEN Inc., Valencia, CA) protocol as described in Markow et al (2013). For *Drosophila*, A 658-bp fragment of COI was amplified using the primer pair LCO1490f and HCO2198r described in Folmer et al 1994, using 5.25 µl DNA as template in a 35 µl reaction. PCR products were amplified starting with one cycle of 5 min at 95 ºC followed by 35 cycles under the following conditions: 30 s at 95ºC, 45 s at 54ºC, 1 min 30 sec at 72ºC, with a final extension step at 72 ºC for 30 min. This segment corresponds to nucleotide positions 1,515–2,172 in the complete mitochondrial genome of *Drosophila yakuba* (GenBank accession no. NC001322).

For mite identification, a 530-bp fragment of the 18S rDNA was amplified using

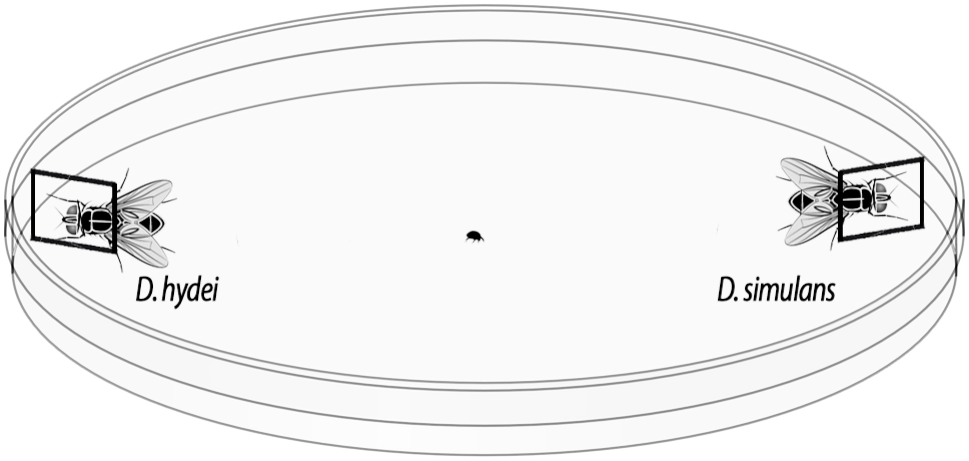
primers FWD1230 (Skoracka & Dabert 2010) and the primer developed in this study consR18S, using 5.25 µl DNA as template in a 35 µl reaction. PCR products were amplified under conditions modified from Dabert et al (2010), starting with one cycle of 3 min at 95 ºC followed by 35 cycles of the following conditions: 30 s at 95ºC, 30 s at 54ºC, 90 s at 72ºC, with a final extension step at 72 ºC for 30 min. Sequencing was performed on a ABI XL3730 at the Core Facility of LANGEBIO, CINESTAV, Irapuato, Guanajuato, México and at GENEWIZ, Inc., South Plainfield, NJ. In total, 124 mites were processed for identification. If more than one mite came from the same fly, and if those were identified as members of the same species using morphological keys, they were pooled together for DNA extraction.

Assessing the role of mite preferences in fly parasitism

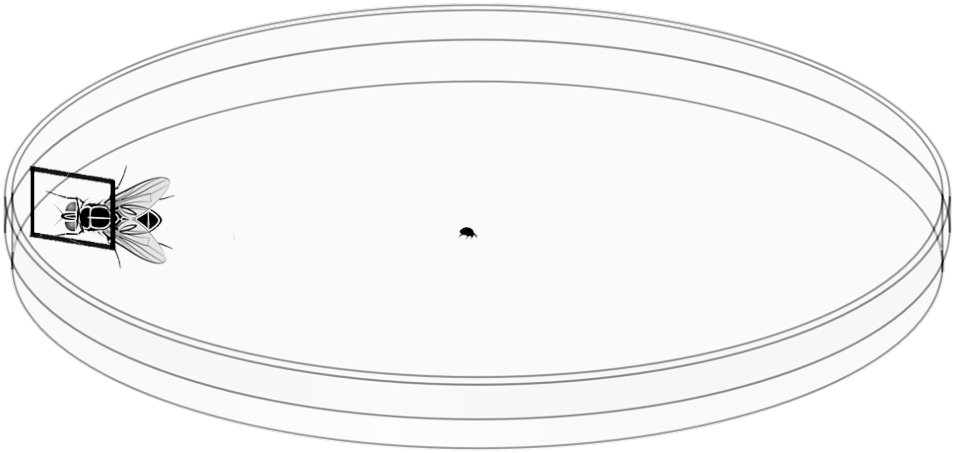
Given the bias observed in parasitism in nature (see Results), I wanted to assess the role of mite preference in the fly species they infest. Observations from flies collected from baits suggested a preference for members of the *repleta* species group over flies belonging to the most abundantly collected subgenus, *Sophophora*. This hypothesis was assesed in the lab by performing choice tests, where two or more host species are simultaneously presented to the test specimen. Here, the response is a measure of the preference for one species in the presence of another species (Withers and Mansfield 2005). From additional baits, mites were harvested from infested flies. I established my protocols based upon similar studies with other organisms because host choice tests with

Drosophila had never been done previously. Under CO_2 anesthesia, mites were detached and transferred to 5cm Petri dishes with 0.5% agar. This provided with enough moisture, to which mites are attracted to in the substrates they colonize (Hunter et al 1988; Polak 1996; Walter and Proctor 2013). Detached mites were maintained separately at 23–25ºC for 72 hours to fully recover from anasthesia. A single mite was then transfered to the middle of another 5cm Petri dish with 0.5% agar, where it was offered the opportunity to choose between lab strains of flies representing the two most abundant subgenra collected in nature; *D. hydei*, member of the *repleta* species group from Subgenus Drosophila and *D. simulans* from Subgenus *Sophophora* (Figure 3a). Because parasite resistance has been observed to be mediated by behavioral forms of defence in the *Drosophila-Macroches* system (Polak 1996; Polak 2003), flies were immobilized inside pipette tips in order to eliminate the possibility of differences in ”front line” forms of defense between subgenera. Mites were observed for 15 minutes and the species of fly to which they attached was recorded. The length of the trial was decided by personal observations on preliminary choice tests based on the time it took for a mite to attach to a fly. This was verified with other choice tests with mites described in the literature, which have a length between three and 20 minutes (Krantz 1991; Krantz et al 1991; Silva-Torres et al 2005; Grossman and Smith 2008).

Figure 3. (a) Choice and (b) no choice setup



(a)



(b)

A second set of behavioral experments was peformed to test for preference without the presence of a second fly species. Will mites equally attach to the two species when only one is present? Both choice and no-choice tests are encouraged to be used in combination when assesing host range because of the difference in information that could be obtained by both experiments (Withers and Mansfield 2005). In the no-choice experiments, mites were presented with either an

immobilized female *D. hydei* or immobilized female *D. simulans* inside a 5cm Petri dish with 0.5% agar and video recorded for one hour using a DinoLite Digital Microscope (AnMo Electronics Corp) at 20X magnification (Figure 3b). In this set of experiments, mites were allowed more time to attach to a host because I wanted to observe and quantify a possible difference in pre-attachment behaviour with respect to fly species as well as the possible difference in behaviour after the mite had made a first contact with the fly. It has been observed that Macrochelids associated to scarab hosts perform a series of behaviors on their host which are different to the behaviours after having mounted a non-host beetles; mites sometimes even leave the host quickly (Krantz 1998). To asses a possible difference of behaviour when in contact with the different *Drosophila* species, the following behaviors were quantified: the number of times a mite approached the fly (to test if once it made contact with a *D. simulans*, the mite was repelled or it approached the fly again), the length of time the mite spent close to the fly (without attaching), the number of times the mite attached, and the length of time the mite stayed attached to the fly. This last measurement was included because if there is a mechanism of defense in *D. simulans* that repells ectoparasites after they have successfully attached, then we would expect to see differences in the length of time the mites stayed attached to each fly species. Videos were analyzed using ImageJ (National Institutes of Health), where a perimeter (5.5mm x 3mm) was delimited around each fly. The number of times the mite entered that area and the time spent inside it was quantified. The Shapiro-Wilk normality test was applied to each data set. For normally distributed data Student's *t* test analyses were performed. Mean times attached to flies were not normally distributed, however,

for comparisons, I used the non-parametric alternative Mann-Whitney test. For differences in number of mites attached, a standard chi square was applied.

Screening of Wolbachia and Spiroplasma

Total genomic DNA from individual flies and mites used for identification was also used for this screening. All infested flies from this study as well as their corresponding mites were screened for both endosymbionts. For *Spiroplasma*, A >500-bp fragment of the 16S ribosomal RNA (rRNA) was amplified using the primer pair 23F and TKSS described in Haselkorn et al (2009), using 5.25 µL DNA as template in a 35 µL reaction. I selected this locus because it is conserved and has been sequenced for numerous strains of *Spiroplasma*. PCR products were amplified starting with one cycle of 3 min at 95ºC, followed by 30 s at 94ºC, 45s at 65ºC, 45 s at 72ºC; the annealing temperature was lowered 1ºC per cycle for 15 cycles, following 20 cycles under the following conditions: 30 s at 94ºC, 45 s at 48ºC, 45 s at 72ºC.

For *Wolbachia*, a 600-bp fragment of the *Wolbachia* surface protein (WSP) gene, which encodes a major cell surface coat protein, was amplified using the primer pair WspF and WspR described in Mateos et al (2006), using 5.25 µL DNA as template in a 35 µL reaction. PCR products were amplified using long PCR conditions described in Jeyaprakash and Hoy (2000), starting with one cycle of 3 min at 94ºC followed by 10 cycles under the following conditions: 10 s at 94ºC, 30 s at 65ºC, 1 min at 68ºC; 25 cycles under the following conditions: 10 s at 94ºC, 30 s at 65ºC, 1 min at 68ºC with an additional 20 s added for every consecutive cycle.

A second pair of primers from Baldo et al (2006) was selected to amplify a 400-bp fragment of COXA, using primer pairs CoxA_F1 and CoxA_R1. Using 5.25 µL DNA as template in a 35 µl reaction, PCR products were amplified starting with one cycle of 2 min at 94 ºC followed by 36 cycles under the following conditions: 30 s at 94ºC, 45 s at 56ºC, 1 min 30 sec at 72ºC, with a final extension step at 72 ºC for 10 min. Primers used to screen endosymbionts are in Table 2. All PCR products were separated by 1% agarose gel electrophoresis and visualized under ultraviolet light.

Table 2. Primers used to for the screening of *Wolbachia* and *Spiroplasma* in *Drosophila* species and mites.

Primer pair	Sequence
Spiroplasma (16S) 23F TKSS	5'-CTCAGGATGAACGCTGGCGGCAT-3' 5'- TAGCCGTGGCTTTCTGGTAA-3'
Wolbachia (WSP) WspF WspR	5'-TGGTCCAATAAGTGATGAAGAAACTAGCTA-3' 5'-AAAAATTAAACGCTACTCCAGCTTCTGCAC-3'
Wolbachia (COXA) CoxA_F1 CoxA_R1	5'-TTGGRGCRATYAACTTTATAG-3' 5'-CTAAAGACTTTKACRCCAGT-3'

RESULTS

Identification of Drosophilid and mite species

Flies were initially sorted to subgenus or species group, or in the case of *Zaprionus indianus*, to species. The most abundant subgenus in cactus and fruit habitats was subgenus *Sophophora*, the second largest subgenus within genus *Drosophila* (Markow and O'Grady, 2005). The subgenus *Sophophora* contains approximately 300 species, including important model systems such as *Drosophila melanogaster* and *D. pseudobscura*. Most species groups within this genus occupy tropical regions although some members are cosmopolitan in distribution (O'Grady and Kidwell 2002). Within the subgenus *Sophophora*, the majority of the flies I found were either *D. melanogaster* or its sister species *D. simulans.* Because females of these two are not always reliably distinguished based on external morphology, I grouped the two species into a category designated MEL-SIM. In collections from cactus or fruit areas I used molecular methods to distinguish the two species. In the subgenus *Drosophila*, the majority of the flies we captured were members of the *repleta* species group, most of which are cactophilic. Because *repleta* group species are morphologically difficult to distinguish, collected flies were grouped into a category called "*repleta*". A small number of *Drosophila busckii* were also collected in both habitats. This is one of three species that make up the small subgenus *Dorsilopha* (Markow and O'Grady 2005). The species *Zaprionus indianus* from family Drosophilidae was also collected from both habitats. This is an invasive pest of fruit which was established itself in the New World within the last 10-15 years (Markow et al 2014).

After identification with CO1, mite parasitism was found in 14 *Drosophila* species, 13 additional to those previously documented (Table 3). While the majority of the mites were *M. subbadius*, as observed earlier in *D. nigrospiracula* (Polak & Markow, 1995; Polak, 1996), I found parasitism by two additional mite species. *Paragarmania bakeri,* another Mesostigmatid mite, was found on four flies of *D. hexastigma.* While *P. bakeri* also is a Mesostigmatid mite, it belongs to a different family, Blattisociidae, than *M. subbadius*. Interestingly, one *D. hexastigma* had a mite of each species. In one case, *a D. hydei* was found to be associated with a second mite, *Lasioseius sp.,* also from family Blattisociidae.

Parasitism was highly biased toward flies of the subgenus *Drosophila.* In the earliest collections, I only keyed the numbers of flies that belonged to the different subgenera. Subsequently, in order to better characterize the distribution of infested species in the *Drosophila* subgenus, I keyed out all collected flies to species group, whether they had mites attached or not. From baits located in cactus as well as fruit habitats (Table 4), the majority of flies collected belonged to the subgenus *Sophophora*, mainly *D. simulans* and *D. melanogaster*. The difference in prevalence of mite infestation between species groups collected near cactus were significant (X^2 =37.97, p <0.00001), as was as the difference between species groups collected near fruit habitats (X^2 =22.08, p <0.0002). Differences in infestation by subgenus in the two habitats are presented in Figure 4. There was no significant difference by subgenus in infestation between both habitats (*Drosophila* U =9*,* p =0.103, *Sophophora* U=1*,* p =0.400).

Table 3. Total species of *Drosophila* found to be parasitized. Mite species and substrate associated to each *Drosophila* species is also included.

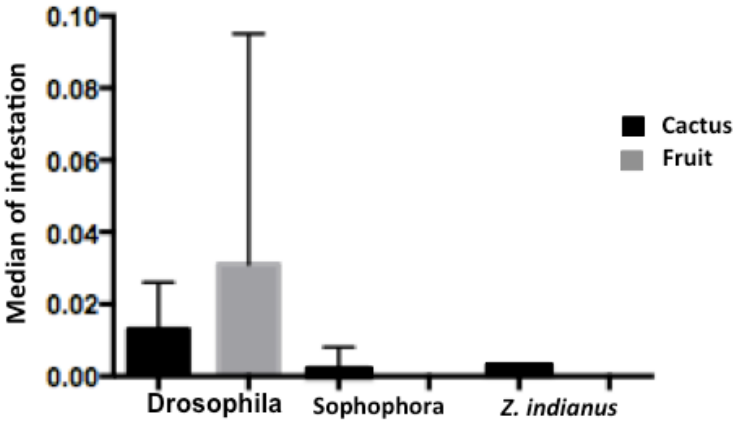
Drosophila species	Mite species	Mite family	Resource
D. arizonae[1]	*M. subbadius*	Macrochelidae	Cacti
D. huichole[1] *			Cacti
D. hexastigma[1]	*P. bakeri* *M. subbadius*	Blattisociidae Macrochelidae	Cacti
D. spenceri[1]	*M. subbadius*	Macrochelidae	Cacti
D. ritae[1]	*M. subbadius*	Macrochelidae	Cacti
D. longicornis[1]	*M. subbadius*	Macrochelidae	Cacti
D. mercatorum[1]	*M. subbadius*	Macrochelidae	Cosmopolitan
D. nigrospiracula[1,2]	*M. subbadius*	Macrochelidae	Cacti
D. hydei[1]	*Lasioseius sp* *M. subbadius*	Blattisociidae Macrochelidae	Cosmopolitan
D. eremophila[1]	*M. subbadius*	Macrochelidae	Soaked soil from columnar cacti
D. mettleri[3]	Not identified	Not identified	Soaked soil from columnar cacti
D. busckii[1]	*M. subbadius*	Macrochelidae	Cosmopolitan
D. melanogaster[1]	*M. subbadius*	Macrochelidae	Cosmopolitan
D. simulans[1]	*M. subbadius*	Macrochelidae	Cosmopolitan
Z. indianus[1]	*M. subbadius*	Macrochelidae	Fruit

[1] Present study
[2] Polak and Markow, 1995
[3] Polak, 1996
* Mite lost prior to identification
Note: Cosmopolitan refers to the guild of *Drosophila* associated with decaying fruits and vegetables in human habitats (Nunney 1996; Markow 2015).

Table 4. Distribution of mite parasitism among *Drosophila* species collected at either cactus or fruit habitats.

HABITAT	Genus	Subgenus	Species	No flies	W/mites	% infestation
CACTUS	*Drosophila*	*Sophophora*	*D. melanogaster* *D. simulans*	2030	2	0.10
			D. pseudoobscura	10	0	0
		Drosophila	*repleta* group spp.	1680	29	1.7
			D. immigrans	63	0	0
		Dorsilopha	*D. busckii*	10	0	0
	Zaprionus		*Z. indianus*	640	1	0.16
FRUIT	*Drosophila*	*Sophophora*	*D. melanogaster* *D. simulans*	521	0	0
			D. pseudoobscura	2	0	0
		Drosophila	*repleta* group spp.	182	6	3.29
			D. immigrans	127	0	0
		Dorsilopha	*D. busckii*	1	1	100
	Zaprionus		*Z. indianus*	15	0	0

Figure 4. Median of infestation of flies collected from either fruit or cactus habitats. Flies of the genus *Drosophila* were grouped by subgenus: *Drosophila* (all *repleta* group species and *D. immigrans), Sophophora* (*D. melanogaster*, *D. simulans* and *D. pseudobscura*) and *Zaprionus indianus* is shown separately. Bars represent median with range. There was no significant difference by subgenus in infestation between both habitats using Mann-Whitney U test (*Drosophila* p =0.103, *Sophophora* p =0.400).



I sampled 14 times in cactus habitats and seven times in fruit habitats. Total numbers of infested and non-infested flies from each habitat were pooled together and, despite the difference in sample size between the two habitat types, mite distribution was not significantly different (Z =0.10, p =0.92, Fisher's exact test). Regardless of which habitat the flies came from, of the 90 total flies with mites, 84 belong to species in the *repleta* group of the subgenus *Drosophila* and the majority were *D. hydei* (Table 5). Two *D. simulans* and one *D. melanogaster* (subgenus *Sophophora*) were found with mites, as well as one *Zaprionus indianus*. While baits from fruit habitats contained a higher number of *D. melanogaster* and *D. simulans* and other non-*repleta* species, none of these flies were parasitized.

Table 5. Total numbers of flies found infested with mites in the present study. The total number of infested flies is 90 instead of 124 because some flies carried multiple mites.

Species	No. Infested flies
D. arizonae	2
D. huichole	1
D. hexastigma	4
D, spenceri	16
D. ritae	1
D. longicornis	1
D. mercatorum	1
D. nigrospiracula	11
D. hydei	46
D. eremophila	1
D. busckii	1
D. melanogaster	1
D. simulans	3
Z. indianus	1

Male and female flies were equally parasitized. In those 80 cases where we determined the sex of the parasitized fly, 43 were male and 37 were female, a difference that was not significant ($X^2 = 0.45$). Attachment sites, however, clearly were not random. The majority of mites were attached to the abdomen near the thorax or at the thorax-head junction (Table 6).

Table 6. Attachment sites of the 124 total mites encountered on flies.

Site of attachment	No. of mites	%
Ventral abdomen	108	87.09
Side of abdomen	7	5.64
Abdomen-thorax junction	2	1.61
Thorax-head junction	4	3.22
Back abdomen	2	1.61
Back thorax	1	0.81
Leg	1	0.81

Assessing the role of mite preferences in fly parasitism

Subsequently, I performed "choice tests" to examine the role of mite preference in the strong bias in parasitism of *repleta* group species (Table 7). At first, all mites performed searching movements, for example edge-walking in the dish before moving towards a fly (Berry and Holtzer 1990). They also performed exploratory walks towards the center of the dish before encountering a fly. When passing close to a fly, they reduced the walking speed. Out of the 40 choice tests, 19 mites attached to *D. hydei* (*repleta* group) while only 2 mites attached to *D. simulans* (subgenus *Sophophora*). In the remaining 19 trials, mites didn't attach to any fly.

Table 7. Results of choice tests. Number of times a mite attached to either fly (*D. hydei* or *D. simulans)* in 15 minute trials.

	D. hydei	*D. simulans*	Not attaching
No. Mites	19	2	19

I then examined the behavior of mites placed with just one fly species or the other (Table 8). For 12 trials with *D. simulans*, 10 mites approached the fly and remained within the delimited area for an average of 9:20 min. Out of those 10 mites, only 4 attached to the fly. The average amount of time a mite remained attached to a *D. simulans* was 13:22 minutes. For the 12 trials with *D. hydei*, 9 mites approached the fly and remained within the delimited area for an average of 11:13 min. Out of the 9 mites, 8 actually attached to *D. hydei*, twice as many as those that attached to *D. simulans*. The amount of time a mite remained attached to a *D. hydei* was 11:35 minutes. Results for the 12 trials for each species in supplementary table S2.

Table 8. Results of no-choice tests. Average results and standard errors of 24 no-choice (12 for *D. hydei* and 12 *D. simulans*) recorded for 1 hour.

Species	**# times approached**	**Close to fly (min:sec)**	**# mites attached**	**Mean time attached to fly (min:sec)**
D. simulans	3.9 ± 0.9 (10)	9:20 ± 2:33 (10)	4	10:09 ± 4:32 (4)
D. hydei	4.3 ± 1.1 (9)	11:13 ± 2:37 (9)	8	13:50 ± 7:11 (8)
	t = 0.31, p = 0.76.	t = 0.70, p = 0.49	$X^2 = 1.33$ $0.25 < p < 0.10$	p > 0.99

Screening of Wolbachia and Spiroplasma

The secondary aim of this project was screening for the two bacterial endosymbionts in all 90 flies collected that were found to have mites attached in nature. I also screened all the corresponding ectoparasitic mites (Table 9).

Out of the 14 *Drosophila* species screened, I was able to identify *Spiroplasma melliferum* only from two specimens of *D. spenceri*. Regarding the mites, only one *Macrocheles subbadius*, originally attached to a *D. hydei* tested positive

also for *Spiroplasma melliferum*. The screening for *Wolbachia* was more problematic. The WSP primers, selected because of the great availability of sequences from this gene in the databases because of its current extensive use for characterizing *Wolbachia* strains, allowed me to identify *Wolbachia* from six *D. nigrospiracula* and one *D. arizonae*. Based on WSP sequences, the strain isolated from all *D. nigrospiracula* and *D. arizonae* is the common strain wMel, originally isolated from *D. melanogaster*. With this set of primers, none of the mites tested positive for *Wolbachia*. Given the technical problems with WSP primers, I tried three more sets of primers, one for the cytochrome C oxidase, subunit I (COXA) and one for ftsZ, a cell division protein, both described in Baldo et al (2006). A third set of primers also for COXA was selected from Reumer et al (2010). Given their efficiency, I selected the primers from Baldo et al (2006) to amplify a 400-bp fragment of the COXA gene, using primer pairs CoxA_F1 and CoxA_R1. I also selected these primers because they were suggested by a multilocus sequence typing (MLST) approach recently implemeted as a genotyping tool for *Wolbachia* (Baldo et al 2006). When I used this set of primers, *Wolbachia* was identified in various mites and in one fly. I was able to identify *Wolbachia* from the ectoparasites of ten specimens of *D. nigrospiracula*, four *D. hydei*, three *D. hexastigma*, and one *D. simulans.* Out of the total of four specimens of *Paragarmania bakeri* found on four *D. hexastigma*, three tested positive for *Wolbachia*. COXA allowed me to identify *Wolbachia* only from one *D. simulans*, whose corresponding mite also tested positive for *Wolbachia* (Table 10). COXA sequence for this *D. simulans* and from its corresponding *M. subbadius,* as well as the sequences obtained from the rest of the infested *M. subbadius* was 100%

identical for 353bp. According to only this sequence, the strain isolated is *Wolbachia pipientis* strain *w*Ri originally isolated from *D. simulans* (Baldo et al 2006; Klasson et al 2009).

Table 9. Frequency of (a) *Spiroplasma* and (b) *Wolbachia* infections in the 90 *Drosophila* screened and their corresponding mites.

Host	*% positive Spiroplasma (n)*
D. spenceri	12.5% (2/16)
Mite from *D. arizonae*	50% (1/2)

(a)

Host	*% positive Wolbachia (n)*
D. arizonae	50% (1/2)
D. nigrospiracula	54.5% (6/11)
D. simulans	33.33% (1/3)
Mite from *D. nigrospiracula*	90.9% (10/11)
Mite from *D. hydei*	8.69% (4/46)
Mite from *D. hexastigma*	75% (3/4)
Mite from *D. simulans*	33.33% (1/3)

(b)

DISCUSSION

Parasitism of *Drosophila* species is much more extensive in taxonomic scope than previously documented. I was able to identify a total of 14 infested *Drosophila* species, out of which 13 had never been reported in the literature as being parasitized by ectoparasitic mites. Despite collecting large numbers of flies of the subgenus *Sophophora*, the great majority of the infested flies were members of the *repleta* species group of the subgenus *Drosophila*. A large number of the infested *repleta* group flies were *D. hydei*, which in addition to using *Opuntia* cactus belongs to the cosmopolitan guild of *Drosophila*, associated with decaying fruits and vegetables in human habitats (Nunney 1996). For other species, only a low number of specimens were found along at the different localities sampled. Unlike other *Drosophila* species that are abundant only at a certain time of the year (*D. virilis or D. pachea*), *D. hydei* is found year-round in association with *Opuntia* species as well as commercial fruits (Nunney 1990). The difference in seasonality of flies is dependent on the temporal and spatial resource availability of their plant host (Breitmeyer and Markow 1998). The differences in the number of infested specimens found for each species in this study might be accounted for by the differential availability of the actual species throughout the year.

Macrocheles subbadius is a well-known generalist species, occupying other substrates besides necrotic cacti (Axtell 1961; 1963; Beresford and Sutcliffe 2009; Mumcuoglu and Braverman 2010), making it unlikely that cactus breeding is the reason why more *repleta* group species have a higher infestation. Besides

collecting infested flies from baits close to rotting cactus, I placed baits around fruit habitats because the generalist ecology of *M. subbadius* allowed me to predict that most likely, this same mite would parasitize additional *Drosophila* species, using substrates other than cacti.

Screening the greatest possible diversity of infested flies was a primary goal of this project. I therefore selected an approach where surveying for infested species was confined to collecting the flies obtained from banana food baits that attract large numbers of species. This approach allowed me to screen a greater diversity of *Drosophila* species than I would have if I had collected flies directly from the substrate. Baits attract dispersing *Drosophila* of a wide range of species (Markow and O'Grady, 2005), as my collections confirm. There are reasons to suspect, however, that flies from baits may have lower mite loads than flies found at their breeding sites. Luong et al. (2015) showed that mite load interferes with aerodynamics of *Drosophila* flight and dispersal. Thus, a smaller population of infested individuals as well as a reduced rate of infestation is expected among those flies able to reach baits. Consequently, although I identified a large number of species affected by mites, it likely is an underestimate. At the same time, owing to the host plant specificity of many *repleta* group species, searching for a cactus rot or other possible substrate would have been a far less efficient means of assessing the diversity of fly species infested. While my data may underestimate the infestation prevalence for a given fly species, I nonetheless discovered a far greater number of cases of parasitized *Drosophila* species and found that those

species tend to be phylogenetically related, i.e. members of the *repleta* species group of the subgenus *Drosophila*.

Regarding the observed bias in phylogenetic distribution, many factors could be involved. For example, the differences in hydrocarbon profiles, underlying mate choice recognition in some Drosophilid species, could underlie the bias. With the exception of a few infested *D. melanogaster* and *D. simulans*, flies of the *Sophophora* subgenus collected were mite free, despite being collected from the same baits or fruits with parasitized *repleta* group flies. *Macrocheles subbadius* is a generalist mite, reproducing in a wide range of substrates from plant material to dung (Axtell 1961; 1963; Beresford and Sutcliffe 2009; Mumcuoglu and Braverman 2010), so it is unlikely that Sophophoran subgenus flies don't encounter mites. In fact, collecting flies from rotting citrus has yielded infested *D. hydei* but not any infested species of the Sophophoran subgenus (unpublished observations). Furthermore, infestation rates were similar in cactus and fruit habitats despite the differences in number of times baits were collected from each habitat.

When placed in the Petri dish for the behavioral tests, all mites performed searching movements characteristic of invertebrate predators (Berry and Holtzer 1990). Most of the time the mites executed edge-walking in the dish before moving towards a fly. They also performed exploratory walks towards the center of the dish before encountering a fly. When passing close to a fly, they reduced the walking speed, as if they could detect some host-related stimulus. This behavior is thought to increase the chances of finding a prey (Takabayashi and Takahashi 1989).

Mites seemed to sense both species of *Drosophila*, but as the choice test results confirm, when given the option to attach to a fly of the *repleta* group such as *D. hydei* in the presence of *D. simulans*, the majority attach to *D. hydei*. Observations within experimental chambers show that as mites approach, flies exhibit reflex behavior in form of movements away from the mites (Polak 2003). Since all flies were immobilized inside a micropipette tip, we can discard differences in pre-attachment defense such as grooming or tarsal flicking (Polak 2003). We can conclude, however, that mites do sense a difference between species that makes *D. hydei* more attractive as a host. Why some mites failed to attach to either fly could be a function of their nutritional condition or age, although I cannot test this hypothesis with my data.

Further insights are revealed by the no choice tests, where preference to attach or not, without the presence of a second fly species was assessed. The pre-attachment behavior of mites wasn't different in the presence of only either a *D. hydei* or a *D. simulans*. If a mite approached either species of *Drosophila* and then walked away, most of the time it came back; therefore, the number of times a mite approached a fly and the amount of time a mite spent close to the fly wasn't different between species. From those mites that approached their potential host, twice as many mites attached to *D. hydei* than to *D. simulans*. In the ten cases where mites approached a *D. simulans*, only four attached, accounting for the 40% of the mites that showed an attraction for *D. simulans.* While low, this rate of parasitism displayed towards *D. simulans* is much higher than that observed in nature. This can be accounted for by the fact that, since the mite didn't have a

second host to chose from, the likelihood of being presented with another option later was unknown. This might have caused the parasite to show greater acceptance for a host it doesn't commonly accept in nature. The non-significant difference between *Drosophila* species in the measurement "number of times attached" in combination with the results of the "length of time attached" suggest that mites can successfully attach to *D. simulans* without an apparent post-attachment defense mechanism forcing them to detach from their host. The fact that members of subgenus *Sophophora* share the same ecological niche as ectoparasitic mites along with the results of the no-choice tests performed in my study, suggest that these flies can become infested in nature. Behavioral tests also suggest that the bias in infestation observed is not differentially mediated between species by a post-attachment immunological response. These results suggest that although generalist *M. subaddius* affects more species tan previously known, parasitism is most likely restricted by a preference of mites for certain hosts mediated by characteristics such as cuticular hydrocarbons.

In nature, behaviors involved in host selection involve the recognition of a habitat, and also recognition and acceptance of a host (Jaenike 1990). The actual host range of parasites is determined by a variety of ecological factors as well as by the suitability of potential hosts for parasite infection and reproduction. While *M. subbadius* is known to be a generalist across broad taxonomic host scales, my data reveal that its host range, at least within the Drosophilids, appears to have some restrictions, which is also the case of a nematode associated with

mycophagous flies. *Howardula aoronymphium* is known to parasitize different *Drosophila* species in Europe and North America. However, in North America, suitable hosts of this nematode fall within a restricted clade within the genus *Drosophila*. Fly collections from nature demonstrate that within this genus, *H. aoronymphium* is successful in infecting flies from the *quinaria*, *testacea*, and *cardini* groups (Jaenike and Perlman 2002). Yet, *D. tripunctata* along with several other species are broadly sympatric with these susceptible species and sometimes even emerge from the same mushrooms, still infected flies belonging to these other species have never been found in nature (Jaenike and Perlman 2002). In general, *Drosophila* species vary in their susceptibility to nematode parasitism, and while there are possible explanations for these differences, the exact mechanism affecting general parasite attractiveness or resistance are still unknown.

My study supports the findings of Jaenike and Perlman (2002); the suitability of a potential host for a specific parasite may depend on host phylogeny, with some clades being more suitable as hosts than others because of their physiological or biochemical characteristics. In the case of susceptibility to nematodes, even sister species of *Drosophila* can differ greatly in infection levels, which might depend on derived characteristics of individual species, such as evolved resistance to infection. Parasite-host interactions represent an arms race with the host acquiring defenses against parasitism and at the same time, the parasite developing mechanisms to overcome the host's defenses. The force exerted by a ubiquitous parasite is a driver of the evolution of its host species, hence more

studies disentangling the differences among Drosophilid species in regard to parasitism will be important to discover the factors delimiting host range.

As a model organism, the genetic tools available for *Drosophila* already have been utilized in studies of host-parasite interactions. For example, numerous candidate genes have been revealed for parasite resistance in *D. melanogaster* (Orr and Irving 1997; Fellowes and Godfray 2000; Carton et al 2005; Sackton et al 2007). The roles of these candidate genes could be examined in other *Drosophila* species for their role in the bias observed between species for mite infestation in nature. Also, biosynthesis of *Drosophila* cuticular hydrocarbons has been well characterized and several genes regulating cuticular hydrocarbon expression have been identified (Foley et al 2007; Sharma et al 2012). As cuticular hydrocarbons are implicated in mated recognition in *Drosophila* (Ferveur and Jallon 1996), their role in mite-*Drosophila* associations is an obvious avenue to pursue.

A secondary goal of my project was to establish if mites contained endosymbionts they could be transferring as vectors between *Drosophila* species in nature. *Wolbachia* and *Spiroplasma* are the only bacterial endosymbionts that have been able to avoid recognition by the robust immune system of *Drosophila* (Mateos et al 2006). Despite technical problems with the amount and quality of DNA obtained from mites, I was able to identify *Drosophila* species containing *Spiroplasma* and even more species infected with *Wolbachia*. Previous surveys of endosymbionts also found that far fewer species of *Drosophila* are infected by *Spiroplasma* than with *Wolbachia* in nature (Mateos et al 2006). Regarding *Spiroplasma*, the fact that

only *D. spenceri* was infested is consistent with the results of Watts et al (2009) who screened 19 species of *Drosophila* from wild populations, eight of which were also included in my screenings. They screened many more specimens from each species than I did (from 50–250) and failed to detect *Spiroplasma* in these species. Previous studies show that *Wolbachia* and *Spiroplasma* appear to be concentrated in certain Drosophilid groups. Mateos et al (2006) found that *Wolbachia* is much more common in the subgenus *Sophophora* than in the subgenus *Drosophila*. I only screened four members of subgenus *Sophophora* and one contained *Wolbachia*, accounting for 25% of all screened Sophophorans, consistent with earlier findings. Furthermore, no cases of co-infection by *Wolbachia* and *Spiroplasma* were observed. This is not surprising as, to date, co-infection has only been reported in *D. melanogaster* (Montenegro et al 2005). The wMel strain detected with WSP in *D. nigrospiracula* and *D. arizonae* belongs to *Wolbachia* supergroup A, were the majority of insect *Wolbachia* strains belong. The detection of very similar or identical *Wolbachia* strains among different species of *Drosophila* is very common (Bordenstein and Werren 2007). More importantly, I am the first to screen mites for endosymbionts and thus the first to detect *Wolbachia* in any species of mite.

In order to establish the possibility of mites being responsible for the horizontal transmission of endosymbionts in nature, as was shown in the lab (Jaenike et al 2007), I first had to find either *Wolbachia* or *Spiroplasma* in a fly as well as in its corresponding mite. I was only able to accomplish this in one specimen of *D. simulans* and its corresponding *M. subbadius*. Although the COXA sequence

obtained was 100% identical in host and parasite, this strain of *Wolbachia* also is found in other *Drosophila* species. Furthermore, due to recombination within and between *Wolbachia* genes (Werren and Bartos 2001; Mateos et al 2006; Baldo et al 2006; Werren et al 2008) a multi-locus approach to strain characterization would be necessary to determine if this is indeed a case of horizontal transmission.

CONCLUSION

Ectoparasitism by mites in Drosophilids is more widespread than previously documented. The most common mite found in the additional Drosophilids is the same *Macrocheles subbadius,* which was found on *D. nigrospiracula*. Finding a second mite species, *P. barkeri*, associated only with *D. hexastigma*, and a third mite, *Lasioseius sp.* associated with *D. hydei* however, is evidence of a more complex system of ectoparasitism in *Drosophila* than was previously thought to exist based upon the earlier work with *D. nigrospiracula* and *M. subbadius* (Polak & Markow, 1995; Polak, 1996). Furthermore, a clear bias in parasitism towards species in the *repleta* group of *Drosophila* was found. My results suggest that the difference in susceptibility to parasitism between species of subgenus *Sophophora* and members of the *repleta* species group is not mediated by a post-attachment immunological response, but rather lies in some external feature of the fly. This can be further investigated thanks to the well-studied genetic toolbox of *Drosophila.* Genes like *desaturase 1* and *2,* which are important for racial and species differences in cuticular hydrocarbon expression (Dallerac et al 2000; Foley et al 2006) and *sept* and *smoq*, which act additively on the production of cuticular hydrocarbons (Ferveur and Jallon 1996; Dallerac et al 2000; Liimatainen and Jallon 2007) are good candidate genes that could be used to further investigate the apparent variation in hydrocarbon expression between species of *Drosophila*. Furthermore, we have whole genomes of different *Drosophila* species available (*Drosophila* 12 Genomes Consortium 2007) that can be used to look for

differences in candidate genes involved in infection and resistance to parasitism between species.

FUTURE DIRECTIONS

- Further screening of additional *Drosophila* species from a wider range of habitats and continents could be performed in order to determine if mite parasitism is wider spread than what was discovered in the present study. This will allow identifying other possible mite species associated with *Drosophila* and, if it is the case, provide more information about the host range of generalist *M. subbadius.* Additional species with and without mites would provide a larger data set to explore possible coevolution.

- Candidate genes like *desaturase 1* and *2, sept* and *smoq* involved in cuticular hydrocarbon biosynthesis could be knocked out with CRISPR/Cas9 in order to test differences in parasitism in experimental and control flies.

- Variation in the cuticular hydrocarbon profiles of *D. hydei* and *D. simulans* could be compared with a technique such as gas chromatography–mass spectrometry. Manipulation of extracted hydrocarbons from flies could be used for behavioral tests.

- Expression patterns of infected and uninfected flies of *D. hydei* and *D. simulans* could be compared to detect potential differences in immune response or defense. While not directly responsible for pre-attachment differences, any differences in infection resistence could have driven the evolution of pre-attachment interactions between the mites and various hosts.

- Differences in the genes involved in infection and immunity could be looked at with the genomes available for several species of *Drosophila*.

- I would encourage a broader screening of endosymbionts using a MLST approach in order to shed light in the poorly understood mechanisms and patters of interspecific transfer of endosymbionts.

SUPLEMENTARY TABLES

S1. Specimens, locality and date of collection

Fly species	Location	Date (month/year)
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. spenceri	La Paz, Baja California Sur	12/12
D. hydei	Guanajuato, Guanajuato	03/14
D. huichole	Guanajuato, Guanajuato	07/14
D. hydei	Juriquilla, Queretaro	11/14
D. hexastigma	San Miguel de Allende, Guanajuato	09/14
D. hydei	San Miguel de Allende, Guanajuato	09/14
D. hexastigma	San Miguel de Allende, Guanajuato	09/14
D. hexastigma	San Miguel de Allende, Guanajuato	09/14
D. hexastigma	San Miguel de Allende, Guanajuato	09/14
D. nigrospiracula	Bahia de Kino, Sonora	10/14
D. arizonae	Bahia de Kino, Sonora	10/14
D. nigrospiracula	Bahia de Kino, Sonora	10/14
D. hydei	Queretaro, Queretaro	10/14
D. hydei	Queretaro, Queretaro	10/14
D. nigrospiracula	Bahia de Kino, Sonora	10/14
D. nigrospiracula	Bahia de Kino, Sonora	10/14
D. nigrospiracula	Bahia de Kino, Sonora	10/14
D. melanogster	Alamos, Sonora	12/14
D.hydei	Alamos, Sonora	12/14
D. arizonae	Alamos, Sonora	12/14
D. hydei	Alamos, Sonora	12/14
D. hydei	Alamos, Sonora	12/14
D. hydei	Alamos, Sonora	12/14

D. simulans	Alamos, Sonora	12/14
D. hydei	Alamos, Sonora	12/14
D. hydei	Alamos, Sonora	12/14
D. hydei	Alamos, Sonora	12/14
D. spenceri	Anza Borrego, California	12/14
D. spenceri	Anza Borrego, California	12/14
D. hydei	Irapuato, Guanajuato	12/14
D. hydei	Irapuato, Guanajuato	12/14
D. hydei	Irapuato, Guanajuato	12/14
D. hydei	San Miguel de Allende, Guanajuato	04/15
D. longicornis	San Miguel de Allende, Guanajuato	04/15
D. hydei	Guanajuato, Guanajuato	04/15
D. hydei	Guanajuato, Guanajuato	04/15
D. hydei	Guanajuato, Guanajuato	04/15
D. busckii	Guanajuato, Guanajuato	04/15
D. hydei	Guanajuato, Guanajuato	04/15
D. hydei	Irapuato, Guanajuato	04/15
D. hydei	Irapuato, Guanajuato	04/15
D. hydei	Irapuato, Guanajuato	04/15
D. eremophila	Metztitlan, Hidalgo	05/15
D. hydei	Metztitlan, Hidalgo	05/15
D. hydei	Irapuato, Guanajuato	05/15
D. hydei	Irapuato, Guanajuato	05/15
D. hydei	San Miguel de Allende, Guanajuato	05/15
D. hydei	San Miguel de Allende, Guanajuato	05/15
D. hydei	San Miguel de Allende, Guanajuato	05/15
D. hydei	San Miguel de Allende, Guanajuato	05/15
D. hydei	San Miguel de Allende, Guanajuato	05/15
D. hydei	San Miguel de Allende, Guanajuato	05/15
D. mercatorum	Irapuato, Guanajuato	06/15
D. hydei	Irapuato, Guanajuato	06/15
D. simulans	San Miguel de Allende, Guanajuato	07/15
D. hydei	Irapuato, Guanajuato	08/15
D. simulans	Irapuato, Guanajuato	08/15
D. hydei	Irapuato, Guanajuato	08/15
D. hydei	Irapuato, Guanajuato	08/15
D. hydei	Irapuato, Guanajuato	08/15
D. hydei	Irapuato, Guanajuato	08/15
D. hydei	Irapuato, Guanajuato	08/15
D. ritae	Irapuato, Guanajuato	08/15
D. hydei	Irapuato, Guanajuato	09/15
D. hydei	Irapuato, Guanajuato	09/15
D. hydei	Irapuato, Guanajuato	09/15

D. hydei	Irapuato, Guanajuato	09/15
D. hydei	Irapuato, Guanajuato	09/15
D. hydei	Irapuato, Guanajuato	09/15
D. hydei	Irapuato, Guanajuato	09/15
Z. indianus	Irapuato, Guanajuato	09/15

S2. Results of individual No-choice tests by species

D. simulans	**# times approached**	**Time close to fly (min:sec)**	**# times attached**	**Mean time attached to fly (min:sec)**
1	0	0:00	0	0:00
2	3	19:34	3	4:50
3	2	4:25	0	0:00
4	1	5:46	2	22:33:00
5	5	3:52	1	4:18
6	5	8:16	0	0:00
7	9	10:23	0	0:00
8	4	22:24	2	7:05
9	8	11:40	0	0:00
10	1	1:01	0	0:00
11	0	0:00	0	0:00
12	1	0:43	0	0:00

D. hydei	**# times approached**	**Time close to fly (min:sec)**	**# times attached**	**Mean time attached to fly (min:sec)**
1	5	0:05	2	6:37
2	1	11:52	1	59:16:00
3	11	10:19	2	4:19
4	3	4:45	0	0:00
5	7	16:19	3	7:52
6	6	17:33	3	5:23
7	1	2:58	1	18:17
8	0	0:00	0	0:00
9	2	20:32	2	2:08
10	0	0:00	0	0:00
11	3	15:30	2	4:36
12	0	0:00	0	0:00

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