FACTORS AFFECTING HOST SELECTION BY MOSQUITOES: IMPLICATIONS FOR THE TRANSMISSION OF VECTOR-BORNE PATHOGENS

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EDITORIAL
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Editorial: Factors Affecting Host Selection by Mosquitoes: Implications for the Transmission of Vector-Borne Pathogens

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Editorial on the Research Topic

Factors Affecting Host Selection by Mosquitoes: Implications for the Transmission of Vector-Borne Pathogens

Host selection by insect vectors, including mosquitoes, represents a key step in the transmission dynamics of vector-borne pathogens by affecting the ecological interactions between infected and susceptible hosts and competent vectors (Yan et al., 2021). But what are the mechanisms underlying these processes in nature? And what are the relative roles of vector-related and host-related factors in shaping host-vector interactions and how pathogens may influence these interactions? Understanding factors governing host-mosquito-pathogen interactions and their potential effects on the transmission of mosquito-borne pathogens is challenging and calls for multidisciplinary approaches. This Research Topic (RT), through seven original research articles and four reviews, addresses some key factors related with both hosts and vectors ultimately determining heterogeneities in host selection by mosquitoes, and their consequences for pathogen transmission. Here, authors explored the effects of host traits on vector attraction, such as their microbiota, the emission of different cues and how all these may be altered by host infection status. The mosquito feeding preferences, their own microbiota and the degree of specialization of pathogens in insect vectors are also addressed here, with special focus on the epidemiological consequences of these processes. In sum, the interrelated questions explored in this Research Topic greatly contribute to expanding our understanding of host-vector-pathogen interactions and importantly, of pathogen epidemiology.

Factors affecting host selection by mosquitoes.

Host selection by mosquitoes is a complex behavior that includes different phases from the location of hosts to blood feeding. Vectors use a diversity of cues to detect their bloodmeal sources (Yan et al., 2021). In this RT, authors tested the role of both chemical and auditory cues potentially affecting the interactions between bloodsucking insects and wild birds. Tomás et al. investigated the effect of bird-derived cues on mosquito attraction using traps baited with either begging calls of nestling hoopoes (*Upupa epops*) or chemical cues derived from birds (i.e., uropygial secretion of hoopoe nestlings or bacteria isolated from uropygial secretions) or nests in different habitats of southern Spain. Although they did not find support for the role of auditory cues affecting mosquito captures, mosquitoes were less abundant in traps baited with bacteria or with nest material than in traps without these stimuli. Moreover, in a blue tit (*Cyanistes caeruleus*) population breeding in

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nest-boxes in central Spain, Castaño-Vázquez et al. studied the role of carbon dioxide (CO_2) and methane (CH_4) as potential cues used by host-seeking haematophagous vectors. The abundance of *Culicoides,* the main vector of the avian malaria-like parasite *Haemoproteus*, positively correlated with the difference in CO_2 between inside and outside nest-boxes, suggesting that *Culicoides* could use this cue to locate their hosts. Altogether, results from these studies provided evidence for the role of avian derived components on vector-bird interactions with both attractive and repellent effects.

The infection status of vertebrate hosts has also been identified as a potential driver determining their susceptibility to mosquito attacks through their effects on host behavior and/or the emission of different cues. The host manipulation hypothesis (Poulin, 1998) argues that parasites are able to modify host traits to increase the chances of parasites to complete their development. Cozzarolo et al. reviewed evidence supporting this hypothesis concerning the interactions between vertebrate hosts and different groups of flying haematophagous insects. Although contradictory results arose, authors found support for enhanced attraction of vectors to infected vertebrates compared to uninfected ones in numerous vertebrate-parasite systems, with examples in, among others, birds and mammals, including humans. This was especially the case for the mosquito-borne *Plasmodium* spp. infecting birds, which were further investigated by Santiago-Alarcon and Ferreira in this RT.

Recently, a novel research area has emerged to assess the role of host microbiota affecting the interactions between vertebrates and parasites, including the contact rates between hosts and mosquitoes. Ruiz-López reviewed the role of host microbiota in mosquito behavior and shows that different host traits including sex and age, genetics, behavior, environmental conditions and, even pathogen infections may determine changes in host microbiota and, potentially, in the odor profiles of vertebrates. The uropygial gland secretion of birds and their volatile compounds are becoming the focus of studies addressing the effect of chemical cues and their interaction with pathogen infection on vector attraction. However, evidence in this regard is not conclusive, as shown in this RT, pointing toward the existence of complex relationships between different host traits, including the microbiota, in shaping their odor profiles.

After reaching their hosts, mosquito females feed on blood to obtain the resources to develop their eggs and complete their life cycle. Different approaches have been used to identify the origin of bloodmeals of mosquitoes captured in the wild (Borland and Kading, 2021). Among them, molecular tools are cost effective, sensitive and specific to accurately identify the hosts of mosquitoes with a bloodmeal in their abdomen. These studies allow researchers to identify interspecific differences in the blood feeding patterns of mosquitoes. For instance, González et al. trapped mosquitoes across an urban-to-wild habitat gradient in northern Spain and found that the species *Culex pipiens*, *Culiseta fumipennis*, and *Culiseta morsitans* fed exclusively on birds, despite the presence of mammals in the area, including humans (*Homo sapiens*) and dogs (*Canis familiaris*). In addition, Hernández-Triana et al. successfully identified the bloodmeal sources of eight species of mosquitoes of the genera *Aedes*, *Culex*, and *Psorophora* from Mexico. In this study, *Cx. quinquefasciatus* was the most frequently sampled species and showed the highest diversity of hosts, revealing its capacity to bite different species of birds such as chickens (*Gallus gallus*) or Great-tailed grackles (*Quiscalus mexicanus*) and mammals. The ability of some mosquito species to feed on different vertebrate groups was further supported by West et al. who found that *Cs. melanura* was able to feed on birds (49.3%), reptiles (34.7%), and mammals (16.0%).

What are the implications of mosquito feeding behavior for the transmission of vector-borne pathogens?

The ability of mosquitoes to feed on hosts from different groups has epidemiological consequences for the transmission of vector-borne parasites, including zoonotic pathogens. For instance, the widespread West Nile virus (WNV) is a flavivirus naturally circulating between birds and mosquitoes, but if an infected mosquito feeds on humans or horses, they can transmit the virus and potentially produce West Nile fever, despite these mammals being dead-end hosts for the virus. Furthermore, the Eastern equine encephalitis virus (EEEV) is a mosquito-borne pathogen infecting birds. As in the case of WNV, horses and humans are susceptible to EEEV infections when bitten by infected mosquitoes, but again, they are considered dead-end hosts. Thus, the contact rates between mosquitoes, susceptible vertebrate hosts and reservoirs represent basic information to be included in epidemiological studies (e.g., calculations of vector capacity). Using their data on mosquito bloodmeal sources in Florida during 2018, together with information such as mosquito abundance, parity, and temperature, West et al. provided support for the links between seasonal variation in vectorial capacity and epizootic spillover of EEEV in the area.

Furthermore, some key parameters included in the calculations of vector capacity of mosquitoes for the transmission of different mosquito-borne pathogens are also affected by mosquito-related factors. Among others, authors have recently found support for the role of mosquito microbiota in the development success of pathogens in mosquitoes and the survival cost of infections (Martínez-de la Puente et al., 2021), potentially affecting the epidemiology of vector-borne pathogens. In spite of its relevance to mosquito-pathogen interactions, there is a clear knowledge gap in the microbiota composition of wild mosquitoes from different areas and their consequences for pathogen transmission. To partially fill this gap, Tainchum et al. studied the abdominal microbiota of different species of *Anopheles* from Thailand and found 24 bacterial genera. The most abundant species captured *Anopheles minimus* presented a higher bacterial diversity than the other sampled *Anopheles* species. Unfortunately, only a single mosquito was infected by *Plasmodium* parasites, so further research on the role of *Anopheles* microbiota in the transmission capacity of mosquitoes in the area was not possible.

The feeding patterns of mosquitoes and the degree of specialization of parasites in their vectors may also have consequences for the ecology and evolution of wild host-parasite interactions. Gutiérrez-López et al. reviewed the importance of the specialization of avian *Plasmodium*, a widespread pathogen infecting birds, in mosquitoes for the transmission of this

parasite. They highlighted the need to study simultaneously the three actors involved in these interactions, i.e., hosts, mosquitoes and parasites, to better understand how host choice by mosquitoes may impact the distribution of parasites in natural settings. Different selective pressures such as those imposed by environmental factors may also contribute to the evolution of mosquito traits affecting their host seeking and blood-feeding behavior, further affecting mosquito population structure and interspecific interactions. López-Mercadal et al. investigated morphological diversity of wing patterns of the invasive Asian tiger mosquito (*Aedes albopictus*) in the Balearic Islands, Spain. These authors found strong evidence of sexual dimorphism of wing shape, which was explained on the basis of ecological and life-history factors, in particular blood-feeding behavior and oviposition.

In sum, the studies included in this RT provide valuable information, including novel research and review articles, on the factors determining the contact rates between vertebrate hosts and insect vectors, with a special focus on mosquitoes. Among others, host microbiota and the infection status by vector-borne pathogens may affect the susceptibility of vertebrate hosts to mosquito attacks by influencing the release of different cues. The biting preferences of mosquitoes to feed on blood from specific host groups or species together with mosquito-related factors such as mosquito's microbiota, may affect the development success of pathogens in mosquitoes and their epidemiology under natural conditions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Bacterial Microbiome in Wild-Caught *Anopheles* Mosquitoes in Western Thailand

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Among the complex microbial community living in the mosquito midgut, some bacteria (e.g., *Enterobacter* spp.) can deliver effector molecules with anti-*Plasmodium* effects suppressing the development of malaria parasites (*Plasmodium falciparum*) before the öokinete can penetrate the mosquito midgut epithelium. Despite knowledge of this phenomenon, only a few studies have defined the diversity of microbiota in wild-caught adult *Anopheles* species. The objective of this study was to analyze and compare the bacterial microbiota in different *Anopheles* species, including representatives of the primary malaria vectors in western Thailand. Wild female *Anopheles* species were sampled from malaria-endemic areas in Tak and Mae Hong Son provinces near the Thai-Myanmar border. Midgut/abdominal bacterial diversity was assessed by examining the 16S rRNA gene, V3 hypervariable region, using PCR-Temporal Temperature Gel Electrophoresis (PCR-TTGE) profiling and sequence analysis. A total of 24 bacterial genera were identified from eight *Anopheles* species. Five bacterial genera were newly reported in *Anopheles* mosquitoes (*Ferrimonas*, *Megasphaera*, *Pectobacterium*, *Shimwellia,* and *Trabulsiella*). Five genera, including *Megasphaera*, were detected exclusively in a single-malaria (*Plasmodium vivax*) infected *Anopheles minimus* and not observed in other non-infected mosquitoes. The use of PCR-TTGE provides the first characterization of the midgut bacterial microbiome present in wild adult *Anopheles* in Thailand. Evidence that microbiota might impact pathogen development (suppression) in *Anopheles* and thereby reduce the risk of pathogen transmission deserves more studies to describe the presence and better understand the biological role of bacteria in natural mosquito populations.

Keywords: *Anopheles* mosquitoes, malaria, bacterial microbiota, biodiversity, Thailand

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INTRODUCTION

Despite significant progress in the control of malaria throughout the country, Thailand remains malaria-endemic, particularly along the international borders with Cambodia, Lao PDR, Malaysia, and Myanmar (DDC, 2018). The vast majority of recorded malaria cases, primarily *Plasmodium vivax* (73%) and *Plasmodium falciparum* (18%), occur along the Thai-Myanmar border (70–80%), especially in Tak and Mae Hong Son provinces (DDC, 2018). Of the 79 recognized *Anopheles* species

present in Thailand, the most important malaria vectors include two sibling species in the Dirus Complex (*Anopheles baimaii* Sallum & Peyton, and *Anopheles dirus* Peyton & Harrison), *Anopheles minimus* Theobald, *Anopheles aconitus* Dönitz, and three members of the Maculatus Group (*Anopheles maculatus* Theobald, *Anopheles pseudowillmori* [Theobald], and *Anopheles sawadwongporni* Rattanarithikul & Green) (Tananchai et al., 2019).

The malaria sporogonic cycle begins when a female mosquito ingests infective-stage (gametocytes) *Plasmodium* parasites from the blood of an infected human host. The ingested parasites undergo syngamy to create motile stage öokinetes that penetrate the mosquito midgut to become oocysts. The oocyst gradually enlarges as parasites multiply to produce many sporozoites. Once released from the oocyst, sporozoites migrate to the salivary glands to await transmission to another host via a mosquito bite. Although many *Plasmodium* gametocytes can be ingested during a single blood-feeding, only a small fraction typically develop to form oocysts (Leroy et al., 2014). The reason for this sharp decrease in potential infection density is multifactorial (genetic and non-genetic) and in large part influenced by the parasite-vector species relationship and individual mosquito susceptibility (competence) to *Plasmodium* infection allowing successful sporogonic development of the parasite while minimizing significant adverse effects on mosquito fitness (survival, fecundity, etc.) (Black and Severson, 2005; Lefevre et al., 2013). One area of research on this relationship has been the modulating effects linked to certain naturally occurring microbiota in the midgut and abdomen of mosquitoes that can suppress or prevent *Plasmodium* development (Cirimotich et al., 2011b). In particular, the role of enterobacteria that influences parasite development and transmission has been investigated in *Anopheles* mosquitoes. Additional investigations on this phenomenon may help to develop novel methods involving bacterial symbionts to arrest malaria from vector to host.

Symbiotic bacteria, such as *Pantoea agglomerans* and *Asaia* spp., have been successfully transformed to express anti-malaria molecules (anti-plasmodia effector proteins) that render host mosquitoes refractory to malaria infection (Favia et al., 2008; Damiani et al., 2010; Wang et al., 2012); in effect, becoming a paratransgenic means for preventing malaria transmission (i.e., transmission-blocking strategy) (Doumbo et al., 2018). Engineered *P. agglomerans* strains can inhibit up to 98% of *P. falciparum* development in infected mosquitoes (Riehle et al., 2007). *Enterobacter* (Esp_Z) is shown to inhibit öokinete, oocyst, and sporozoite formation of *P. falciparum* in *Anopheles gambiae* by up to 99% (Cirimotich et al., 2011b). Co-infections with *Serratia marcescens* and *P. vivax* in *Anopheles albimanus* have resulted in only 1% of mosquitoes being able to develop oocysts and complete sporogonic development (Minard et al., 2013). In another study, *Serratia marcescens* Y1 strain isolated from field-collected female *Anopheles*, induced anti-plasmodia factors that activated the immune system in *Anopheles stephensi* effectively rendering the mosquito resistant to *Plasmodium berghei* infection (Bai et al., 2019).

The bacterial biodiversity in nine species of field-collected *Anopheles* in Thailand and Vietnam demonstrated complex microbiota in the mosquito midgut and abdomen, primarily Gram-negative bacterial rods, including *Serratia marcescens*, *Klebsiella ozaenae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterobacter* spp. (Manguin et al., 2013). Other studies have reported the majority of adult mosquito midgut microbiota were Gram-negative species in the phylum *Proteobacteria* (Tandina et al., 2016; Zoure et al., 2020). At least three mosquito-specific bacterial species have been isolated from the midgut of African malaria vectors in the Gambiae Complex, including *Thorsellia anophelis*, *Janibacter anophelis* (Kampfer et al., 2006) and *Elizabethkingia anophelis* (Kampfer et al., 2011). Despite a large amount of work done on malaria vectors over many decades, few studies have examined the natural diversity of microbiota in adult mosquitoes (Manguin et al., 2013; Bassene et al., 2018). Therefore, the objective of this study is to use a sensitive molecular method to evaluate the natural bacterial diversity in wild-caught adult *Anopheles* in a malaria-endemic region of western Thailand.

MATERIALS AND METHODS

Ethics Statement

Human use protocol (human-landing collections) for this study was reviewed and approved by the Ethical Research Committee, Chulalongkorn University, Bangkok, Thailand (No. 0961/56).

Mosquito Collections and Species Identification

In 2011, adult *Anopheles* mosquitoes were collected from two hypoendemic malaria locations along the Thai-Myanmar border in Tak and Mae Hong Son provinces, respectively (**Figure 1**), using a standard human-landing collection technique (Tainchum et al., 2014). **Table 1** provides geographic locations, prevailing climatic factors, environment biotype, and crude malaria incidence rate (2010–2012). The abundance of the primary malaria vectors, *An. minimus*, *An. dirus* complex, and *An. maculatus* group, by month of collection (from February to November 2011) and location, is presented in **Figure 2**. Live mosquitoes were initially sorted using discriminating morphological criteria for species, complex or group level identification (Rattanarithikul et al., 2006). For further mosquito identification, *Anopheles* specimens were assayed using the appropriate allele-specific PCR technique by species complex or group (Tainchum et al., 2014). Each mosquito was divided in two parts, head-thorax for mosquito species identification and detection of malaria (*Plasmodium*) infection, and abdomen retained for bacteriological analysis. Abdomens were stored at −80°C until further processing.

Plasmodium Infection in Mosquitoes

Plasmodium detection used an aliquot of the head-thorax DNA extraction used for *Anopheles* species identification. A Roche LightCycler480 (Software Version LCS480 1.5.0.39)

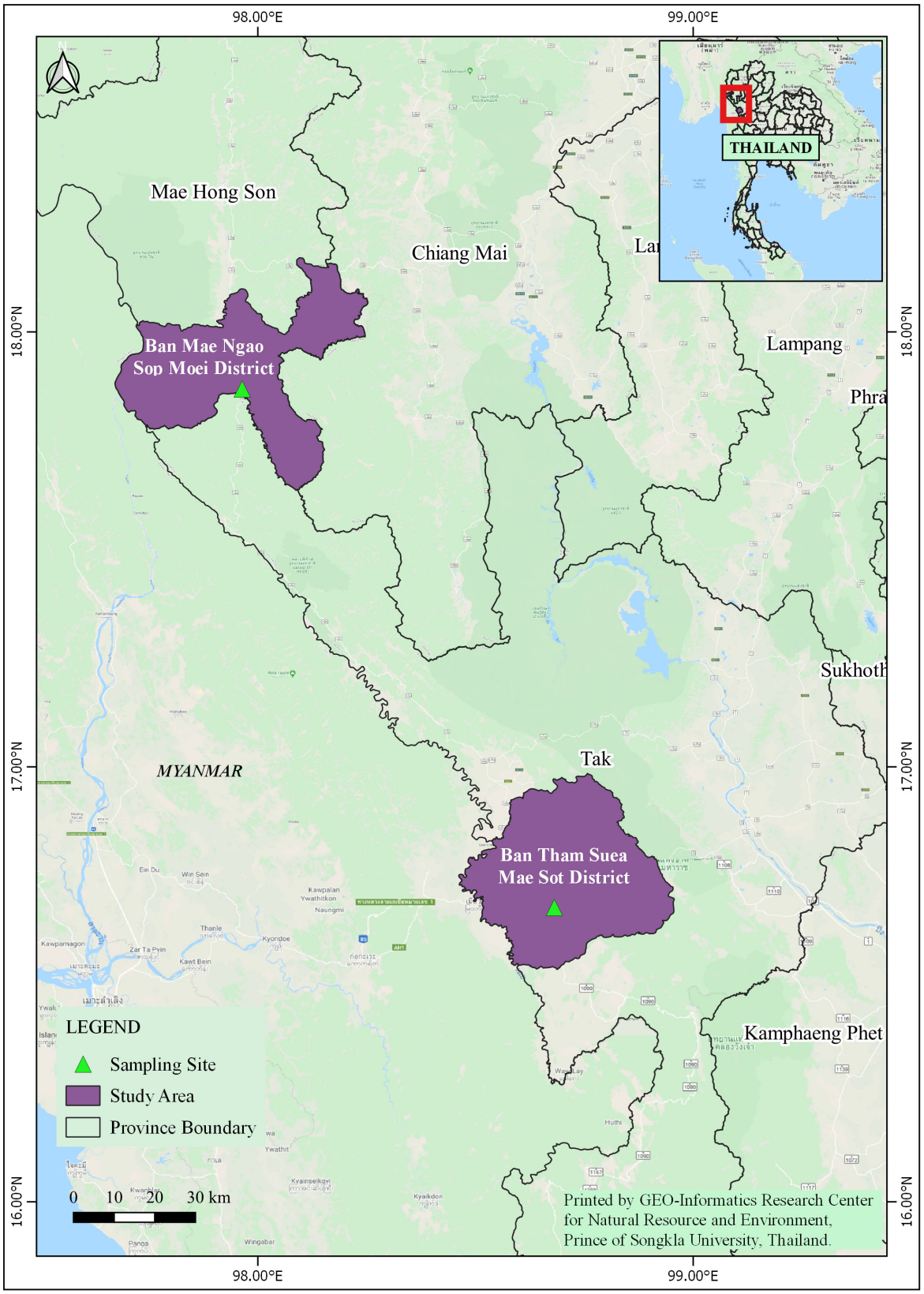

FIGURE 1 | Study site locations in Mae Sot District (Tak Province) and Sop Moei District (Mae Hong Son Province), western Thailand.

TABLE 1 | Background information on sampled locations with malaria transmission.

Location	Province	Tak	Mae Hong Son
	District	Mae Sot	Sop Moei
	Subdistrict	Phra That Pha Daeng	Mae Suat
	Village	Ban Tham Suea	Ban Mae Ngao
	GPS coordinates	16°67′ N, 98°68′ E	17°51′ N, 97°57′ E
	Altitude (meter above sea level)	196 m	157 m
Climatic parameters (diel range)	Temperature (°C)	15.5 to 36.9	15.7 to 33.5
	Humidity (% RH)	76.5 to 95.0	79.8 to 95.3
	Mean annual rainfall (mm)	2,053	2,060
Environment	Main vegetation type	Mixed forest/agriculture	Native forest
Total population (2010)		525,684	284,138
Number of reported malaria cases in province per 100,000 population*	2010	1,608.3	569.1
	2011	1,027.2	548.9
	2012	765.3	438.2

**Bureau of Vector-Borne Disease, Ministry of Public Health, Nonthaburi, Thailand. Available source: https://apps.boe.moph.go.th/boeeng/annual.php.*

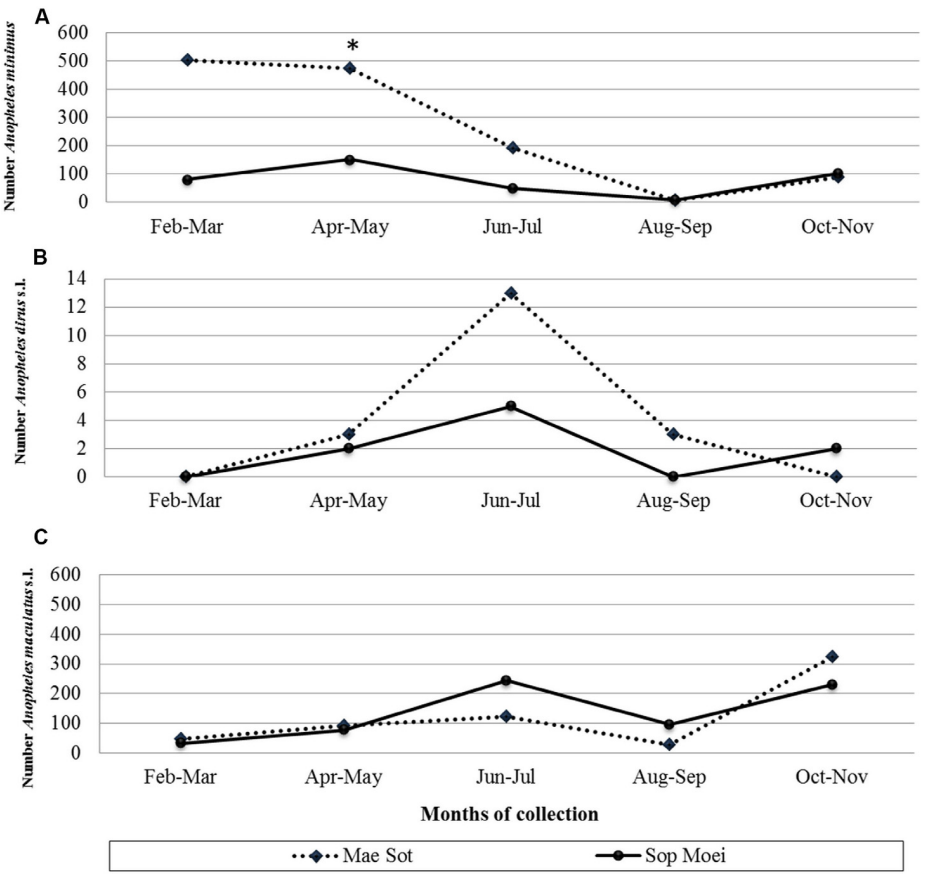

FIGURE 2 | Number of **(A)** *Anopheles minimus* (scale from 0 to 600 specimens), **(B)** *An. dirus* complex (scale from 0 to 14 specimens), and **(C)** *An. maculatus* group (scale from 0 to 600 specimens) mosquitoes collected by location and month (February to November 2011). *, month (April) and location (Mae Sot) where the vivax-infected *An. minimus* was collected.

was performed for real-time PCR with TaqMan reagents and hydrolysis probes for the detection of *P. falciparum*, *P. vivax,* and *P. knowlesi*, following a slightly modified methodology of Divis et al. (2010).

DNA Extraction From Abdominal Contents

Each abdomen was rinsed twice in purified water (from sealed injectable solution) before complete disruption using

a tissue crusher device in 150 μL of TE buffer. DNA was extracted using the Master Pure Gram Positive DNA purification kit following supplier instructions (Epicentre Biotechnologies, Madison WI, United States).

Polymerase Chain Reaction

For each sample, the V2–V3 region of the 16S rRNA bacteria gene was amplified using the primers HDA1/HDA2 (Roudière et al., 2009); HDA1: 5′-ACTC CTA CGG GAG GCA GCA GT-3′, HDA2: 5′-GTA TTA CCG CGG CTG CTG GCA-3′. A 40-bp clamp, named GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3′) flanked the 5′ extremity of HDA1 (Ogier et al., 2002) to form HDA1-GC. PCR was performed using an Eppendorf thermal cycler® (Eppendorf, Le Pecq, France) in sterile 0.5 ml tubes. The reaction mixture (50 μL) contained 2.5 units of Taq DNA Polymerase (FastStart High Fidelity PCR System, Roche, Meylan, France), 0.2 mM of each primer and 1 μl of DNA in the appropriate reaction buffer. The amplification cycle was 95°C for 2 min, 35 cycles at 95°C for 1 min, 62°C for 30 s, 72°C for 1 min, and 7 min at 72°C for the final extension step. Solutions were prepared using sterile DNA-free water with the addition of template DNA and gel electrophoresis of PCR products carried out in separate rooms to avoid any possible contamination. PCR amplification of DNA was detected by 2% agarose gel electrophoresis containing ethidium bromide and visualized under ultraviolet light.

Temporal Temperature Gel Electrophoresis (TTGE) Migration

Temporal temperature gel electrophoresis was performed using the DCode universal mutation detection system (Bio-Rad Laboratories, Marne-la-Coquette, France) with 16 cm × 16 cm × 1 mm gels (Roudière et al., 2009). The 60 ml gels were composed of 8% (w/v) bisacrylamide (37.5:1), 7 M urea, 60 μl of *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and 0.1% (w/v) ammonium persulfate. Gels were run with 1X Tris-acetate-EDTA buffer at pH 8.4. A 5 μl PCR product was loaded on the gel with 5 μl in-house dye marker (50% saccharose 50%, 0.1% bromophenol blue) using capillary tips. Denaturing electrophoresis was performed at 46 V with a temperature ramp from 63 to 70°C during 16 h (increment 0.4°C/h) following a pre-migration step of 15 min at 20 V and 63°C. Gels were stained with ethidium bromide solution (5 μg/ml) for 20 min, washed with de-ionized water, and viewed using a UV transillumination system (Vilber Lourmat Sté, France) and photographed.

TTGE Band Sequencing and OTU Identification

On each TTGE gel, approximately 50% of the bands were sequenced, while other bands were assigned to an affiliated operational taxonomic unit (OTU) by comparing their migration distance with that of sequenced bands. TTGE bands were excised and DNA eluted with 50 μl of elution buffer using Qiaquick PCR purification kit (Qiagen, Courtaboeuf, France) overnight at 37°C before PCR amplification with HDA1/HDA2 without GC clamp. The reaction conditions were identical to those described above. PCR products were sequenced using an ABI 3730xl sequencer (Cogenics, Meylan, France). Each sequencing chromatograph was visually inspected and corrected as appropriate.

The sequences were quality-checked using the SEQMATCH program in the 16S rDNA-specialized database, Ribosomal Database Project (RDP[1]). The 16S rDNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) from the GenBank database[2] and/or the RDP (Cole et al., 2005), for initial sample identifications. The reference sequence with the highest percentage was used for OTU affiliation. Clustered OTUs are based on 97% DNA sequence identity threshold of the 16S gene sequences to distinguish between bacteria at the genus level. A sequence was affiliated to a species-level OTU when the percent of sequence similarity was > 99% (as proposed by Drancourt et al., 2000). This value is above the recognized cut-off value standard for the delineation of species (Stackebrandt and Goebel, 1994), but warrants high stringency for species-level OTU affiliation. Below 99% similarity, the sequence is affiliated to the genus of the reference sequence having the highest percentage. When different species reference sequences match (or near equal), affiliation was done to the genus level. For example, 99.5% sequence similarity between species *Aeromonas caviae* and *Aeromonas hydrophila* was only assigned to the genus *Aeromonas*. Low cut-off is not defined for the genus delineation as affiliation to a higher taxonomic rank, e.g., family or order, was to be done considering the taxonomic frame of the clade using Greengenes database (McDonald et al., 2012).

Statistical Analysis

The OTU means between the two different regions were compared using a Mann–Whitney U-test for the non-parametric test with the statistical significance level set at $p < 0.05$ using SPSS for Windows version 16 (Chicago, IL, United States).

RESULTS

Bacteria in *Anopheles* and Diversity Index

A total of 190 *Anopheles* specimens, representing eight *Anopheles* species, were collected from Mae Sot (Tak Province) and Sop Moei (Mae Hong Son Province) (**Table 1** and **Figure 1**). The peak collection numbers of mosquitoes (*An. minimus*, *An. dirus* complex, and *An. maculatus* group) occurred during the same periods in both locations but differed by species (**Figure 2**). For instance, in Mae Sot *An. minimus* had higher captures during February and April, while a more modest increase was seen in Sop Moei in May. *Anopheles dirus* s.l. peaked in June and July in Mae Sot and Sop Moei, respectively. For *An. maculatus* s.l., between both locations greater numbers were captured in June-July and October-November timeframes compared to other

[1]http://rdp.cme.msu.edu

[2]http://www.ncbi.nlm.nih.gov/blast/

months (**Figure 2**). Within the Minimus Complex, only *An. minimus* was identified in association with very few specimens ($n = 2$) of a closely related species, *An. aconitus* (Funestus Group) (**Table 2**). Two species in the Dirus Complex, *An. dirus* and *An. baimaii,* and four Maculatus Group species, *An. maculatus*, *An. sawadwongporni*, *An. pseudowillmori* and *An. dravidicus* were molecularly identified (**Table 2**).

From 104 *Anopheles* assayed from Mae Sot and 86 from Sop Moei, only one (0.53%) mosquito, *An. minimus*, was found with malaria (*P. vivax*) sporozoites. This specimen was collected in Mae Sot (Tak Province) in April 2011 during the typical warm-dry season (**Figure 2**). The abdominal microbiota findings of all 190 assayed specimens, including the malaria-infected sample (**Figure 3**), was based on isolating the 16S rRNA gene using PCR-TTGE. In total, 107 sequences were obtained from 56 mosquitoes (30% of total sample) (**Table 2**).

A raw diversity index that globally reflects the bacterial diversity in a sample is typically reflected by counting the resulting bands in TTGE profiles. The number of bands ranged from one to 10, suggesting that the bacterial diversity per mosquito also ranged from one to 10 OTUs. However, subsequent sequencing showed that bands with different migration distances could belong to the same OTU. This atypical phenomenon was observed for bacteria displaying sequence heterogeneity among the 16S rRNA gene copies. For example, most members of genera in the large family *Enterobacteriaceae* displayed a high level of 16S rRNA gene heterogeneity, thus producing complex banding patterns. Considering that *Enterobacteriaceae* were relatively common in our samples, the raw diversity index overestimated the actual bacterial diversity. Therefore, a refined diversity index was calculated after affiliation of each band to an OTU by sequencing or by a comparative analysis approach. The resulting index showed a bacterial diversity with an average of 1.7 OTU per specimen. The number of OTUs per specimen did not differ between mosquitoes in the two locations, with an average OTU of 1.69 and 1.7 per specimen in Mae Sot and Sop Moei, respectively ($p = 0.345$). *Anopheles minimus* hosted the majority of OTU identified in this study, 50% and 29% of OTU identified in Mae Sot and Sop Moei, respectively; however, it was also the most abundant *Anopheles* species captured (**Figure 4**).

Bacterial Diversity in *Anopheles* Mosquitoes in Western Thailand

The 16S rRNA gene PCR-TTGE that focused on the hypervariable V3 region produced sequences of approximately 200 bp, which are generally of size not informative enough for species-specific affiliation. Consequently, this study presents the bacterial diversity at the genus level. However, probable species affiliation was proposed for several genera when the phylogenetic signal of the V3 region was regarded significant. Contrasting with the low diversity per individual (average 1.7), collectively the OTU diversity in the entire sampling was high with 24 different bacterial genera distributed among three phyla; *Proteobacteria* (71%), *Firmicutes* (21%), and *Bacteroidetes* (8%) (**Table 3**). *Proteobacteria*, the predominant microbiota, encompassed the Alpha, Beta, and Gamma superclasses.

Within the seven *Anopheles* species analyzed (*An. aconitus* excluded), *An. minimus* was more microbiota diverse with 16 bacterial genera identified, followed by *An. dirus* and *An. maculatus*, with half the number of genera (8) detected per species. However, *An. minimus* was a dominant species captured ($n = 35$) and analyzed based on PCR-TTGE (21 specimens out of 56) (**Figure 4** and **Table 3**). Nineteen and 15 genera were identified in Mae Sot and Sop Moei respectively, with 10 shared genera (**Figure 4**). The comparison of bacterial diversity between the four *Anopheles* species collected at both locations (*An. minimus*, *An. dirus*, *An. maculatus,* and *An. sawadwongporni*) revealed in three instances the number of genera was higher in Mae Sot than Sop Moei.

Among the 24 bacterial genera identified, 14 (58%) were shared by at least two specimens and 10 were identified in only one mosquito (**Table 3**). Among these 14 genera, two (*Elizabethkingia* and *Serratia*) were shared by five *Anopheles* species out of seven, one (*Shimwellia*) by four species, six

TABLE 2 | Sample number and species of *Anopheles* assayed for abdominal bacteria, those found PCR-TGGE bacteria positive, and bacteria designated either Gram-negative or Gram-positive.

Collection sites	Mae Sot					Sop Moei				
***Anopheles* species**	***N***	**No. positive by PCR-TTGE**	**No. sequences**	**Gram-negative**	**Gram-positive**	***N***	**No. positive by PCR-TTGE**	**No. sequences**	**Gram-negative**	**Gram-positive**
An. minimus	23	13	30	20	3	12	8	12	9	–
An. aconitus	1	–	–	–	–	1	–	–	–	–
An. maculatus	23	4	10	7	–	5	2	2	2	–
An. sawadwongporni	7	2	4	3	–	12	8	10	9	–
An. pseudowillmori	1	–	–	–	–	34	4	6	4	1
An. dravidicus	5	1	1	1	–	1	–	–	–	–
An. dirus	19	5	15	7	–	8	3	9	4	–
An. baimaii	25	4	6	4	0	13	2	2	1	–
	104	29	66	42	3	86	27	41	29	1
Total*	190	56	107	71	4					

**Total for both study sites, Mae Sot and Sop Moei.*

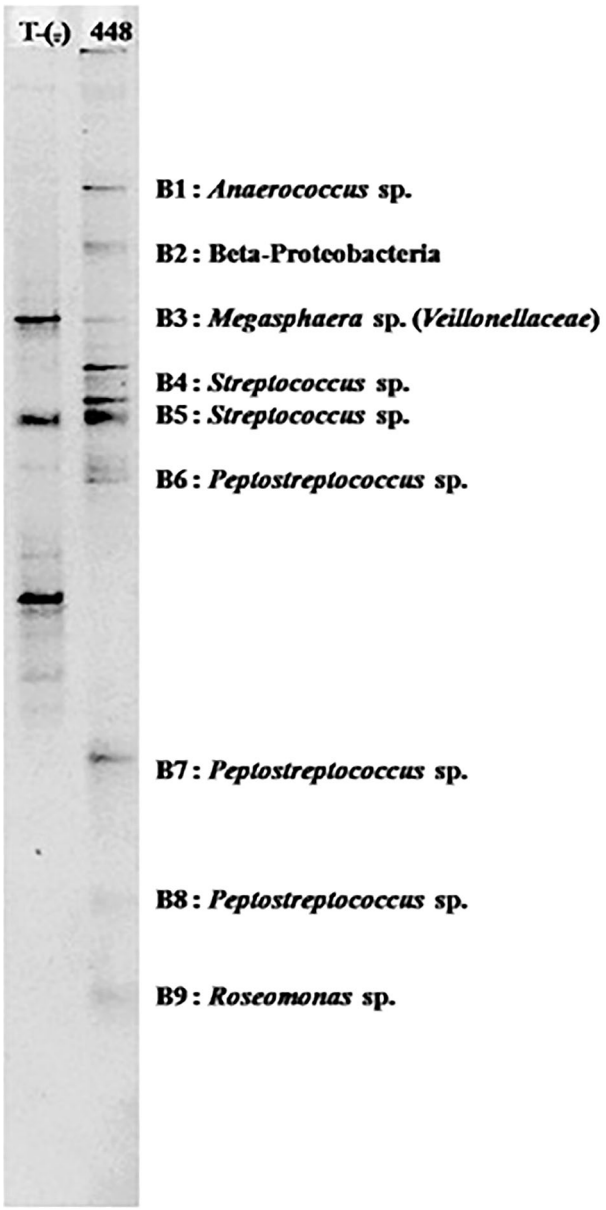

FIGURE 3 | PCR-TTGE banding patterns for bacterial genera detected in the abdomen of one naturally *Plasmodium vivax* infected *An. minimus*.

(*Acinetobacter*, *Enterobacter*, *Klebsiella*, *Pantoea*, *Raoultella*, *Trabulsiella*) in three species, two (*Aeromonas*, *Pseudomonas*) in two species, and three (*Chromobacterium*, *Ferrimonas*, *Herbaspirillum*) identified in at least two specimens of the same species (**Table 3** and **Figure 4**).

Nine of the 24 genera (37.5%) belonged to *Enterobacteriaceae*, including species within the genus *Enterobacter*, *Erwinia*, *Klebsiella*, *Pantoea*, *Pectobacterium*, *Raoultella*, *Serratia*, *Shimwellia,* and *Trabulsiella* (**Table 3**). The *Anopheles* microbiota included four primary species, *Serratia marcescens* (n = 14), *Shimwellia blattae* (n = 10), *Enterobacter cloacae* (n = 9), and *Klebsiella pneumoniae* (n = 9). *Shimwellia* was the dominant genus identified in Mae Sot (n = 9 specimens), while *Serratia* was dominant in Sop Moei (n = 11 specimens) (**Figure 4**). *Elizabethkingia*, which belongs to the *Bacteroidetes* phylum, was the second most common genus identified, present in the abdomen of 13 *Anopheles* specimens among five species (71.5%) – *An. minimus*, *An. dirus*, *An. maculatus*, *An. sawadwongporni,* and *An. dravidicus.* Sequences affiliated with the genus *Elizabethkingia* could not be definitively assigned to either *E. anophelis* or *Elizabethkingia meningoseptica* as the V3 region cannot discriminate between the *Anopheles*-specific bacterium and the bacterium as human pathogen. Among the 10 genera identified in only one *Anopheles* adult, six were found in *An. minimus* (*Aerococcus*, *Erwinia*, *Megasphaera*, *Peptostreptococcus*, *Roseomonas,* and *Streptococcus*). The other four genera were *Staphylococcus* and *Stenotrophomonas* in *An. pseudowillmori, Pectobacterium* in *An. dirus,* and *Chryseobacterium* in *An. sawadwongporni.*

In all, 24 bacterial genera were identified in this study, of which 14 have been reported previously in another study (Manguin et al., 2013). Five genera are newly identified in wild-caught adult *Anopheles*, including *Megasphaera* found only in a malaria-infected *An. minimus* in Mae Sot (**Figures 3**, **4** and **Table 3**). In addition, this single malaria-infected mosquito hosted five genera not identified in the remaining non-infected *Anopheles* assayed, including four genera within the phylum *Firmicutes* (*Aerococcus*, *Megasphaera*, *Peptostreptococcus, Streptococcus*) and one genus, *Roseomonas*, in the phylum *Proteobacteria* (**Figure 3** and **Table 3**). Interestingly, three of these five genera are Gram-positive cocci, out of the total four cocci genera identified in this study. Of the 10 genera that were observed only once, half were identified in the only *Plasmodium*-infected mosquito from the 190 sampled (**Table 3**).

DISCUSSION

Native adult *Anopheles* species were sampled from malaria-endemic areas in Tak and Mae Hong Son provinces near the Thai-Myanmar border. Bacterial diversity was assessed by the 16S rRNA gene, V3 hypervariable region, PCR-Temporal Temperature Gel Electrophoresis (PCR-TTGE) profiling and sequence analysis. The method provides the first estimation of the abdominal bacterial biodiversity present in field-collected *Anopheles* mosquitoes in Thailand. A total of 24 bacterial genera were identified from eight *Anopheles* species. *Anopheles minimus* presented a higher bacterial diversity than the other sampled *Anopheles* species identified in this study. Five bacterial genera were detected from a single malaria (*P. vivax*) infected *An. minimus*. Five genera are newly reported from field-collected *Anopheles* mosquitoes (*Ferrimonas*, *Megasphaera*, *Pectobacterium*, *Shimwellia,* and *Trabulsiella*), suggesting that the diversity of bacteria in *Anopheles* remains largely underestimated. *Elizabethkingia* spp., a relatively common midgut bacterium in *Anopheles* was also detected. This genus appears to have a role in increasing iron metabolism necessary for bacterial growth and *Plasmodium* development (Clark et al., 2014;

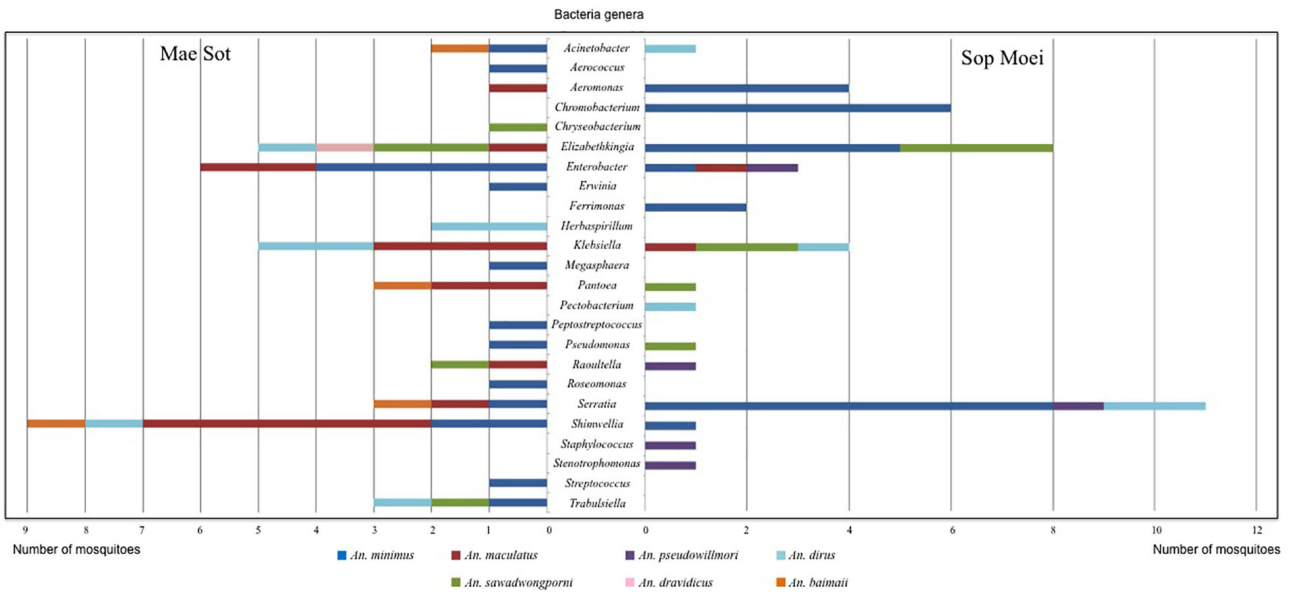

FIGURE 4 | Bacterial genera identified in *Anopheles* species by PCR-TTGE.

Sharma et al., 2019). Bacterial microbiota in *Anopheles* and their effects on the development of *Plasmodium* parasites as a potential means of controlling malaria transmission have been reported in various *Anopheles* species, but the precise mechanisms of activity remain poorly understood (Dong et al., 2009; Meister et al., 2009; Abdul-Ghani et al., 2012; Gendrin and Christophides, 2013; Minard et al., 2013; Hughes et al., 2014; Sharma et al., 2014; Villegas and Pimenta, 2014; Romoli and Gendrin, 2018). The PCR-TTGE described bacteria for 30% of *Anopheles* (56/190) analyzed and appears an efficient tool to investigate bacterial diversity in large samplings of mosquitoes. This method is appropriate to detect bacterial communities with low to moderate diversities as seen in wild-caught *Anopheles* samples in this study and other investigations (Manguin et al., 2013; Ngo et al., 2015, 2016). However, the method presents some limitations due to the restricted number of bands that can be separated within the length of the gel. Optimization of TTGE conditions allows the separation of bands with a minimum distance of 0.1 mm. Therefore, the TTGE procedure would present difficulties to interpret bacterial diversity that exceeds 25 to 30 OTUs per mosquito sample (Roudière et al., 2009).

In this study, the anopheline microbiota displayed TTGE profiles that did not exceed 10 bands; however, the profiles have been interpreted with some difficulty due to inherent heterogeneities in rRNA genes for many bacterial species present in the mosquito ecosystem. At the genomic level, rRNA genes are generally organized in multigene families (Acinas et al., 2004) and sequences show low variability within species, subspecies or genome level (Liao, 2000). That aside, TTGE remains useful by providing an accurate 'snapshot' of microbiota in different populations of hosts. Results obtained with TTGE fingerprinting compared to pyrosequencing or Next-Generation Sequencing have demonstrated good correlation for the detection of the majority of OTUs in complex microbiotic communities (Manguin et al., 2013; Li et al., 2014).

Nineteen of 24 bacterial genera detected have been reported from field-collected *Anopheles* (Abdul-Ghani et al., 2012; Gendrin and Christophides, 2013; Minard et al., 2013; Hughes et al., 2014; Sharma et al., 2014; Villegas and Pimenta, 2014). From western Thailand, the abdominal microbiota presented large inter-specimen variability but was dominated by *Serratia*, *Elizabethkingia, Shimwellia*, *Enterobacter* and *Klebsiella,* particularly members in the family *Enterobacteriaceae*. These results demonstrate that PCR-TTGE has fairly low detectability of the minority and/or low-density bacteria populations. This low resolution is a limitation but alternatively can be beneficial for this type of work as the majority of taxa detected by TTGE probably corresponds to true symbiotic endo-colonizers in *Anopheles* and are not likely to be transient or incidental contaminant bacteria.

The presence of enterobacteria is of particular interest because mosquitoes harboring abundant commensal *Enterobacteriaceae* appear more likely to be infected by *P. falciparum* suggesting a possible protective role of these bacteria for natural *Plasmodium* infection (Boissière et al., 2012). On the contrary, some species of *Enterobacteriaceae*, particularly *S. marcescens* and *Enterobacter* (*Esp_Z*), are able to inhibit *Plasmodium* development in *Anopheles* midguts (Gonzalez-Ceron et al., 2003; Cirimotich et al., 2011a; Bai et al., 2019). For instance, *S. marcescens* in *An. albimanus* is associated with inhibition of *P. vivax* oocyst development (Gonzalez-Ceron et al., 2003). In the same phylum (*Proteobacteria*, family *Neisseriaceae*), the presence of *Chromobacterium Csp_P* is associated with a significant reduction in susceptibility of *An. gambiae* to *P. falciparum* (Ramirez et al., 2014).

TABLE 3 | Bacterial genera detected in adult *Anopheles* abdomens collected in Mae Sot and Sop Moei districts, western Thailand.

Bacterial genera (number of *Anopheles* species)	Bacterial phyla	*An. minimus*		*An. dirus*		*An. baimaii*		*An. maculatus*		*An. sawadwongporni*		*An. dravidicus*		*An. pseudowillmori*	
		M[a] (*n* = 13)	S[b] (*n* = 8)	M (*n* = 5)	S (*n* = 3)	M (*n* = 4)	S (*n* = 2)	M (*n* = 4)	S (*n* = 2)	M (*n* = 2)	S (*n* = 8)	M (*n* = 1)	S (*n* = 0)	M (*n* = 0)	S (*n* = 4)
Acinetobacter (3)	*Proteobacteria*	1	–	–	1	1	–	–	–	–	–	–	–	–	–
Aerococcus# (1)	*Firmicutes*	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Aeromonas (2)	*Proteobacteria*	–	4	–	–	–	–	1	–	–	–	–	–	–	–
Chromobacterium (1)	*Proteobacteria*	–	6	–	–	–	–	–	–	–	–	–	–	–	–
Chryseobacterium (1)	*Bacteroidetes*	–	–	–	–	–	–	–	–	1	–	–	–	–	–
Elizabethkingia (5)	*Bacteroidetes*	–	5	1	–	–	–	1	–	2	3	1	–	–	–
Enterobacter (3)	*Proteobacteria*	4	1	–	–	–	–	2	1	–	–	–	–	–	1
Erwinia (1)	*Proteobacteria*	1	–	–	–	–	–	–	–	–	–	–	–	–	–
*Ferrimonas** (1)	*Proteobacteria*	–	2	–	–	–	–	–	–	–	–	–	–	–	–
Herbaspirillum (1)	*Proteobacteria*	–	–	2	–	–	–	–	–	–	–	–	–	–	–
Klebsiella (3)	*Proteobacteria*	–	–	2	1	–	–	3	1	–	2	–	–	–	–
Megasphaera# (1)	*Firmicutes*	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Pantoea (3)	*Proteobacteria*	–	–	–	–	1	–	2	–	–	1	–	–	–	–
*Pectobacterium** (1)	*Proteobacteria*	–	–	–	1	–	–	–	–	–	–	–	–	–	–
Peptostreptococcus# (1)	*Firmicutes*	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Pseudomonas (2)	*Proteobacteria*	1	–	–	–	–	–	–	–	–	1	–	–	–	–
Raoultella (3)	*Proteobacteria*	–	–	–	–	–	–	1	–	1	–	–	–	–	1
Roseomonas# (1)	*Proteobacteria*	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Serratia (5)	*Proteobacteria*	1	8	–	2	1	–	1	–	–	–	–	–	–	1
*Shimwellia** (4)	*Proteobacteria*	2	1	1	–	1	–	5	–	–	–	–	–	–	–
Staphylococcus (1)	*Firmicutes*	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Stenotrophomonas (1)	*Proteobacteria*	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Streptococcus# (1)	*Firmicutes*	1	–	–	–	–	–	–	–	–	–	–	–	–	–
*Trabulsiella** (3)	*Proteobacteria*	1	–	1	–	–	–	–	–	1	–	–	–	–	–

**Five genera or OTU newly identified in Anopheles mosquitoes.* [#]*Five bacterial genera only detected from one Anopheles minimus infected with Plasmodium vivax.* [a]*M = Mae Sot (Tak Province);* [b]*S = Sop Moei (Mae Hong Son Province).*

Members of the genus *Elizabethkingia* detected in assayed mosquitoes from Thailand could not be definitively identified as *E. anophelis* given its close relatedness in the 16S rRNA gene sequence with *E. meningoseptica*. This latter bacteria species is generally widely dispersed in the environment and recognized as an occasional serious bacterial pathogen in humans giving rise to meningitis and pneumonia (Xie et al., 2009). Previous studies reported that *E. anophelis* is a dominant midgut bacterium of laboratory-reared *An. stephensi* and *An. gambiae* (Boissière et al., 2012; Ngwa et al., 2013). Sharma et al. (2019) reported an interesting microbial interaction and immune modulation, whereby the presence of *P. vivax* in *An. stephensi* provoked a metabolic alteration of the availability of iron and nutritional physiology required for ideal bacterial growth resulting in a reduction of the microbiota present in the midgut, particularly the predominant bacteria *Elizabethkingia* and *Pseudomonas*. In addition to the parasite suppressing gut immunity in the mosquito host, by retarding bacterial growth, a greater amount of time is then allowed for the parasite to successfully cross the midgut wall and develop into the oocyst stage.

Within the genus *Staphylococcus*, *S. sciuri* was detected in *An. pseudowillmori*, a bacterium also isolated in the abdomen of other *Anopheles* species in Vietnam, such as *Anopheles crawfordi* and *Anopheles barbumbrosus* (Ngo et al., 2015). *Enterobacter* and *Staphylococcus* are among the genera showing trans-stadial maintenance in *Anopheles* and have been found in *An. albimanus* (*Enterobacter*) and mainly in male *An. stephensi* (*Staphylococcus*) (Rani et al., 2009; Minard et al., 2013; Galeano-Castaneda et al., 2020). Two other genera, *Aerococcus* and *Peptostreptococcus,* were found in *An. minimus* from Thailand, similar to findings from Vietnam (Ngo et al., 2016).

In this study, one *An. minimus* from Mae Sot was found naturally infected with *P. vivax* (Tainchum et al., 2014). This mosquito had at least five genera (*Aerococcus*, *Megasphaera, Peptostreptococcus, Roseomonas, Streptococcus*), bacteria not present in the malaria-free *Anopheles*. Moreover, the malaria-infected mosquito was devoid of *Enterobacteriaceae* and had three of the Gram-positive cocci out of the four total cocci genera detected in the study. Cocci bacteria are commonly present in larval habitats, which might explain their presence in *Anopheles* mosquitoes (Rejmankova et al., 2000; Piyaratne et al., 2005). Although no reasonable extrapolation or conclusion can be drawn from the findings in a single infected mosquito, the larger microbiota diversity found in this specimen is concordant with observations on microbiota detected in African *An. gambiae* s.l. and *Anopheles funestus* in which microbial diversity was greater in *P. falciparum*-infected samples than in non-infected ones (Bassene et al., 2018). Some bacterial symbionts were absent in the infected *An. minimus* specimen, notably *Elizabethkingia* and *Serratia*, which were commonly present in non-infected mosquitoes. Again, the single malaria-infected *Anopheles* was insufficient to allow a comparative analysis of bacterial species biodiversity between malaria-infected and non-infected mosquitoes. One possible limitation of the study design was only focusing on detection of infectious stage sporozoites (post-oocyst) present in the head-thorax portion of the mosquito – the final stage of the *Plasmodium* sporogony. We acknowledge a potential methodology flaw in which additional mosquito infections present as developing oocysts, would have escaped detection. However, having detected *Plasmodium* in the head-thorax indicates that we found sporozoites and the infected mosquito was a malaria vector, while detecting parasite stages in the abdomen is not a guaranty that the mosquito is a vector.

Although, the infected *An. minimus* was collected during a peak abundance period characteristic for this species, malaria-infected mosquitoes are becoming relatively rare findings due to a substantial decrease of malaria transmission in Thailand. A recent study in western Thailand showed *P. vivax*-infected *An. minimus* during an April period with an overall 0.76% sporozoite rate for the Minimus Complex (Tainchum et al., 2014; Sriwichai et al., 2016), comparable to this study (0.53%) and higher than the 0.092% reported by Tainchum et al. (2014). Therefore, in order to investigate and compare the natural microbiota between *Plasmodium*-infected and non-infected *Anopheles*, further collections and analyses are required. The mosquitoes found without *Plasmodium* displayed relatively high enterobacteria diversity, especially the genus *Serratia*. Identification of enterobacteria species in more samples will be the next step in the search for *Enterobacter* (*Esp_Z*) and *Chromobacterium* (*Csp_P*), both known to inhibit *P. falciparum* development (Cirimotich et al., 2011a).

CONCLUSION

The analysis of the microbiota detected in the abdomens of eight field-caught *Anopheles* species from Thailand resulted in 24 bacterial genera, among which five were only detected from one *P. vivax*-infected *An. minimus* specimen. A total of five genera were newly reported in *Anopheles* mosquitoes of which *Megasphaera* was only found in the malaria-infected mosquito. *Serratia* and *Elizabethkingia* were the most frequent bacteria found in the anopheline abdomens. Findings of low bacterial diversity, ranging from one to five genera per *Anopheles*, contrasted with a high overall OTU diversity in the entire sampling of species from both localities, which included three major bacterial phyla, *Proteobacteria*, *Firmicutes,* and *Bacteroidetes*. An analysis of the bacterial biodiversity in mosquitoes infected by malaria parasites compared with non-infected specimens was not possible due to the insufficient number of *Plasmodium* infections detected. Five genera identified in the infected specimen were not detected in other specimens, including three Gram-positive cocci. The PCR-TTGE method with bacterial 16S rRNA provided the first estimation of bacterial biodiversity present in *Anopheles* in Thailand. These findings suggest that bacterial diversity in *Anopheles* remains underestimated and requires further investigation. As some microbiota can suppress or block human pathogen development in *Anopheles* vectors, thus reducing the risk of transmission, more studies are needed to better understand the role of naturally occurring bacteria in wild mosquito populations as a potential method of disease control.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

KT collected the specimens, made the mosquito identification, participated in the bacterial analyses and result's interpretation, and wrote the draft. CD made the bacterial analyses, their interpretation and improved substantially the manuscript. EJ-B supervised the study design and was involved in the bacterial sequence analyses. TC supervised the mosquito collections and analyses done in Thailand. MB improved the manuscript and participated in the data analyses. SM was at the origin of the study design, was highly involved in data analyses and writing the manuscript. All the authors contributed to the manuscript redaction.

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Nest Gasses as a Potential Attraction Cue for Biting Flying Insects and Other Ectoparasites of Cavity Nesting Birds

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The presence of nestlings and other nest dwelling organisms in cavity nests alters the composition of gasses inside the cavity. Differential concentrations of gasses could be used by some parasites as a cue to localize their hosts. Here, we explored temporal variation in the concentration and isotopic signature of carbon dioxide (CO_2) and methane (CH_4) inside nest boxes of blue tits (*Cyanistes caeruleus*) during the nestling period (on days 3, 8, 13, 20, and 21 post-hatching) as well as several variables potentially affecting such variation. Finally, we checked whether differences in gas concentrations affect the abundance of different types of parasites affecting nestlings. Gas concentration and isotopic signature were significantly different between nest boxes and the forest during the nestling period. The CO_2 concentration was higher inside nests than in the forest air, whereas CH_4 concentration was lower. We expected to observe a positive correlation between the abundance of parasites actively seeking nests (i.e., blackflies, biting midges, and blowflies) and differences in gas concentration for those species that use these differences as a cue for host location. We observed that biting midge abundance was positively related to differences in CO_2 between nest and forest air at day 20 of nestling age, indicating that this species can use these differences to locate hosts. We also found a positive relationship between blackfly abundance and differences in CH_4 concentration. However, we hypothesize that the concentration of this gas inside nests may be related with bacterial activity; therefore, this relationship may be due to an effect of bacteria on blackflies and not to the effect of CH_4 as an attraction cue for blackflies.

Keywords: CH_4, CO_2, ectoparasites, gasses, nesting period, relative humidity

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INTRODUCTION

Several invertebrate species act as hosts and vectors for different pathogens (Marquardt et al., 2000). Vector borne diseases are usually more virulent that those transmitted by direct contact (Ewald, 1994) and mosquitoes are considered the deadliest animals in the world due to the diseases they transmit (GatesNotes, 2014). A pathogen's necessity to reach both a host and a vector to complete

its life cycle is obviously a challenge. However, evolution promotes important adaptations in pathogens to solve these challenges. Selection for such adaptations is facilitated by the advantages that flying vectors can provide such as increased dispersion, which can then lead to a significant increase in the pathogen's fitness. In this respect, the pathogen's successful transmission directly depends on the success of the vector to locate its host. What cues do mosquitoes and other vectors use to locate their hosts? Several studies have investigated mechanisms used by insects and other invertebrate vectors to locate their hosts. For instance, morphological studies of the sense organs of blood sucking insects have shown the kinds of substances that could act as host-specific attraction cues and have indicated that the gasses produced by host respiration may be potential cues for biting flying vectors (reviewed in Lehane, 2005). However, very little is known on the potential use of host-emitted gasses as an attraction cue for vectors in wild animal populations.

It is well known that mosquitoes, biting flies and ticks can use even slight fluctuations of CO_2 as a directional cue to find their hosts (Bogner, 1992; Grant et al., 1995; Mboera and Takken, 1997; Takken and Knols, 1999). In fact, Wright and Kellogg (1962) suggested that mosquitos moved to a CO_2 source specifically due to a change in CO_2 concentration. Grant and Kline (2003) showed that female mosquitoes, and many other biting insects, are equipped with an array of sensors (chemoreceptor neurons) that are highly sensitive to different concentrations of CO_2. Therefore, a higher CO_2 fluctuation is probably the main stimulus for mosquitoes to approach their hosts (McPhatter and Gerry, 2017).

Several studies in birds have measured the concentration of CO_2 during the incubation period (Lamson and Edmond, 1914; Wangensteen et al., 1971; Walsberg, 1980), however, few have investigated the effect of gas composition on vector attraction during nestling development. For example, White et al. (1978) found a higher concentration of CO_2 in nest chambers of the European bee-eater (*Merops apiaster*) compared with outside air during the nestling period. In other study, Mondain-Monval and Sharp (2018) found a higher concentration of CO_2 in occupied Sand Martin (*Riparia riparia*) burrows than in unoccupied ones during the nestling period. However, neither of these works explored the potential effect of these changes on vector attraction. In terms of other gasses, methane (CH_4) production by domestic and wild animals, including arthropods and insects, has been studied by many authors (see e.g., Crutzen et al., 1986). However, to our knowledge, none have examined the relationship between gasses (e.g., CO_2 and CH_4) produced in the nest during the nesting period and parasite abundance. In fact, the effects of CH_4 on birds and parasites in nests are completely unknown. In order to determine whether CH_4 concentration in nests is involved in parasite attraction, it is necessary to study the evolution of this parameter during the nestling period.

Geochemical tracing using the stable carbon isotopic signature $\delta^{13}C$ is a very useful tool for understanding biogeochemical processes in ecosystems (Peterson and Fry, 1987). $\delta^{13}C$ is the molecular ratio between the heavy and light carbon isotopes ^{13}C and ^{12}C, respectively (Earth's average relative concentration: ^{12}C, 98.9% and ^{13}C, 1.1%). This ratio allows for the identification of multiple carbon-producing (and removing) sources and mechanisms of CO_2 and CH_4 transference in natural ecosystems (Cerling et al., 1991; Yakir and Sternberg, 2000; Maseyk et al., 2009; Garcia-Anton et al., 2017). Processes that remove or produce CO_2 and CH_4 are characterized by a process-specific fractionation of carbon isotopes, owing to the isotope-dependent rates of the chemical reactions involved. As a result, the isotopic signature of CO_2 and CH_4 reflects the relative contribution of these processes. In a nest environment, variations in the concentration of CO_2 and CH_4 may be directly related to the metabolic activity of birds and the microbial activity in the nest. For instance, methane oxidation by methane-oxidizing bacteria (MOB) preferentially removes $^{12}CH_4$ and, therefore, increases $\delta^{13}C$ -CH_4 (Fernandez-Cortes et al., 2015). To our knowledge, CH_4 concentration has not, until now, been analyzed throughout the entire nestling period in any cavity-nesting bird. By measuring the concentration and isotopic signature of CH_4 in nests during this period, we can determine the cause of these variations and whether they are related to the presence and abundance of parasites in the nests.

The microclimate inside a nest cavity may vary considerably between nests due to differences in nest material composition and the level of bird activity (Mainwaring, 2017; Maziarz, 2019). Nestling development also affects the microclimate by adding different wastes (i.e., feces and feather debris) to nests, leading to increased heat and changes in relative humidity by evapotranspiration, which could both affect the gas composition inside nests. All these changes may affect the attraction and development of parasites and other arthropods inside nests. Here, we assess the effect of different variables (brood size, hatching date, nestling age, humidity, and temperature) on the concentration of gasses inside nest boxes and evaluate differences in gas concentrations between nest boxes and the forest air. We expect that nests with larger broods and older nestlings to show higher concentration differences. We also examine whether some ectoparasites (fleas, blowflies, mites, biting midges, and blackflies) potentially use CO_2 and CH_4 concentration differences as attraction cues. Finally, we determine whether ectoparasite abundance is positively correlated with gas concentration differences between nest boxes and forest air at any nestling age.

MATERIALS AND METHODS

Study Population

The study was conducted during the 2016 bird breeding season in a Pyrenean Oak (*Quercus pyrenaica*) deciduous forest located within a protected area of Sierra de Guadarrama National Park (Montes de Valsaín) near Valsaín (Segovia, central Spain, 40° 53′ 74 N, 4° 01′ W, 1,200 m.a.s.l). The geographic area of Valsain (Segovia) is characterized by a Mediterranean climate, a subtype of a warm temperate climate with a dry warm summer and a cool mild winter (Csb climate type, Köppen–Geiger Classification slightly modified, AEMET-IM, 2011). Annual mean temperature in 2016 at the study area was 10.2°C, and

total annual precipitation was 556 mm[1]. A blue tit *C. caeruleus* population breeding in wooden nest boxes has been studied in this area since 1991 (Sanz, 2002; Tomás et al., 2006). Nest boxes were 17.5 cm high, 11.7 cm wide, and 12.5 cm deep. The entrance hole of each box was 4.5 cm in diameter, and for airing the nest, there was a small uncovered hollow space (11.7 cm × 1.3 cm) located just under the nest-box roof. Total nest volume was 2,559 cm^3. A plastic tube with a diameter of 5 cm was attached to the outside of the entrance hole to prevent nestling predation by genets. The tubes likely reduce gas exchange with the outside air when birds enter or exit the nests because of the limited space around the bird's body and the fact that they are forced to move slowly while in the tube. Each breeding season, nest boxes are periodically inspected to determine reproductive parameters such as laying date, clutch size and hatching date (Merino et al., 2000; Tomás et al., 2007). The present study is considered observational work as it only involves ringing and measuring the birds. Ringing permits were granted by the Directorate General of the Natural Environment and the Junta of Castille and León (permit numbers: EP/SG/705/2015 and PNSG_SG_2015_0323). No other animal use permits were required.

Measuring Gasses

Between 24 May and 20 June 2016, we monitored and sampled 44 nest boxes occupied by blue tits. First, the ecosystem was characterized from both a climatic (meteorological data during the 2016 monitoring period see footnote 1) and a geochemical perspective (CO_2 and CH_4 concentration and $\delta^{13}C$-CO_2 and $\delta^{13}C$-CH_4 from oak forest air). For this purpose, we measured the atmospheric air, soil air and air inside nest boxes during the nesting period at a specific location in the middle of the study area. Four samples of each soil and forest air were taken each sampling day and used to calculate the average measurement for that day. In addition, a total of 148 samples of air from inside nest boxes were taken during the nestling period at different nestling ages (44 samples, one from each nest at 3, 8, and 13 days post-hatching, and 16 samples at 20 days post-hatching as nestlings had already fledged from the other 28 nests). The air inside four empty nest boxes was also sampled prior to their occupation (one box had a nest but no eggs or nestlings and the other three were completely empty). These samples were collected on May 18 (7:00–9:00 AM) in order to have a reference value for the concentration of gasses in empty nest boxes. Once the nestlings fledged (20 or 21 days post-hatching), a final sample was taken from the 44 nest boxes. Nest boxes were hanging from tree branches about 5 m above the ground. For gas collection, nest boxes were first carefully taken down from the tree branches and placed on the ground. Gasses were extracted through the entrance hole of the nest boxes. After gas collection, nest boxes were opened and the number of nestlings counted. Then, nest boxes were closed and returned to the same position on the tree branch until the next sampling. Sampling of gasses was always conducted between 7:00 and 10:00 AM for forest air and soil and between 8:30 AM and 16:30 PM for nest-box air. Sampling time had no effect on the results in our analyses.

[1]http://climaguadarrama.es/valsain/NOAA/NOAA-2016.txt

For the extraction of gasses from nest boxes and soil, we used a micro-diagraph gas pump (NMP-830-KNDC-12V; KNF, Germany) at 1.5 l min^{-1} at atmospheric pressure. Gas extraction was carried out slowly and for the same duration (20 s) in order to obtain approximately 0.5 L of air from each nest box. Similarly, atmospheric forest air was collected using a battery operated portable air pump (Aquanic S790, 0.010 Mpa 0.4 l/min Battery 1.5V; ICA, S.A, Spain). Grab air samples were collected through a tube connected directly to the pump, while another tube expelled the air from the pump and stored it in a 1-L Tedlar® gas sampling bag (Supelco; United States). The bags were sealed and subsequently analyzed in the laboratory within 24 h. The same sampling method and pump were used in other studies (e.g., Garcia-Anton et al., 2014, 2017; Fernandez-Cortes et al., 2015, 2018). CO_2 and CH_4 molar fractions and stable isotope ratios of carbon in both gasses were measured with a G2201-i analyser (Picarro Inc., United States) that used cavity ring-down spectroscopy (CRDS) technology to identify and quantify the compounds contained in the air being analyzed (Crosson, 2008). Stable isotope ratios of carbon are reported in standard δ-notation with units of ‰ ($\delta^{13}C$). The CRDS analyser measures the molar fractions and $\delta^{13}C$ with a dynamic range of 100 to 4,000 ppmv for CO_2 and 0 to 1,000 ppmv for CH_4 and a precision greater than 0.16‰ for $\delta^{13}C$-CO_2 and 1.15‰ for $\delta^{13}C$-CH_4.

Measuring Temperature and Relative Humidity

Environmental conditions (i.e., temperature and relative humidity) were recorded inside and outside nests boxes during the nestling period (days 3, 8, 13, and 20 post-hatching). Sensors that register both variables were fitted to the inner wall of the nest box just below the rim (iButton Hygrochron Temperature/Humidity Logger with 8KB Data-Log Memory DS 1923; 6 mm × 17 mm, temperature range: −20–85°C; resolution 0.0625°C; humidity range: 0–100% with a resolution 0.04%; Maxim Integrated, CA, United States). Temperature and relative humidity were recorded every 45 min during the nesting period. Similarly, four external sensors (HOBO U23-001; data logger; 10.2 cm × 3.8 cm, temperature range: −40–70°C; resolution 0.02°C; humidity range: 0–100% with a resolution 0.03%; Onset Data Loggers, Massachusetts, United States) were located underneath empty nest boxes to register temperature and relative humidity in the study area during the nesting period.

Quantifying Nest Ectoparasites

Several biting flying insects and nest-dwelling ectoparasites, many acting as vectors for blood parasites, are commonly found in this bird population. Biting midges (*Culicoides* spp.) are very small dipterans whose females need bird blood to lay eggs (although some autogenous species can lay the first batch of eggs without a blood feed). Biting midges are vectors of haemosporidian parasites of the genus *Haemoproteus* (Valkiunas, 2005; Martínez-de la Puente et al., 2011), which are known to have detrimental effects on bird reproduction and survival (Merino et al., 2000; Martínez–de la Puente et al., 2010). Blue tit nests are also visited by blackflies (Diptera: Simuliidae) looking

for bird blood to complete their life cycle. Blackflies are vectors of another common blood parasite of birds, *Leucocytozoon* spp. (Order Haemosporida), which cause chronic diseases in infected birds (Merino et al., 2000; Martínez–de la Puente et al., 2010). Mosquitoes are scarcely found in the bird nests of our population; however, the transmission of *Plasmodium* species to blue tits in this area has been previously reported (Martínez-de la Puente et al., 2011).

Nest-dwelling parasites typically found in blue tit nests in Valsain include a dipteran flying insect, the blowfly *Protocalliphora azurea*. Only blowfly larvae feed on blood and their role as vectors is unclear. Fleas (*Ceratophyllus gallinae*) are also common in nests. The presence of adult fleas is mainly evidenced by the presence of their larvae as adults typically remain attached to the birds or easily escape when the nest is collected to quantify parasites (see below). Only adult fleas feed on blood, and it is unclear whether they are vectors of any diseases in blue tits. Haematophagous mites (*Dermanyssus* spp.), which can act as vectors for trypanosomes (Macfie and Thomson, 1929), also attack blue tits. Both fleas and mites reach new nests by attaching to adult birds as they inspect other cavities. Mites may also reach nests by phoresy on midges and blackflies (Marshall, 1981), although this is unlikely their main means of transport.

Biting midges and blackflies parasitizing nests were collected by using traps located inside the nest boxes (see Tomás et al., 2008). The traps consisted of a plastic petri dish (8.5 cm in diameter; 55.67 cm^2) containing a commercially available baby oil gel (Johnson's Baby Oil Gel with Camomile; Johnson & Johnson, Dusseldorf, Germany). The petri dishes were placed within nest boxes at day 10 post-hatching and retrieved at day 13. Afterward, petri dishes were observed under a magnifying glass and the number of biting midges and blackflies that adhered to the gel were counted. Once nestlings fledged (20 or 21 days post-hatching), the sensors were removed, and the nest material was collected in a sealed labeled plastic bag and transported to the laboratory to quantify ectoparasite abundance. Nests were stored at 4°C for 2–4 days prior to ectoparasite extraction using Berlese funnels. Nests were kept in funnels for 48 h under constant temperature and light conditions provided by a lamp placed above the nests (see Tomás et al., 2007). Small nest ectoparasites (mites and fleas) were collected in vials containing 70% ethanol and examined under a stereomicroscope (OLYMPUS-SZX7; ACH1x, Tokyo, Japan; see Merino and Potti, 1995). Their abundance was estimated from this material. The nest material was then manually searched for blowfly pupae.

We also evaluated the relationship between ectoparasite abundance and bird body condition. For this, 75 blue tit adults (37 males and 38 females) were captured at 13 days post-hatching with traps mounted in the nest boxes. Adults were measured and, when necessary, ringed with numbered aluminum rings. Nestlings were also measured and ringed at 13 days post-hatching. Adult and nestling body mass was measured with an electronic balance (±0.1 g). Tarsus length was measured with a digital caliper (±0.1 mm), and wing length with a ruler (±0.5 mm). The body condition index in adults and nestlings was calculated as the residuals of body mass on tarsus length.

Statistical Analyses

Our first aim was to select a number of potential explanatory variables for the variation in gas concentration in nests. We hypothesized that the number of nestlings (brood size) and nestling age would significantly affect gas concentration (i.e., the greater the nestling number or the larger the nestlings, the greater the CO_2 concentration). The time in the season as measured by hatching date may also influence gas concentration, although this variable could be correlated with brood size. Temperature and relative humidity could influence gas concentration, for example, by favoring dilution effects. However, these two variables have also been shown to be negatively correlated with each other (see e.g., Castaño-Vázquez et al., 2018). We checked the variance inflation factor (VIF) for these variables (brood size, hatching date, temperature and relative humidity) in multiple correlation analyses with each of the gas concentration variables. VIFs estimate how much the variance of a coefficient is "inflated" because of linear dependence with other predictors. In all cases, the VIF values were low (less than 2). Thus, we included all of these variables in a mixed model analysis with gas concentration (CO_2/δ^{13}C-CO_2 or CH_4/δ^{13}C-CH_4 measured both inside nest boxes and in the forest) as the dependent variable (variables were log transformed in order to comply with normality assumptions), measurement location (inside nest box or forest) and nestling age as fixed factors and brood size, hatching date, temperature and relative humidity as covariables. The interaction of measurement location × nestling age was included as a repeated measures effect on nests. Then, we conducted a backward stepwise procedure to reduce the model to the significant variables.

To explore the relationship between gas concentration and ectoparasite abundance, we used generalized linear models (GzLM) with a negative binomial distribution and log link function. The number of each parasite type (flea larvae, mites, blowfly pupae, biting midges, and blackflies) was used as the dependent variable with the following independent variables: the differences in the concentration of CO_2 and CH_4 (i.e., the concentration inside the nest box less the concentration in the forest air), the differences in relative humidity inside nest boxes and in the forest air and brood size. We conducted one analysis for each day the gasses were measured, thus analyzing the relationship between the abundance of each parasite and the difference in gas concentration at the different ages independently. We assumed that the abundance of the different parasites measured at the end of the nestling period was directly related to their abundance at other stages of nestling development (see e.g., Dube et al., 2018). We selected gas concentration differences between nest boxes and forest air to reduce the number of variables in the model and because it could be the cue used by some ectoparasites to locate potential hosts. We expected that a lack of clear gas concentration differences between forest and nest boxes would result in a lack of signal for parasites; however, if concentration differences were observed from hatching to fledging stages, then we assumed that there was a potential attraction cue for ectoparasites. We first used an Omnibus test to check that the analysis could be performed. Omnibus tests assess the significant of

the overall model by calculating whether explained variance is significantly higher than unexplained variance. A positive Omnibus test suggested that the model fitted the data. In this case, at least one independent variable was significant and the other independent variables could be included in the model under the assumption of non-colinearity between independent variables. Conversely, a negative test suggested that the model was not sufficient to determine model fit for the predictors and, therefore, the analysis could not be performed. The Omnibus test used to compare the current versus the null (in this case, intercept) model was a likelihood-ratio chi-square test. Only when this test was significant did we explored the effect of the significant independent variables. Models were simplified by backward stepwise elimination of those variables with the highest *P*-value. Thus, only significant variables remained in the final models.

In addition, we used multiple linear regressions to test the relationship between parasite abundance and body condition of adult and nestling blue tits. Statistical analyses were performed in STATISTICA 7[2] and SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY, United States: IBM Corp).

Data Deposition

Data are available at the Digital CSIC repository.

RESULTS

Concentration of Gasses in the Ecosystem

During the nesting period (24 May–20 June), the study area experienced eight rainy days with a total rainfall of 39.8 mm, and the average temperature was 13.7°C (**Table 1**). The mean concentration of CO_2 of the forest air ranged from 411 to 547 ppm, and the isotopic signal ($\delta^{13}CO_2$) from −8.56 to −12.21‰. The highest CO_2 values for forest air were recorded on the warmest days due to increased flow from the soil: during drier periods, soil humidity decreases, which induces increased gas diffusivity (Werner et al., 2006; Garcia-Anton et al., 2017). CO_2 mean values from soil air ranged from 887 to 15,025 ppm, and the isotopic signature ($\delta^{13}CO_2$) varied from −16.5 to −22.4‰. These data are consistent with the local climate and C3 vegetation. The $CO_2/\delta^{13}C\text{-}CO_2$ results of the forest and soil air sample analyses were studied using the Keeling approach. This method is widely used to characterize the $\delta^{13}C\text{-}CO_2$ of ecosystem respiration (Garcia-Anton et al., 2014). The intercept value of the Keeling plot ($\delta^{13}C_s$) for the samples collected over the monitoring period was −22.77‰, indicating a prevalence of C3 plant activity in the oak woodland study area (C3 plant activity reference value is around −27‰, Amundson et al., 1998), plus a 4.4‰ diffusional enrichment from soil (Yakir and Sternberg, 2000).

[2]www.statsoft.com

Gas Concentrations, Relative Humidity, Age and Brood Size

At the start of the sampling period (May 18, 2016), we did not observe any significant difference in the concentration of CO_2 between the air inside empty nest boxes (without eggs or nestlings) and the forest air (unpaired *t*-test: $t = 1.27$, df = 5, $p = 0.259$; **Tables 1**, **2**). However, there was a significant difference in their isotopic signal, indicating that nest air was isotopically lighter than forest air [$\delta^{13}CO_{2(‰)}$; unpaired *t*-test: $t = -2.90$, df = 5, $p = 0.033$; **Tables 1**, **2**]. CH_4 concentration was significantly lower inside the empty boxes compared with the forest air (unpaired *t*-test: $t = -5.73$, df = 5, $p = 0.002$); however, no significant difference was observed in the CH_4 isotopic signal [$\delta^{13}CH_{4(‰)}$; unpaired *t*-test: $t = 2.27$, df = 5, $p = 0.072$; **Tables 1**, **2**].

CO_2 concentration was higher inside nest boxes than in the forest air during the nesting period (ANOVA mixed model, $F_{1,250.96} = 219.10$, $p < 0.0001$); this difference was maintained at all nestling ages (ANOVA mixed model, measurement location × nestling age interaction: $F_{3,259.65} = 21.34$, $p < 0.0001$, **Figure 1**). Moreover, CO_2 concentration inside nests differed significantly according to nestling age with the highest concentrations observed at 8 and 20 days of age and the lowest at 3 and 13 days of age (ANOVA mixed model, $F_{3,250.77} = 30.12$, $p < 0.0001$; **Figure 1**). Likewise, CO_2 concentration was positively and significantly related to relative humidity (ANOVA mixed model, $F_{1,263.32} = 13.71$, $p < 0.0001$) and to brood size ($F_{1,57.65} = 4.51$, $p = 0.038$). The results of the CO_2 isotopic signal [$\delta^{13}CO_{2(‰)}$] analysis gave similar but inverse results (lower level inside nests than outside; data not shown) (Measurement location: ANOVA mixed model, $F_{1,268.60} = 1134.56$, $p < 0.0001$; Nestling age: $F_{3,263.75} = 38.10$, $p < 0.0001$; Measurement location × nestling age interaction: $F_{3,247.37} = 22.43$, $p < 0.0001$]. Temperature, instead of relative humidity, was negatively and significantly related to $\delta^{13}CO_{2(‰)}$ (ANOVA mixed model, $F_{1,270.95} = 4.13$, $p < 0.043$), whereas brood size was positively related to $\delta^{13}CO_{2(‰)}$ ($F_{1,51.26} = 13.60$, $p = 0.001$).

CH_4 concentration was lower inside occupied nests boxes than in the forest air (ANOVA mixed model, $F_{1,240.53} = 659.99$, $p < 0.0001$) at all nestling ages (ANOVA mixed model, measurement location × nestling age interaction: $F_{3,240.86} = 3.30$, $p = 0.021$, **Figure 2**). Moreover, CH_4 concentration inside nests differed significantly according to nestling age (ANOVA mixed model, $F_{3,249.13} = 12.19$, $p < 0.0001$). The highest concentration was observed at day 3 and the lowest at day 20. In addition, CH_4 concentration was negatively and significantly related to hatching date (ANOVA mixed model, $F_{1,51.24} = 9.40$, $p = 0.003$) and positively to relative humidity ($F_{1,223.18} = 16.10$, $p < 0.0001$). The results of the CH_4 isotopic signal [$\delta^{13}CH_4$ (‰)] analysis showed similar but inverse patterns (a higher isotopic signal in nests that in the forest; see also **Figure 3**) (Measurement location: ANOVA mixed model, $F_{1,265.55} = 427.87$, $p < 0.0001$; Nestling age: $F_{3,259.21} = 4.11$, $p = 0.007$; Measurement location × nestling age interaction: $F_{3,241.77} = 11.82$, $p < 0.0001$). $\delta^{13}CH_{4(‰)}$ was positively related to temperature and relative humidity (ANOVA mixed model, $F_{1,281.50} = 10.82$, $p = 0.001$ and

TABLE 1 | Average data of environmental conditions of the study area (Valsain; Segovia, central Spain, 40° 53′ 74 N, 4° 01′ W, 1,200 m a.s.l) during blue tit nesting period.

			Forest air				Soil air			
Date	**T (°C)**	**Rain (mm)**	$CO_{2(ppm)}$	$\delta^{13}CO_{2(‰)}$	$CH_{4(ppm)}$	$\delta^{13}CH_{4(‰)}$	$CO_{2(ppm)}$	$\delta^{13}CO_{2(‰)}$	$CH_{4(ppm)}$	$\delta^{13}CH_{4(‰)}$
18-May-2016	15.4	0	405	−8.34	1.98	−51.28	31765	−29.12	0.24	−16.03
24-May-2016	14.9	0	411	−9.39	1.97	−51.55	15025	−21.39	0.20	−26.52
25-May-2016	12.1	0	442	−9.35	2.00	−54.91	3194	−21.13	1.39	−50.56
26-May-2016	13.6	0	480	−10.61	2.07	−54.09	9671	−22.26	0.13	−51.87
27-May-2016	12.9	0	428	−8.56	2.09	−61.27	8846	−21.99	0.14	–
28-May-2016	9.5	6	428	−9.28	1.98	−51.50	7919	−21.94	0.32	−12.84
29-May-2016	8.7	10.2	445	−9.21	2.12	−62.94	9253	−22.34	0.12	–
30-May-2016	9.3	1.2	424	−9.25	2.02	−53.50	3108	−21.54	1.44	−46.30
31-May-2016	10.3	0	439	−10.05	2.13	−54.23	10845	−22.42	0.13	−10.82
01-June-2016	11.5	0	424	−9.63	2.00	−50.71	9910	−22.25	0.17	−31.32
02-June-2016	13.2	0	473	−11.06	1.98	−53.51	9523	−22.16	0.20	−15.88
03-June-2016	15.5	0	488	−10.31	2.06	−57.98	8686	−21.93	0.19	−48.26
04-June-2016	14.5	0	436	−9.64	2.09	−55.45	8257	−21.84	0.20	−17.13
05-Jun-2016	15.5	0	447	−9.93	2.00	−53.42	8004	−21.93	0.18	–
06-June-2016	16	0	487	−11.34	2.02	−56.18	7616	−21.88	0.24	−26.96
07-June-2016	17.8	0	463	−10.32	2.05	−56.12	4003	−19.21	1.04	−32.12
08-June-2016	19.4	0	542	−12.21	2.03	−55.03	3056	−20.90	1.24	−42.99
09-June-2016	19.7	0	547	−12.08	2.00	−54.65	3819	−21.28	1.02	−42.89
10-June-2016	16.7	0.2	456	−10.92	2.04	−56.06	1907	−19.39	1.45	−45.41
11-June-2016	16	0	460	−10.6	2.01	−55.09	887	−16.51	1.75	−45.41
12-June-2016	16.4	0	474	−10.77	2.04	−55.58	1518	−18.90	1.56	−45.32
13-June-2016	18.1	0	448	−10.50	1.99	−55.31	1666	−19.19	1.52	−45.33
14-June-2016	14.9	0	424	−10.22	1.97	−50.64	4790	−21.43	0.63	−34.64
15-June-2016	10.1	9.4	430	−10.09	1.95	−53.44	3565	−21.08	0.96	−47.87
16-June-2016	7.9	11.4	429	−10.27	1.94	−53.10	3319	−21.47	1.16	−47.94
17-June-2016	10.3	1.2	435	−9.99	1.99	−56.64	3085	−21.19	1.32	−52.11
18-June-2016	11.3	0.2	427	−9.91	2.03	−53.34	8155	−21.88	0.27	−36.09
19-June-2016	11.9	0	437	−10.11	2.01	−54.95	8375	−21.52	0.18	−33.06
20-June-2016	16.3	0	411	−8.97	1.96	−58.19	8259	−21.51	0.18	−40.24

Temperature (°C), CO_2 concentration (ppm), CO_2 isotopic signal [$\delta^{13}CO_{2(‰)}$], CH_4 concentration (ppm), and CH_4 isotopic signal [$\delta^{13}CH_{4(‰)}$] are shown. Data from May 18 and June 20 correspond to nestboxes without nestlings.

$F_{1,273.79} = 9.95$, $p = 0.002$, respectively) and negatively to hatching date ($F_{1,47.65} = 11.27$, $p = 0.002$).

Once nestlings fledged, CO_2 concentration did not differ between nest and forest air (unpaired *t*-test: $t = 0.60$, df = 16, $p = 0.554$); however, the isotopic signal [$\delta^{13}CO_{2(‰)}$] was lower inside nests (unpaired *t*-test: $t = -2.59$, df = 16; $p = 0.019$). By contrast, CH_4 concentration was significantly lower and the isotopic signal [$\delta^{13}CH_{4(‰)}$] heavier inside nests once nestlings fledged compared with the forest air (unpaired *t*-test: $t = -8.07$, df = 16, $p < 0.001$ and unpaired *t*-test: $t = 3.22$, df = 16, $p = 0.005$, respectively; see **Tables 1**, **2**).

Nest Ectoparasites

The abundances of the different parasites collected in the blue tit nest boxes are shown in **Table 3**. We found a significant positive relationship between the abundance of biting midges (*Culicoides* spp.) and differences in CO_2 levels between forest air and air inside nest boxes at day 20 of nestling age ($B = 0.001$, $F_{1,14} = 5.78$, $p = 0.031$; **Figure 4**). The presence of an influential data point could drive this relationship; however, the binomial approach was used to control for the aggregated distribution of parasites in the wild. The presence of high numbers of *Culicoides* in a few nests is frequently observed in our study area and, therefore, is part of the natural variation. When we excluded this data point from the comparison, the relationship was no longer significant ($B = 0.001$, $F_{1,13} = 0.33$, $p = 0.577$).

We also found a significant positive relationship between blackfly abundance and CH_4 differences at day 8 of nestling age ($B = 11.26$, $F_{1,39} = 10.78$, $p = 0.002$; **Figure 5**). As in the case of the biting midges, it is not uncommon to observe high numbers of blackflies in some nests in the wild. In this case, however, the relationship was still significant after excluding the potentially influential data point ($B = 11.28$, $F_{1,38} = 17.38$, $p < 0.001$). Blowfly abundance was not significantly related to any of the variables at any nestling age (data not shown, $p > 0.05$ in all cases).

We observed a significant positive relationship between mite abundance and CO_2 differences at days 3 and 20 of nestling age ($B = 0.002$, $F_{1,38} = 9.59$, $p = 0.004$ and $B = 0.001$, $F_{1,12} = 6.75$,

TABLE 2 | Average values of concentration (ppm) and isotopic signals (‰) of CO_2 and CH_4 from nest-boxes without nestlings.

Date	CO_2	δ^{13}C-CO_2	CH_4	δ^{13}C -CH_4
18 May	410	−8.82	1.95	−48.70
10 June	441,74	−10,90	1,87	−45,76
11 June	429,61	−10,19	1,88	−46,39
13 June	420,81	−10,00	1,88	−46,44
14 June	431,48	−10,91	1,89	−54,36
15 June	439,27	−10,89	1,88	−50,18
16 June	458,72	−12,08	1,90	−52,18
17 June	461,18	−11,17	1,91	−50,89
18 June	466,10	−11,23	1,91	−53,41
19 June	433,78	−10,65	1,91	−52,57

Data from the 18 of May are from three empty nestboxes and one nestbox with a nest completed but previous to egg laying. Data from June are from nests once nestlings fledged.

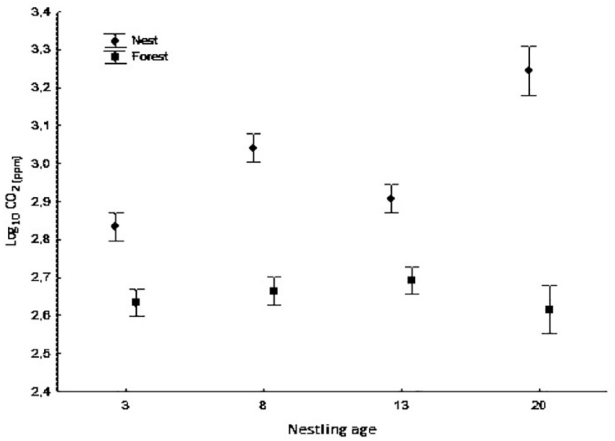

FIGURE 1 | Average concentration of CO_2 (log_{10}) inside nest-boxes and in forest air at different nestling ages (days 3, 8, 13, and 20). Bars show mean ± SE.

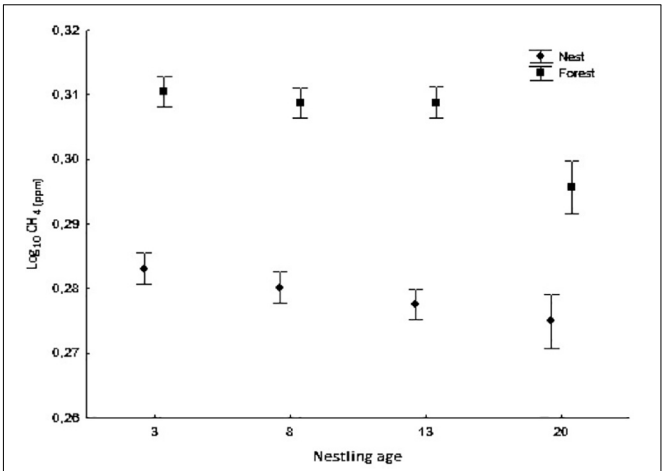

FIGURE 2 | Average concentration of CH_4 (log_{10}) in nest boxes and forest air at different nestling ages (days 3, 8, 13, and 20). Bars show mean ± SE.

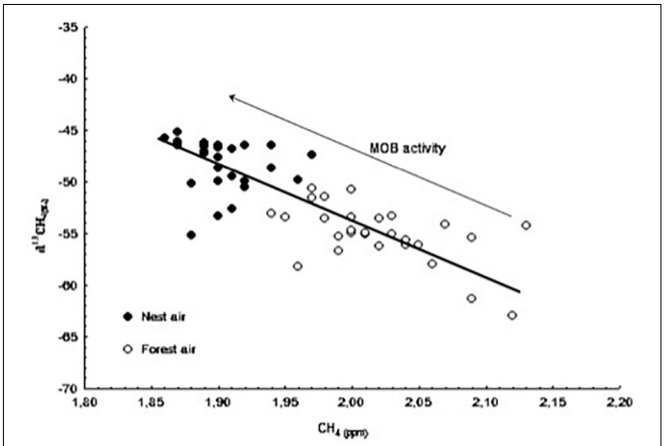

FIGURE 3 | Concentration values of methane (CH_4) and δ^{13}C-CH_4 from samples taken at the nest-boxes (black dots) and from the outdoor air samples (white dots) throughout the monitoring period. The direction of increase in methane oxidizing bacteria (MOB) activity is shown by an arrow.

$p = 0.023$, respectively) and between mite abundance and CH_4 differences at day 20 of nestling age ($B = 35.86$, $F_{1,12} = 14.37$, $p = 0.003$). Mite abundance also appeared to have a significant positive relationship with brood size at day 3 of nestling age ($B = 0.287$, $F_{1,38} = 4.43$, $p = 0.042$) but a negative one at day 20 ($B = -0.475$, $F_{1,12} = 4.93$, $p = 0.046$).

Ceratophyllus gallinae (flea) larval abundance was significantly and negatively related to brood size at days 13 and 20 of nestling age ($B = -0.613$, $F_{1,40} = 9.32$, $p = 0.004$ and $B = -0.493$, $F_{1,14} = 7.33$, $p = 0.017$, respectively), and positively to relative humidity at day 13 ($B = 11.26$, $F_{1,40} = 9.38$, $p = 0.004$). The relationships among the other variables including gasses, humidity, brood size and ectoparasites were not significant according to the results of an Omnibus test ($P > 0.05$, data not shown).

The female body condition was not related to any of the variables except blowfly pupal abundance, with which it had a significant negative relationship (multiple linear regression, $F_{1,33} = 10.17$, $p = 0.003$). Male and nestling body condition were not significantly related to the abundance of any of the ectoparasites ($p > 0.05$ in all cases).

DISCUSSION

The concentration of gasses (i.e., CO_2 and CH_4) inside bird breeding cavities has been poorly studied, much less the effects of these gasses on the attraction of parasites to nests, and their growth once inside the nests. In this study, we showed that CO_2 and CH_4 concentration and isotopic signal [$\delta^{13}CO_{2(‰)}$ and $\delta^{13}CH_{4(‰)}$] inside blue tit (*C. caeruleus*) nest boxes differed from forest air during the nestling period (from 24 May to June 20) in an ecosystem dominated by the activity of C3 plants. Air inside nest boxes had a higher concentration of CO_2 and a lighter $\delta^{13}CO_{2(‰)}$ than forest air. A similar result has also been

TABLE 3 | Abundance and standard deviation of the different ectoparasites collected in blue tit nest-boxes.

Parasite	Average	*SD*
Culicoides spp.	136.42	271.82
Simuliidae	30.28	31.33
Protocalliphora azurea	26	12.27
Dermanyssus spp.	117.98	176.93
Ceratophyllus gallinae larvae	31.1	78.75

observed in studies of burrow nesting birds [e.g., the European bee-eater *M. apiaster* (White et al., 1978) and the Sand Martin *R. riparia*, (Mondain-Monval and Sharp, 2018)]. Before and after the nestling period, we did not find any significant differences between the concentration of CO_2 in nest-box and forest air. Thus, as expected, nestlings increase the concentration of CO_2 inside nests and more nestlings imply a higher concentration of this gas. Slight but significant differences in the isotopic signal of CO_2 in the absence of nestlings suggest the presence of microbial activity in the nesting material.

In our study, the greatest differences in CO_2 concentration between air inside nest boxes and forest air were observed at days 8 and 20 of nestling age. Morganti et al. (2017) showed that blue tit nestlings have a greater mass gain between days 4 and 8 of nestling age. Therefore, changes in the CO_2 concentration observed on day 8 may have been due to an increase in growth and, in turn, metabolic rate after hatching, resulting in a higher level of CO_2 inside nest boxes. An increase in the metabolic rate of nestlings may also be due to the initiation of thermoregulatory development, a process that usually begins around 8 days of age in passerine birds (Visser, 1998; Pereyra and Morton, 2001). In addition to higher CO_2 production by nestlings due to thermoregulation is the respiratory effect of adult females that may have been still brooding small chicks and staying inside the nests for long periods. The greatest difference in CO_2 concentration between nest and forest air was found on day 20 of nestling age. This difference could have been due to the higher level of activity that nestlings display just before leaving the nest. Previous studies have also found a relationship between nestling age and gas concentration in nests. For example, Wickler and Marsh (1981) reported increases in CO_2 concentration as nestlings aged in the nest chambers of bank swallows (*R. riparia*), and Mersten-Katz et al. (2012) found that woodpecker nestlings have little influence on the concentration of CO_2 inside nests until they reach 15 days of age and become more active within the nests.

To our knowledge, this study is the first to analyze the concentration of CH_4 in nest boxes throughout the nestling period. In contrast to our results for CO_2, we observed a lower concentration of CH_4 and a significantly heavier isotopic signal [$\delta^{13}CH_{4(‰)}$] inside nest boxes compared with forest air. This difference was evident even in the nests without nestlings, indicating that methane-oxidizing bacteria (MOB), which are thought to be the main consumer of CH_4 (Hanson and Hanson, 1996), are active inside nest boxes from the very beginning of the breeding season. MOB activity may be more intense and slightly

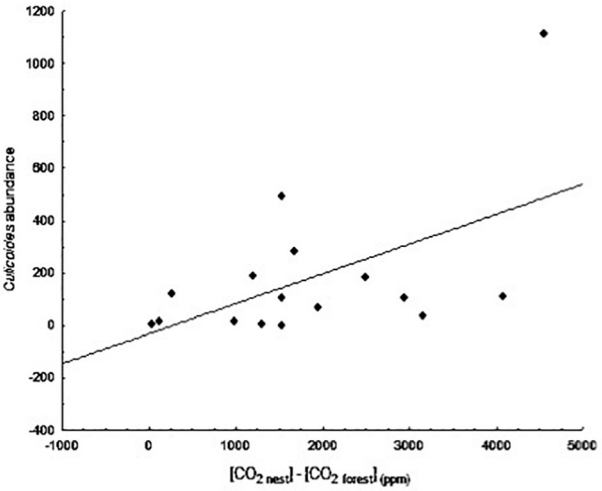

FIGURE 4 | Relationship between the difference in concentration of CO_2 between nest and forest ([CO_2 nest] – [CO_2 forest]) at day 20 of nestling age and abundance of biting midges at day 13.

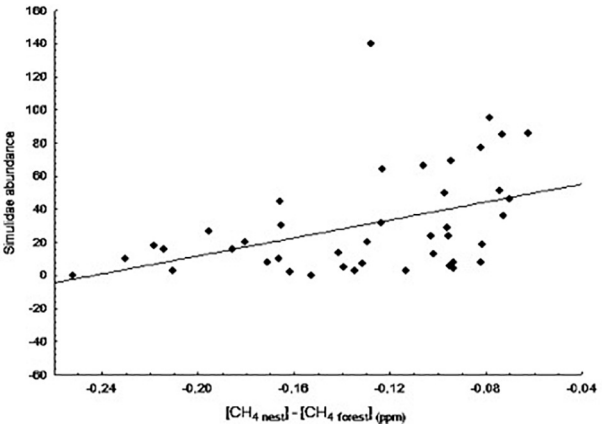

FIGURE 5 | Relationship between the difference in concentration of CH_4 between nest and forest ([CH_4 nest] – [CH_4 forest]) at day 8 of nestling age and abundance of Simuliidae at day 13.

modified in nests due to the insulating effect of the boxes and the metabolic activity of the birds inside (probably related to the accumulation of waste and feces). Also, the negative relationship observed between CH_4 concentration and hatching date indicates that MOB activity increases as the season progresses. **Figure 3** shows that the progressive decrease in CH_4 concentration in nest air during the nesting period is linked to the increase in $\delta^{13}C$-CH_4 values. This result shows that the pattern of CH_4 oxidization occurring in the ecosystem due to the presence of MOB in the forest soil is the same but less intense as the one observed in the nests. In other words, it is proof of intense microbial activity within nests, which may be related to bird health or the presence and/or abundance of parasites. Finally, compared with nest and forest air, the concentration of CH_4 in soil air was always lower and the isotopic ratio was always lighter (see **Table 1**).

These data are consistent with the removal of CH_4 from aerobic soils by bacterial oxidization (Conrad, 1996) and the subsequent consumption of the atmospheric methane in the ecosystem.

We are unaware of any studies investigating the relationship between gasses inside nest cavities and abiotic factors (i.e., relative humidity and temperature) during the nestling period. In our study, CO_2 concentration inside nest boxes was positively and significantly related to relative humidity at different nestling ages. The presence of nestlings (which increase CO_2 levels) may increase transpiration, leading to an increase in relative humidity inside nests. The concentration of CH_4 was also positively related to relative humidity, perhaps due to an indirect effect of humidity on bacterial activity. However, studies that experimentally test this potential effect are needed to confirm the relationships among these factors. In the case of the isotopic signals, temperature showed a negative relationship with $\delta^{13}CO_{2(‰)}$, whereas temperature and relative humidity were both positively related to $\delta^{13}CH_{4(‰)}$. Nestlings may increase the temperature inside nests, which could favor the dilution of gasses. The nature of these relationships, however, is still unclear and merit more research to be fully understood.

Our starting hypothesis was that differences in gas concentrations between nests and the forest could be an attraction cue for ectoparasites. The significant difference observed in the concentration of both CO_2 and CH_4 between nest and forest air support that possibility. In this respect, we expected to find a positive relationship between changes in gas concentration and ectoparasite abundance, particularly of those parasites actively seeking hosts (i.e., blackflies, biting midges, and blowflies). The positive relationship observed between biting midge abundance and CO_2 concentration differences between nest and forest air at day 20 of nestling age clearly indicates that biting midges can use CO_2 concentration inside nests as a cue to locate their hosts (**Figure 4**). We also observed a positive relationship between blackfly (Simuliidae) abundance and CH_4 concentration differences between nest and forest air. That is, blackflies were more abundant in nests that had a high CH_4 concentration and lower concentration differences between nest and forest air (**Figure 5**). A low concentration of CH_4 inside nests implies a high level of bacterial activity, which could be, in some way, detrimental to blackflies. In fact, bacteria have been used to infect larvae as a means to control blackfly populations (De Barjac, 1978; Hougard and Back, 1992); however, the bacteria used in this system could be different than those growing in the nests. Further investigation of the bacterial composition of the nests would be needed to confirm this hypothesis. It is also possible that blackflies are initially attracted to hosts based on visual cues and only use host odors (e.g., ether extracts and CO_2) once a host has been located (Fallis and Smith, 1964). Alternatively, Tomás and Soler (2016) have proposed that acoustic signals produced during nestling begging behavior attract blood-feeding insects to nests. We did not observe any significant relationship between blowfly abundance and gas concentration differences at any nestling age, indicating that this fly species does not use CO_2 or CH_4 concentration to locate their hosts, but rather relies on other attraction cues.

We observed some correlation between differences in gas concentration and mite abundance. In contrast to other ectoparasites, mites must be transported to new nests by way of adult birds, for instance, when they visit old nests or cavities containing mites (Proctor and Owens, 2000). In some cases, mites can also reach a new nest by phoresy on other arthropods (Marshall, 1981, p. 274). Thus, it is possible that some mites reached the nests via other arthropods that use CO_2 concentration as an attraction cue. Alternatively, the positive relationship between CO_2 differences and mite abundance at day 3 of nestling age may be associated with brood size: a high number of nestlings (and hence higher CO_2 production) may be needed at the beginning of nestling development to enable mite population growth within nests. Mites go through several life cycle stages before reaching adulthood. In this context and given their short life span, mite population growth might depend more on the initial number of nestlings rather than on nestling age. Another possibility is that mites may have disturbed brooding females at the beginning of the nestling period, causing them to produce more CO_2, thus accounting for the positive relationship between mite abundance and CO_2 concentration at day 3 of nestling age. The positive relationship between mite abundance and CH_4 concentration differences at the end of the nestling period (day 20) might be related to bacterial activity, as suggested in the case of blackflies. The negative relationship observed between brood size and flea abundance at days 13 and 20 of nestling age and between brood size and mite abundance at day 20 could be due to the fact that some of the nestlings at those ages abandoned some of the more infested nests (see e.g., Berggren, 2005).

Temperature and humidity are important factors for ectoparasite development. For example, several studies have shown that a high humidity environment can favor an increase in the number of ectoparasites (Heeb et al., 2000; Moyer et al., 2002). The positive relationship between relative humidity and larval flea abundance inside nests could be explained by the importance of humidity for their development. Indeed, an experimental increase in temperature inside blue tit nest boxes leads to a reduction in relative humidity and in the number of blowfly pupae in nests (Castaño-Vázquez et al., 2018).

Blowfly pupal abundance was negatively and significantly related to the condition of blue tit females. Tomás et al. (2005) also found a negative effect of blowfly pupae on the mass of blue tit females in the same study area. This relationship is indicative of an extra effort made by females trying to compensate for the effect of blowflies on nestlings, supporting the idea that females pay part of the cost of parasitism on nestlings (Tripet and Richner, 1997; Bouslama et al., 2002).

Differences between the atmospheric gas composition of nest boxes and the surrounding forest appear to be a cue, among other potential ones, used by, at least, biting midges to locate hosts. On the basis of our results, we cannot conclude that gas concentration differences act as an attraction cue for the other parasites, which may use other cues. Clearly, more studies are needed to better understand the relationships among nest cavity environment, hosts, parasites and even bacteria living in nests. In this respect, studies that analyze gas composition, and its

variation, inside nest boxes would provide key insights on the host–parasite relationships of these organisms.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Dirección General de Agricultura, Ganaderia y Alimentación, Comunidad de Madrid.

AUTHOR CONTRIBUTIONS

FC-V and SM conducted the field-work and parasite analyses. SC and SS-M conducted the analyses of gasses. All authors contribute to the design of the research and analyses of data as well as writing of MS.

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Vectorial Capacity of *Culiseta melanura* (Diptera: Culicidae) Changes Seasonally and Is Related to Epizootic Transmission of Eastern Equine Encephalitis Virus in Central Florida

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Vectorial capacity is an equation that integrates the major aspects of vector biology to predict the number of new mosquito-borne disease infections. Developed for studying transmission of malaria, vectorial capacity is rarely applied to zoonotic vector-borne diseases and is not often adjusted to account for seasonal changes in vector ecology. We used field data from Florida, United States, to expand the understanding of how vectorial capacity of *Culiseta melanura* (Coquillett), the primary enzootic vector of eastern equine encephalitis virus (EEEV), changes seasonally and its effect on EEEV risk. We determined parity via dissection and identified bloodmeals by PCR for field-collected *Cs. melanura* females from Central Florida. We used density of the vector, proportion of avian hosts fed upon, parity state of the vector, and mean temperature of the study area to quantify vectorial capacity as a function of season. The calculated values of vectorial capacity shifted significantly with season, with highest values observed in the summer with an additional peak in December. Linear regression revealed a strong positive correlation between vectorial capacity values and Florida EEEV equine cases in 2018, as well as cases reported during the last decade. The relationship between virus infections in equids and vectorial capacity lends support to the large effect that enzootic transmission has on epizootic outbreaks of zoonotic vector-borne pathogens.

Keywords: vectorial capacity, seasonal, arbovirus, zoonosis, epizootic

INTRODUCTION

The ecology of zoonotic vector-borne pathogens is inherently complex, given the diversity of vectors and/or multiple hosts that are involved in their transmission (Kuno et al., 2017). Host, vector, and pathogen populations interact within the physical environment (e.g., vegetation, climate, season), such that pathogen transmission is driven by overlapping spatial-temporal

distributions of hosts and vectors (Reisen, 2010). Quantifying the influence of key biological and environmental variables and mathematically integrating their relationships is useful for understanding the relative importance of each component in the spread of the pathogen (Smith et al., 2014) and illuminates opportunities for targeted interventions to disrupt transmission (Garrett-Jones, 1964b; Brady et al., 2016). Vectorial capacity (*C*) is an equation that accounts for the major factors of pathogen transmission by mosquitoes and is defined as the average number of new vertebrate infections per day resulting from an initial index case (Garrett-Jones, 1964b). The origins of this equation are rooted in efforts to better understand *Plasmodium falciparum* (human malaria parasite) transmission and assess the effectiveness of malaria control in sub-Saharan Africa (Smith et al., 2014). Nevertheless, with modifications vectorial capacity can be applied to zoonotic vector-borne disease systems and is especially relevant to pathogens vectored by mosquitoes or insects with similar life cycles (Macdonald, 1957; Garrett-Jones and Shidrawi, 1969; Smith et al., 2014). Vectorial capacity is calculated by equation 1, where *ma* represents the man-biting rate, *a* is the man-biting habit, *p* is the vector's daily probability of survival, *n* is the extrinsic incubation period of the pathogen, and *v* is the vector competence (Macdonald, 1957; Garrett-Jones, 1964b):

$$C = \frac{ma^2 p^n v}{-lnp} \quad (1)$$

The man-biting rate (*ma*) for transmission cycles in which humans are the primary host (e.g., malaria) can be estimated by human landing catch (Garrett-Jones, 1964a; Almeida et al., 2005). Because non-human animals serve as amplifying hosts of zoonotic mosquito-borne pathogens, estimating mosquito density and biting rate from human landing catches is not relevant. The parameter *a* also presents challenges, as it is the product of the host bloodmeal index and the feeding frequency. Polymerase chain reaction (PCR)-based methods enable measuring the former, while the latter is often estimated as the inverse of the length of the gonotrophic cycle.

Despite its potential utility in understanding zoonotic vector-borne disease, few studies of zoonotic pathogens are able to measure all of the components of vectorial capacity (Dye, 1986). As a consequence, the relationship between vectorial capacity and spillover of zoonotic mosquito-borne disease is not fully established.

Eastern equine encephalitis virus (EEEV; family Togaviridae, genus Alphavirus) is a vector-borne pathogen found in the eastern United States that causes severe disease in humans, with high case fatality rates in symptomatic persons (Bigler et al., 1976). The mosquito *Culiseta melanura* (Coquillett) (Diptera: Culicidae) is the primary enzootic vector of EEEV throughout eastern North America (Howard and Wallis, 1974), although a number of other species serve as bridge vectors (Armstrong and Andreadis, 2010). The distribution of *Cs. melanura* closely matches that of EEEV, occurring along the eastern coast of the United States and stretching west to Kansas (Darsie and Ward, 2005). Virus infection in this species has been recorded from transmission foci in many states, including Maine (Lubelczyk et al., 2013), Vermont (Molaei et al., 2015b), Connecticut (Armstrong and Andreadis, 2010), Maryland (Saugstad et al., 1972), Virginia (Molaei et al., 2015a), Alabama (Cupp et al., 2003), and Florida (Bingham et al., 2014). *Culiseta melanura* is an efficient biological vector of the virus (Howard and Wallis, 1974; Scott and Burrage, 1984; Vaidyanathan et al., 1997), and EEEV disseminates rapidly in this species, with biological transmission occurring in as few as 3 days at 25–30°C (Scott and Burrage, 1984). In addition, many bird species that are commonly bitten by *Cs. melanura* serve as amplifying hosts for the virus throughout its range (**Figure 1**). American robin and northern cardinal were the first or second most frequently bitten avian hosts (**Figure 1**) in 10 independent PCR-based studies of *Cs. melanura* host use, spanning seven eastern states (Molaei et al., 2006, 2013, 2015a,b, 2016; Estep et al., 2011; Bingham et al., 2014; Burkett-Cadena et al., 2015; Blosser et al., 2017; West et al., 2020). Transmission of EEEV in the United States is seasonal, and epizootic activity primarily occurs in the summer months and declines in autumn (Tenbroeck et al., 1935; Bigler et al., 1976; Scott and Weaver, 1989). In temperate regions, the first heavy frost in November or December ends virus transmission in mosquitoes (Scott and Weaver, 1989). Cases in equids can also occur during winter in the southern United States because of its warmer climate (Bigler et al., 1976; Bingham et al., 2015; Burkett-Cadena et al., 2015). In southern states, mosquitoes can be present year-round and are thought to maintain EEEV transmission in the enzootic cycle even when epizootic transmission is not observed (Burkett-Cadena et al., 2015). In Florida, reported equine cases are sporadic and occur in isolated foci (Day and Shaman, 2011). Sixty-six percent of EEEV equine cases in Florida from 2009 to 2018 were reported in summer (Florida Department of Health, 2019). Although yearly number of cases may be low in some years and regions of Florida, horse cases are the most reliable indicator of virus activity in the southeast (Bigler et al., 1976). The seroconversions of sentinel chicken flocks that are used for detection of arboviruses have a similar trend of summer (May–September) transmission in Florida (Heberlein-Larson et al., 2019). The summer peak in transmission also coincides with increased mosquito abundance and EEEV antibody seroprevalence in young birds (Tenbroeck et al., 1935; Elias et al., 2017).

The epizootic transmission of EEEV in Florida fluctuates from year to year and varies in severity between regions within the state of Florida. A large and widespread equine epidemic occurred in 2003 with 207 confirmed cases. Between 2004 and 2018, there were 6 years with fewer than 30 equine cases and 4 years with more than 90 equine cases. In the past decade (2009–2018), 41.4 mean equine cases per year were reported in Florida. Equine cases typically peak in June (mean = 11.5 cases) and are lowest in February (mean = 0.7 cases).

The goal of this study was to quantify vectorial capacity (*C*) of *Cs. melanura* for EEEV as a function of season in Central Florida, by measuring *Cs. melanura* abundance, avian host use, and parity throughout the year. These variables were incorporated into the vectorial capacity equation using temperature data and published data on extrinsic incubation period (*n*) and duration

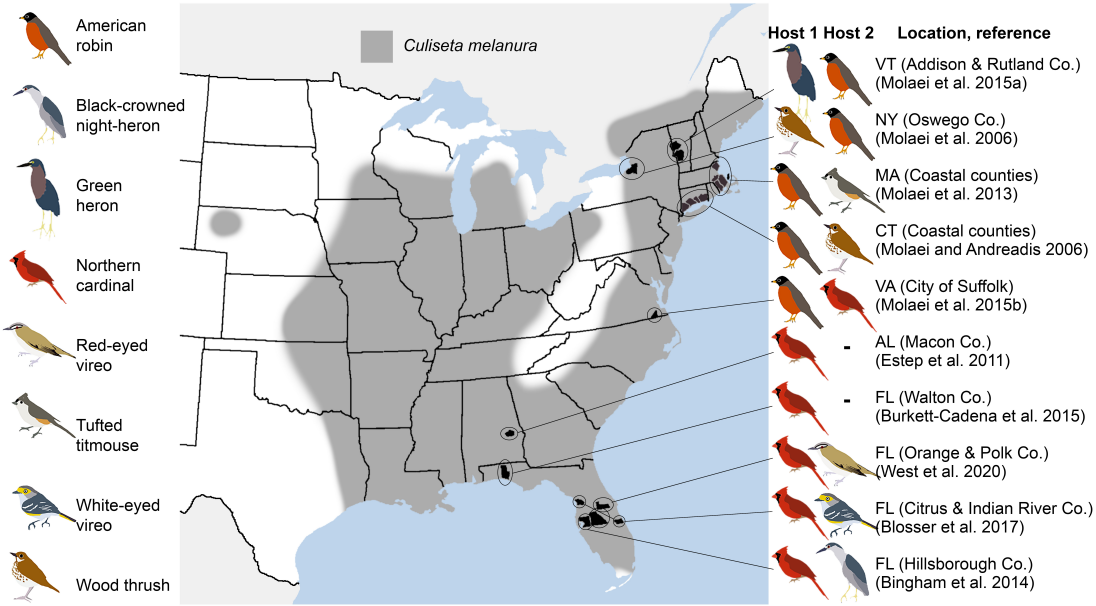

FIGURE 1 | Geographical patterns of avian host use by *Cs. melanura*. The first and second most commonly bitten avian hosts are presented for PCR-based studies of bloodmeal analysis of *Cs. melanura* in North America. Distribution of *Cs. melanura* after Darsie and Ward (2005). Dash represents several equally fed upon secondary avian hosts.

of gonotrophic cycle (g_c) for this species. Importantly, although g_c is not often included as a parameter of vectorial capacity, it is a biologically meaningful value that can be used to estimate a and p from field data. Quantifying vectorial capacity of *Cs. melanura* and comparing values with EEEV cases in equines will expand the knowledge of how *Cs. melanura* ecology affects EEEV transmission.

MATERIALS AND METHODS

Sampling was conducted in Central Florida with six sites in Orange (3) and Polk counties (3), with evidence of prior EEEV activity (**Table 1**). Weekly mosquito collections were made at these six sites from January 2018 to December 2018 using six artificial resting shelters at each site for a total of 36 resting sites. Resting shelters serve as dark, protected refuges that attract blood-engorged females seeking a place to rest and digest their meals and are an effective method to collect blood-fed *Cs. melanura* females (Bingham et al., 2014; Blosser et al., 2017). The resting shelters used were made by placing a fitted black trash compactor bag over a cylindrical frame made with PVC pipes (Burkett-Cadena et al., 2019; **Supplementary Video S1**). Mosquitoes were collected by aspiration with a modified vacuum (BDH1800S Ni-Cd 18V Dustbuster, Black & Decker, MD) and collection cup (BioQuip Products, Rancho Dominguez, CA) (Blosser et al., 2017; **Supplementary Video S2**). Collected female mosquitoes were identified to species by examining morphological traits using a dissecting microscope and updated dichotomous keys (Darsie and Morris, 2003; Darsie and Ward, 2005; Burkett-Cadena, 2013). Samples were stored in Thermo Scientific Microcentrifuge tubes at −20°C for subsequent parity and bloodmeal analysis. Field staff conducting this research used appropriate protective clothing (long sleeves, pants) and repellent to minimize risks associated with EEEV infection.

After mosquitoes were sorted and identified, unfed or freshly blood-fed *Cs. melanura* females were dissected to determine parity. To make this diagnosis, ovaries were extracted into a drop of water on a slide and left to dry, making the tracheoles visible for inspection (Detinova, 1962). This method was tested on *Aedes vigilax* (Skuse) for accuracy and was 83.7–89.8% reliable (Hugo et al., 2014). *Culiseta melanura* females whose parity could not be determined were excluded from the total for the parity calculation.

The hosts fed on by *Cs. melanura* were previously determined by bloodmeal analysis consisting of DNA extraction, PCR, and sequencing (West et al., 2020). Extraction was performed using InstaGene Matrix (Bio-Rad, Hercules, CA, United States) followed by PCR assays using a series of five primer pairs to amplify vertebrate host bloodmeals in a series of PCRs as previously described. Details regarding primer sequences, PCR mixtures, and cycling conditions are provided in West et al. (2020).

VECTORIAL CAPACITY CALCULATIONS

The vectorial capacity values per month were calculated following Macdonald (1957) with modifications including adjustments for density and biting rate of an ornithophilic mosquito species, calculating p for a wild field population, and incorporating the monthly temperature of the study area. With the assumption that host density is equal between sites and seasons, the variable m was calculated as the mean number of female *Cs. melanura* per resting

TABLE 1 | Mosquito sampling location details. Orange and Polk County, FL, in 2018.

Site name	County	Coordinates	Surveillance type	Habitat
Deen Still Road	Polk	28.2568°, -81.6850°	CDC light trap	Forest
Fussell Road	Polk	28.2163°, -81.7720°	CDC light trap	Forest, pasture, and residential
Green Pond Road	Polk	28.3693°, -81.8185°	CDC light trap	Pasture, forest, and wetland
New Independence	Orange	28.4663°, -81.5982°	Former sentinel chicken flock	Forest, wetland, and residential
Kelly Park	Orange	28.7583°, -81.5022°	Sentinel chicken flock	Forest, wetland
Tibet-Butler Park	Orange	28.4437°, -81.5432°	Sentinel chicken flock	Forest, wetland, and residential

TABLE 2 | Monthly numbers of *Cs. melanura* and other mosquito species. Mosquitoes were collected using resting shelters in Orange and Polk County, FL, in 2018.

Species	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	% of total
Cx. erraticus	150	94	46	47	196	343	476	452	252	185	358	121	2,720	44.6
Cs. melanura	31	19	47	74	163	166	170	199	33	45	59	30	1,036	17.0
An. crucians s.l.	38	69	67	51	96	137	95	119	63	51	95	36	917	15.1
Cx. cedecei	0	2	3	1	22	128	129	27	5	0	0	0	317	5.2
Cq. perturbans	0	1	9	6	9	20	111	64	22	22	3	0	267	4.4
Cx. territans	11	7	30	35	45	43	11	8	4	4	18	6	222	3.6
Ur. sapphirina	1	0	0	1	23	0	62	32	1	5	4	1	130	2.1
Cx. nigripalpus	4	8	1	2	13	18	21	34	14	7	3	2	127	2.1
Cx. pilosus	0	1	0	0	1	1	14	73	22	7	1	0	120	2.0
An. quadrimaculatus s.l.	0	0	3	5	3	12	10	9	5	6	2	3	58	1.0
Cx. quinquefasciatus	0	5	8	6	15	10	1	1	0	1	0	1	48	0.8
Cx. peccator	0	0	0	0	0	0	7	11	7	2	1	0	28	0.5
Mn. titillans	0	1	3	0	2	0	3	2	2	2	7	0	22	0.4
Ur. lowii	1	0	0	0	1	2	1	7	1	0	0	0	13	0.2
Ae. atlanticus/tormentor	0	0	0	0	0	1	0	6	0	0	0	0	7	0.1
Ae. albopictus	0	0	0	0	0	0	3	1	2	0	0	0	6	0.1
Unidentifiable	0	0	0	0	1	0	3	10	17	0	0	0	31	0.5
Minor species ($n < 5$)	0	1	2	1	5	7	5	2	1	0	0	0	24	0.4
Total	236	208	219	229	595	888	1122	1057	451	337	551	200	6093	100

shelter per month. Host density is likely to be higher during spring and autumn during bird migrations but was not measured or incorporated into the vectorial capacity calculation. To calculate *a,* the proportion of *Cs. melanura* feeding on competent (avian) hosts was divided by the length of the gonotrophic cycle (g_c) (Rubio-Palis, 1994). The gonotrophic cycle length of laboratory reared *Cs. melanura* varies from 23.2 days at 10°C to 4.5 days at 28°C (Mahmood and Crans, 1997). Estimation of g_c was done using the equation $g_c = 1/V$ where $V = \frac{t-6.4}{95.87}$ and t is temperature in Celsius (Mahmood and Crans, 1997). The values of t for the study period were the mean daily temperature taken from historical weather data from the National Climatic Data Center weather stations at Gilbert Airport in Winter Garden, FL, for the samples from Polk County and at Executive Airport in Orlando, FL, for the samples from Orange County (National Climatic Data Center, 2019). The value of p was estimated with $p = \sqrt[g_c]{M}$, where M is equal to the proportion parous and g_c is the length of the gonotrophic cycle (Almeida et al., 2005). The variable M is the proportion of parous mosquitoes of the females with a positive parity determination. The mosquitoes with undetermined parity were not included in the C calculation and included only gravid females, females with a bloodmeal >2 days old, and females with damaged ovaries. EIP (n) was estimated for our study using data from published laboratory studies of vector competence of *Cs. melanura* under different temperatures. The Excel TREND function was used to define a relationship between competence (n) and temperature values along an extrapolated trend line, using ambient temperatures at each of the six field sites in Florida. The n of EEEV in *Cs. melanura* (50% transmission) was found to be 3 days when kept at 28°C (Scott and Burrage, 1984) and 5 days when held at 24°C (Weaver et al., 1990).

When these calculations were complete, the determined value of C for each period was compared to the mean monthly number of EEEV equine cases in Florida from the last decade and the number of cases from 2018 (Florida Department of Health, 2019). Linear regression analyses were used to determine the relationship of C values and epizootic EEEV transmission in R (R Core Team, 2014).

RESULTS

Mosquito Abundance and Host Use

During the 2018 sampling year, 6,093 female mosquitoes, comprised of 24 different species, were collected from six sites in Orange and Polk counties, Florida (**Table 2**). The most abundant mosquito species were *Cx. erraticus* (n = 2,720)

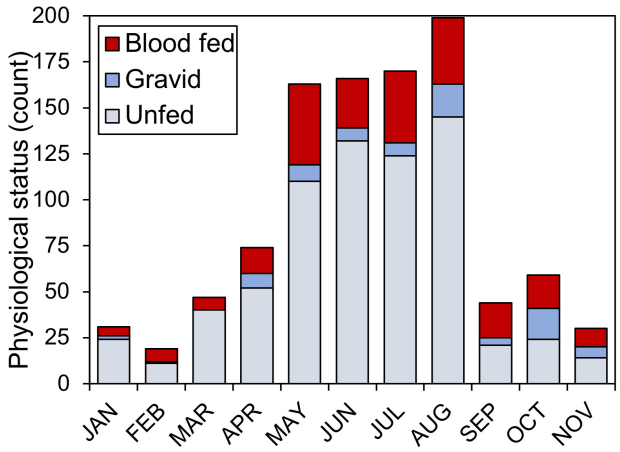

FIGURE 2 | Physiological status of *Cs. melanura* females from Central Florida. Females were sampled using resting shelters at 12 total sites in Orange and Polk County, FL, United States, in 2018.

and *Cs. melanura* (n = 1,036). Unfed females constituted the majority (67.5–85.1%) of total females collected throughout the spring and summer (**Figure 2**). In autumn, however, blood-fed and gravid females (combined) accounted for more than half (53.3–59.3%) of total females (**Figure 2**). Abundance of *Cs. melanura* showed a seasonal pattern of increasing numbers in summer months, which was three times larger than winter months (**Figure 3A**). The density of *Cs. melanura* peaked in July, with a mean of 1.29 females captured per shelter (n = 170, **Table 3**). In August, the density remained high but a large decrease in density to 0.29 occurred in September. The smallest density occurred in February with 0.18 females per shelter. Abundance and density of *Cs. melanura* were significantly correlated (R^2 = 0.86, df = 13, $p < 0.0001$).

Host use by *Cs. melanura* in 2018 was previously determined by bloodmeal analysis and resulted in 105 avian (49.3%), 74 reptilian (34.7%), and 34 mammalian (16.0%) bloodmeals. Avian host use varied by month (**Figure 3B**), from a low of 31% of total bloodmeals to a high of 86% of total bloodmeals. Bird bloodmeals were most common every month except April, May, and June, when reptile bloodmeals were frequent (**Figure 3B**). In September, only three bloodmeals were identified, which corresponded to a period of very low *Cs. melanura* abundance (**Figure 3A**). Avian species identified from bloodmeals consisted of 9 orders (**Figure 3B**) and 22 families of birds. The avian hosts most fed upon were northern cardinal, red-eyed vireo, mourning dove, Carolina wren, barred owl, pine warbler, and house wren. The largest number of bloodmeals came from songbirds (Order Passeriformes) with 79 positive identifications (75% of avian hosts), with other avian meals derived from Columbiformes (n = 10), Strigiformes (n = 7), Galliformes (n = 4) and single meals (n = 1) from four other avian orders (Charadriiformes, Coraciiformes, Cuculiformes, Gruiformes and Pelecaniformes).

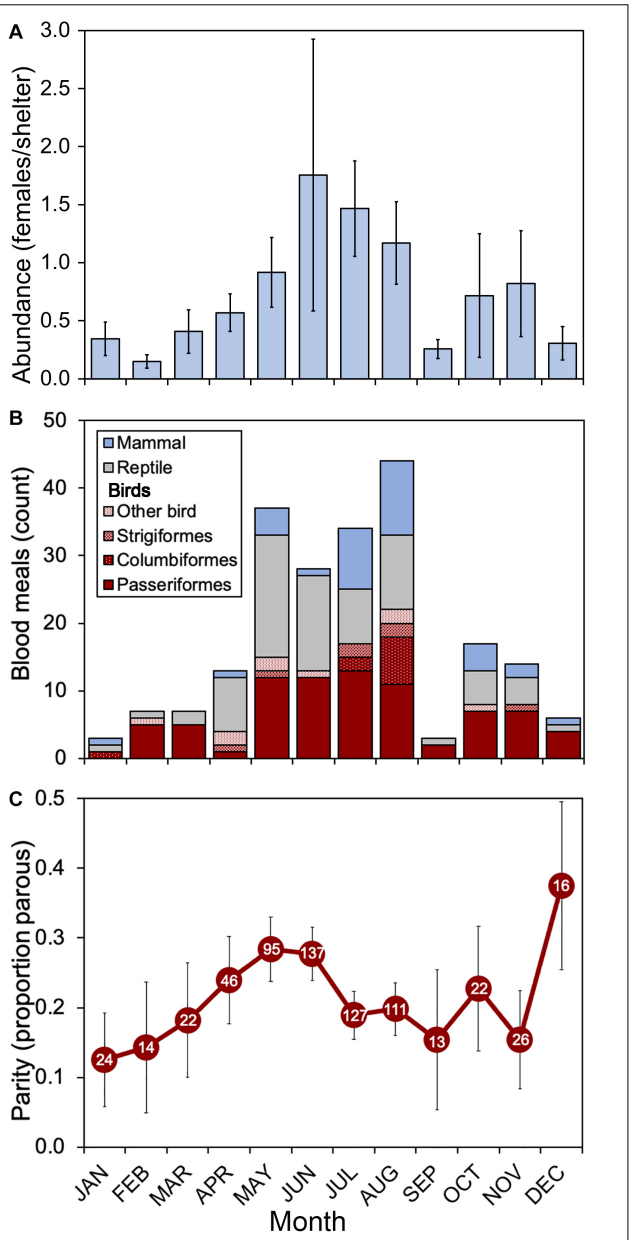

FIGURE 3 | Seasonal patterns of abundance, host use and parity of *Cs. melanura* in Central Florida. **(A)** Abundance represents the mean number of female mosquitoes per artificial resting shelter. Bars indicate standard errors of means. **(B)** Vertebrate hosts (class level) determined by PCR and Sanger sequencing of blood-engorged females. "Other birds" includes Galliformes (n = 4) and one each of Charadriiformes, Coraciiformes, Cuculiformes, Gruiformes, and Pelecaniformes. **(C)** Parity represents the proportion of females with tracheal skeins. Sample sizes appear within markers. Bars represent SE. *Culiseta melanura* collected from Orange and Polk County, FL, United States, in 2018.

Parity, Gonotrophic Cycle, Extrinsic Incubation, and Survival

Parity was determined for 653 *Cs. melanura* females of 1,036 collected (63.0%). The proportion of parous females ranged from

TABLE 3 | Vectorial capacity components of *Cs. melanura* for EEEV. Mosquitoes were collected in Orange and Polk County, FL, in 2018.

Month	*m*	*H*	g_c	*t*	a^2	*M*	*p*	*n*	*C*
Jan	0.34	0.33	11.44	14.8	0.00	0.13	0.83	9.61	0.0003
Feb	0.18	0.86	5.95	22.5	0.02	0.14	0.72	5.75	0.0017
Mar	0.38	0.71	7.51	19.2	0.01	0.18	0.80	7.42	0.0028
Apr	0.59	0.31	5.64	23.4	0.00	0.24	0.78	5.31	0.0018
May	0.88	0.41	5.05	25.4	0.01	0.28	0.78	4.31	0.0078
Jun	1.06	0.46	4.38	28.3	0.01	0.28	0.75	2.85	0.0177
Jul	1.29	0.50	4.32	28.6	0.01	0.19	0.68	2.71	0.0160
Aug	1.28	0.50	4.25	28.9	0.01	0.20	0.68	2.53	0.0177
Sep	0.29	0.67	4.18	29.4	0.03	0.15	0.64	2.32	0.0058
Oct	0.42	0.47	4.77	26.5	0.01	0.23	0.73	3.75	0.0041
Nov	0.82	0.57	6.29	21.6	0.01	0.15	0.74	6.18	0.0036
Dec	0.38	0.67	8.32	17.9	0.01	0.38	0.89	8.04	0.0081

m, density of mosquito vector (mean no. of mosquitoes/shelter); H, proportion of avian hosts from Cs. melanura; g_c, gonotrophic cycle = 1/V, V = (t - 6.4)/95.87; t, mean daily temperature of Polk and Orange County in Celsius; a = H/g_c, the biting rate of the vector; M, proportion of parous mosquitoes; p= $\sqrt[g_c]{M}$, vector's daily probability of survival; n, extrinsic incubation period; C, vectorial capacity.

0.13 in January to 0.38 in December of 2018 (**Figure 3C**). From January to May, the proportion of parous females increased gradually to reach relatively high levels (0.28) in May and June, followed by a substantial decrease in July and August (0.19–0.20). From September through December, low numbers of females (n = 13–26) were available for parity determination, resulting in fluctuating parity for the remainder of the year. Of 257 blood engorged *Cs. melanura* females from Orange and Polk County, only 65 (25.3%) were successfully determined for parity, due to maturation of egg follicles. Of these, 7 (10.8%) were found to be parous.

Incorporating the mean monthly temperatures from the study sites enabled the calculation of n and g_c, the latter of which is also used to estimate a and p. The lowest mean temperature of Orange and Polk counties was recorded at 14.8°C in January, and the highest mean temperature of 29.4°C was recorded in September. The relationships between temperature and each of the parameter estimates for n, g_c, and p are shown in **Figure 4**. The monthly temperatures in Orange and Polk County were similar, with a mean difference of 0.3°C. At these temperatures, the g_c calculated for *Cs. melanura* ranged from 4.2 to 11.4 days in length (**Table 3**). The estimated n for *Cs. melanura* ranged from 2.3 days in September to 9.6 days in January (mean = 5.1 days). Daily probability of survival varied modestly over the study period, compared to other variables (**Table 3**), ranging from a low of 0.63 in September to a high of 0.89 in December.

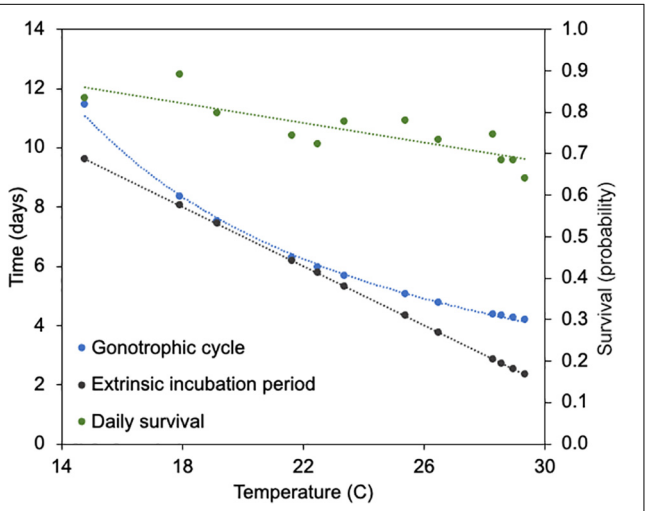

FIGURE 4 | Relationship between temperature, gonotrophic cycle length, extrinsic incubation period for eastern equine encephalitis virus and daily survival of *Cs. melanura* from Central Florida. Temperature represents average monthly temperature in Orange and Polk County, FL, United States, in 2018.

EEEV Transmission in Florida

A total of 55 equine cases (**Figure 5**; FDOH data) was recorded in Florida in 2018, which was somewhat higher than the 10-year average of 41.4 cases per year. Epizootic transmission of EEEV was particularly high in winter and spring of 2018 (January to May) with more than twice as many cases as the annual mean for the same period over the past ten years. The numbers of equine cases declined sharply after June of 2018, with just four cases in August, one case each in September and October, and no cases reported in November or December. The 10-year average shows low numbers of cases (0.7–1.9) from September through April (8 consecutive months), with the bulk of cases (10.8–11.5/month) occurring in June and July. In Orange and Polk counties in 2018, 2 horse cases and 13 sentinel chicken seroconversions to EEEV were reported. In the last decade, Orange and Polk counties have reported 2 and 12 total EEEV horse cases, respectively.

Vectorial Capacity

Using data from mosquito abundance, parity, bloodmeal source, and temperature, vectorial capacity (C) values of *Cs. melanura* for EEEV were calculated for the months of 2018 (**Table 3**). The m variable is traditionally calculated as the number of vectors per host. Because the density variable used in this study is the number of mosquitoes per shelter, the m is

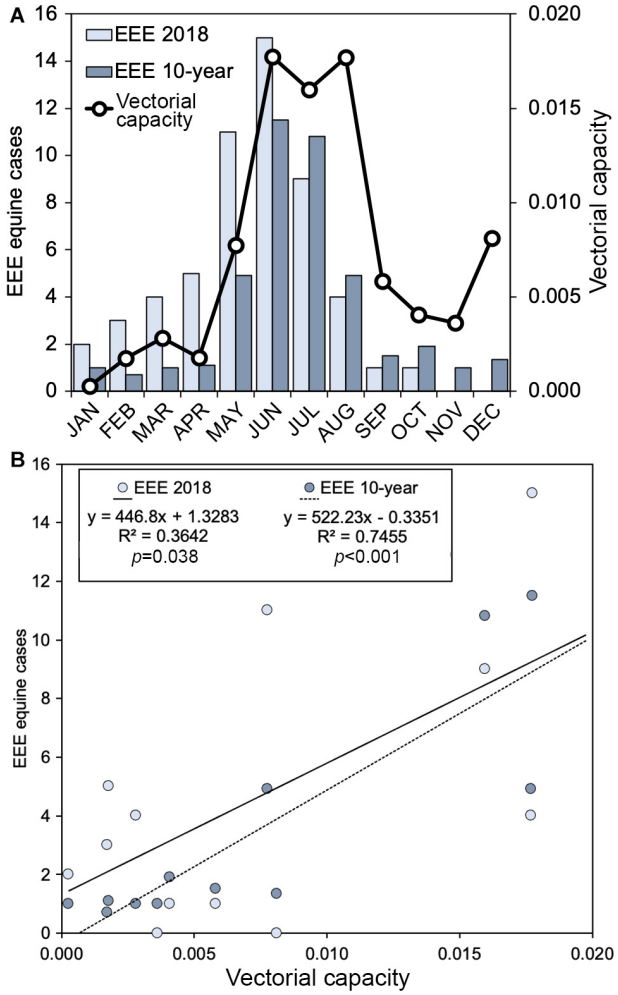

FIGURE 5 | Relationship of vectorial capacity of *Cs. melanura* for EEEV and equine cases from Florida. **(A)** Monthly values of vectorial capacity (black line) and EEEV cases in equines (bars); **(B)** Scatterplot of vectorial capacity and EEEEV cases in equines with best fit linear regression lines. Values of the vectorial capacity (*C*) were calculated using *Cs. melanura* collected from two Central Florida counties. EEEV cases are monthly aggregates for the year of *Cs. melanura* sampling (2018) or monthly averages over the prior decade.

likely underestimated and *C* values are relatively smaller than previous studies (Garrett-Jones and Shidrawi, 1969). Estimated values of *C* were lowest (0.0003) in January and remained low throughout winter and early spring (**Figure 5A**). *C* increased in May, reaching highest values (0.0160–0.0177) in June, July and August (**Figure 5A**). In September, C decreased substantially and continued to lower until December, when a small resurgence was observed (**Figure 5A**).

Equine cases of EEEV generally reflected seasonal changes in estimated values of *C* for *Cs. melanura*, with higher numbers of EEEV cases observed during months with greater *C* (**Figure 5A**). Linear regression revealed a significant positive relationship between *C* and Equine EEEV cases for the year 2018 ($R^2 = 0.36$, df = 10, $p = 0.038$) and the mean monthly number of cases from the prior decade ($R^2 = 0.75$, df = 10, $p < 0.001$) (**Figure 5B**). During 2018, 55 positive EEEV cases were reported from equids as well as five emu flocks. Epizootic transmission from January to May was more than twice as high in 2018 than the mean of the last decade.

DISCUSSION

Mosquito Abundance and Host Use

Culiseta melanura density in Central Florida was highest in the late spring and summer (June–August) which contrasts with results from Edman et al. (1972) and Blosser et al. (2017) where decreases of abundance in late spring were reported. These contrasting patterns suggest that phenology of this important vector species is plastic and likely varies from year to year. Studies from the northeastern United States have shown that hydrological conditions drive *Cs. melanura* abundance (Skaff et al., 2017), but that temperature shapes the rate of larval development (Mahmood and Crans, 1997). Interestingly, high ambient temperature during larval development caused a dramatic decrease in adult emergence success, from 59.6% emergence at 28°C, to 1.0% emergence at 32°C, in laboratory studies (Mahmood and Crans, 1997). September was the warmest month in 2018 with a mean daily temperature of 29.4°C and a mean daily high temperature of 33.9°C. The high temperatures observed in September corresponded with very low *Cs. melanura* abundance (**Figure 3A**) during a period of otherwise relatively high mosquito density. Importantly, other commonly collected mosquitoes, including EEEV bridge vectors *Cx. erraticus, Cx. nigripalpus,* and *Cq. perturbans,* did not undergo similar decreases in abundance (**Table 2**), suggesting that high temperatures adversely affect *Cs. melanura* uniquely and may have cascading effects on EEEV, as high *Cs. melanura* abundance is significantly correlated with EEEV transmission (Skaff et al., 2017). Our finding that the increase in *Cs. melanura* abundance observed in the spring (**Figure 3A**) closely parallels the increase in EEEV cases in horses that occurred in May 2018 supports a relationship between *Cs. melanura* abundance and EEEV transmission.

Our finding that the biting rate by *Cs. melanura* varies throughout the year (**Table 3**) has important implications for understanding how *C* changes seasonally, and perhaps how vector abundance influences host use. Morris et al. (1980) found that non-avian feedings by *Cs. melanura* occurred simultaneously with high populations, irrespective of distance from the breeding swamp. Similarly, we observed that during the period of peak *Cs. melanura* abundance (May–August), feedings upon reptiles and mammals were at their greatest (**Figures 3A,B**). It is unclear how abundance and host use are related; however, increasing numbers of attacking mosquitoes (*Cx. nigripalpus*) have been shown to result in increasing levels of defensive behaviors exhibited by birds and a corresponding reduction in the proportions of successfully feeding female mosquitoes (Edman et al., 1974). Large numbers of questing *Cs. melanura* females could be thwarted from successfully obtaining a bloodmeal from preferred avian hosts and shift to biting available mammals or reptiles in

the environment. Under this scenario, *Cs. melanura* could serve as an epizootic vector of EEEV.

Parity, Gonotrophic Cycle, Extrinsic Incubation, and Survival

Our finding that parity rates varied throughout the year is expected, given the natural cycles of emergence, blood-feeding and oviposition. The relatively high parity rates observed in spring (**Figure 3C**), followed by a decrease in summer is reflective of patterns observed in New York (Morris et al., 1980). However, much higher parity rates (>75% in some samples) were observed in New York, compared to Florida (generally <30%). Parity does not directly factor into the vectorial capacity equation, but it contributes to estimates of the age of the vector population, an important determinant of the potential infectiousness of the population. Increasing parity indicates an older vector population with prior host contact, which is thought to correspond with higher infection rates in the vector and host (Lines et al., 1991).

Gonotrophic cycle length and the extrinsic incubation period of *Cs. melanura* were not measured directly but were estimated using ambient mean temperature at area weather stations, and data from published studies. In general, both g_c and n have negative relationships with temperature, with longer gonotrophic cycles and extrinsic incubation periods at lower temperatures. Interestingly, because of differences in the shape of the relationships of these two variables and temperature (linear versus non-linear) g_c and n are virtually identical at moderate temperatures (18–22°C) but differ by nearly 2 days at both upper and lower temperature extremes (**Figure 4**). The effect of this discordance between g_c and n on transmission of EEEV is not known, yet could have dampening effect on transmission at temperature extremes. Females that have completed a gonotrophic cycle, but with incomplete extrinsic incubation periods would not be "ready" to transmit virus upon the next bite. A better understanding of the time between oviposition and blood-feeding by *Cs. melanura* would help to determine whether this temperature-variation discordance is important in nature.

Daily survival probability (p), as estimated using parity and length of the gonotrophic cycle (Almeida et al., 2005) also has a negative relationship with temperature, which is not surprising given its derivation. The lowest estimated daily survival was observed in September (0.64) when very high temperatures were observed, a relationship which was supported by very low abundances during that month (**Figure 5A**). Estimated values of p in this study align with findings of Chamberlain and Sudia (1955) that mosquitoes which were incubated at lower temperatures survived longer.

EEEV Transmission in Florida

The epizootic transmission of EEEV in Florida can fluctuate greatly from year to year and the number of equine cases was greater than average in 2018, with a total of 55 equine cases (**Figure 5A**). The phenology of epizootic transmission in 2018 was fairly representative of the 10-year average with one notable exception. The peak in equine cases in 2018 occurred earlier in the year, with greater than normal cases in May, and fewer than normal cases in July (**Figure 5A**). The reason behind the early peak in EEEV is unclear. However, our data from Central Florida show that during May *Cs. melanura* had relatively high parity (c.f. **Figure 3C**) and the species took a relatively large fraction of bloodmeals from non-avian hosts during this period (**Figure 3B**). Surprisingly, *Cs. melanura* abundance was not particularly high in May 2018, although the population had been increasing steadily since February.

Vectorial Capacity

Our study represents an important step in demonstrating links between seasonal variation in vectorial capacity and epizootic spillover of a zoonotic vector-borne pathogen and indicates that concepts developed to describe relationship between entomologic factors and anthroponotic vector-borne disease also apply to vector-borne zoonoses. Interestingly, vectorial capacity of *Cs. melanura* explained a larger fraction of the variation in historical (10-year average) EEEV epizootic cases (75%) than that of the concurrent year (36%). Notable departures from the relationship between epizootic EEEV cases and C occurred in August and December, when C was estimated to be relatively high, but the numbers of equine cases were relatively low (**Figure 5A**). The disparity in December is likely due to low sample sizes of mosquitoes (<20) used to calculate relative avian host use (66.6%) and proportion of parous females (38%) in that month (**Figure 3A**), possibly artificially inflating the estimates of C for December. Conversely, C values in August were based upon robust numbers of females used to determine host use (n = 44) and parity (n = 111). The low numbers of equine cases in August is therefore perplexing. Bridge vector species, such as *Cx. erraticus* and *Cq. perturbans*, were also common during August, and would have presumably contributed to EEEV transmission, further confounding the low numbers of equine cases. Vaccination rates in horses could increase in response to the outbreak, lowering the numbers of cases reported. Additional work should be done to understand how C of a zoonotic pathogen vector might fundamentally differ from that of vectors of anthroponotic pathogens.

This work has several limitations, many of which are the result of difficulties studying field populations of mosquitoes that feed principally on wild vertebrate hosts. Using the number of females per resting shelter is an indirect method of estimating density, in contrast to measuring incidence of bites per host per day. Previously, measuring density indirectly resulted in three to four times lower density of *Anopheles* than using direct measurements (Garrett-Jones, 1964a). If density of *Cs. melanura* was measured directly using landing rates on birds, density values would likely be higher and better reflect the actual contact rates between vectors and amplifying hosts. One assumption of vectorial capacity is that the pathogen is transmitted by only one species of vector to one type of host (Dye, 1986). The a for our calculation assumes that all avian hosts are equally competent. But avian EEEV competence has not been measured in all avian hosts found in this study. The host competence of an avian species may differ depending on family, size, recent

exposure to an exotic species, or could differ between individuals (Komar et al., 1999). The parity rates of winter and autumn were based upon low sample sizes (<30 per month) which may have affected the accuracy of parity estimates, and variables that were calculated from it. In addition to environmental factors such as predation, infection with EEEV may decrease the survival rate of mosquitoes (Scott and Lorenz, 1998; Moncayo et al., 2000; Kramer and Ciota, 2015). Importantly, few data are available to accurately estimate the relationship between temperature and EIP for *Cs. melanura*. These relationships are likely population/strain dependent and correlated with not just mean, but also min/max temperatures. Future experimental studies with the population used here over a range of temperatures regimes would be invaluable to parameterize models.

CONCLUSION

In conclusion, a positive association between monthly EEEV infections in equids and vectorial capacity of the vector was observed, supporting the hypothesis that enzootic transmission by *Cs. melanura* has a large effect on epizootic outbreaks. This study reveals how aspects of the biology of an important enzootic arbovirus vector, such as abundance, host use and longevity, change throughout the year, and how these changes possibly drive to spillover of a human pathogen. This information may aid in the prevention, monitoring, and control of arboviruses, including EEEV, by providing detailed information on the relationships between key biological variables of the vector and seasonal patterns of pathogen transmission.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

RW, CB, and NB-C carried out the methodology. RW and NB-C analyzed the data. All authors contributed to the study design and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Ecology and Evolution

REVIEW
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Enhanced Attraction of Arthropod Vectors to Infected Vertebrates: A Review of Empirical Evidence

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A large diversity of parasites manipulates their hosts in various ways to complete their own life cycle. Enhancing the attractiveness of their host to vectors has been suggested as a strategy allowing vector-borne parasites to increase their transmission. Indeed, a higher attraction of hematophagous, arthropod vectors to infected vertebrates compared to uninfected individuals has been found in many systems (e.g., *Trypanosoma*-tsetse flies, *Leishmania*-sand flies, *Borrelia*-ticks) but was most often verified in the *Plasmodium*-mosquitoes model. However, a number of studies found no difference in attractiveness, or a higher attractiveness of uninfected hosts. In this review, we present studies reporting a comparison of the attractiveness and/or the biting rate of infected and uninfected vertebrates. We then discuss several biological factors and experimental design aspects that can explain discrepancies between studies. Finally, we stress the importance of investigating the mechanisms of parasite-induced increased attractiveness of infected hosts to conclude that such observations are cases of adaptive manipulation.

Keywords: attractiveness, extended phenotype, hematophagous arthropods, host-choice, host-seeking, manipulation, vector-borne parasites

INTRODUCTION

Host manipulation by parasites has been fascinating parasitologists for decades (Thomas et al., 2005), especially when this manipulation results in dramatic changes in host's physiology (Cordaux et al., 2011; Kageyama et al., 2012; Fayard et al., 2020), morphology (Bakker et al., 1997; Yanoviak et al., 2008; Fayard et al., 2020) or behavior (Berdoy et al., 2000; Thomas et al., 2002; Weinersmith, 2019; Fayard et al., 2020). This host exploitation strategy, described in many phylogenetically distant host-parasite systems, usually involves phenotypic changes in the infected host (extended phenotype, Dawkins, 1982). These alterations can be mediated by direct or indirect mechanisms (Thomas et al., 2005). Parasites can produce, store and release active manipulation factors that act directly on host tissues such as the nervous system or muscles. These manipulative factors are part of a complex mixture of molecules called the secretome (Adamo, 2013; Biron and Loxdale, 2013; Berger and Aubin-Horth, 2020). On the other hand, the mere physical presence of parasites in particular tissues or simply the pathological by-products of infection can influence the development and/or the metabolism of the host, secondarily leading to an alteration of its phenotype (Dingemanse et al., 2009; Thomas et al., 2012).

Vector-borne parasites have evolved different angles of attack to increase their own transmission by manipulating their vectors and hosts (Lefèvre and Thomas, 2008). Indeed, infected vectors

show alterations in host-seeking and feeding behaviors, fecundity and longevity [reviewed in Hurd (2003), Cator et al. (2012), Murdock et al. (2017), Stanczyk et al. (2017)], microhabitat preference (Fialho and Schall, 1995) and selection of host species (Vantaux et al., 2018 but see Nguyen et al., 2017; Vogels et al., 2017), that all seem beneficial for the transmission of the parasites they harbor. For example, impaired ingestion forcing the vector to bite several times to achieve a complete blood-meal has been reported in *Trypanosoma*-infected tsetse flies and kissing bugs, *Leishmania*-infected sand-flies, *Plasmodium*-infected mosquitoes and plagued fleas [reviewed in Hurd (2003)]. Although this example can be considered as a simple pathological consequence of the infection with coincidental benefit for transmission, the convergent appearance of this phenomenon was hypothesized to reflect adaptive manipulation (Poulin, 1995).

In addition, parasites seem able to make their vertebrate hosts more attractive to their vectors. From this point of view, the vectors can be considered as "exploited", because their host-seeking behavior is indirectly influenced by an alteration of the infected vertebrate's phenotype. Although increased attractiveness of infected hosts has been reported in various host/parasite systems, many studies have obtained conflicting results. Here, we first propose a review of the current knowledge on this topic by gathering all the studies that have empirically investigated the impact of infection of vertebrate hosts on their attractiveness to arthropod vectors. We also take stock of current knowledge on the mechanisms potentially involved in attraction bias. Then, based on a review of the materials and methods used to study attraction bias, we seek to identify crucial aspects of the experimental designs that could explain the contradictory results frequently observed.

CURRENT KNOWLEDGE ON THE ATTRACTION OF ARTHROPOD VECTORS TO INFECTED VERTEBRATES

Integration of Vector Feeding Bias in Epidemiological Models

For over a century, the epidemiology of vector-borne diseases was addressed using mathematical models describing the life cycle of pathogens in vertebrate and invertebrate (vector) hosts. Although the early models included several strong assumptions, which greatly simplified the complex host-vector interactions (Ross, 1916; Macdonald, 1956, 1957; Bailey, 1975), they highlighted the impact of different host and vector life-cycle parameters on parasite transmission and, in particular, they pointed out that the vector's biting rate could have major consequences on transmission dynamics. Then, epidemiological models became more and more complex by integrating new parameters such as vector feeding bias. Whereas early models assumed that vectors chose hosts and fed on them randomly, and therefore independently of the absence or presence of infection, empirical data acquired in the early 1980s have overturned this paradigm. It was observed that mosquitoes preferentially fed on malaria-infected rather than uninfected mice (Day and Edman, 1983; Day et al., 1983). Based on this observation, Kingslover was the first to develop and analyze a model for the dynamics of parasite transmission that incorporates nonrandom feeding behavior by the vector (Kingsolver, 1987). He demonstrated that increasing the preference of vectors for infected hosts leads to an easier maintenance of a stable infection, compared to the model where vectors feed on their host in a random manner. Subsequent studies confirmed this result and showed that the preference of vectors for infected hosts can strongly reinforce the transmission of the parasite at the beginning of the epidemic (McElhany et al., 1995; Hosack et al., 2008; Sisterson, 2008; Chamchod and Britton, 2011; Zeilinger and Daugherty, 2014; Gandon, 2018). Parasite that are able to manipulate their vertebrate hosts to make them more attractive to vectors should be therefore favored by natural selection. However, an extreme preference of vectors for infected hosts may also limit or stop the transmission of parasites (Kingsolver, 1987; McElhany et al., 1995; Sisterson, 2008; Zeilinger and Daugherty, 2014; Gandon, 2018). Indeed, when levels of host infection in a population are very high, vector feeding bias results in most of the bites occurring on already infected hosts. Natural selection should then favor parasites that induce either an intermediate level of attractiveness or a conditional change in the behavior of the vector depending on its own infection status (see Gandon, 2018).

Empirical Evidence of Parasite Manipulation of Host Attractiveness

We gathered the studies that investigated the manipulation of host attractiveness to vector-borne parasites, by searching Google Scholar and Web of Science databases with combinations of the terms "vector" and "parasite" or "virus" or "bacteria" and "attraction" or "attractiveness" or "choice" or "host selection" or "olfactometer" or "preference." All studies that report an observational or experimental comparison of the numbers of arthropod vectors that were attracted to and/or bit infected and uninfected vertebrates were selected. We also included experiments using only the odor of the vertebrates. These studies are summarized in **Table 1** (Culicidae) and **Table 2** (other invertebrate vectors). The majority of studies focused on Culicidae vectors and *Plasmodium* infection, finding in most cases, but not always, that mosquitoes are more attracted to *Plasmodium*-infected hosts (12/18; **Table 1**, **Figure 1**).

Only two studies looked for a biased attractiveness of hosts infected with filarial worms and found none (Burkot et al., 1989; Kruppa and Burchard, 1999). More tsetse flies (*Glossina pallidipes*) bit the Orma Boran oxen infected with *Trypanosoma congolense*, but not those infected by *Trypanosoma vivax* (Baylis and Nambiro, 1993; Baylis and Mbwabi, 1995), and only hybrids of the *Triatoma dimidiata* complex preferred *Trypanosoma cruzi*-infected mice over uninfected ones (Ramirez-Sierra and Dumonteil, 2016). *Leishmania infantum* made their hamster hosts more attractive to *Lutzomyia longipalpis* (O'Shea et al., 2002; Nevatte et al., 2017), but *Leishmania braziliensis* did not enhance attractiveness of mice for *Nyssomyia neivai* (da Rocha Silva et al., 2019). The probability that a bat fly chose a host was negatively

TABLE 1 | Summary of studies comparing attractiveness of infected and uninfected hosts to mosquitoes.

Host	Vector	Parasite	Most attractive hosts	Method	Compared treatments	Infection	Setup	Vector infection status	Defensive behavior, activity	Comment	Reference	Citations
Rana clamitans	*Culex pipiens*	*Hepatozoon clamatae*	Infected	Comparison of biting rates	Infected vs uninfected individuals	Natural	Lab	Uninfected	Possible		Ferguson et al., 2013	8
	Culex territans		None	Comparison of biting rates	Infected vs uninfected individuals	Experimental						
			Infected	Choice, biting	Infected vs uninfected individuals	Natural						
			None	Choice, probing	Infected vs uninfected individuals	Experimental						
Thamonophis sirtalis	*Culex pipiens*	*Hepatozoon sipedon*	Infected	Choice, biting	Infected vs uninfected individuals	Natural						
Homo sapiens	*Anopheles darlingi*	*Plasmodium vivax*	Infected	Comparison of attraction rates in olfactometer (individuals)	During infection vs after anti-parasite treatment	Natural	Lab	Uninfected	Impossible	Only gametocyte carriers were more attractive	Batista et al., 2014	25
			Infected	Comparison of attraction rates in olfactometer (individuals)	Infected vs uninfected individuals							
Homo sapiens	*Anopheles farauti*	*Plasmodium spp.*	None	Comparison of biting rates	Infected vs uninfected individuals	Natural	Wild	Uninfected	Possible but unlikely (sleeping)		Burkot et al., 1989	23
	Anopheles punctulatus	*Plasmodium spp.*	Uninfected	Choice, biting	Infected vs uninfected individuals							
		Wuchereria bancrofti	None	Choice, biting	Infected vs uninfected individuals							
Homo sapiens	*Anopheles coluzzii*	*Plasmodium falciparum*	None	Comparison of attraction rates in olfactometer (skin odor)	Infected vs uninfected individuals	Experimental	Lab	Uninfected	Impossible	Effect was present in one of two experimental blocks	de Boer et al., 2017	15
			Uninfected		Before infection vs during infection							
			Uninfected		During infection vs after anti-parasite treatment							
			None		Infected vs uninfected individuals							
			None		Before infection vs during infection							
			None		During infection vs after anti-parasite treatment							
Homo sapiens	*Anopheles gambiae*	*Plasmodium falciparum*	Infected	Comparison of attraction rates in olfactometer (individuals)	During infection vs after anti-parasite treatment	Natural	Lab	Uninfected	Impossible	Only gametocytes carriers were more attractive	Lacroix et al., 2005	171

(Continued)

TABLE 1 | Continued

Host	Vector	Parasite	Most attractive hosts	Method	Compared treatments	Infection	Setup	Vector infection status	Defensive behavior, activity	Comment	Reference	Citations
			Infected	Dual-choice olfactometer (individuals)	Infected vs uninfected individuals							
Homo sapiens	*Anopheles gambiae*	*Plasmodium falciparum*	None	Dual-choice olfactometer (individuals)	NA	Natural	Lab	Unknown	Impossible	Only two men compared, based on their symptoms	Mukabana et al., 2007	5
Homo sapiens	*Anopheles gambiae*	*Plasmodium falciparum*	Infected	Comparison of attraction rates in olfactometer (individuals)	During infection vs after anti-parasite treatment	Natural	Lab	Uninfected	Impossible	Only microscopic gametocyte carriers were more attractive	Busula et al., 2017a	19
			Infected	Comparison of attraction rates in olfactometer (individuals)	Infected vs uninfected individuals							
Homo sapiens	*Anopheles gambiae*	*Plasmodium falciparum*	Infected	Dual-choice olfactometer (skin odor)	During infection vs after anti-parasite treatment	Natural	Lab	Uninfected	Impossible	Both gametocytes carriers and asexual stages carriers were more attractive	Robinson et al., 2018	25
Mus musculus	*Aedes aegypti*	*Plasmodium yoelii*	Infected	Comparison of attraction rates	Before infection vs during infection	Experimental	Lab	Uninfected	Possible and monitored: infected less active		Coleman et al., 1988	9
			None	Comparison of biting rates	Infected vs uninfected individuals							
		Leishmania mexicana amazonensis	None	Comparison of attraction rates	Before infection vs during infection							
			None	Comparison of biting rates	Infected vs uninfected individuals							
		Plasmodium yoelii and Leishmania mexicana amazonensis	Infected	Comparison of attraction rates	Before infection vs during infection					Only during peak of infection were the hosts more attractive		
			Infected	Comparison of biting rates	Infected vs uninfected individuals							

(Continued)

TABLE 1 | Continued

Host	Vector	Parasite	Most attractive hosts	Method	Compared treatments	Infection	Setup	Vector infection status	Defensive behavior, activity	Comment	Reference	Citations
Mus musculus	*Aedes aegypti*	*Plasmodium bergei*	Infected	Comparison of biting rates	Infected vs uninfected individuals	Experimental	Lab	Uninfected	Possible and monitored: infected less defensive	Only just after the acute phase were the hosts more attractive; was linked with defensive behavior.	Day et al., 1983	48
			Infected	Choice, biting								
		Plasmodium chabaudi	Infected	Comparison of biting rates								
			Infected	Choice, biting								
Mus musculus	*Anopheles stephensi*	*Plasmodium chabaudi*	Infected	Dual-choice olfactometer (individuals)	Infected vs uninfected individuals	Experimental	Lab	Uninfected	Impossible	The hosts were more attractive right after the acute phase, when gametocytes were still present.	de Moraes et al., 2014	61
			Infected	Dual-choice olfactometer (volatiles)								
Mus musculus	*Anopheles stephensi*	*Plasmodium chabaudi*	Infected	Comparison of biting rates	Infected vs uninfected individuals	Experimental	Lab	Uninfected	Impossible		Ferguson et al., 2003	35
Serinus canaria	*Culex pipiens*	*Plasmodium relictum*	Infected	Comparison of biting rates	Before infection vs during infection	Experimental	Lab	Uninfected	Impossible	More attractive only during chronic stage	Cornet et al., 2013b	63
			Infected	Choice, biting	Infected vs uninfected individuals							
Serinus canaria	*Culex pipiens*	*Plasmodium relictum*	Infected	Choice, biting	Infected vs uninfected individuals	Experimental	Lab	Infected and uninfected	Impossible		Cornet et al., 2013a	29
Gallus gallus domesticus	*Aedes aegypti*	*Plasmodium gallinaceum*	Uninfected	Choice, biting	Infected vs uninfected individuals	Experimental	Lab	Uninfected	Impossible		Freier and Friedman, 1976	37
			Uninfected									
Parus major	*Culex pipiens*	*Plasmodium spp.*	Uninfected	Dual-choice olfactometer (individuals)	Infected vs uninfected individuals	Natural	Lab	Uninfected	Impossible		Lalubin et al., 2012	14

(Continued)

TABLE 1 | Continued

Host	Vector	Parasite	Most attractive hosts	Method	Compared treatments	Infection	Setup	Vector infection status	Defensive behavior, activity	Comment	Reference	Citations
Coloeus monedula	*Culex pipiens*	*Plasmodium spp.*	None	Comparison of biting rates	Infected vs uninfected individuals	Natural	Lab	Uninfected	Impossible		Gutiérrez-López et al., 2019	5
	Ochlerotatus caspius		None									
Passer domesticus	*Culex pipiens*		None									
	Ochlerotatus caspius		None									
Passer domesticus	*Culex pipiens*	*Plasmodium spp.*	Infected	Dual-choice olfactometer (headspace)	Infected vs uninfected individuals	Natural	Lab	Uninfected	Impossible		Díez-Fernández et al., 2020	0
			None	Dual-choice olfactometer (uropygial gland extract)								
Passer domesticus	*Culex pipiens*	*Plasmodium spp.*	Infected	Comparison of biting rates	During infection vs after anti-parasite treatment	Natural	Lab	Uninfected	Possible	The effect was correlated to the parasite density.	Yan et al., 2018	5
			None	Choice, biting	Infected vs uninfected individuals							
Passer domesticus	*Culex quinquefasciatus*	St. Louis encephalitis virus	None	Comparison of attraction rates in olfactometer (individuals)	Infected vs uninfected individuals	Experimental	Lab	Uninfected	Impossible		Scott et al., 1990	27
	Culex tarsalis	Western equine encephalomyelitis virus	None									
Gallus gallus domesticus	*Culex annulirotris*	Sindbis virus	Infected	NA	NA	Experimental	Wild	NA	NA		Mahon and Gibbs, 1982	25
			Infected	Choice, biting								
Ovis aries	*Culex pipiens*	Rift Valley fever virus	Infected	Choice, biting	Infected vs uninfected individuals	Experimental	Lab	Uninfected	Possible and monitored: infected less active		Turell et al., 1984	33
	Aedes taeniorhynchus		None									

Google Scholar and Web of Science databases were searched with combinations of the terms "vector" and "parasite" or "virus" or "bacteria" and "attraction" or "attractiveness" or "choice" or "host selection" or "olfactometer" or "preference." The numbers of citations were retrieved from Web of Science on May 5th, 2020. The "infection" column refers to the way the infection was initiated; "Natural" means the vertebrate host was already harboring an infection when caught, while "Experimental" means the experimenters performed the inoculation in the lab.

TABLE 2 | Summary of studies comparing attractiveness of infected and uninfected hosts to non-mosquito vectors.

Host	Vector	Parasite	Most attractive hosts	Method	Infection	Setup	Vector infection status	Defensive behavior, activity	Comment	Reference	Citations
Orma Boran oxen	*Glossina pallidipes*	*Trypanosoma congolense*	Infected	Comparison of biting rates	Natural	Wild	Unknown	Possible		Baylis and Nambiro, 1993	21
			None	Choice, no biting							
Orma Boran oxen	*Glossina pallidipes*	*Trypanosoma congolense*	Infected	Comparison of biting rates	Natural	Wild	Unknown	Possible and monitored: did not differ	The effect did not depend on parasite density.	Baylis and Mbwabi, 1995	16
		Trypanosoma vivax	None								
Mus musculus	*Triatoma dimidiata group 1*	*Trypanosoma cruzi*	None	Dual-choice olfactometer	Experimental	Lab	Uninfected	Impossible		Ramirez-Sierra and Dumonteil, 2016	5
	Triatoma dimidiata group 2		None								
	Triatoma dimidiata 1x2 hybrids		Infected								
Homo sapiens	*Simulium damnosum complex*	*Onchocerca vozvuzu*	None	Choice, biting	Natural	Wild	Unknown	Impossible		Kruppa and Burchard, 1999	5
Myodes glareolus	*Ixodes ricinus*	*Borellia afzelii*	Infected	Dual-choice olfactometer	Natural	Lab	Unknown	Impossible		van Duijvendijk *et al.,* 2016	4
			Infected	Choice, biting				Possible	Only males were more attractive.		
Myotis daubentonii	*Nycteribia kolenatii*	*Polychromophilus murinus*	Uninfected	Choice, biting	Natural	Lab	Unknown	Possible	The effect was correlated to the parasite density.	Witsenburg et al., 2015	9
Mesocricetus auratus	*Lutzomyia longipalpis*	*Leishmania infantum*	Infected	Dual-choice olfactometer	Experimental	Lab	Uninfected	Impossible		O'Shea et al., 2002	34
Mesocricetus auratus	*Lutzomyia longipalpis*	*Leishmania infantum*	Infected	Dual-choice olfactometer, choice between scents of individual before and during infection	Experimental	Lab	Uninfected	Impossible	The hosts were more attractive only during late stage of infection.	Nevatte et al., 2017	4
Mus musculus	*Nyssomyia neivai*	*Leishmania braziliensis*	None	Comparison of attraction rates in olfactometer	Experimental	Lab	Uninfected	Impossible		da Rocha Silva et al., 2019	0
			None	Choice, biting							

Google Scholar and Web of Science databases were searched with combinations of the terms "vector" and "parasite" or "virus" or "bacteria" and "attraction" or "attractiveness" or "choice" or "host selection" or "olfactometer" or "preference." The numbers of citations were retrieved from Web of Science on May 5th, 2020.

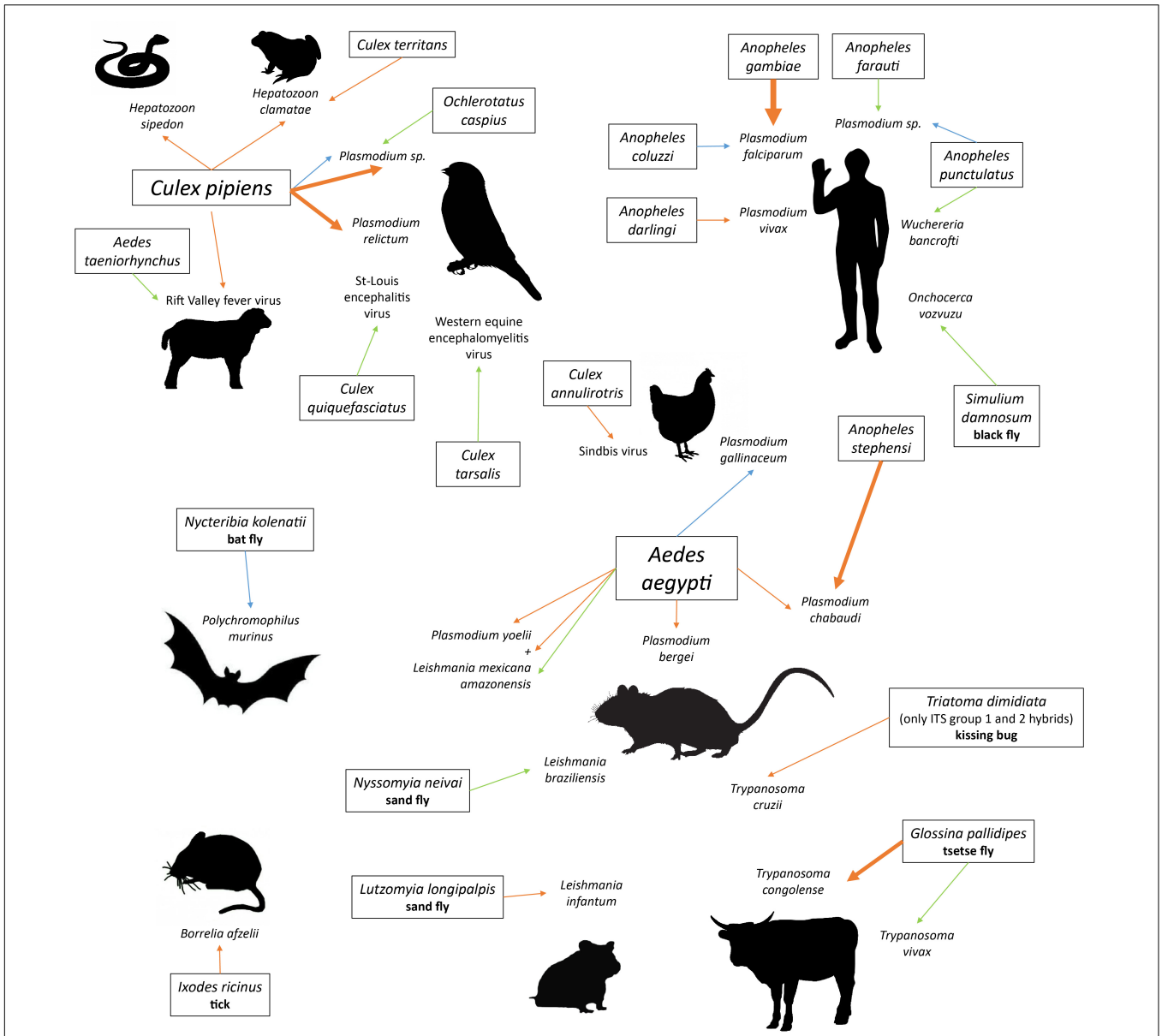

FIGURE 1 | Pairs of vector-parasite species that have been tested for differential attractiveness of infected versus uninfected hosts. Line width is proportional to the number of studies finding this result. Orange lines: higher attractiveness of infected hosts; blue lines: higher attractiveness of uninfected hosts; green lines: no preference found. Species names in boxes are vectors; latin names that are not followed by a vernacular name are mosquitoes (Culicidae).

correlated with *Polychromophilus murinus* parasitaemia in the Daubenton bat (*Myotis daubentonii*) (Witsenburg et al., 2015). The only non-insect study that we found showed that male bank voles infected with the bacteria *Borrelia afzellii* harbored more ticks (van Duijvendijk et al., 2016). Finally, a few early studies investigated the attractiveness of virus-infected animals and found mitigated results (Mahon and Gibbs, 1982; Turell et al., 1984; Scott et al., 1990).

Mechanisms of Enhanced Attraction

Vectors of endoparasites infecting vertebrates are usually hematophagous arthropods, mostly insects and ticks. Insects perceive olfactory molecules thanks to receptor neurons in sensilla, distributed on their antennae, maxillary palps and labia (Keil, 2012; Suh et al., 2014). Ticks sense volatile organic compounds (VOC) through a specific organ called Haller's organ located on their first pair of legs. Although not well known yet, it seems that chemoreceptors on Haller's organ are not similar to those on insects' sensilla (Carr et al., 2017). Molecules found to trigger responses in hematophagous arthropods mostly fall into the following categories: short chain carboxylic acids, aldehydes or low molecular weight nitrogenous compounds like ammonia (Syed, 2015). Indole and 1-octen-3-ol are often identified as attractant among the studied

insects, and carbon dioxide attracts all vectors (triatomines, cimicidae, ticks, tsetse flies, sand flies and mosquitoes; Syed, 2015). Carbon dioxide is also used in long-distance detection by host-seeking insects (Gillies, 1980). Mosquitoes can perceive very small variations of CO_2 concentration, like for instance *Aedes aegypti,* which has a detection threshold between 150 and 300 ppm and can perceive increments of 50 ppm (Grant et al., 1995). Cues used by vectors to select hosts are scattered in the air through the breath, the skin, and the excretions (Takken, 1991).

Recent studies have identified how VOC profiles differ between infected and uninfected hosts, and which compounds are involved in influencing attractiveness. Profiles of VOCs produced by humans and mice infected by *Plasmodium falciparum* and *Plasmodium chabaudi*, respectively, show they produce some compounds in higher quantities, while other compounds are suppressed in infected hosts (de Moraes et al., 2014; de Boer et al., 2017; Robinson et al., 2018). Some of these compounds (e.g., hexanoic acid, 2- and 3-methyl butanoic acid and tridecane in mice, heptanal, tetradecanoic acid, 3-methyl-1-butanol, and butan-1-amine in humans) have been shown to elicit a preference when added to the scent of a non-infected host (de Moraes et al., 2014; Robinson et al., 2018). Octanal, nonanal and decanal are among VOCs that showed significant variations between healthy dogs and dogs infected with *Leishmania infantum* (Magalhães-Junior et al., 2014), and were attractive to male sand fly vectors *Lutzomyia longipalpis* (Magalhães-Junior et al., 2019).

These attractive molecules can be produced by the infected host, by the parasite itself, or by the host microbiota. There is evidence that *Plasmodium* can synthesize terpenes, to which mosquitoes respond (Kelly et al., 2015), although they were not identified from infected mice or infected human skin and breath emissions (de Moraes et al., 2014; Berna et al., 2015; Robinson et al., 2018). Some malaria-associated VOCs are known to be produced by skin bacteria (de Moraes et al., 2014; de Boer et al., 2017). *Plasmodium* infection could cause a change in host microbial species composition, possibly mediated by immunological or endocrine systems (Busula et al., 2017b). Emami et al. (2017) have shown that a metabolite produced by *Plasmodium falciparum* ((E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; HMBPP) induces red blood cells to produce molecules involved in mosquito attraction (CO_2, aldehydes, and monoterpenes). The mechanisms involved in these potential parasite-induced phenotypic alteration (Poulin, 1995) are yet not all identified and several unknowns remain to be discovered (Busula et al., 2017b; Joice Cordy, 2020).

EXPERIMENTAL DESIGNS AND CONFLICTING RESULTS

Although most of the investigated vector-borne parasite genera are featured in at least one study finding that infected hosts attract more vectors or are more often bitten than uninfected hosts (**Figure 1**), it is difficult to determine whether this is a widespread phenomenon. One of the reasons for this is that the methods used to study vector attraction bias vary considerably from one study to another, making it impossible to draw an overall picture. In this second part, we have sought to identify aspects of experimental designs that may explain why, for the same parasite/vector pair, studies come to different conclusions.

Infection Stage and Parasite Density

A source of discrepancies between conclusions of studies might come from the parasite developmental stages in the timeframe of the experiments. Enhanced attractiveness of the infected vertebrate host should coincide with stages when the parasites are transmissible to vectors. This is confirmed by most studies checking for parasite developmental stages, which found that only *Plasmodium* gametocytes (sexual, transmissible stage) carriers were more attractive to *Anopheles* mosquitoes [Lacroix et al., 2005; Batista et al., 2014; de Moraes et al., 2014; Busula et al., 2017a, reviewed in Busula et al. (2017b), but see Robinson et al. (2018)]. Most studies finding a preference for the uninfected hosts did not verify whether the infected host exhibited transmissible stages (Freier and Friedman, 1976; Burkot et al., 1989; Lalubin et al., 2012). de Boer et al. (2017) did control but found no gametocytes in the tested individuals. The exception comes from Witsenburg et al. (2015) who found that bat flies that switched hosts ended up more often on the one with the lowest density of *Polychromophilus murinus* gametocytes, a malaria-like parasite. While attracting vectors when harboring transmissible stages seems like a selective advantage for parasites, it would bring no benefit during non-transmissible stages. Premature blood meals might even be deleterious, for example if they bring new parasites that may compete for resources, trigger a stronger immune response or kill their host before their own transmission.

Plasmodium infection dynamics in the blood is in general composed of two stages: the acute phase, a few days after infection, characterized by a peak in parasite density, followed by the chronic phase, with low parasite density (Garnham, 1966). Hosts harboring high parasite densities during the acute stage of *Plasmodium* infections were sometimes found to be less or equally attractive than uninfected hosts, and hosts became more attractive once the peak passed (Cornet et al., 2013b; de Moraes et al., 2014). Authors suggested that, as blood characteristics linked to immune response suppress infectivity (Mendis et al., 1987; Naotunne et al., 1993; Ramiro et al., 2011), it makes it useless for the parasite to attract vectors when the proportion of gametocytes relative to asexual stages is low, even if the absolute quantity of gametocytes may be higher than during other infection stages. In addition, the strong reduction in red blood cells counts following erythrocytic meronts rupture makes the blood less nutritious, and thus potentially less attractive for hematophagous arthropods. This hypothesis could be tested by examining the attraction of hematophagous species to individuals infected with parasites that they do not transmit. A recent study suggests that at least *Amblyomma* tick nymphs do not avoid birds infected with haemosporidian parasites (Fecchio et al., 2020). However, ectoparasites that are relatively less mobile, such as ticks, might

not be the best models to test this, as the cost of being too selective might be high, especially if the chances to encounter several potential hosts are low.

Conversely, Day et al. (1983) found that peak parasitaemia of *P. chabaudi* was associated with a peak of biting rate by *Aedes aegypti*, probably because infected mice exhibited fewer defensive behaviors due to illness. Similarly, *P. berghei*-infected mice were more bitten in the end of infection before mice died (but also right before the increase in parasitaemia), which can also be associated with the decrease in anti-mosquito behavior. Sickness behavior-linked reduction in anti-mosquito behavior could also explain Coleman et al. 's results (1988), who found that *Plasmodium yoelii* and *Leishmania mexicana amazonensis* co-infected mice were more bitten by *Aedes aegypti* than single- or un-infected mice, especially when the infection symptoms were the most severe.

Experimental Setup and the Importance of Controlling for Host Behavior

Successful transmission to vectors is required to conclude that an increased attractiveness of infected hosts is an adaptive strategy of the parasite. Three main steps lead to transmission and could be influenced by parasites: attraction of vectors, vectors' decision to bite, and vectors' ability to bite, determined mostly by hosts' defensive behavior. Experiments aiming at determining whether parasites make their host more attractive to vectors use diverse methods, which sometimes does not allow addressing, or differentiating between, each step of this process. **Supplementary Figure S1** summarizes study main results in function of experimental characteristics.

A major difference between studies is whether uninfected and infected hosts attractiveness is compared through dual-choice experiments or through comparisons of biting/attraction rates. In dual-choice experiments, vectors are offered equal numbers of infected and uninfected hosts (in general, one of each) simultaneously, either in a dual-choice olfactometer (e.g., Lalubin et al., 2012), where vectors are not allowed to bite, or in a cage (e.g., Cornet et al., 2013a,b) or arena (e.g., van Duijvendijk et al., 2016). Dual-choice olfactometers have the advantage of allowing a distinction between attraction and vectors' ability to bite and providing direct evidence that one category of hosts is more attractive than the other. They also allow using only host odor, carbon dioxide emission or individual volatile compounds and test the relative attractiveness of each molecule. Nevertheless, dual-choice olfactometer experiments should, when possible, be coupled with dual-choice experiments where vectors are able to bite. Indeed, a higher attraction does not necessarily translate into more blood meals. We can conclude that the apparent manipulation is adaptive only by showing that infected hosts get more bites and transmit their parasites.

Allowing animals to defend themselves against vectors during experiments admittedly provides a better representation of the process in the way it would happen in the wild. However, when infected hosts differ in their behavior due to sickness, they become in general less active and less defensive, which facilitates vector attacks (Day et al., 1983; Turell et al., 1984; Coleman et al., 1988). Thus, in this setting, a higher biting rate of infected host cannot be conclusively assigned to either sickness behavior, which may be associated with a decrease in defensive behavior, or parasite manipulation of host attractiveness. For example, *Plasmodium yoelii*-infected mice and *P. yoelii* + *Leishmania mexicana* – infected mice were less active during the peak infection and this coincided with a peak in the number of mosquitoes able to take a blood-meal (Coleman et al., 1988). Day et al. (1983) found an association between gametocyte density, defensive behavior and mosquito biting success in *P. chabaudi*- and *P. berghei*-infected mice. This type of change in behavior of infected animals is sometimes regarded as a type of parasite manipulation (Lefèvre and Thomas, 2008). While it can be coincidentally advantageous for parasite transmission, sickness behavior may have evolved as an adaptive strategy in hosts, increasing the probability to eliminate or recover from the infection (Hart, 1988; Johnson, 2002), rather than a part of parasite extended phenotype. Indeed, sickness behavior is triggered by the immune response, in particular cytokines, and can be induced by the injection of lipopolysaccharide without the actual presence of parasites (Dantzer, 2001). Thus, this response is not specific to any parasite, which supports the idea that this consequence of infection has not evolved as a parasite adaptation but is under the genetic control of the host. Adamo (2014) has formulated the hypothesis that sexually transmitted pathogens suppress sickness behavior, which generally includes loss of sexual motivation, thus favoring their transmission. It would seem that inhibition of sickness behavior is more likely to result from parasite manipulation than the opposite.

When trying to determine whether a parasite adaptively manipulates the attractiveness of its host, experiments should as much as possible decompose each step of the process to properly identify whether infected hosts are really more attractive, whether they really get more bites and whether bites really result in transmission. One way to test whether infected hosts get more bites, while avoiding the confounding factor of defensive behavior, is to anesthetize the tested animals (da Rocha Silva et al., 2019) or to mechanically immobilize them (Cornet et al., 2013a,b).

Natural Versus Experimental Infections

The possibility of observing parasitic manipulation of host attractiveness may depend on the level of co-adaptation in the host-vector-parasite trio. If being infected bears a fitness cost for vectors, they might evolve parasite avoidance strategies. Evidence for impacts of parasites on their arthropod vector reproductive parameters exist, especially in mosquitoes, although they are sometimes contradictory. Using lab-bred *Culex pipiens* and *Plasmodium relictum*, infection was shown to decrease mosquito fecundity (Vézilier et al., 2012; Pigeault and Villa, 2018) and to increase their survival under *ad libitum* food conditions (Vézilier et al., 2012). On the opposite, using mosquitoes reared from egg rafts collected in the field, the same infection was shown to slightly increase fecundity (Delhaye et al., 2016) and to decrease survival under a food restriction regime (Lalubin et al., 2014). Independently of the systems used,

most of these studies did not find an effect of the infection on host longevity when food was given *ad libitum* (Lalubin et al., 2014; Delhaye et al., 2016; Pigeault and Villa, 2018). Moreover, *Plasmodium falciparum* increases fecundity and do not reduce survival in *Anopheles gambiae* (Alout et al., 2016). Evidence for cost of infection in other vector groups are less numerous; *Trypanosoma cruzii* infection decreases fecundity and survival of the triatomine bugs *Meccus pallidipennis* (Cordero-Montoya et al., 2019). Conflicting results regarding the manipulation of host choice by vector-borne parasites could result from differences in the associations of species tested (natural or not), as well as in the origin of the tested individuals, wild or laboratory-bred (Ferguson and Read, 2002; Tripet, 2009).

It may thus be possible that vectors and parasites face off in an evolutionary arms race between parasite avoidance and deceptive signaling. If this is the case, when we observe interactions between populations that are co-evolving, vectors' potential anti-infection mechanisms might counteract parasite manipulation, depending on what stage of this arms race they are experiencing. In addition, we need to consider the release from selective pressure allowed to lab strains as potentially enhancing or reducing the effect of parasite on attractiveness. Indeed, lab-bred mosquito populations that have not been under selective pressure for parasite resistance/avoidance for hundreds of generations – as they are usually fed on uninfected blood sources – might show exacerbated attraction to hosts whose attractiveness has been manipulated by parasites. Alternatively, they might have lost some host-seeking skills by being constantly offered effortless food. Similarly, parasite strains that have been passaged across several years without the contribution of vectors (e.g., by intraperitoneal injections of infected host blood into another host) might have lost their capacity to make their hosts attractive to vectors. This might explain the discordant results of Lalubin et al. (2012) and Cornet et al. (2013b), who found a lower attraction of *Culex pipiens* toward wild great tits (*Parus major*) naturally infected by *Plasmodium sp.* and a higher biting rate in canaries infected with a lab strain of *Plasmodium relictum* (SGS1), respectively. In addition to the infection origin and the variable measured, these experiments differ with regards to the mosquito origins, as Lalubin et al. (2012) used mosquitoes emerged from freshly collected egg rafts, present at one of the sites where the tested great tits were caught, while Cornet et al. (2013b) used a lab colony initiated 10 years before the experiment. The generally reduced phenotypic variation of lab strains and the release from co-evolutionary selection pressures (Tripet, 2009) can explain the diverging results of these two experiments.

The expectation that mosquitoes should experience selection for parasite avoidance needs to be mitigated by two considerations. First, if the cost of seeking a new – hopefully uninfected – host exceeds the cost of being infected, evolution for parasite avoidance is unlikely. This could depend, among other factors, on host density and availability, and on parasite prevalence. Secondly, if the only effects of infection perceivable by vectors are quantitative variations of existing cues always used when host seeking, for instance the increased production of some VOCs (de Moraes et al., 2014; de Boer et al., 2017; Robinson et al., 2018), it might be more difficult for vectors to evolve parasite avoidance. They could rely on other cues, for instance the detection of a particular skin microbiota composition after landing on host, however, the mechanisms of this hypothetic detection is unknown to the best of our knowledge.

The Importance of Controlling for Co-infections

When comparing the attractiveness of uninfected and infected hosts, it is crucial to take into account the possibility of co-infection with other parasites. It is especially important when using naturally infected individuals, in particular if several parasites are locally transmitted by the same vector species. For example, none of the studies examining the attractiveness of wild *Plasmodium*-infected birds to *Culex pipiens* shown in **Table 1** tried to determine whether the birds were harboring West Nile Virus (WNV), which is also transmitted by *Culex pipiens*. This could be a concern especially if these parasites co-occur in a non-random way, as it was found to be the case in Chicago, where there was a negative association between *Plasmodium* sp. infections and WNV serostatus (Medeiros et al., 2014). By ignoring other potential infections, we might under- or overestimate the capacity of the focal species to increase its hosts' attractiveness.

On Using Anti-malarial Treatment to Have Uninfected Controls

Most studies comparing infected individuals and individuals after anti-parasite treatments found a preference for non-treated individuals (Lacroix et al., 2005; Batista et al., 2014; Busula et al., 2017a; Robinson et al., 2018; Yan et al., 2018; but see de Boer et al., 2017). Using treated individuals as uninfected controls is convenient for assessing the attractiveness of naturally infected hosts when uninfected hosts are difficult to find. It also allows avoiding a pre-existing bias, in the sense that naturally uninfected individuals might be so because they are, for other causes unrelated to the focal infection, less attractive to vectors (Mukabana et al., 2002). However, anti-parasitic drugs can have an effect on biting insects, independently of their infection status, either through a direct effect or through an alteration of blood characteristics. A preference for infected hosts in this type of experiments might actually indicate an avoidance of drug-treated hosts. In a recent study, de Boer et al. (2019) evaluated the effect of artemether-lumefantrine (AL) on mosquito host-seeking behavior and fitness. Using traps baited with socks worn by AL-treated men (before, during and after treatment) they found more *Anopheles coluzzi* attracted to the socks worn after treatment, but no difference between before and during treatments. In a semi-natural setting, *Anopheles gambiae* did not show a preference for either AL treatment time-points. However, a comparison of the attractiveness of socks worn the same day by AL-treated and untreated humans would have been useful to avoid an effect of "time since odor collection". Membrane-feeding with human blood containing a high concentration of dissolved AL resulted in no difference

in survival, oviposition rate, timing before oviposition and number of eggs. This tends to suggest that it is appropriate to use AL-treated individuals as "uninfected" hosts in mosquito choice trials. Nevertheless, studies allowing mosquitoes to feed on infected+treated, uninfected+treated, infected+untreated and uninfected+untreated humans are necessary to conclude, because metabolization of AL might induce changes in blood characteristics that would not occur in membrane-feeders. Lacroix et al. (2005) found that former gametocyte carriers treated with sulfadoxine pyrimethamine (SP) seemed to repel mosquitoes, although it was not the case of treated individuals that were not gametocyte-carriers. As high concentrations of SP can strongly reduce *Anopheles stephensi* survival (Kone et al., 2010), mosquitoes might evolve the capacity to detect and avoid SP-treated humans.

Potential for Publication Bias

Parasitic manipulation of host attractiveness is a seducing hypothesis and publication bias is likely to occur, as was suggested by Poulin (2000). Among the studies presented here, those that found a preference for infected hosts were on average more cited than the studies finding a preference for the uninfected host, and both were more cited than studies finding no preference (**Supplementary Figure S2**). This might discourage researchers to submit (and publishers to publish) manuscripts reporting results that do not support the hypothesis of increased attractiveness of infected hosts.

PERSPECTIVES

Future work on this topic could use the selection of studies reviewed here to conduct a meta-regression aiming to quantify the magnitude of the effects. Indeed, while we discussed the potential influence of different experimental designs on effect directionality – i.e., the preference of vectors for infected versus uninfected hosts – a meta-regression would provide a formal testing of their impacts on the strength of the observed effect in a quantitative way. This would also allow a more accurate identification of possible publication bias. Finally, a meta-regression could be used to assess to what extent different transmission cycle characteristics or parasite life history strategies affect vector-borne parasite manipulation of host.

CONCLUSION

This review highlights that manipulation of host attractiveness is widespread among different groups of vector-borne parasites. However, contradictory results show that it cannot be considered as a rule. Manipulation of host attractiveness might be one side of an evolutionary arms race against parasite avoidance, and different pairs of vector and parasite populations could find themselves at one or the other stage of a co-evolutionary timeline. In addition, parasites other than *Plasmodium sp.* deserve that we put more effort into investigating their ability to trigger attractant molecules production in their hosts, especially vector-borne viruses and filarial worms. Experiments trying to assess the generality of this phenomenon should ideally design their experiment in a way that (1) verifies the presence of transmissible stages in host blood, (2) separates attraction from capacity to bite, and (3) uses parasite and vector strains that have not been released from co-evolutionary selective pressures for many generations. Finally, we stress the importance to investigate the proximate mechanisms responsible for a higher attractiveness of infected hosts in order to determine which partner has the genetic control of the situation, as well as its impact on parasite fitness, before concluding on adaptive manipulation (Herbison et al., 2018).

AUTHOR CONTRIBUTIONS

C-SC, OG, PC, and RP contributed to the original idea and the final version of this manuscript. C-SC and RP collected the data and contributed to the first draft of this manuscript. C-SC conceived the figures and analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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FIGURE S1 | Study main results in function of experimental characteristics. This figure summarizes the number of studies that found a greater attraction of infected vertebrate hosts according to different experimental characteristics (natural versus experimental infection, host defensive behavior possible or not). This figure does not account for effect sizes.

FIGURE S2 | Number of citations of the reviewed studies in function of the year of their publication. The regression lines were plotted using the "ggplot" package (Wickham, 2016), using values predicted by a negative binomial generalized linear model, using the number of citations as the response variable, and the year of publication and the main result as predictors (orange: at least one result of the study shows a higher attractiveness of infected hosts; blue: at least one result of the study shows a higher attractiveness of uninfected hosts; green: no difference found). We used the function *glm.nb* in the "MASS" package (Venables and Ripley, 2002) to fit the model. The likelihood ratio test was used to assess the significance of both predictors: "year of publication": $\chi^2_1 = 3.73$, $p = 0.053$; "main result": $\chi^2_2 = 6.69$, $p = 0.035$. We also fitted a model with an interaction between the year of publication and the main result, and the interaction was not significant ($\chi^2_2 = 2.15$, $p = 0.34$). Analyses were performed on R v.3.5.1 (R Core Team, 2018).

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Is Host Selection by Mosquitoes Driving Vector Specificity of Parasites? A Review on the Avian Malaria Model

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Parasites and hosts are in a complex evolutionary arm race where host compatibility represents a key obstacle for successful infections. The degree of parasite specialization on a host varies along a continuum: on one end, extreme specialist parasites may be restricted to a single host species, and on the other, generalist parasites are able to infect a diverse set of hosts. Multiple intrinsic factors associated to the host, such as their immune system and physiological condition, can contribute to the evolution of host specificity of parasites and have been well-studied and documented in vertebrates. In contrast, vector specificity of parasites has been largely overlooked, especially in natural conditions. While a few studies suggested that insect vectors do not play an important role in shaping the parasite community structure since they may feed widely from the bird community, current studies have demonstrated the importance of vector feeding behavior on transmission dynamics for several vector-borne pathogens. Here, we provide a review on the specialization of avian *Plasmodium* in vectors, emphasizing the necessity to study simultaneously the three players to further understand how host choice by the vectors might influence the distribution of parasites in the wild. In addition, we suggest specific research directions making use of both empirical data gathered in the field and controlled experiments on vector host-feeding preferences.

Keywords: avian *plasmodium*, feeding preference, insect-vector, ornithophilic, parasite specialization

INTRODUCTION

The degree of parasite specialization for a host varies along a continuum: on one end, extreme specialist parasites may be restricted to a single host species, and on the other, generalist parasites are able to infect a diverse set of hosts, with a large diversity of intermediate states (Vázquez et al., 2005; Poulin, 2007; Hellgren et al., 2009; Mccoy et al., 2013). Both ecological and evolutionary mechanisms are currently acting to maintain this high diversity in the range of host specialization (Poisot et al., 2015). Understanding how parasite specialization has evolved and how different strategies can co-occur is challenging and remains a debated topic. It has been suggested first that

specialization represents an evolutionary "dead end," which limits further evolution (Kelley and Farrell, 1998; Snyder and Loker, 2000; Nosil, 2002). However, several later studies have indicated that generalists can repeatedly evolve from specialist lineages, albeit with a relatively low frequency (Poulin et al., 2006; Johnson et al., 2009; Gomez et al., 2010).

The probability for a parasite of infecting a suitable host depends on many factors, including the host-parasite compatibility, rate of encounter, the parasite life cycle, the population density (Combes, 1997) as well as host intrinsic factors such as its immune system, age, or sex (Loiseau et al., 2008; Bichet et al., 2014; Calero-Riestra and Garcia, 2016). For parasites such as avian *Plasmodium*, with a complex life cycle that involves two hosts, one vertebrate (bird) and one insect (mosquito; Valkiunas, 2005), additional parameters linked to the insect, such as its feeding behavior or immune system, may also influence parasite specialization (Billingsley and Sinden, 1997; Gutiérrez-López et al., 2020). Interactions with the insects, commonly called vectors (Wilson et al., 2017), are likely important for parasite evolution since mosquitoes are the definitive host, in which sexual reproduction occurs. Thus, as in the vertebrate hosts (hereafter the term "host" will be used), the parasite ingested in a mosquito blood meal must elude the immune system of the vector and complete all the developmental stages while the mosquito remains alive long enough to feed on another host (Marquardt et al., 2005).

Avian malaria parasites are considered an excellent model for investigating parasite transmission dynamics in ecological and evolutionary studies (LaPointe et al., 2012; Rivero and Gandon, 2018). They have been studied extensively in birds because of their high diversity of hosts and nearly worldwide distribution (Valkiunas, 2005). To date, parasite specialization for their vertebrate hosts has been much more studied compared to their vector specificity. Studies have investigated host-parasite associations using phylogenetic analyses and indices of host specialization (e.g., Ishtiaq et al., 2008; Hellgren et al., 2009; Loiseau et al., 2012; Ricklefs et al., 2014; Lauron et al., 2015; Fecchio et al., 2019) and have revealed that several lineages of *Plasmodium* exhibit extreme host generalist parasitism strategies, whereas other lineages appear to have been constrained to certain host families or individual bird species (Ricklefs and Fallon, 2002; Ricklefs et al., 2004; Beadell et al., 2009). Interestingly, in the discussion of a large number of these publications, authors suggest a key role for vectors in the system, but very few studies are exploring this vector role. In fact, the number of publications using the bird-avian *Plasmodium* system has considerably increased with on average 25 articles published per year since the last 10 years, but the number of publications that integrates the vector in their study remains much lower (3.5 per year since 2010; see **Supplementary Figure 1**). A number of reasons are responsible for the fact that vector specificity of avian parasites has been largely overlooked, especially in natural conditions: (i) researchers studying avian malaria are often trained ornithologists with little or no entomological expertise, (ii) insect vectors are difficult to capture compared to birds, (iii) they are also more difficult to identify, especially in tropical countries, (iv) the parasite prevalence in mosquitoes is usually low (10% on average with values ranging from 0.003 to 30%; Aruch et al., 2007; Glaizot et al., 2012; Lalubin et al., 2013; Zélé et al., 2014; Martínez-de la Puente et al., 2015, 2016, 2020) compared to birds where prevalence often reaches up to 80% (Bichet et al., 2014).

Different ecological and behavioral factors determine the co-evolution of avian *Plasmodium* and mosquitoes (Ferraguti et al., 2016). Among them, the vector feeding behavior is an important parameter that can affect the specialization of parasites both in the vector and the vertebrate host (**Figure 1**). A hypothesis on parasite vertebrate host specificity is that if the vector is not selective and feeds on a large variety of avian hosts, then the malaria parasite should have an advantage in being an avian host generalist, maintaining compatibility with a wide set of birds, even if some of them are not optimal hosts. This should increase its encounter rate with suitable avian hosts and, therefore, its overall prevalence in the community (Dobson, 2004; Hellgren et al., 2009). On the other hand, if the vector is selective and feeds on specific bird species (Kilpatrick et al., 2006; Hellgren et al., 2008; Rizzoli et al., 2015), the parasite should have an advantage in being host specialist and optimize its host compatibility, development and transmission efficiency. Interestingly, the host specificity of parasites may not reflect their vector specificity. As shown in **Figure 1**, a parasite that is a vector specialist (i.e., able to complete its sexual cycle in one mosquito species only) could present different host strategies, specialist or generalist, depending on the host selection by vectors. If the parasite is a vector generalist (i.e., able to complete its sexual cycle in various mosquito species), the diversity in the range of host specialization can be even more important i.e., from highly host specialist to extremely host generalist (**Figure 1**). Therefore, from a parasite perspective, it would seem more advantageous to be a vector generalist since this would enable a broader gradient of host specificity (considering always that those vectors are competent to transmit the parasite). On the other hand, being a vector or host generalist may come at the expense of optimal parasite compatibility with host or vector.

Here, we review the current knowledge on parameters that influence host selection by mosquitoes and its potential repercussions on vector-parasite co-evolution and vector specificity. Using the MalAvi database (Bensch et al., 2009), which currently comprises 1276 unique *Plasmodium* lineages found in around 1,200 bird species and 40 mosquito species and data from the literature, we aimed at (i) evaluating the feeding preference of those mosquito species that could be potential avian malaria vectors and (ii) exploring the phylogenetic relationships of avian malaria lineages and their vector and bird specificity. We selected mosquito species where *Plasmodium* lineages have been isolated from the head/thorax or from salivary glands as a proxy of competent vector. We excluded lineages found in whole mosquitoes since they may correspond to parasites that will experience abortive developments in the vector midgut (Valkiunas et al., 2013). Finally, we address various challenges and limitations when working with vectors and suggest lines of research for the future, both in the field and in laboratory conditions.

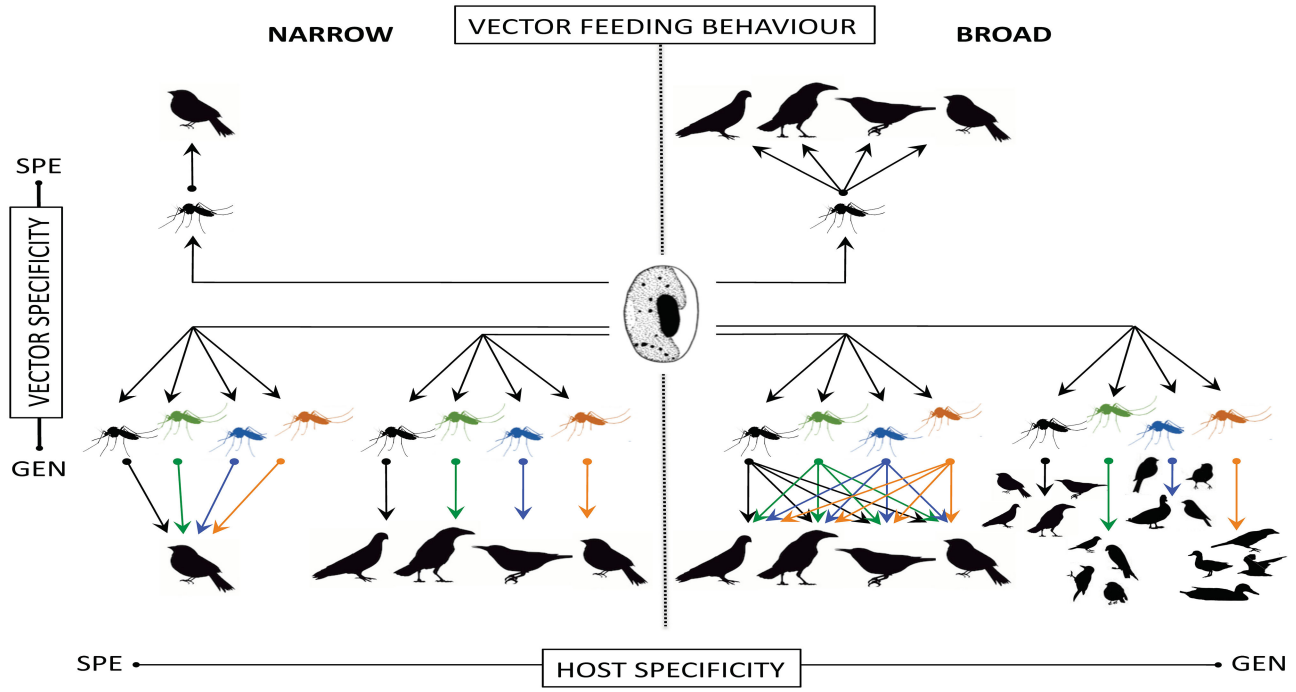

FIGURE 1 | Diagram of the potential interactions between *Plasmodium* parasite and the mosquito vector and the bird host in function of the feeding preference of the mosquito (narrow or broad: feeding on only one or four bird species, respectively). On the top half part, the parasite is a vector specialist (infecting one mosquito; SPE) and on the bottom part, the parasite is a vector generalist (infecting four mosquitoes; GEN). In each case (Top left: vector specialist and narrow feeding preference; Top right: vector specialist and broad feeding preference; Bottom left: vector generalist and narrow feeding preference; Bottom right: vector generalist and broad feeding preference), only extreme results on the parasite host specificity continuum (bottom line from specialist (SPE) to generalist (GEN) are illustrated. In the case of mixed community of vectors with both narrow and broad feeding preferences (not represented here), the number of infected hosts will vary from 4 to 13. Credit for Plasmodium gametocyte drawing: Valkiunas and Iezhova, *Malaria Journal* (2018).

HOW DO MOSQUITOES SELECT THEIR HOSTS?

Large Variation in Feeding Preferences

The transmission dynamics of avian *Plasmodium* is going to be strongly influenced by the contact rates between mosquitoes and susceptible hosts (Takken and Verhulst, 2013). Under natural conditions, mosquitoes show different innate feeding preferences at different levels. First, while some species feed mostly on mammals (i.e., mammophilic species), others prefer to feed on birds (i.e., ornithophilic species), or amphibians and reptiles, while yet other species show a real opportunistic behavior (Molaei et al., 2007; Burkett-Cadena et al., 2008; Viana et al., 2010; Muñoz et al., 2012; Martínez-de la Puente et al., 2015). Second, in addition to this broad tendency for particular host groups, mosquitoes may feed on certain host species at higher rates than expected from their abundance (Hamer et al., 2009; Lura et al., 2012). For instance, in Europe, *Cx. pipiens* seems to show a preference for blackbirds (*Turdus merula*) compared to European starlings (*Sturnus vulgaris*; Rizzoli et al., 2015). These two levels (group/species) of selection by the mosquito could be altered by the availability of preferred hosts that depends on seasonality and habitat (Wekesa et al., 1997; Kilpatrick et al., 2006; Thiemann et al., 2011). As an example, *Culex pipiens* and *Culex tarsalis*, known to be predominantly ornithophilic, shift their diet to include more mammals when the availability of certain bird species decreases at the end of the summer (Kilpatrick et al., 2006). Seasonality in temperate regions affects also mosquito abundance and in consequence parasite prevalence, with a typical peak in August or early autumn (Ferraguti et al., 2013; Lalubin et al., 2013). In addition, more or less effective host anti-mosquito behavior and other intrinsic host factors, developed below, influence feeding patterns of mosquitoes (Takken and Verhulst, 2013).

Host Characteristics Influencing Feeding Preferences

Female mosquitoes detect their vertebrate hosts by a combination of different cues [e.g., visual cues (body size), carbon dioxide (CO_2), temperature, moisture, and/or body odor], which influence the attractiveness of individuals to vectors (Eiras and Jepson, 1994; Lehane, 2005). Some individuals (males or females) depending on their physiological state (e.g., hormone levels), age-class groups (young or older individuals) or infectious status (infected vs. non-infected) may be more attractive than others. Those individuals are therefore more likely to receive more mosquito bites (Dye and Hasibeder, 1986; Liebman

et al., 2014) and acquire new infections from vectors, but also to pass on parasites to subsequent vectors with whom they come in contact. These super-receivers and super-spreaders increase the contact rates between hosts and vectors, and may influence the vector specificity of parasites. Both empirical studies and theoretical models (Woolhouse et al., 1997; Perkins et al., 2013) showed that inter-individual heterogeneity has considerable implications for the epidemiology of mosquito-borne parasites.

First, larger hosts may receive more mosquito bites (Yan et al., 2017), probably due to (i) the higher amounts of cues (e.g., CO_2) released by larger individuals or (ii) the greater skin surface available for mosquito bite (Kleiber, 1947; Yan et al., 2017). Different studies have reported a positive relationship between the host body mass and the feeding rate of different blood-searching insect vector (Martínez-de la Puente et al., 2010; Schönenberger et al., 2016). In addition, Yan et al. (2017) found that *Cx. pipiens* fed more frequently on birds with a longer tarsus, suggesting that larger areas of exposed skin are important in determining feeding patterns. However, experimental studies have observed contrasting results. While Simpson et al. (2009) found differences in *Cx. pipiens* attraction among bird species, with American robins (*Turdus migratorius*) being more attractive than house sparrows (*Passer domesticus*), Gutiérrez-López et al. (2019) found no difference in attraction between jackdaws (*Coloeus monedula*) and house sparrows. Furthermore, in both studies, differences in body mass between individuals of the same species did not determine mosquito attraction, suggesting that, at short distances (as happens in an experimental setup), other signals such as heat, humidity or odor could be more important than intraspecific differences in body mass (Raji and DeGennaro, 2017).

Sex-associated characteristics could also cause differences in the mosquito attraction (Zuk et al., 1990). Sex-biased individual attraction could explain why several studies found that male birds present usually a higher prevalence of blood parasites than females (Skorping and Jensen, 2004; Zuk and Stoehr, 2010; Calero-Riestra and Garcia, 2016). As an example, Burkett-Cadena et al. (2014) found that mosquitoes had a higher feeding preference for male birds, but this result was observed mainly in mammophilic mosquitoes. On the other hand, several studies failed to find any significant sex-biased feeding pattern in ornithophilic mosquitoes (Simpson et al., 2009; Yan et al., 2018; Cozzarolo et al., 2019; Díez-Fernández et al., 2019; Gutiérrez-López et al., 2019). It has also been suggested that the composition of volatile substances from the uropygial gland secretions, which differ between male and female birds (Jacob et al., 1979; Amo et al., 2012), could affect the attraction of blood-sucking insects (Russell and Hunter, 2005; Martínez-de la Puente et al., 2011). However, Díez-Fernández et al. (2020) found no difference between feeding pattern in *Culex pipiens* and the sex of the birds. Therefore, other factors such as the degradation of the uropygial secretions by the microbiome present in the feathers could act as an attractive to mosquitoes (Allan et al., 2006; Díez-Fernández et al., 2020).

Parasite Manipulation and Vector Adaptive Avoidance

According to the parasite manipulation hypothesis (Poulin, 2000), the infection with the parasite should increase the incidence of feeding by mosquitoes (Cornet et al., 2013), either by modifying its defense behavior or increasing its attractiveness (Heil, 2016). It could also modify the behavior of the mosquito, making it more aggressive. These parasite manipulations should increase the contact rates between the host and the vector and, therefore, favor the parasite transmission (Hurd, 2003; Lefévre and Thomas, 2008).

To date, experimental tests of this hypothesis using the *Plasmodium relictum—Cx. pipiens* system have obtained contradictory results. While some studies found that mosquitoes were attracted to, or fed on chronically infected birds at a higher rate than uninfected hosts (Cornet et al., 2013; Díez-Fernández et al., 2020), other studies have reported the opposite pattern (Lalubin et al., 2012) or an absence of significant differences between infected and uninfected birds (Yan et al., 2018; Gutiérrez-López et al., 2019). This discrepancy may come from the different experimental procedures used (e.g., either dual-choice olfactometer or direct contact with immobilized hosts) and therefore further studies are required to clarify how the infection status may influence the feeding behavior of mosquitoes.

The parasite load in the vertebrate (i.e., parasitemia) may also play an important role on the mosquito feeding patterns. According to the vector adaptive avoidance (Lalubin et al., 2012), mosquitoes should avoid highly infected individuals due to the costs induced by parasite in the vectors. However, Yan et al. (2018) found the opposite with mosquitoes feeding with a higher rate on those individuals that had a higher *Plasmodium* parasite load, suggesting a pre-eminence of parasite manipulation over vector avoidance. Comprehensive studies controlling for multiple factors are needed to better understand the interplay between these two opposite factors in the host selection by vectors.

WHAT ARE THE FEEDING PREFERENCES OF MOSQUITOES TRANSMITTING AVIAN MALARIA?

To illustrate the diversity of mosquito feeding preference patterns, we used the MalAvi database and a set of publications to gather information on blood meals for all the vectors collected in the field with avian *Plasmodium* in their head/thorax or salivary glands ($N_{vector} = 22$ and $N_{lineage} = 58$; **Figure 2** and see **Supplementary Materials**, **Table S1**). We classified the diet, using the percentage of blood meals derived from birds, in three categories as (i) mammophilic (from 0 to 33%), (ii) opportunistic (from 33 to 66%), or (iii) ornithophilic (more than 66%).

According to the literature, *Cx. pipiens* was the most studied mosquito species for avian malaria and was found to feed on more than 74 different bird species. In fact, 82% of the blood meals found in *Cx. pipiens* were from birds, confirming its ornithophilic feeding preference (Kilpatrick et al., 2006;

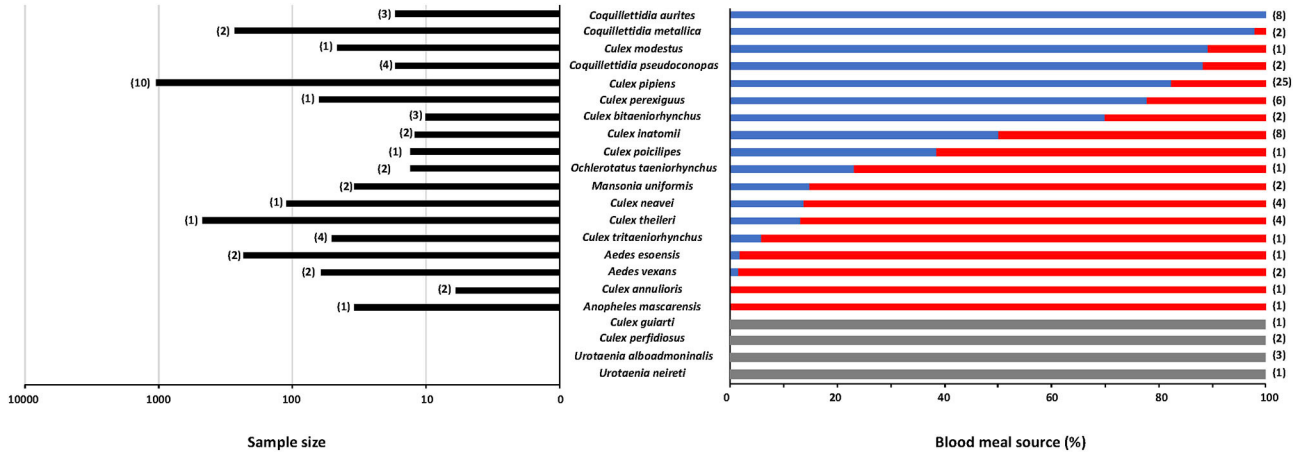

FIGURE 2 | Percentage of bird (blue) and mammal (red) derived blood meals from female mosquitoes species that have shown avian malaria parasites. Mosquito species without information about their blood-feeding behavior are represented in gray ($N = 4$). Numbers in bracket on the right represent the number of different lineages isolated in the head-thorax of each mosquito species. Sample size (log-transformed) of each mosquito species analyzed is shown in black. Numbers in brackets on the left represent the number of studies (see **Supplementary Table 1**) that have analyzed blood meals in each mosquito species.

Hamer et al., 2008; **Figure 2**). It also harbors the highest number of avian *Plasmodium* lineages in the head/thorax ($N = 25$; **Supplementary Figure 2**). Therefore, its feeding preference together with its wide distribution around the world, its ecology (Farajollahi et al., 2011) and the diversity of lineages harbored, make this mosquito species one of the main vectors of avian *Plasmodium*. Among other vector species known to harbor avian *Plasmodium*, we found species that exhibit mammophilic ($N = 9$; e.g., *Culex theileri* and *Culex neavei*), opportunistic ($N = 2$; *Cx. inatomii*, *Cx. poicilipes*), and ornithophilic preferences ($N = 7$; **Figure 2**). We therefore observe a large variation in mosquito behavior that should lead to different vector specificity strategies and parasite transmission efficiency. For instance, avian *Plasmodium* lineages should enter in contact at higher rate with ornithophilic mosquito species, which preferentially feed on birds, than with opportunistic or mammophilic mosquito species. This in turn should cause avian *Plasmodium* lineages to become better adapted to ornithophilic mosquitoes and develop specificity for this vector type.

WHAT DO WE KNOW ABOUT VECTOR SPECIFICITY OF AVIAN *PLASMODIUM*?

Ten years ago, some studies began to use molecular markers and phylogenetic analyses to examine the distribution of avian blood parasite lineages in wild-caught arthropod vectors. One of the first studies showed that different parasite lineages found in different mosquito genera were sharing a common vertebrate host (Gager et al., 2008). Their data imply that the co-occurrence of two parasite lineages in the same vertebrate host is not necessarily explained by access being provided by a shared mosquito. Other studies showed that different mosquito species could harbor and share closely related or identical parasite lineages (Ishtiaq et al., 2008; Kimura et al., 2010; Inci et al., 2012), suggesting that these lineages did not have high vector specificity. However, these studies identified lineages from whole mosquitoes or head-thorax and discussed the fact that without salivary gland dissection or experimental demonstration of the transmission cycle, it was not possible to establish the vector competence of mosquitoes for parasite lineages and therefore to fully assess the vector specificity. Indeed, an important percentage of parasite DNA amplification could come from abortive stages of parasite and not from infective stages (i.e., sporozoites; Valkiunas, 2011; Valkiunas et al., 2013). More recently, researchers performed experimental work to identify competent vector species for different avian *Plasmodium* lineages by studying sporogonic development in salivary glands or saliva (Valkiunas, 2005; Kazlauskiene et al., 2013; Palinauskas et al., 2016; Carlson et al., 2018; Gutiérrez-López et al., 2020). These studies highlighted variation in the mosquito ability to transmit different avian *Plasmodium* lineages, with some mosquito species being completely refractory to some parasite lineages or some being able to transmit different parasite lineages and/or species.

These experimental results combined with empirical data from field surveys demonstrate that avian *Plasmodium* lineages, which show extensive variation in host range (from one to more than 100 bird species) and specialization (host specificity index calculated as in Hellgren et al., 2009; **Figure 3**), also show variation in vector specificity (from one to 6 mosquito species; **Figure 3**). It is worth noting that the most host generalist parasite (*Plasmodium relictum* SGS1) that has been found to infect around 120 bird species is not the lineage that has been found in the most vector species so far (**Figure 3**). Some host specialist lineages (e.g., CXPIP10) or with an intermediate host range (e.g., PADOM02) were found in the same number of vectors as *Plasmodium* SGS1 (**Figure 3**), reflecting differential host and vector specificity for a given parasite lineage. Overall however, the parasite host specificity was positively correlated to

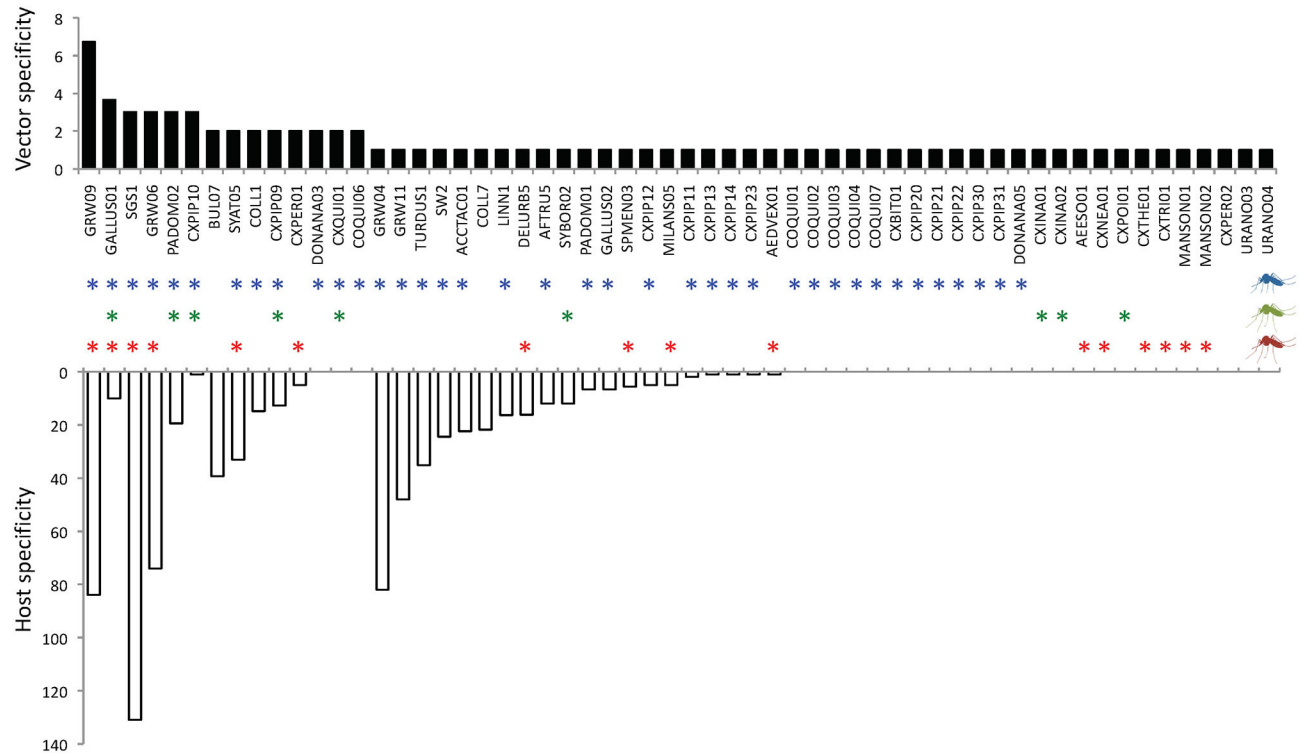

FIGURE 3 | Vector specificity of parasites of the 58 *Plasmodium* lineages found in head and thorax of vectors and the associated host specificity of lineages that were found in birds ($N = 31$). Blue, green, and red asterisks correspond to lineages that were found in ornithophilic, opportunistic and mammophilic mosquitoes, respectively.

its vector specificity (Pearson coefficient R = 0.47; $P = 0.008$; see **Supplementary Figure 3**).

In order to explore the phylogenetic relationships of *Plasmodium* lineages and their associations with mosquito species, we performed Bayesian phylogenetic analyses with 58 *Plasmodium* lineages plus another 16 morphospecies described and deposited in MalAvi (**Figure 4**; see methods in **Supplemental Materials**). In the tree, we were able to determine 12 clades that correspond to groups of lineages closely related (Clades A to L; represented by a triangular tip with a node support higher than 0.85), most of them including a known morphospecies. First, we observe that most lineages found in the same mosquito species were not necessarily closely related but rather were found all across the phylogenetic tree, suggesting no evidence for strong coevolutionary relationships between these lineages and their vectors. At the lineage level, it appears that around 75% of the lineages were found in only one vector species, which probably does not always reflect strict vector specificity but rather a sampling effort bias. The other lineages were found in two to six different mosquito species sometimes from different genus (e.g., *Plasmodium elongatum* GRW06, *Plasmodium gallinaceum* GALLUS1, *Plasmodium* GRW09). These three lineages are most likely vector generalists since they shared different mosquito genus that also had different feeding preferences (**Figure 4**). Interestingly, at the clade level, we found more variation in vector specificity. If we consider a gradient of specialization, from generalist to specialist, we could discern: (i) clades associated with two or three mosquito genera (2–6 species; clades B, D, E, H, J, and L) with different feeding preferences (i.e., vector generalists), (ii) clades associated with only one genus (*Culex*) but 2–4 different species with different feeding preferences as well (i.e., intermediate vector specificity; clades C, F, and I), and (iii) clades associated with only one or two ornithophilic mosquito species (i.e., vector specialists; Clades A, G, and K).

DOES HOST SELECTION BY THE MOSQUITO INFLUENCE PARASITE VECTOR SPECIFICITY?

Depending on the feeding preference of the vectors, parasites should have different strategies of transmission and specificity. We predicted that avian *Plasmodium* lineages found in ornithophilic vectors should be more numerous and on average more vector specialists than lineages found in opportunistic or mammophilic mosquitoes since they have a higher chance of ending up in avian hosts. First, we found that ornithophilic mosquitoes harbor more *Plasmodium* lineages ($N = 38$) than opportunistic or mammophilic vectors as predicted (9 and 16 lineages, respectively; **Figure 3**). In addition, all the clades were associated with at least one ornithophilic mosquito, which

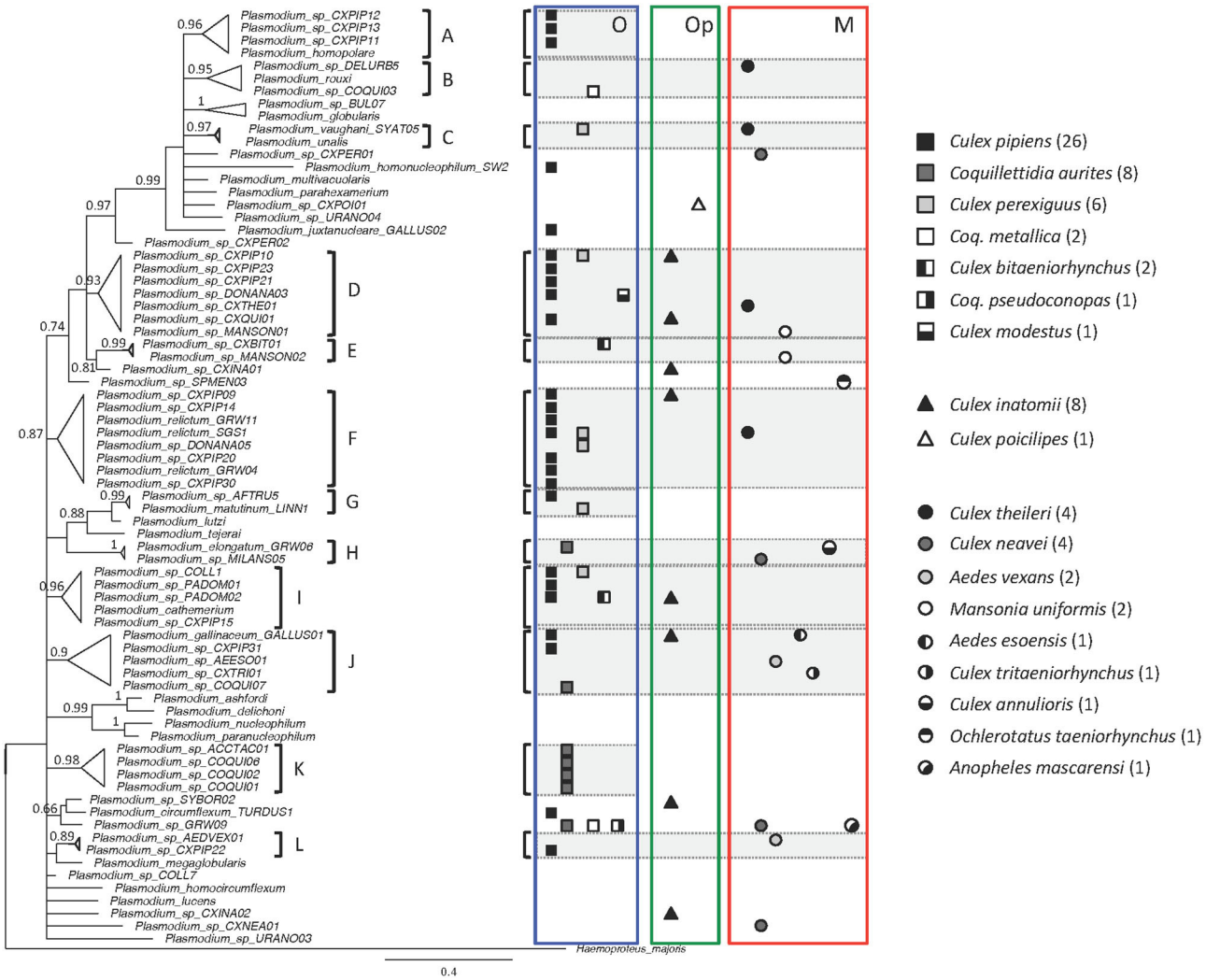

FIGURE 4 | Phylogenetic relationships based on cytochrome *b* sequences of the 58 *Plasmodium* lineages found in mosquitoes (head and thorax), as well as 16 morphospecies described in the literature. Morphospecies are shown with their full Latin name while lineages are shown with their MalAvi code (e.g., *Plasmodium CXPIP12*). Numbers located on the top of the branches indicate Bayesian posterior probability values (only values above 0.6 are shown). Twelve clades are represented by a triangular tip with a node support higher than 0.85 and a vertical bar (clades A–L) and correspond to groups of lineages closely related. For each mosquito species, we indicated the feeding preference: ornithophilic (O; square), opportunistic (Op; triangle), mammophilic (M; circle). The four-mosquito species with unknown feeding preferences are not represented in the figure. *Haemoproteus majoris* was used as the outgroup; scale bar: 0.4 estimated substitutions per site.

was not the case for opportunistic or mammophilic species. However, the feeding preference of each mosquito species (i.e., the percentage of blood meal from birds, ranging from 0 to 100%; **Figure 2**) was not significantly associated with the number of *Plasmodium* lineages that they carried (Pearson coefficient R = 0.39; $P = 0.11$; see **Supplementary Figure 4**). We also failed to find statistical associations between the vector specificity index and feeding preference, either at the lineage level ($F_{2,62} = 0.64$; $P = 0.53$) or clade level ($F_{2,22} = 1.83$; $P = 0.18$; see **Supplementary Figure 5**). However, this could be due to insufficient data being available from scarce studies on mosquito-parasite interactions. Most of the lineages are probably transmitted by different mosquito species and therefore the vector specificity of *Plasmodium* lineages calculated here does not reflect the true parasite specialization. Therefore, it seems difficult to have a clear answer to our question at present since we cannot decipher if host selection is driving or not the vector specificity of avian *Plasmodium*. Nonetheless, some of these limitations can be addressed by doing extensive mosquito surveys and experimental work in the field that could fill the current knowledge gaps.

HOW CAN WE DETERMINE THE VECTOR SPECIFICITY OF AVIAN *PLASMODIUM*? CHALLENGES AND FUTURE RESEARCH

Experimental studies have provided essential information about the vector competence of different mosquito species for

avian *Plasmodium* (Santiago-Alarcon et al., 2012; Palinauskas et al., 2016; Gutiérrez-López et al., 2020). Nonetheless, it also seems essential to carry out more holistic studies in natural conditions to allow a better understanding of all the potential interactions between avian *Plasmodium*, their vectors and their vertebrate hosts. Today, molecular techniques greatly facilitate the identification of blood meal origin in mosquitoes (Alcaide et al., 2009), as well as the presence of blood parasites in different parts of the mosquito body (Njabo et al., 2011; Gutiérrez-López et al., 2016; Palinauskas et al., 2016). However, the number of studies combining both the feeding behavior of mosquitoes and the transmission of avian *Plasmodium* are still very rare (Ejiri et al., 2011; Ferraguti et al., 2013; Martínez-de la Puente et al., 2016), mainly due to the difficult task of capturing engorged mosquitoes during short field seasons. Other vector-parasite systems suffer from the same fieldwork limitations, and to date, very few studies investigated the vector specificity in closely related haemosporidian parasites (Leucocytozoon: Hellgren et al., 2008; Haemoproteus: Martínez-de la Puente et al., 2011) or other blood parasites (e.g., Trypanosoma: Bennett, 1961; Svobodová et al., 2017). More efforts should be put on regular and constant surveys using various resting boxes to increase resting insects' collections and blood fed females (Panella et al., 2011) even though it is particularly time consuming. One unusual but appealing solution to overcome this field work limitation and to perform more efficient research is to foster multidisciplinary collaborations between research teams and centers for diseases control to (i) mutualize material and human resources, and (ii) answer different questions using the same specimens. More collaborative work would avoid double laborious field surveys while greatly enhancing the results since one mosquito can be used by different researchers and be screened for a great variety of parasites. For example, the feeding behavior of mosquitoes has been extensively studied in North America. However, due to the spread of West Nile Virus [CDC (Centers for Disease Control and Prevention) (2019)], studies mainly focused on arboviruses (Savage et al., 2007; Farajollahi et al., 2011) while to our knowledge very few studies have investigated avian malaria in vectors (Kimura et al., 2010; Carlson et al., 2018). Something similar can be observed in Central Africa for example, where the main entomological studies have been based on anthropophilic mosquitoes and human malaria or other zoonotic diseases of medical importance (Kamgang et al., 2012; Mayi et al., 2020), while the role of mosquito species in the transmission of avian *Plasmodium* is practically unknown (Njabo et al., 2009, 2011). If researchers with different research interests but working in the same region or localities were willing to combine their sampling effort, material and data, it would be beneficial for everyone and likely add value to everybody's data. One obvious advantage would be to determine natural co-infection rates in mosquito species and their consequences on pathogen transmission. Although this idea may seem utopian in the ever more specialized research world, it would be a very interesting prospect to greatly increase mosquitoes sampling size from field surveys while exploring mosquito-avian parasite interactions in more details.

To date, *Cx. pipiens* has been considered as the main vector of avian malaria in temperate regions. This mosquito species has a wide geographical distribution and is certainly the easiest ornithophilic species to catch and raise in laboratory, which makes it a perfect study model for experimental work. Studies have shown so far that 12 morphospecies of avian *Plasmodium* are able to develop sporozoites in *Cx. pipiens* (Santiago-Alarcon et al., 2012; Palinauskas et al., 2016; Gutiérrez-López et al., 2020). However, other less abundant species that share habitats with *Cx. pipiens*, such as *Cx. perexiguus, Cx. restuans*, or *Cx. inatomii*, among others, may play an important secondary role as vectors of avian malaria parasites. Further research work should therefore focus on less studied mosquitoes that present different feeding behaviors or restricted geographical distribution compared to *Cx. pipiens*, although some other potential vectors may be difficult to raise and study. For example, opportunistic mosquitoes might feed on a smaller number of bird species, and thus, come into contact with a smaller number of avian *Plasmodium* parasites, leading to different co-evolutionary processes between mosquito and avian *Plasmodium*. In addition, according to the MalAvi database, studies that have identified *Plasmodium* lineages in insects have been carried out in 20 countries only, mostly in Europe. Thus, future research projects could be conducted in geographical areas that are poorly studied up until now, such as tropical regions and migratory bird wintering areas in Africa, that may present very distinct host selection patterns and vector specificity due to the highly diverse communities of hosts and vectors. Undoubtedly, the development of field surveys and experimental studies on vector competence of poorly sampled or little-known mosquito species is essential to better understand the vector specificity and transmission dynamics of avian *Plasmodium*.

Lastly, detection of avian *Plasmodium* DNA in mosquito's head and thorax, where the salivary glands are located, provides valuable information on interactions between parasites and potential vectors. However, this does not fully guarantee the vector competence for avian malaria since DNA amplification could come from abortive stages of the parasite (Valkiunas, 2011; Valkiunas et al., 2013). To determine the transmission capacity of avian *Plasmodium* by the mosquito, visualization of sporozoites in the salivary glands or the extraction of saliva from the mosquito are more reliable methods (Valkiunas, 2005; Gutiérrez-López et al., 2016; Palinauskas et al., 2016). In order to dissect the salivary glands or obtain a saliva sample, the mosquito must be fresh or alive, and must not have been previously frozen. However, mosquito dissection is not always compatible with field conditions and the number of engorged mosquitoes that fed on infected birds that show parasites in their salivary glands or saliva is usually very low (between 1 and 5%; Alves, 2012; Gutiérrez-López et al., 2020). In addition to fieldwork, experimental studies in laboratory conditions seem therefore essential to evaluate the competent mosquito species for avian *Plasmodium* lineages, even if it requires complex logistics to (i) test several lineages at the same time, and (ii) keep mosquitoes in colonies and birds in captivity. Among many lines of research that could improve our understanding of vector specificity, future projects should focus on (i) questioning the parasite

transmission capacity of mammophilic mosquitoes since, so far, *Plasmodium* lineages have been found in head/thorax only and not in salivary glands, (ii) testing the vector competence of mosquitoes that share the same habitat as *Cx. pipiens* but are present in lower abundance, (iii) determining if the infection status of the mosquitoes influences their host selection, using a wide range of mammals and bird species, or, (iv) evaluating if lineages with different host specialization strategies (specialists vs. generalists) are transmitted equally by mosquitoes.

CONCLUSION

Research projects based on a global approach to host-parasite relationships that integrates the pivotal role of vectors in avian malaria transmission are still very rare, but essential to understand the evolutionary strategies of parasites. Although molecular ecology tools helped make great strides, there is still a long way to go to understand parasite-vector-host relationships, especially given the high diversity of known parasites and potential vectors. To date, data from the literature suggest that avian *Plasmodium* lineages present a gradient of host specificity from highly specialist to highly generalist, and relatively high vector specificity (i.e., each lineage uses only a few vectors). However, true vector specificity of avian *Plasmodium* needs to be clarified by obtaining larger datasets about vector-parasite interactions in the field and by demonstrating vector competence. Among factors that could influence interactions between vectors and their hosts and parasites, the feeding behavior of mosquitoes may be a key factor that influences vector specialization, although this remains to be proven. Multidisciplinary teams, including ecologists, ornithologists, parasitologists and entomologists, should work hand in hand to understand fully the range of parasite evolutionary strategies in both hosts and vectors.

DATA AVAILABILITY STATEMENT

The raw data of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

RG-L and CL conceived the idea for this study, extracted the data from MalAvi, and wrote the first draft. CL carried out the statistical analyses. VB commented on the setup and assisted during the writing of the paper and revisions. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.569230/full#supplementary-material

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ORIGINAL RESEARCH
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Effects of Chemical and Auditory Cues of Hoopoes (*Upupa epops*) in Repellence and Attraction of Blood-Feeding Flies

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Research on the mechanisms involved in host location by parasites is of paramount importance and may aid in developing protective measures against them. This topic attains far-reaching repercussions for human and animal welfare regarding parasites transmitting vector-borne pathogens, such as blood-feeding flies. Very few studies have evaluated the effect of bird-derived cues on attraction of vectors in field conditions. We here explored the attraction of different groups of blood-feeding flies (mosquitoes, blackflies and biting midges) to auditory cues produced by begging hoopoe (*Upupa epops*) nestlings, and to three chemical cues derived from hoopoe nestlings or nests (uropygial secretion, symbiotic bacteria isolated from the secretion, and nest material) in the field. We deployed insect traps baited with the different stimuli at the beginning and at the end of the hoopoe breeding-season in four different habitats. Abundance of blood-feeding flies varied depending on habitat and sampling period. Begging auditory cues of nestling hoopoes did not affect abundance of flies. However, chemical stimuli affected abundance of mosquitoes, which were less abundant in traps baited with bacteria or with nest material than in control traps. Abundance of biting midges in traps also depended on the chemical stimulus but in interaction with sampling period or habitat. Fewer biting midges were collected in traps baited with bacteria and with secretion in the habitats where abundance of biting midges is higher. Our results suggest that uropygial secretion of hoopoes, and symbiotic bacteria living in this secretion, may repel blood-feeding flies from their nests.

Keywords: begging, Ceratopogonidae, Culicidae, ectoparasite repellent, *Enterococcus faecalis*, host location mechanisms, Simuliidae, uropygial gland

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INTRODUCTION

Parasitism is one the most important selection pressures influencing evolution across most taxa (Combes, 2001; Poulin, 2011). Thus, understanding the processes underlying and governing parasite-host interactions is paramount from ecological, epidemiological and evolutionary points of view (Schmid-Hempel, 2011; Webster and Cardé, 2017; Chandrasegaran et al., 2020). For efficient

parasitism, the parasite first needs to establish contact with the host. The study of factors mediating attraction of the parasite to locate and colonize a host have long been searched for (e.g., Sutcliffe, 1986, 2010; Allan et al., 1987), especially for some selected parasite types of medical and veterinary importance (Mullen and Durden, 2002). However, it is recognized that after decades of research, our comprehension of these factors is still limited and incomplete for many parasite taxa (Tomás et al., 2008b; Tomás and Soler, 2016; Webster and Cardé, 2017; Veiga et al., 2020).

Birds and their ectoparasites continue to be fruitful model systems to shed light on many aspects of parasite-host interactions (Loye and Zuk, 1991; Clayton and Moore, 1997). Among all ectoparasites of birds, our knowledge on free-living blood-feeding flies still lies far behind that of nest-dwelling ectoparasites, mainly due to the more ephemeral contact with their host of the former, which poses subsequent challenges for their sampling and study (Tomás et al., 2008a; López-Rull and Macías-García, 2015). Yet among all sort of blood-feeding flies affecting birds, three dipteran families have received the most attention by researchers, mainly because of their nearly world-wide distribution and of their importance in the transmission of vector-borne pathogens. These vectors are commonly named mosquitoes, blackflies and biting midges.

Mosquitoes (Culicidae) are the best-known family of blood-feeding flies due to their major importance as vectors of human diseases (Foster and Walker, 2002). They are also vectors of infection for birds all over the world, being the main responsible for transmission of malaria parasites of the genus *Plasmodium*, and of several viruses (Naugle et al., 2004; Valkiûnas, 2005; Becker et al., 2010). Despite being relatively large-sized and reaching high abundances in many areas, the direct impact on wild birds caused by blood-sucking and associated nuisances of these mainly crepuscular or nocturnal insects is scarcely known. Blackflies (Simuliidae) are small flies present in all continents except Antarctica. Blackflies can build up huge populations specially in boreal ecosystems (Malmqvist et al., 2004). These mainly diurnal flies can cause nestling mortality (Hunter et al., 1997; Smith et al., 1998), complete nest failures (Solheim et al., 2013) and even provoke mass desertions of bird colonies (Bukaciński and Bukacińska, 2000). They are known vectors of filarial nematodes, bacteria, viruses and haemosporidians such as *Leucocytozooon* or *Haemoproteus* malarial parasites (Adler and McCreadie, 2002; Adler et al., 2004). Biting midges of the genus *Culicoides* (Ceratopogonidae) are tiny flies also globally distributed that can reach huge abundances in certain areas, especially in tropical and subtropical regions (Mullen, 2002). They are mainly crepuscular or nocturnal, and have detrimental effects on nestling birds (Tomás et al., 2008b). Biting midges are known to transmit viruses, filarial worms, and several malarial parasites of the genus *Haemoproteus* and *Plasmodium* (Borkent, 2005; Valkiûnas, 2005; Martínez-de la Puente et al., 2011a).

Host seeking by these blood-feeding insects relies on the detection of host associated cues, which include body heat, carbon dioxide (CO_2), and different odors (Lehane, 2005; Takken and Knols, 2010; Cardé, 2015; Lazzari, 2019; Castaño-Vázquez et al., 2020; Greppi et al., 2020). Long range detection of hosts is assumed to rely on chemical cues, which are combined with heat and visual cues when in closer proximity to hosts (Sutcliffe, 1986; Allan et al., 1987; Gibson and Torr, 1999; Cardé, 2015; Greppi et al., 2020). It has also been suggested that ectoparasites might use bird host auditory cues for host location (Tomás and Soler, 2016), though this hypothesis has not yet been tested. Members of several ectoparasite groups locate their hosts by means of host auditory cues (Tomás and Soler, 2016). These include Sarcophagidae and Tachinidae parasitoid flies attracted to calling crickets and cicadas (Lehmann, 2003; Farris et al., 2009), and also Chaoboridae phantom midges, Corethrellidae frog-biting midges and Culicidae mosquitoes attracted to frog calls (Borkent, 2008; Toma et al., 2019). Anecdotal evidence further suggests that Corethrellidae and mosquitoes can be attracted to bird calls or songs (Camp, 2006; Bartlett-Healy et al., 2008; see Tomás and Soler, 2016). Overall, current knowledge on the array and specificity of cues used by blood-feeding flies to locate hosts is still incomplete for many parasite groups. Even different related parasites may not be affected by the same array of cues for host location, a possibility that has received little attention. Regarding chemical cues derived from birds that may be involved in host detection by blood-feeding flies, several odor sources have been investigated. For instance, mosquitoes (Cooperband et al., 2008) and biting midges (Fernandes-Rios et al., 2020) are attracted to traps baited with bird feces. Yet, odors emitted or derived from the uropygial gland of birds have been repeatedly suggested as major candidates involved in attraction of biting flies. Thus, extracts of uropygial glands of crows *Corvus brachyrhynchus* and common loons *Gavia immer* have been shown to attract mosquitoes (Russell and Hunter, 2005) and blackflies (Fallis and Smith, 1964; Bennett et al., 1972), respectively. Birds continuously rub their uropygial secretion onto their plumage and skin, which confers them specific odor signatures. This may explain that mosquitoes and blackflies are also attracted to chemical cues derived from bird feathers, in combination with CO_2 (Allan et al., 2006) or with visual cues provided by bird decoys (Weinandt et al., 2012), respectively. Mosquitoes are also attracted to different chemical compounds prevalent in the odorant profile of birds (and humans) (Syed and Leal, 2009). In contrast, other studies have not found attraction of blood-feeding flies to uropygial secretions of birds. Biting midges and blackflies were not attracted to uropygial secretions of blue tits *Cyanistes caeruleus* and feral pigeons *Columba livia* (Martínez-de la Puente et al., 2011b), while mosquitoes were not attracted to uropygial secretions of house sparrows *Passer domesticus* (Garvin et al., 2018a; Díez-Fernández et al., 2019, 2020). Alternatively, certain odors released or derived from the uropygial gland may be employed by birds as repellents of ectoparasites (Moyer et al., 2003).

The hoopoe (*Upupa epops*) can be regarded as a quite special case in relation to its uropygial secretion. The uropygial gland of this bird is unusually large and, only in reproducing females and nestlings, produces a dark and pungent, malodorous secretion (Soler et al., 2008; Martín-Vivaldi et al., 2009, 2010). The hoopoe (and two related species) is unique in harboring symbiotic Enterococci bacteria in their uropygial secretion, which produce antibiotic substances (Soler et al., 2008). *Enterococcus faecalis*

is by far the most prevalent bacteria present when aerobically culturing hoopoe uropygial secretions (Soler et al., 2008; Ruiz-Rodríguez et al., 2012). Analysis of bacterial abundance by different methodologies revealed consistently high bacterial loads in uropygial secretions (Soler et al., 2008; Rodríguez-Ruano et al., 2018), and similar bacterial community composition in birds from different nests (Rodríguez-Ruano et al., 2018). This secretion is rubbed into plumage and eggs through preening (Soler et al., 2014; Martínez-García et al., 2015), and it has been shown to increase hatching success (Martín-Vivaldi et al., 2014). The uropygial gland microbiome is partially acquired from the environment, and nest material is thought to be a source of bacteria (Martín-Vivaldi et al., 2018; Díaz-Lora et al., 2019; Campos-Cerda and Bohannan, 2020). Hoopoes do not add materials to the cavity, so nest materials mainly comprise rotten wood from cavity walls, old nest debris and feces and prey remains (Martín-Vivaldi et al., 2016). Besides the antimicrobial function, uropygial secretions may play a role in sexual selection (Whittaker et al., 2011; Amo et al., 2012; Díaz-Lora et al., 2020) or deterring predators (Jacob and Ziswiler, 1982) or ectoparasites (Soler et al., 2010).

The abundance and composition of vector and host species in the community vary across time and space, geographical variability being influenced for instance by environmental variables (e.g., Ferraguti et al., 2018; van Hoesel et al., 2019; Nourani et al., 2020). Vector species composition and abundance may affect host investment in antiparasite or immunological defenses (e.g., Moyer et al., 2002). Both factors, variation in vector species and in host traits, might in turn influence the cues employed by blood-feeding flies to locate their avian hosts in any given habitat (Webster and Cardé, 2017). It is important therefore to take into account seasonal and spatial variability when researching on host location mechanisms by vectors.

In this study, we assessed the potential role of auditory and chemical cues of hoopoes in the attraction of blood-feeding flies to insect traps in the field. Specifically, we assessed the potential role of nestling begging auditory cues, and of chemical cues released by the uropygial secretion, by bacteria living in the uropygial secretion, and by the nest substrate. We expected that these auditory and chemical cues were used by blood-feeding flies for host location and acted as attractants. Alternatively, traps baited with these auditory or chemical stimuli may render fewer captures if these stimuli acted as repellents for blood feeding ectoparasitic flies.

MATERIALS AND METHODS

Study Area

The study was carried out during the breeding season of 2019 in the Hoya de Guadix (37° 18′ N, 38° 11′ W), located in the south east of Spain (e.g., Martín-Vivaldi et al., 1999). The experiment was performed in four different habitats representative of the main variability found in the study area. These habitats were: (1) *irrigated* (irrigated crops where fruit trees, including olive trees, as well as vegetables and greens are grown); (2) oak *savannah* (Mediterranean savannah, with scattered holm-oak (*Quercus ilex*) trees); (3) *steppe* (high-altitude arid plateaux with scarce vegetation represented mainly by a few *Retama sphaerocarpa* and crops of *Prunus dulcis*); and 4) *pine* forest (*Pinus pinaster* and *P. halepensis* orchards scattered within the arid plateaux). Distance between these sampled locations at different habitats ranges from 3.5 to 15 km. Proximity to waterbodies suitable for oviposition of vectors (small irrigation channels and ponds) is higher in the irrigated habitat, intermediate in the savannah and pines habitat, and lower in the steppe habitat. Hoopoe nests are scattered in the area, with higher densities in the pines habitat, intermediate in the savannah and irrigated habitat, and lower densities in the steppe.

Sampling of Blood-Feeding Flies

We carried out two samplings of vectors of three consecutive days each. The first sampling (May 4th–May 7th) was conducted coinciding when most bird species in the area start reproduction, and the second sampling (June 3rd–June 6th) was conducted when most birds in the area are raising nestlings. Weeks with similar and stable weather, and with no rain, were selected for both sampling periods. For each of the sampling periods and in each of the four habitats, we installed four CDC miniature downdraft suction traps (model 1212; John W. Hock, Gainesville, FL) but with the lights removed to focus exclusively on attraction or repulsion caused by the tested stimuli (see Russell and Hunter, 2005). The traps were placed at 1.5 m above ground, in the shadow provided by vegetation cover, and were operated for 24 h periods (Silver, 2008a; Becker et al., 2010). Distance between the four traps within each habitat was 25 m. Location of the traps was the same in both sampling periods. Traps were baited with a source of CO_2 and a combination of chemical and auditory stimulus. Dry ice was used as a source of CO_2. For that, 800 g of dry ice maintained in a cool box were used in each trap per 24 h, to ensure the continued emission of CO_2 from a standard cannula (ref. G8581, Entomopraxis, Barcelona, Spain) located close to the fan of the trap (Veiga et al., 2018).

Auditory Stimulus

As auditory stimulus, we played back a recording of begging calls of hoopoe nestlings. This recording was constructed with 20 sequences of begging, each lasting between 0.5 and 2 min, recorded from five nests (four sequences from each nest) when nestlings were 2/3–3/3 grown. These sequences were played back in random order but alternated with a 2 min silence, thus simulating a maximum of one provisioning visit by a parent every 2.5 min. This 60 min-recording was played back in a MP3 device at 65 dB (measured at 20 cm of the speaker, which was hung close to the trap fan entrance) in a continuous loop for 6 h (the duration of the battery of the MP3 device). A speaker with no sound was used as the control auditory treatment. Traps were deployed at 19:00 h and collected insects for 24 h periods. All traps were checked in the morning, to replace batteries and MP3 devices, so that auditory stimuli were displayed for 12 of the 24 h periods.

Chemical Stimuli

As chemical stimuli, we used one of three different products derived from hoopoe nestlings (or nests) presented in a Petri

dish inside a black and opaque cotton bag hung close to the fan entrance of the trap (an empty Petri dish was used as *Control* treatment): (1) *Secretion*: malodorous uropygial secretion of hoopoe nestlings. The secretion of nestlings when 2/3–3/3 grown was collected from active nests in the area following the protocol described in Soler et al. (2008). Briefly, the secretion was collected using an automatic 1–10 μl micropipette with a previously autoclaved tip that was gently introduced into the opening of the papilla of the uropygial gland and directly pipetted the secretion. The collected secretion was introduced in sterile microcentrifuge tubes that were kept frozen at −20°C until usage. For the experiment, the secretion of all nestlings in a nest was collected with a cotton swab that was then fixed inside a Petri dish (see Russell and Hunter, 2005). Secretion from different nests was used for different traps and days. (2) *Nest*: nest material and debris collected the day before the experiment from active hoopoe nests and kept at 4°C until usage. The amount needed to fill a Petri dish was used. Nest material from different nests was used for different traps and days. (3) *Bacteria*: a culture from our laboratory collection of the bacterium *Enterococcus faecalis (MRR-10.3)*, isolated from hoopoe uropygial secretions. The bacterium was grown on Brain-Heart Infusion (BHI) medium at 37°C for 12 h and then BHI containing bacteria was spread onto TSA (Tryptic Soy Agar) plates by streaking technique and incubated again at 37°C for 24 h before being used as stimulus.

Within each habitat, two traps were baited with one of the three chemical treatments and two traps were controls, while one trap with each treatment was presented also with sound stimulus and the other with control sound (**Table 1**). After 24 h of sampling, the chemical treatment corresponding to the area was changed with a second chemical treatment. Again the next day, the third chemical treatment was applied to the area. Therefore, both auditory and chemical stimuli were always presented with their respective control treatments, while the chemical experimental treatment was changed from the first to the third day within the same sampling period, so that all habitats had the three chemical treatments in three consecutive days (**Table 1**). The order of treatment presentation and whether each trap within each habitat had control or experimental stimuli were randomly assigned. Thus, by assuming that abundance of blood-feeding flies does not differ within the same sampling period, we got a full factorial design of the auditory and chemical stimuli presented.

Statistical Analyses

Abundance of mosquitoes, blackflies and biting midges were Box-Cox transformed before analyses. A multivariate analysis of variance (MANOVA) was used to first explore the potential effects of habitat and sampling period (as categorical predictors) on abundance of blood-feeding flies, with abundance of mosquitoes, blackflies and biting midges as dependent variables. Similar MANOVAs were used to explore differences in abundance of these blood-feeding flies between auditory and chemical stimuli separately, with habitat and sampling period as categorical predictors. Two-order interactions between sampling period, habitat and treatment (either auditory or chemical stimuli) were also explored. In all analyses, main effects were explored in models that did not include the interactions, while the interactions were estimated in models that included main effects. Fisher LSD tests were used for *post hoc* comparisons between the different experimental treatments and the control treatment. Analyses were conducted with Statistica 12 (StatSoft Inc, 2007). Residuals of the models were checked for normality. Means ± 95% confidence intervals (CI) are shown.

RESULTS

Abundance of Blood-Feeding Flies

Mosquitoes were collected in 41 out of 96 traps (42.7%), ranging from 0 to 18 individuals, with an average of 3.1 ± 1.3 mosquitoes (excluding traps with no captures). Blackflies were collected in 64 out of 96 traps (66.7%), ranging from 0 to 242 individuals, with an average of 20.9 ± 10.5 blackflies. Biting midges (*Culicoides* spp.) were collected in 40 out of 96 traps (41.7%), ranging from 0 to 24 individuals, with an average of 5.1 ± 1.8 biting midges. Seventy-eight traps captured at least one blood-feeding fly, while 18 traps rendered zero captures. For the three groups of blood-feeding flies, most traps rendered a few vectors or none at all, while others have many, which resulted in typical aggregated right-skewed distributions of parasite abundance.

Habitat and Seasonal Differences

Abundance of blood-feeding flies varied depending on habitat and sampling period (**Table 2**). Blackflies, but not mosquitoes or biting midges, were more abundant at the beginning than at the end of the bird breeding season (**Table 3** and **Figure 1**). Seasonal differences for these three blood-sucking vectors however depended on habitat types (**Table 3**). Mosquitoes were more abundant in the irrigated habitat, mainly during the second sampling period in early June (**Table 2**). The lower abundance of mosquitoes was detected in the steppe habitat (**Table 2**). Blackflies were more abundant in pine forest and in oak savannah, but mainly during the first sampling period, at early May (**Table 2**). Again, independent of the sampling period, the lower abundances were detected in the steppe habitat (**Table 2**). Biting midges were also more abundant in the pine forest and, during the second sampling period, they were also abundant in the irrigated habitat (**Table 2**).

Effects of Auditory and Chemical Stimuli

Auditory stimuli did not affect abundance of blood-feeding flies in the traps after controlling for the effects of habitat and sampling period, neither as main effect nor in interaction with habitat or sampling period (**Table 3**). Chemical stimuli however affected abundance of mosquitoes and biting midges in the traps, but did not affect blackfly attraction (**Table 3**). Mosquitoes were less abundant in traps baited with bacteria or nest material odors than in control traps (Fisher LSD tests: bacteria *vs.* control: $p = 0.016$; nest *vs.* control: $p = 0.026$), and this effect was more apparent in the irrigated habitat (main effect in **Table 3** and **Figure 2**). Abundance of mosquitoes did not differ between traps baited with the secretion and control traps ($p = 0.670$). Thus, bacteria from hoopoe secretion as well as nest material may

TABLE 1 | Assignment of hoopoe stimuli (chemical + auditory) to traps for capturing blood-feeding flies in relation to day of sampling and habitat in Guadix, south eastern Spain.

		Habitat			
	Trap	**Irrigated**	**Savannah**	**Steppe**	**Pines**
Day 1	1	Control+Begging	Nest+Silence	Nest+Begging	Control+Silence
	2	Bacteria+Begging	Control+Begging	Nest+Silence	Secretion+Silence
	3	Bacteria+Silence	Nest+Begging	Control+Begging	Control+Begging
	4	Control+Silence	Control+Silence	Control+Silence	Secretion+Begging
Day 2	1	Secretion+Begging	Control+Begging	Control+Silence	Nest+Begging
	2	Secretion+Silence	Bacteria+Begging	Secretion+Silence	Control+Silence
	3	Control+Silence	Control+Silence	Secretion+Begging	Nest+Silence
	4	Control+Begging	Bacteria+Silence	Control+Begging	Control+Begging
Day 3	1	Control+Silence	Secretion+Begging	Bacteria+Silence	Control+Begging
	2	Control+Begging	Secretion+Silence	Control+Begging	Bacteria+Begging
	3	Nest+Begging	Control+Silence	Control+Silence	Bacteria+Silence
	4	Nest+Silence	Control+Begging	Bacteria+Begging	Control+Silence

TABLE 2 | Abundance of mosquitoes, blackflies, and biting midges [mean and 95% confidence intervals (CI)] collected in traps in four different habitats (irrigated, oak savannah, steppe and pine forest) and in two sampling periods (May and June).

Habitats		**Mosquitoes**	**Blackflies**	**Biting midges**
	N	**Mean (95%CI)**	**Mean (95%CI)**	**Mean (95%CI)**
May				
Irrigated	12	1.42 (0.50–2.33)	5.08 (0.89–9.28)	0.83 (0.08–1.59)
Savannah	12	0.58 (0.16–1.01)	17.83 (6.25–29.42)	1.42 (-0.29–3.12)
Steppe	12	0.08 (-0.10–0.27)	0.08 (-0.10–0.27)	0.08 (-0.10–0.27)
Pines	12	0.50 (0.17–0.83)	77.83 (31.64–124.02)	10.17 (5.01–15.33)
All habitats	48	0.65 (0.37–0.92)	25.21 (11.25–39.17)	3.13 (1.42–4.83)
June				
Irrigated	12	7.08 (3.14–11.02)	3.17 (0.95–5.38)	2.67 (1.10–4.23)
Savannah	12	0.75 (0.20–1.30)	3.58 (-0.38–7.55)	0.08 (-0.10–0.27)
Steppe	12	0.08 (-0.10–0.27)	0.42 (-0.16–0.99)	–
Pines	12	0.25 (-0.14–0.64)	3.67 (1.43–5.91)	1.58 (0.48–2.68)
All habitats	48	2.04 (0.81–3.27)	2.71 (1.51–3.90)	1.08 (0.55–1.62)
Both periods				
Irrigated	24	4.25 (2.03–6.47)	4.13 (1.91–6.34)	1.75 (0.86–2.64)
Savannah	24	0.67 (0.35–0.99)	10.71 (4.30–17.12)	0.75 (-0.09–1.59)
Steppe	24	0.08 (-0.04–0.20)	0.25 (-0.04–0.54)	0.04 (-0.04–0.13)
Pines	24	0.38 (0.13–0.62)	40.75 (14.15–67.35)	5.88 (2.82–8.93)
All habitats	96	1.34 (0.71–1.98)	13.96 (6.71–21.21)	2.10 (1.20–3.01)

Sample size (N = number of traps) and mean values of habitat-pooled information for each sampling period and for both periods together are also shown.

repel mosquitoes from hoopoe nests. Moreover, abundance of biting midges depended on the used olfactory stimulus but in interaction with either sampling period or habitat (**Table 3**). Abundance of biting midges did not differ between control traps and traps baited with any of the olfactory stimuli, in first and second sampling periods (all $p > 0.05$). During the first sampling period, biting midges were more abundant in traps baited with bacteria stimulus than in those with nest material, while the opposite was found during the second sampling period (**Figure 1**). The effects of olfactory stimuli on biting midge abundance also depended on the habitat (**Table 3**). Traps baited with bacteria in the irrigated habitat, and with secretion in the pine forest, significantly captured less biting midges than control traps (bacteria *vs.* control in irrigated: $p = 0.019$; secretion *vs.* control in pines: $p = 0.008$; **Figure 2**). However, in the pine forest, traps baited with nest material captured more biting midges than control traps ($p = 0.015$). Given that biting midges were more abundant in the irrigated and pine habitats (**Table 2**), these results suggest that uropygial secretion of hoopoes, and symbiotic bacteria living in this secretion, might repel biting midges from their nests depending on habitat. Chemical cues released by nest material instead may attract biting midges to hoopoe nests, also depending on habitat.

DISCUSSION

Research on the factors and mechanisms involved in host location by ectoparasites is of paramount importance and may aid in developing protective measures against these parasites (Webster and Cardé, 2017; Chandrasegaran et al., 2020). This topic attains far-reaching repercussions for human and animal

TABLE 3 | Results from multivariate and univariate ANOVA exploring the effects of sampling period and habitat, together with the experimental effect of auditory and chemical stimuli, on abundance of blood-feeding flies collected in traps.

	MANOVA results				Univariate results						
						Mosquitoes		Blackflies		Biting midges	
	Wilks	F	df	P	df	F	P	F	P	F	P
Sampling period (1)	**0.864**	**4.66**	**3, 89**	**0.005**	1,91	0.52	0.471	**14.24**	**0.0003**	0.97	0.328
Habitat (2)	**0.283**	**16.40**	**9, 216.8**	**<0.0001**	3,91	**16.97**	**<0.0001**	**24.80**	**<0.0001**	**15.81**	**<0.0001**
(1) × (2)	**0.632**	**4.837**	**9, 209.5**	**<0.0001**	3,88	**3.23**	**0.026**	**6.77**	**0.0004**	**4.54**	**0.005**
Auditory stimuli											
Sampling period (1)	**0.864**	**4.61**	**3, 88**	**0.005**	1,90	0.52	0.471	**14.10**	**0.0003**	0.97	0.327
Habitat (2)	**0.281**	**16.31**	**9, 214.3**	**<0.0001**	3,90	**16.95**	**<0.0001**	**24.57**	**<0.0001**	**15.89**	**<0.0001**
Begging (3)	0.965	1.06	3, 88	0.371	1,90	0.85	0.358	0.13	0.722	1.48	0.226
(1) × (2)	**0.625**	**4.67**	**9, 197.3**	**<0.0001**	3,83	**3.18**	**0.028**	**6.59**	**0.0005**	**4.47**	**0.006**
(1) × (3)	0.969	0.87	3, 81	0.460	1,83	1.58	0.213	1.74	0.190	0.02	0.876
(2) × (3)	0.945	0.52	9, 197.3	0.860	3,83	0.38	0.766	0.27	0.846	0.68	0.565
Chemical stimuli											
Sampling period (1)	**0.855**	**4.85**	**3, 86**	**0.004**	1,88	0.56	0.456	**14.66**	**0.0002**	0.94	0.334
Habitat (2)	**0.270**	**16.59**	**9, 209.5**	**<0.0001**	3,88	**18.13**	**<0.0001**	**25.54**	**<0.0001**	**15.4**	**<0.0001**
Odor (3)	0.859	1.50	9, 209.5	0.149	3,88	**3.06**	**0.032**	1.90	0.136	0.28	0.843
(1) × (2)	**0.559**	**5.19**	9, 172.9	**<0.0001**	3,73	**3.35**	**0.023**	**6.76**	**0.0004**	**6.09**	**0.0009**
(1) × (3)	0.817	1.67	9, 172.9	0.100	3,73	0.70	0.552	0.92	0.438	**3.13**	**0.031**
(2) × (3)	**0.562**	**1.68**	27, 208.0	**0.023**	9,73	0.73	0.676	0.62	0.775	**3.82**	**0.0005**

Statistical significant effects are shown in bold.

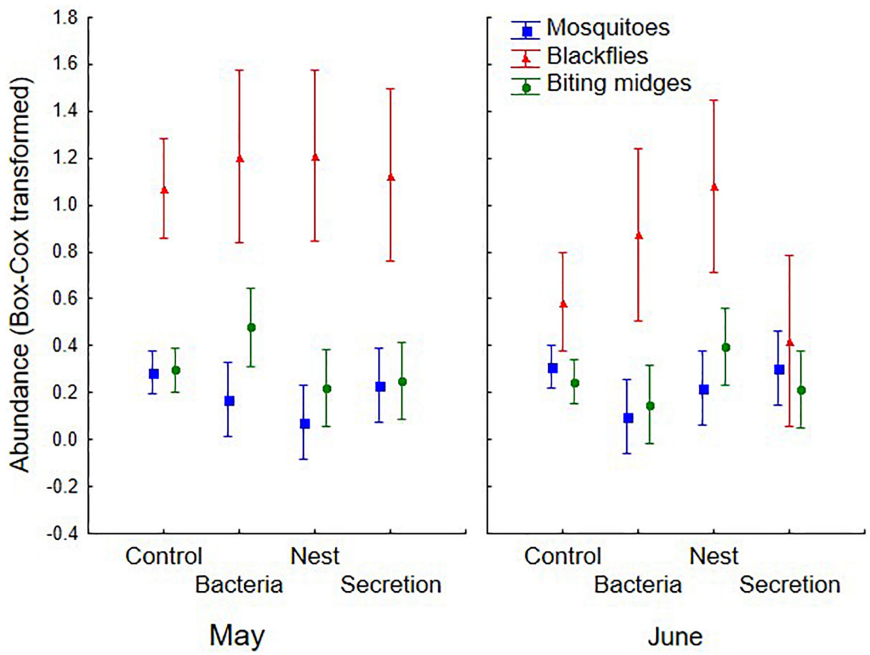

FIGURE 1 | Abundance of blood-feeding flies (mosquitoes, blackflies, and biting midges) collected in traps baited with different chemical stimuli derived from hoopoe nestlings or nests (uropygial *secretion*, *nest* material, or *bacteria* found in the secretion), or without stimulus (*control*), and in two sampling periods (May and June). Mean and 95% confidence intervals (CI) are shown.

welfare regarding those parasites transmitting vector-borne pathogens, such as blood-feeding flies which include mosquitoes, blackflies and biting midges (Mullen and Durden, 2002; World Health Organization [WHO], 2020). We have found seasonal and habitat differences in abundance of blood-feeding flies that also varied depending on the vector considered.

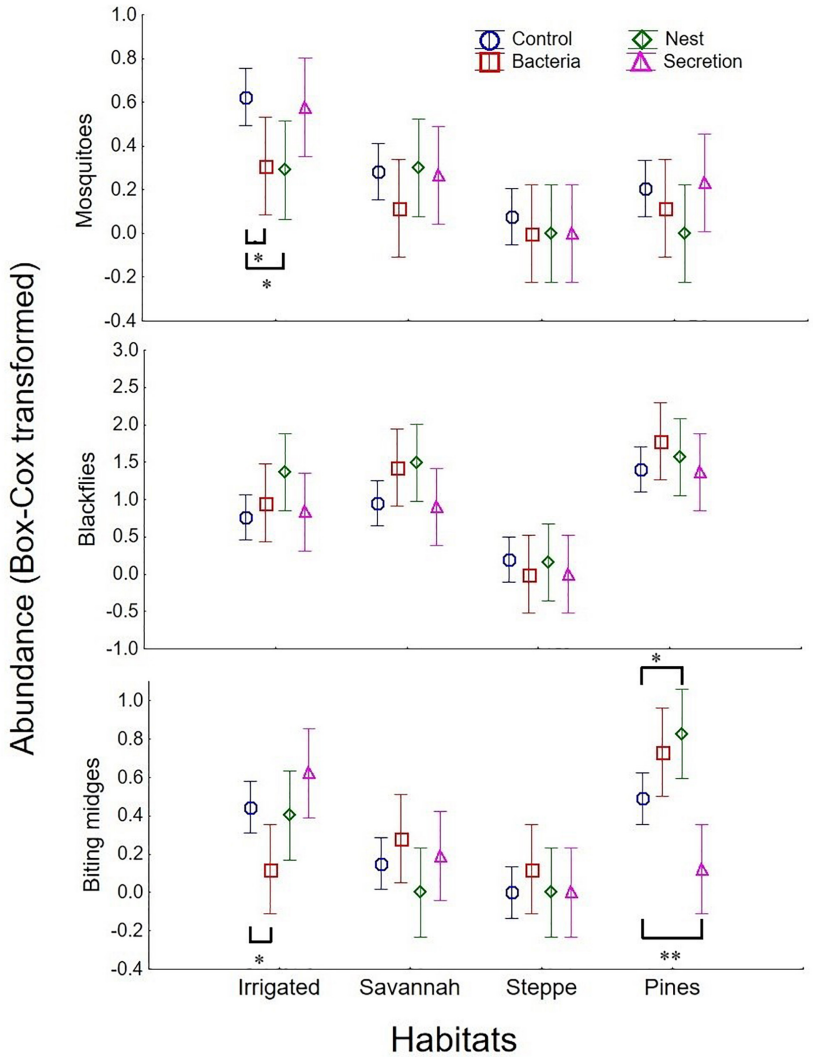

FIGURE 2 | Abundance of blood-feeding flies (mosquitoes, blackflies, and biting midges) collected in traps baited with different chemical stimuli derived from hoopoe nestlings or nests (uropygial *secretion*, *nest* material, or *bacteria* found in the secretion), or without stimulus (*control*), and in four different habitats (irrigated, oak savannah, steppe and pine forest). Mean and 95% confidence intervals (CI) are shown. Statistically significant differences between the different chemical stimuli and the control in each habitat are also shown (*$p < 0.05$; **$p < 0.01$).

Blackflies were more abundant at the beginning than at the end of the birds breeding season. When sampled inside avian nest-boxes in an oak forest in central Spain, both blackflies and biting midges were more abundant at nestling than at incubation stages, as well as in later than in early nests (Tomás et al., 2008a, 2012; Martínez-de la Puente et al., 2009a). Besides differences in sampling method, if we assume that obtained abundances reflect real abundances in our study area, this discrepancy in seasonal patterns may be partly due to habitat differences. Abundance of certain blood-feeding flies such as blackflies is greater at forested habitats (Martin et al., 1994). In accordance with this, we have found that blood-feeding flies are more abundant in the pines and irrigated habitats, and less abundant in the steppe habitat, which is less forested. This pattern might also match the relative abundance of hoopoes in the different habitats.

Current knowledge on dispersal patterns in blood-feeding flies in general is rather poor (e.g., Veiga et al., 2020). Apparently, dispersal of blackflies is concentrated within a few kilometers of the natal waters (Baldwin et al., 1975). Nevertheless, blackflies, by being active during the day when air convection is higher than during the night, may disperse over longer distances than other blood-feeding flies with more restricted nocturnal activity patterns (Service, 1980). This may explain that blackflies were the most abundant blood-feeding flies in the steppe habitat, which is situated further from potential oviposition sites. However, abundance of blackflies (and biting midges) in nests was not directly related with distance to water sources (i.e., oviposition sites) in another area (Tomás et al., 2008a). Besides dispersal behavior, environmental conditions may also explain the observed habitat differences in abundance of blood-feeding

flies. Their activity is affected by weather variables including wind speed, temperature and humidity (e.g., Service, 1980; Garvin and Greiner, 2003; Martínez-de la Puente et al., 2009a), some of which may show marked differences among sampled habitats, even though distance among them is relatively small.

It has been suggested that ectoparasites might take advantage of auditory cues of their hosts for host location, likely in combination with other host cues (Tomás and Soler, 2016). The present study represents the first formal test of this hypothesis. However, we did not find that auditory cues of begging hoopoe nestlings attract mosquitoes, blackflies or biting midges to traps, even though auditory cues were presented in combination with CO_2 and with several chemical stimuli. We cannot rule out the possibility that sound plays a role in host detection under different experimental conditions in other host-ectoparasite systems (Tomás and Soler, 2016). This hypothesis was mainly postulated for ectoparasites detecting auditory cues of begging offspring. Besides the potential attraction of auditory cues derived from begging nestlings, other host auditory cues may be detected by different ectoparasites (Tomás and Soler, 2016). In the past, for example, sound of wing beats of female mosquitoes was evaluated as attractant for increasing trap catches of various species of mosquitoes (Bidlingmayer, 1967; Silver, 2008b), so further research is necessary to fully test the hypothesis.

Regarding olfactory cues, we have found that different chemical stimuli affected attraction of blood-feeding flies to traps. First, fewer mosquitoes were collected in traps baited with bacteria and with nest material than in control traps. This effect was especially apparent in the irrigated habitat, where mosquitoes were more abundant. Second, abundance of biting midges collected in traps varied in relation to chemical stimuli, but in interaction with sampling period and with habitat. Depending on the sampling period, either traps baited with bacteria or with nest material collected fewer biting midges. This suggests that the main odor source in nests that is relevant for host detection by vectors might change during the breeding period of the birds. However, fewer biting midges were collected in traps baited with bacteria in the irrigated habitat and with uropygial secretion in the pines habitat compared to control traps. On the other hand, in the pines habitat, more biting midges were collected in traps baited with nest material than in control traps. It is noteworthy that the larger abundances of biting midges were detected in the irrigated and the pines habitats. Thus, results suggest that the uropygial secretion of hoopoes, as well as the chemical cues delivered by symbiotic bacteria living in this secretion, are not attractants for blood-feeding flies but might instead act as repellents for mosquitoes and biting midges.

Although all chemical stimuli came from hoopoes, they did not have the same effects attracting or repelling blood-feeding flies. Blackflies were not apparently affected by any of the stimuli presented, and CO_2 may be a major attractant for this group (Sutcliffe, 2010). Nest material repelled mosquitoes, but attracted biting midges in the pines habitat. Uropygial secretion repelled biting midges in the pines habitat, while a culture of Enterococci isolated from hoopoe secretion repelled both mosquitoes and biting midges in the irrigated habitat. That the effect of the different chemical stimuli varies depending on the habitat may be explained by habitat differences in the abundance (or species composition) within these different blood-feeding flies. Additionally, it might suggest that different vector species may follow different cues for host location (e.g., Mullens and Gerry, 1998; Russell and Hunter, 2005; Allan et al., 2006; Gerry et al., 2009). Identifying blood-feeding flies to species level would be a next step to shed light on these possibilities. In addition, visual and olfactory associative learning of the vectors may affect host preferences (Takken and Verhulst, 2013). Uropygial secretion of hoopoes is special in harboring Enterococci symbiotic bacteria, especially in females during reproduction and in nestlings (Soler et al., 2008; Martín-Vivaldi et al., 2009, 2010). Hoopoes spread this secretion onto their plumage, brood patch and nest contents when preening (Martín-Vivaldi et al., 2014, 2018; Soler et al., 2014), while the nest environment is also an important source of bacteria (Ruiz-Rodríguez et al., 2014; Martínez-García et al., 2015, 2016; Rodríguez-Ruano et al., 2015; Martín-Vivaldi et al., 2018; Díaz-Lora et al., 2019). Therefore, multiple sources interact to create the microbiome assembly of hoopoe nesting environments. This microbiome may be a major component of chemical cues produced (Caro et al., 2015), with a prominent role of the nest, the parents and the offspring in integrating what has been termed the nidobiome (Soler et al., 2016; Campos-Cerda and Bohannan, 2020).

Different odors emitted by birds have been evaluated as attractants of vectors, from feces that attract mosquitoes (Cooperband et al., 2008) and biting midges (Fernandes-Rios et al., 2020), to bird feathers and skin that attract mosquitoes (Allan et al., 2006) and blackflies (Lowther and Wood, 1964; Weinandt et al., 2012). It has been also shown that mosquitoes are attracted to birds (Cornet et al., 2013a,b; Yan et al., 2018), or to odor from birds (Díez-Fernández et al., 2020), infected by avian malaria over non-infected or less infected birds (but see Tomás et al., 2008b; Martínez-de la Puente et al., 2009b; Lalubin et al., 2012 for contrasting results). However, chemical cues from the uropygial secretion apparently are not responsible for this mosquito response (Díez-Fernández et al., 2020).

Only a handful of studies have evaluated the influence of uropygial secretion of birds in host location by blood-feeding flies, and experimental research in field conditions is also scarce. Overall, the influence of uropygial secretion in host location remains controversial. Several studies have found that uropygial secretion attracts mosquitoes (Russell and Hunter, 2005; Garvin et al., 2018b) and blackflies (Fallis and Smith, 1964; Lowther and Wood, 1964; Bennett et al., 1972), either alone or in combination with other stimuli. However, other studies have failed to find any attractant effect (Martínez-de la Puente et al., 2011b; Garvin et al., 2018a; Díez-Fernández et al., 2019, 2020) or have found attraction to the secretion only in certain bird or mosquito species, while not in other bird (Garvin et al., 2018b) or mosquito species (Russell and Hunter, 2005). On the other hand, it has even been suggested that uropygial secretion may confer protection against ectoparasites such as lice (Moyer et al., 2003). Our results would suggest that uropygial secretion and derived odors may protect hoopoes from blood-feeding flies and the pathogens they vector by having a repellent or masking effect impairing host detection. Likewise, further evidence suggesting that uropygial secretions

may act as repellent of blood-feeding flies comes from the finding that house sparrows with larger uropygial glands are less infected by a vector-borne malarial parasite (Magallanes et al., 2016). However, this relationship may be driven by overlooked third variables, because for instance gland size is positively correlated with body condition (Magallanes et al., 2016; Moreno-Rueda, 2017).

Evidence of chemical deterrents of ectoparasites in birds is reduced to a handful of bird species, and it does not include uropygial secretions but compounds produced in the skin or feathers (reviewed by Dumbacher and Pruett-Jones, 1996; Moyer and Clayton, 2004; Rajchard, 2007, 2010; Caro et al., 2015). Crested auklets (*Aethia cristatella*) emit a characteristic citrusy odor, which is associated from specialized wick feathers in the interscapular region. These chemical compounds are effective in repelling or killing lice, ticks and mosquitoes (Douglas et al., 2004, 2005a; Douglas, 2013; but see Douglas et al., 2005b). Similarly, the feathers and skin of birds of the genus *Pitohui* and *Ifrita* from New Guinea contain a potent toxin that has been suggested to deter lice (Dumbacher et al., 1992, 2000; Mouritsen and Madsen, 1994; Poulsen, 1994; Dumbacher, 1999). Hoopoe uropygial secretion is special among birds as well. Besides the known adaptive functions of uropygial secretion, our results suggest a role in protection against ectoparasites that deserves further research, also to ascertain to what degree it can be generalized to other bird species.

DATA AVAILABILITY STATEMENT

Data used in this paper are available from the CSIC Institutional Repository at: http://dx.doi.org/10.20350/digitalCSIC/12618.

AUTHOR CONTRIBUTIONS

GT, CZ-M, and JS conceived and designed the research. GT, CZ-M, MB, and JS conducted fieldwork. GT, CZ-M, MM-V, MB, CR-C, and JS contributed to reagents and materials. GT analyzed the parasite samples. JS and GT analyzed the data. GT and JS wrote the article. All authors contributed to revisions.

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Avian Feeding Preferences of *Culex pipiens* and *Culiseta* spp. Along an Urban-to-Wild Gradient in Northern Spain

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Mosquitoes (*Diptera*: *Culicidae*) are regarded as annoying biting pests and vectors of disease-causing agents to humans and other vertebrates worldwide. Factors that affect their distribution and host choice are not well understood. Here, we assessed the species abundance, community composition, and feeding patterns of mosquitoes in an urban-to-wild habitat gradient in northern Spain. Adult mosquitoes from four habitats (urban, periurban, rural, and wild) were collected by aspiration from mid-July to mid-September, 2019. Thirteen species were represented among the 268 specimens (132 females and 136 males) trapped, including six new records reported for the first time in the region. *Culex pipiens* was the most abundant species in all habitats except in the wild, where *Culex territans* was dominant. The highest mosquito diversity was recorded in the wild habitat [species richness (S) = 10 and Shannon/Margalef-Diversity Indices (H'/MI = 1.51/1.36)] and the lowest in the urban habitat (S = 3; H'/MI = 0.24/0.41). Blood-engorged specimens (n = 65) represented 49.2% of the total female collections. Eighty percent of the blood-meals (n = 52) were successfully identified based on cytochrome c oxidase I subunit (COI) DNA barcoding. Nine species of birds were identified in blood meals from the three ecological forms of *Cx. pipiens* (n = 48), *Culiseta fumipennis* (n = 3), and *Culiseta morsitans* (n = 1) collected along the four sampling habitats. Four dominant bird species were recorded in *Cx. pipiens*, i.e., *Parus major* (35.4%), *Turdus merula* (18.7%), *Pica pica* (18.7%), and *Passer domesticus* (10.4%). Despite the availability of dog and human hosts in the sampling sites located in the urban habitat, *Cx. pipiens* seemed to have a preference to feed on birds. *Culiseta fumipennis* blood-meal host records are reported for first time in Europe. These findings on mosquito blood-feeding preferences and habitat community changes will help to better understand vector-host associations and pathogen transmission paths.

Keywords: *Culex pipiens*, *Culiseta*, blood meals, avian hosts, cytochrome c oxidase I, biodiversity, urban-wild gradient

INTRODUCTION

Mosquitoes (*Diptera*: *Culicidae*) are recognized as the most important arthropod group negatively impacting human and animal health worldwide. Numerous mosquito species serve as enzootic, bridge, and/or epidemic vectors of human pathogens. Although tropical countries are more exposed to mosquito-borne diseases, Europe is experiencing an increasing number of human cases. In southern Europe, local autochthonous transmission of important arbovirus diseases has been recorded [dengue virus (DENV), Chikungunya virus (CHIKV), and Zika virus (ZIKV)]. Other arboviruses [West Nile virus (WNV); Usutu virus (USUV); Sindbis virus (SINV); Tahyna virus (TAHV); Batai virus (BATV); Inkoo virus (INKV); and Snowshoe Hare virus (SSHV)] but also protozoans (*Plasmodium* spp.) and nematodes (*Dirofilaria* spp.) have been notified in the continent with variable distribution and effect on humans (Calzolari, 2016). Specifically in Spain, WNV, USUV, and DENV viruses have been detected in native and non-native mosquito species (Vázquez et al., 2011; Aranda et al., 2018). Zoonotic parasitic diseases, such as the canine heartworm and other filarioid nematodes, have also been found in native mosquito species (Bravo-Barriga et al., 2016). Protozoan parasites of avian species are also prominent in Spain, i.e., *Haemoproteus* and *Plasmodium* are commonly found in mosquitoes fed on birds (Gutiérrez-López et al., 2016; Martínez-de la Puente et al., 2016).

Among native mosquito species, the genus *Culex* has deserved great attention, due to their biting nuisance and potential as bridge vector for different pathogens (Vázquez et al., 2011; Martínez-de la Puente et al., 2016; Brugman et al., 2018). Mosquitoes of the *Culex pipiens* complex have a cosmopolitan distribution, and include several species, subspecies, forms, races, physiological variants, or biotypes (Becker et al., 2012). No consensus exists on the taxonomic status of the members of this complex. In Europe, *Cx. pipiens* is presented into three intraspecific forms: *Cx. pipiens molestus*, *Cx. pipiens pipiens*, and *Cx. pipiens* hybrids (Brugman et al., 2018). Hybrids between *Cx. pipiens* and *Cx. quinquefasciatus* have also been reported from the Mediterranean Basin (Shaikevich et al., 2016). Both *Cx. pipiens* forms (*pipiens* and *molestus*) and their hybrids are present in the Iberian Peninsula (Martínez-de la Puente et al., 2016). *Cx. pipiens* has a good tolerance to human-altered environments and is an opportunistic feeder on birds and mammals, including humans (Brugman et al., 2018). Another native genus, namely *Culiseta*, includes some species that have been moderately implicated in the transmission of important arboviruses, such as USUV and WNV, in Europe (Martinet et al., 2019). Nevertheless, up to date no report of virus transmission has been noticed in Spanish mainland.

Abbreviations: BATV, Batai virus; CHIKV, Chikungunya virus; COI, Cytochrome c Oxidase Subunit 1; DENV, Dengue virus; DOI, Digital object identifier; H', Shannon-Wiener Diversity Index; INKV, Inkoo virus; ITS-2, Internal Transcribed Spacer 2; MI, Margalef Diversity Index; OTU, perational taxonomic units; SINV, Sindbis virus;S, Species richness; SSHV, Snowshoe Hare virus; TAHV, Tahyna virus; UMI, Universal molecular identifiers; USUV, Usutu virus; USUV, Usutu virus; WNV, West Nile virus; ZIKV, Zika virus.

Mosquitoes inhabit diverse types of habitats in urban, periurban, and wild environments, where they undertake host-seeking activity and search for suitable substrates for the development of their progeny. Several factors are involved in the mechanisms that affect spatial-temporal distribution and host selection. The urban-to-wild gradient is composed of distinct environmental habitats that influence distribution, diversity, and abundance of mosquito species (Ferraguti et al., 2016). In Europe, urbanization processes created suitable habitats for a small number of anthropophilic species, mostly *Cx. pipiens* (Becker et al., 2012), and *Aedes albopictus* (Li et al., 2014). Conversely, permanent water bodies in natural environments serve as favorable breeding sites for a wide range of species, such as *Anopheles maculipennis* s.l., *Aedes vexans, Aedes sticticus*, *Aedes caspius,* and *Aedes detritus* (Medlock and Vaux, 2015). The elucidation of how mosquito communities change along urban-to-wild habitats provides valuable information to characterize the environmental and ecological processes that significantly define the mosquito populations and host-related interactions.

Feeding patterns of hematophagous arthropods are also a critical component of the ecological cycles of pathogen transmission. Identifying the host affinity of mosquito vector species under field conditions is particularly important given the role of wild and domestic animals as reservoir hosts of several important arboviruses (Failloux et al., 2017). In addition, since not all avian species are competent hosts of arboviruses (Kilpatrick et al., 2007), the identification of blood meals is a first step for the detection of key reservoir species. Consequently, an enhanced understanding of the factors involved in the distribution and host-choice of mosquitoes, particularly disease vectors, would improve the efficiency of surveillance and control programs (Ferraguti et al., 2016).

Mosquito surveillance and screening efforts have been focused on the southern and eastern regions of Spain (Muñoz et al., 2011; Ferraguti et al., 2016; Martínez-de la Puente et al., 2016) while the northern regions have been comparatively overlooked due to their less favorable climatic conditions (harsh winters and short summers) for the proliferation of mosquitoes, bites and their diseases (Eritja et al., 2005; Ruiz-Arrondo et al., 2019). However, climate change and anthropogenic factors are beginning to affect the distribution and incidence of mosquito vectors and their pathogens, an example being the recent arrival and establishment of the exotic species *Ae. albopictus* (Goiri et al., 2020) and *Ae. japonicus* in northern Spain (Eritja et al., 2019).

The main goal of the present study was to ascertain the species diversity including the screening of potential exotic species, abundance, and feeding patterns of mosquito species along an urban-to-wild gradient in northern Spain. In addition, we determined the success of identification of female mosquitoes based on digestion status, and the gonotrophic stages distribution. Finally, the efficacy of active suction trapping as monitoring tool is discussed.

MATERIALS AND METHODS

Study Area and Sampling Sites

This study was conducted in the municipality of Vitoria-Gasteiz (*ca.* 260,000 inhabitants), capital of the Autonomous Community of the Basque Country (northern Spain). Four types of habitats, located 1.0, 2.5, 4.5, and 12.0 km from the city center, were selected following a gradient from a high-anthropogenic site (urban) to a highly conserved natural site (wild). The human population density for each habitat was an estimation calculated from demographic data from 2018 (Gobierno Vasco-Eusko Jaurlaritza, 2019) and the final selection of the study site was based on environmental characteristics (**Figure 1A**). The urban habitat (42.853397, -2.682756, and *ca.* 860 habitants/km^2) was a densely populated area with buildings, schools, and multiple gardens containing hedges of *Prunus cerasifera* and *Chamaecyparis lawsoniana*, and several tree species, including *Aesculus hippocastanum, Magnolia grandiflora, Liriodendron tulipifera*, and *Paulownia tomentosa*. The periurban habitat (42.855776, -2.710002, and *ca.* 150 habitants/km^2) consisted of an industrial environment mixed with buildings, wastelands and rows of *Quercus ilex* and *Fraxinus excelsior*. Both urban and periurban settings had sewer systems in the vicinity. The rural habitat (42.841991, -2.717480, and *ca.* 40 habitants/km^2) comprised of a green patch (with paths, small lagoons, and diverse vegetation) surrounded by country homes and agricultural fields. The wild habitat (42.890751, -2.532650) where human activities were very limited or non-existent, was located in an ornithological park at the edge of a large water reservoir (swamp) which contained aquatic plants, thick vegetation, grasslands, and small woodlands.

Mosquito Collection and Host Counting

We employed a battery-powered Prokopack aspirator (John W. Hock, Florida, United States) to collect outdoor-resting mosquitoes across the four habitats. This quiet, low-cost, and easy handling aspirator allowed working in both open and densely vegetated areas without disturbing or attracting attention of humans and animals. Collections were conducted between 9:00 a.m. and 12.00 p.m. along a 100 m line transect, and lasted approximately 30 min per habitat. Each habitat was sampled fortnightly from mid-July to mid-September 2019, giving a total of five sampling periods. Transects were selected according to the presence of natural resting sites, such as humid and shadow shelters (tree-lines, hedgerows) and corners in concrete walls. Insect collection was carried out by passing the aspirator up and down and from side to side, so that the entire surface was sampled. Insects escaping from their resting place were also collected by aspirating them out of the air. Collection cups that became full or clogged were replaced as necessary during each collection event. Immediately after collection, the cups were stored at −30°C until further taxonomical and molecular analyses.

The number of hosts encountered in each sampling transect was scored concurrently to the mosquito aspiration along each sampling transect. In the case of mammals (i.e., humans and dogs), count was done at the beginning, middle and end of each transect, during 5-min point counts with a limited observation radius (30 m), unless the features of the landscape minimized the visual capacity of the operator. For avian species, counting was performed in a similar way by determining the number of individuals that were seen either walking, perched on vegetation/trees or took off after the walking activity of the operator within the visual radius (30 m). In this regard, no determination was done at species level. Those birds flying up in the sky were excluded from the counting (**Figure 1B**).

Morphological Identification

Prior to taxonomical identification, specimens were separated by sex, and females sorted by their physiological status based on abdomen condition (blood-fed, gravid, and unfed). Species identification was done using appropriate taxonomic keys (Schaffner et al., 2001; Becker et al., 2010) based on morphological features of females and male genitalia. In order to identify blood-meal host species, abdomens of blood-engorged females were removed and individually stored in 2 ml screw-top vials after being classified according to the scale of Sella (II-VII) by observation of the degree of digestion of the blood and developing eggs (gravid stage = Sella stage VII) (Martínez-de la Puente et al., 2013).

Molecular Identification of Blood-Fed Mosquitoes and Their Hosts

Cytochrome c oxidase I subunit (COI) DNA barcoding was used to identify damaged or morphologically indistinguishable mosquito specimens (n = 2), as well as vertebrate host species of gravid (n = 11), and blood fed females (n = 65) at the Centre for Biodiversity Genomics, University of Guelph (Guelph, ON, Canada). Standard barcoding methods were used for mosquito identification (Hebert et al., 2003) while a modified version that accounts for DNA degradation, as well as the possibility of mixed feeding, was used for blood-meal vertebrate host identification. In all cases, abdomens were removed using sterile forceps and placed individually in 2-ml screw-tubes. DNA extraction was performed by immersing the abdomen in a lysis buffer containing proteinase-K followed by incubation at 56°C for 18 h. Blood-engorged abdomens were purposefully disrupted during transfer into screw-tubes to facilitate DNA extraction from the blood meal. DNA purification employed a glass fiber-based bind-wash-elute method (Ivanova et al., 2006). Briefly, the lysate was mixed with two volumes (120 μL) and a binding mix, and then applied to a well of a 96-well silica membrane plate. The plate was centrifuged at 5000 *g* for 5 min, and washed two times. Traces of wash buffer were removed by incubating the plate at 56°C for 30 min, after which 40 μL of elution buffer was applied directly to the membrane and allowed to incubate at room temperature for 1 min. DNA was eluted from the membrane into a clean plate via centrifugation at 5000 *g* for 5 min.

For mosquito identification, the entire barcode region of COI was amplified using the primers C_LepFolF + C_LepFolR (Hernández-Triana et al., 2014) and the products were used directly for Sanger sequencing (Hajibabaei et al., 2006). For

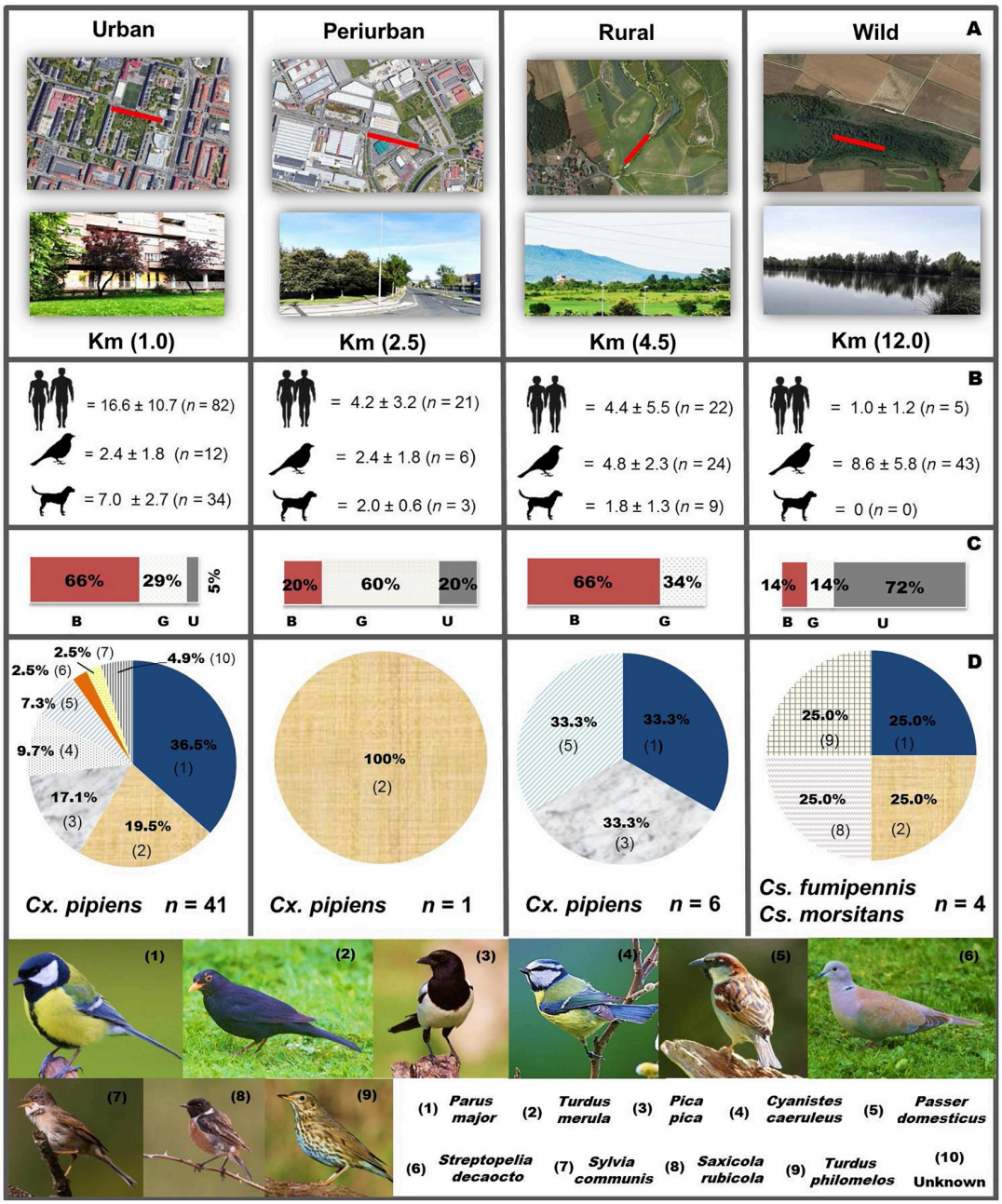

FIGURE 1 | Summary of sampling sites and host blood-meal sources for *Cx. pipiens* and *Culiseta* spp. **(A)** Sampling habitats gradient (urban, periurban, rural, and wild) with dismissing degree of anthropogenic modification (NASA Google Earth images). Red continuous line within the colored image denotes the 100 m linear transect sampling area. The images below show a general view of the sampled sites along with the distance (kilometers) from the city center. **(B)** Mean (±SEM) host abundance of humans, birds and dogs (*n* = total host abundance) recorded across sampling period. **(C)** Proportion of blood-engorged (B), gravid (G), and unfed (U) females in each habitat. **(D)** Host blood meal sources for mosquitoes collected (numbers in parenthesis refer to host species showed below; *n* = successful blood meals identifications).

blood meal analysis, PCR amplification consisted of a two-stage fusion approach: the first step amplified a short region of the COI barcode using primers specific to vertebrates (C_BloodmealF1_t1 + Mod.Mam.R_t1) (Estrada-Franco et al., submitted), while the second step added sample-specific universal molecular identifiers (UMI) and Ion Torrent sequencing adapters. Specific conditions of PCR, thermocycling, and purification procedures are detailed in Estrada-Franco et al. (submitted). Briefly, the PCR reaction (12.5 μL) consisted of 2.0 μL of Hyclone ultra-pure water (Thermo Scientific), 1.25 μL of 10X Platinum Taq buffer (Invitrogen), 0.625 μL of 50 mM MgCl2 (Invitrogen), 0.125 μL

of each primer cocktail (10 μM), 0.0625 μL of 10 mM dNTP (KAPA Biosystems), 0.060 μL of 5 U/lL Platinum Taq DNA Polymerase (Invitrogen), 6.25 μL of 10% D-(+)-trehalose dihydrate (Fluka Analytical), and 2 μL of DNA. The forward primer cocktail contained the primers BloodmealF1_t1 (TGTAAAACGACGGCCAGTACCACWATTATTAAYATAAAR CCMC), BloodmealF2_t1 (TGTAAAACGACGGCCAGTACTAC AGCAATTAACATAAAACCMC), while the reverse primer cocktail contained the primers VR1_t1 (CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCAAAG AATCA), VR1d_t1 (CAGGAAACAGCTATGACTAGACTTCTG GGTGGCCRAARAAYCA), and VR1i_t1 (CAGGAAACAGCTATGACTAGACTTCTGGGTGICCIAAIAA ICA). The forward primers were designed to bind to vertebrate – but not mosquito – DNA, so that only vertebrate blood meal host DNA is amplified. These primers targeted a 185 bp fragment of the COI barcode region, allowing them to amplify highly degraded blood meal DNA. The thermocycling profile consisted of initial denaturation at 95°C for 2 min, followed by 60 cycles of 95°C for 40 s, 56°C for 40 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. After confirming amplification on a 2% E-gel (Invitrogen), the PCR products were diluted two-fold with sterile water and used as a template for a second round of PCR. This second PCR (PCR2) served to index the PCR1 amplicons with IonXpress UMI tags and Ion Torrent sequencing adapters. The M13F and M13R tails of the PCR1 primers served as universal binding sites for PCR2. PCR2 reactions consisted of the same components as PCR1. The PCR2 thermocycling consisted of an initial denaturation at 95°C for 2 min, followed by 5 cycles of 95°C for 40 s, 45°C for 40 s, and 72°C for 30 s, and then 35 cycles of 95°C for 40 s, 51°C for 40 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR2 products were pooled in equal volumes and purified using SpeedBeads (Sigma Aldrich) by incubating 400 μL of pooled PCR2 product with 400 μL of beads. The DNA-bead mixture was incubated at room temperature for 8 min, after which the beads were collected on a magnetic rack. The bead pellet was washed three times with 1 mL of freshly prepared 80% ethanol and air dried for 10 min. DNA was released from the beads by mixing the pellet with 200 μL of sterile water for 1 min at room temperature. The beads were collected on a magnetic rack and 180 μL of the supernatant was transferred to a clean 1.5 mL tube. The purified library was quantified using a Qubit 2.0 fluorometer, adjusted to 26 pM with sterile water, and loaded onto an Ion Chef automated platform (Thermo Scientific) following manufacturer's instructions. Sequencing was performed using 400 bp chemistry on an Ion Torrent S5 (Thermo Scientific) using a 530 chip. Raw sequence reads were automatically demultiplexed following sequencing using the Torrent Browser sequencing software. The demultiplexed reads were further processed using standalone bioinformatics tools (see section "Bioinformatic, sequence information and statistical analysis" below for details). The efficacy of the technique to amplify both mammalian and avian hosts was validated prior to the analysis of the samples by testing the specificity of the primers by the inclusion of positive (DNA extracted from blood-fed insects known to have fed on certain vertebrate hosts), and negative controls. The amplified products were sequenced to confirm that they originated from the target vertebrate species and not the insect.

Molecular Identification of Sibling Species and Forms

The identification of the members of the *Anopheles maculipennis* complex (n = 5) and blood fed/gravid *Cx. pipiens* (a total of 64 out of 76 gravid/blood-fed female mosquitoes submitted to the molecular methods described in the previous section) ecological forms were carried out from DNA extracted from the thorax of individual specimens in the Animal Health Department of NEIKER (Spain). In both cases, genomic DNA extraction was carried out by NZY Tissue gDNA isolation kit (NZYTech, Lisboa, Portugal). Identification of the specimens of the *An. maculipennis* species complex was carried out by a PCR-RFLP assay targeting polymorphisms in the Internal Transcribed Spacer 2 (ITS-2) of the ribosomal DNA (Vicente et al., 2011). Amplicons were digested first with *Hha*I and secondly with *Hpa*II restriction enzymes (Invitrogen/Thermo Scientific, Vilnius, Lithuania). Identification of the different *Cx. pipiens* female forms (*Cx. pipiens pipiens, Cx. pipiens molestus*, and its hybrids) was performed by PCR amplification of the flanking region of the CQ11 microsatellite, following the protocol already described (Bahnck and Fonseca, 2006). Amplicons from both PCR methods were separated by 2% agarose gel electrophoresis using GelRed Nucleid Acid Stain (Biotium, Hayward, CA, United States) as staining solution, and with a 100 bp DNA ladder (Thermo Scientific, Vilnius, Lithuania) as a molecular weight marker.

Bioinformatic, Sequence Information and Statistical Analysis

Raw sequence reads were filtered (based on a minimum length of 100 bp and minimum quality of QV20) and cleaned (removal of adapter and primer tails) to ensure that only high-quality reads were included in the final dataset. Reads passing these filters were clustered into operational taxonomic units (OTUs) with 97% identity and a minimum of 10 reads per OTU. Each OTU sequence was compared using BLAST tool[1] and BOLD Systems[2], which are composed of global vertebrate COI sequences. Identifications were considered valid only if the query sequence matched a reference sequence with at least 95% identity with 100 bp of coverage between the queried sequence and the reference sequence. Furthermore, mosquito identifications were manually vetted in order to ensure that they conformed with local fauna sighting records.

Detailed specimen records and sequence information (including trace files) were uploaded to the Barcode of Life Database (BOLD-http://www.boldsystems.org) and can be found within the Working Group 1.4 Initiative "Human Pathogens and Zoonoses" container "MCBCS-Surveillance of mosquitoes and *Culicoides* in the Basque Country, Spain." The Digital Object Identifier (DOI) for the publicly available projects in BOLD is doi:dx.doi.org/10.5883/DS-MQBMBC. All generated

[1] www.ncbi.nlm.nih.gov/genbank

[2] http://www.boldsystems.org/index.php/

sequences have been submitted to GenBank (accession numbers: MT519609-MT519682).

The diversity indices of Shannon and Margalef (H'/MI), and species richness (S) were used as a measure of mosquito community heterogeneity. Host counting was obtained from the sum of the five trapping periods. Both mean abundances of mosquitoes within habitats and gonotrophic stages were compared by non-parametric Kruskal–Wallis test followed by Mann–Whitney *U* test pairwise comparisons (adjusting significance levels). All tests were conducted with IBM SPSS statistics v 23.0 software package.

RESULTS

A total of 268 mosquitoes (132 females and 136 males; mean ± SEM = 13.4 ± 2.9 specimens/habitat) belonging to four genera and 13 species were identified during the five sampling periods (**Table 1**). The highest number of mosquitoes was recorded in mid-August (**Supplementary Table 1**). Six new records (*Culiseta annulata, Cs. fumipennis, Cs. morsitans, Cx. territans*, *Cx. theileri,* and *Uranotaenia unguiculata*) are reported for first time in the Basque Country region. No exotic invasive mosquitoes were recorded in the study area.

Mosquito Community Composition and Abundance

Cx. pipiens was the most predominant species (200 specimens, 74.6% from the total collections), followed by *Cx. territans* (32, 11.9%), *Cs. longiareolata* (11, 4.1%), and other less frequent species (25, 9.3%; **Table 1** and **Figure 2A**). *Cx. pipiens* was the only species collected along the four habitats and the most predominant in all sampled settings except in the wild habitat, where *Cx. territans* was the dominant species. Despite mosquito abundance being greater in the urban habitat, the difference was not significant (χ^2 = 6.4, df = 3, and p = 0.094). Higher mosquito S and biodiversity was recorded in the wild habitat (S = 10, H'/MI = 1.51/1.36) compared to the other habitats (S ≤ 4; H'/MI ≤ 0.40/0.75; **Table 1**).

Molecular identification of blood-fed/gravid females by means of PCR (COI gene) and sequencing, confirmed the identity of *Cx. pipiens* specimens, thus this species is referred in the text as *Cx. pipiens* (100% match identity to bank identification number in BOLD: AAA4751, accession number GU908075). Note that molecular identification allowed the separation of *Cx. torrentium* females from the sibling *Cx. pipiens* females.

The pattern of *Cx. pipiens* ecological forms was successfully determined in 89.1% (57/64) of the blood-fed/gravid *Cx. pipiens* analyzed, showing the presence of the three ecological forms in different proportions [*Cx. pipiens pipiens* (*n* = 50; 87.7%; in urban, periurban and rural habitats); *Cx. pipiens molestus* (*n* = 3; 5.3%; in urban and rural habitats); and *Cx. pipiens* hybrids (*n* = 4; 7.0%; solely in urban habitat)]. *Cx. pipiens pipiens* was predominant in all the habitats but the wild, in which no *Cx. pipiens* blood-fed specimens were collected.

Morphologically similar members of the subgenus *Culiseta* were separated into three species (*Cs. fumipennis* voucher specimen = 100% identity, accession number KM258139; *Cs.*

TABLE 1 | Species diversity of mosquitoes along an urban-to-wild gradient in northern Spain.

Species	Sampled habitats															
	Urban				Periurban				Rural				Wild			
	F	M	T	%	F	M	T	%	F	M	T	%	F	M	T	%
Cx. pipiens	77	36	113	94.2	7	42	49	90.7	5	12	17	85.0	4	17	21	28.4
Cx. territans	0	0	0	0	0	0	0	0	0	0	0	0	13	19	32	43.2
Cs. longiareolata	3	3	6	5.0	1	1	2	3.7	0	0	0	0	2	1	3	4.1
Cs. fumipennis	0	0	0	0	0	0	0	0	0	0	0	0	6	0	6	8.1
An. maculipennis s.s.	0	0	0	0	0	0	0	0	0	0	0	0	5	0	5	6.8
Cx. hortensis	0	1	1	0.8	1	1	2	3.7	1	0	1	5.0	0	0	0	0
An. claviger s.l.	0	0	0	0	1	0	1	1.9	0	0	0	0	0	2	2	2.7
Cs. morsitans	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	2.7
Cx. theileri	0	0	0	0	0	0	0	0	1	0	1	5.0	0	0	0	0
An. plumbeus	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1.4
Cs. annulata	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1.4
Cs. litorea	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1.4
Ur. unguiculata	0	0	0	0	0	0	0	0	1	0	1	5.0	1	0	0	0
Total	80	40	120		10	44	54		8	12	20		34	40	74	
Species richness (S)	3				4				4				10			
Mean (SEM)	23.6 (6.4)				10.8 (35.4)				4.2 (1.3)				14.4 (6.2)			
Shannon-Index (H')	0.24				0.40				0.39				1.51			
Margalef-Index (MI)	0.41				0.75				0.66				1.36			

**F = females, M = Males, and T = total.*

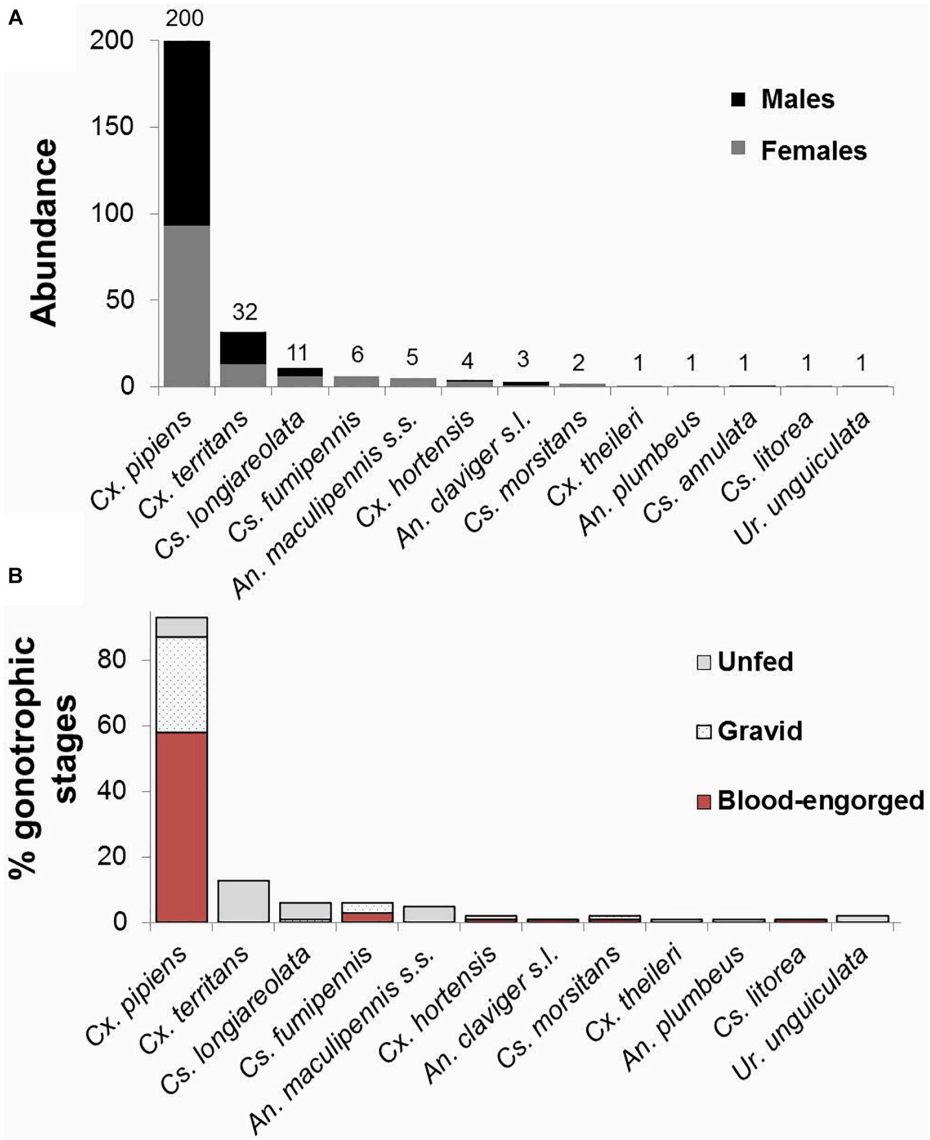

FIGURE 2 | Catches of mosquito species recorded from all the sampling settings. **(A)** Males and females. **(B)** Gonotrophic stages of females.

litorea = 98.8% identity, accession number MK402821; and *Cs. morsitans* = 99.7% identity, accession number KM258135).

Mosquitoes of the *Anopheles maculipennis* complex (n = 5) were identified as *An. maculipennis* sensu stricto (bands at 300 bp after *Hha*I digestion, and 200 bp after *Hpa*II digestion).

Analysis of Gonotrophic Stages

The distribution of the gonotrophic stage categories for each habitat and species is represented in **Figures 1C**, **2B**, respectively. Overall, blood-engorged females represented the highest percentage of the catches, accounting for 49.2% (65/132) of the total (**Figure 3A**). However, no statistically significant differences were detected in the abundance of the three gonotrophic stages (χ^2 = 0.002, df = 2, and p = 1.2) (**Figure 3A**). In contrast, significant differences were found in the number of blood-fed (χ^2 = 10.64, df = 3, and p = 0.014) and unfed females (χ^2 = 11.84, df = 3, and p = 0.008) within habitats (**Supplementary Table 2** and **Figure 3B**). The statistical analysis revealed that significantly more blood-engorged females were aspirated in the urban habitat, while unfed individuals constituted the dominant gonotrophic stage in the wild habitat. Regarding gravid females, no differences were detected among sampling sites.

Host-Derived Blood-Meals Identification

Blood-meal host identification was successful for 80.0% (n = 52) of the 65 engorged females analyzed in this study. All the stages within the scale of Sella (II–VI) yielded high number of successful identifications regardless their blood-digestion degree [II = 9/13

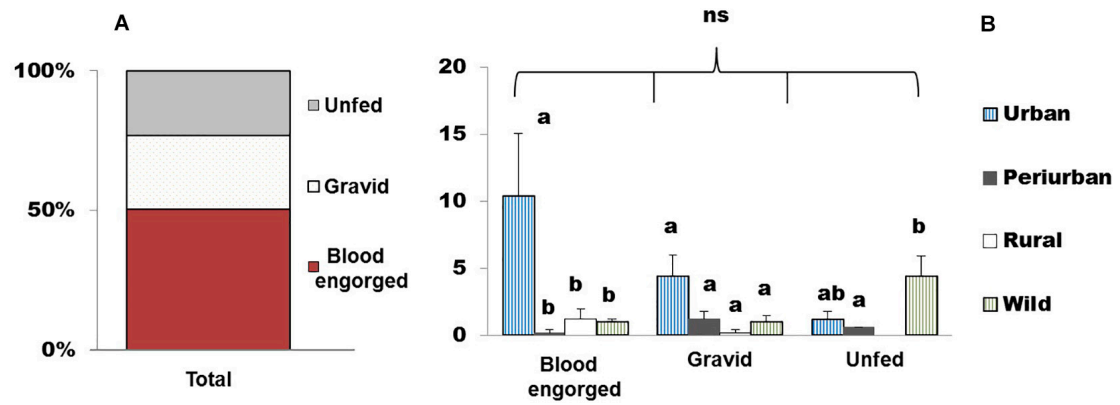

FIGURE 3 | Distribution of gonotrophic stages in female mosquitoes in the four sampling habitats **(A)** Total frequency of gonotrophic stages (blood-engorged, gravid, and unfed). **(B)** Abundance (Mean + SEM) of gonotrophic stages per habitat. Columns with different letters (a,b) within each gonotrophic stage are statistically different, while "ns" denotes non-significant differences in the total number of females between gonotrophic stages (Kruskal–Wallis test followed by Mann–Whitney *U* test).

(69.2%), III = 6/7 (85.7%), IV = 6/6 (100%), V = 20/22 (90.9%), and VI = 11/17 (64.7%)] while those scored as gravid stages (stage VII, $n = 11$) failed to produce sequences.

A total of 52 bird hosts of at least nine species were identified for the three blood-fed mosquito species. *Cx. pipiens* ($n = 48$) fed on the great tit (*Parus major*, 35.4%), blackbird (*Turdus merula*, 18.7%), magpie (*Pica pica,* 18.7%), house sparrow (*Passer domesticus,* 10.4%), blue tit (*Cyanistes caeruleus*, 8.3%), Euroasian collared dove (*Streptopelia decaocto*, 2.1%), whitethroat (*Sylvia communis*, 2.1%), and two unspecific bird species (one belonging to Order Accipitriformes, 95% sequence match) (**Figure 1D**).

Culiseta spp. ($n = 4$) fed upon the European stonechat *Saxicola rubicola*, the song thrush *Turdus philomelos* and *P. major* for *Cs. fumipennis,* and *T. merula* for *Cs. morsitans* (**Figure 1D**). Blood-engorged specimens from three other species (*Cx. hortensis*, *Cs. litorea*, and *An. claviger* s.l.) tested negative to amplification. Mixed host feeding was not detected in any of the analyzed specimens.

DISCUSSION

The results of this study revealed that *Cx. pipiens* mosquitoes were the dominant species in urban, periurban, and rural habitats, where they showed a preferred tendency to feed on avian hosts. This feeding pattern was clearly observed in the urban setting, where a higher number of blood-fed *Cx. pipiens* females were trapped. Taking into account the predominance of non-avian hosts (humans and dogs) in the urban environment, it is highly remarkable that the blood-feeding pattern recorded for *Cx. pipiens* was strongly biased to bird hosts. In contrast, the wild habitat did not yield blood-fed specimens of the two predominant *Culex* species (i.e., *Cx. pipiens* and *Cx. territans*), though blood-fed *Culiseta* spp. were demonstrated to have fed also on bird hosts.

In this study, neither human nor dog sequences were obtained in blood from *Cx. pipiens* mosquitoes from the urban habitats despite the relative high abundance of these hosts (64% and 26%, respectively). Similarly, humans were highly present in periurban and rural habitats (70% and 40%, respectively). On the basis of relative host abundance measures and mosquito diet, our results suggest a prevailing affinity of *Cx. pipiens* to feed on avian hosts in these habitats, however, it is not possible to definitively prove that the mosquitoes were not feeding on certain mammalian hosts (i.e., a failure to detect a host could be due to DNA degradation, primer binding issues for certain taxa, etc.). In Europe, the ornithophilic preference of *Cx. pipiens* has been observed in some studies (Muñoz et al., 2012; Roiz et al., 2012; Gomes et al., 2013; Radrova et al., 2013; Brugman et al., 2017), however, other authors have also highlighted the importance of mammals as a relevant feeding source (Alcaide et al., 2009; Muñoz et al., 2011; Rizzoli et al., 2015; Börstler et al., 2016; Martínez-de la Puente et al., 2016). Both human and non-human vertebrates (i.e., *Capreolus capreolus*, *Sus scrofa*, *Lepus europaeus*, *Canis lupus familiaris*, *Felis silvestris*, *Equus ferus caballus*, *Bos taurus*, *Oryctolagus cuniculus*, *Rattus rattus*, and some reptiles) from *Cx. pipiens* have been reported in variable proportions depending on the study and regardless of the *pipiens* forms (Alcaide et al., 2009; Muñoz et al., 2011; Rizzoli et al., 2015; Börstler et al., 2016; Martínez-de la Puente et al., 2016). Five out of the eight species of birds identified in the blood-meals from the urban setting were included in the bird census undertaken by ornithologists in the same park (Ayuntamiento de Vitoria-Gasteiz, 2019). They noted *Columbia livia* and *P. domesticus* as the most frequent birds, followed by *Apus apus, P. pica*, *Cyanistes cauruleus*, *P. major*, *T. merula* and, to a lesser extent, three other species. This marked feeding tendency of *Cx. pipiens* to feed on *T. merula* and *P. domesticus* was also observed in other European studies (Muñoz et al., 2012; Roiz et al., 2012; Gomes et al., 2013; Rizzoli et al., 2015). It is noteworthy that synanthropic and abundant bird species such as the rock pigeon *C. livia* could have been underestimated, since their abundance in the urban setting did not reflect the proportion of blood meals taken, which is consistent with other studies carried

out in Europe and United States (Rizzoli et al., 2015; Kothera et al., 2020). Analyses of gravid/blood-fed *Cx. pipiens* showed that they fed mostly on birds regardless of the ecological form, which is in agreement with similar studies carried out in Spain, United Kingdom, and Portugal (Gomes et al., 2013; Martínez-de la Puente et al., 2016; Brugman et al., 2017). Consequently, mosquito populations that mostly feed on birds would have a larger capacity to amplify WNV due to the fact that humans and other mammals do not support viremias high enough to infect mosquitoes (Platt et al., 2007).

In the wild habitat, the few blood-meals recorded corresponded to bird hosts from *Cs. fumipennis* and *Cs. morsitans*, which is in agreement to the relative bird density observed (89% of visible host). According to literature, *Culiseta* species feed on birds and mammals, including humans (Brugman, 2016), but in United States they have also been found feeding on reptiles (Blosser et al., 2017). To best of our knowledge, the *Cs. fumipennis* blood-meal identification represents the first report of the feeding pattern of this species in Europe. In Germany and United Kingdom, *Cs. morsitans* have been recorded feeding on birds but also on mammals, including humans (Börstler et al., 2016; Brugman et al., 2017). Further studies are necessary to establish solid conclusions about the feeding preferences of both species.

According to our findings, the highest S was found when the distance was increased from the urban sampling site, with the wild site harboring the highest number of species. This relatively low S is tightly linked to the negative effect of the urbanization processes on the diversity of mosquito communities (Ferraguti et al., 2016). Non-disturbed wild habitats, in contrast, are constituted by a plethora of suitable heterogeneous microhabitats, leading to a favorable scenario for enhancing mosquito diversity. Environmental factors including land use, vegetation, and hydrological characteristics are known to affect mosquito abundance and community composition (Ferraguti et al., 2016). Among them, the spatial heterogeneity gradient is reported as one of the most important key indicators with relevant influence in mosquito species diversity and distribution (Overgaard et al., 2003; Leisnham et al., 2014). For instance, urbanized environments are organized in small patches (buildings, green areas, and streets) which are highly structured with many interfaces between them. Conversely, natural environments habitats are structured in larger patches and less interfaces, resulting in high species diversity. As stated by McDonnell and Pickett (1990), suburban areas are usually prone to congregate higher S and diversity. Indeed, we observed that fewer species were found in urbanized environments compared to rural/wild environments. However, the number of catches did not follow a well-defined pattern, as the highest abundance was observed in the anthropogenic habitat. This contrasts with previous reports (Ibañez-Justicia et al., 2015; Ferraguti et al., 2016; de Valdez, 2017; Honnen and Monaghan, 2017) but the collection method employed was different among surveys (i.e., CDC-mini light traps, BG-Sentinel traps baited with CO_2, and aspiration). One possible explanation to the observed discrepancies is that mosquitoes could be more concentrated on the sampling site of the urban setting where fewer resting sites were available to use compared to wild areas, which in turn offer a wide variety of features for mosquito activity (Burkett-Cadena et al., 2013). These discrepancies are the result of the presence of substantial numbers of *Cx. pipiens* (mostly the *pipiens* form) and *Cs. longiareolata*, highly anthropophilic and container-breeding species. Both species find in urban areas their preferred oviposition sites, i.e., artificial habitats such as urban sewage and ground water systems (Bueno Marí, 2010), and other temporary water bodies (gardens, terraces, etc.). *Cx. pipiens* abundance decreased as the distance from the urban setting increased, being replaced by other species, such as *Cx. territans*, *Cs. fumipennis,* and *An. maculipennis* s.s. The lower abundance of mosquitoes in the natural habitat might have been caused by the presence of a dense vegetated barrier between the sampling transect and swamp land, which could have prevented the dispersion of mosquitoes from breeding sites. Indeed, mosquito community composition and habitat selection are highly variable among countries (Möhlmann et al., 2017). The combination of various collection methods would allow a better representation of the mosquito and host community fauna, however, the use of other routine traps, i.e., suction traps are inappropriate as they are subject to vandalism and logistic constraints in public urban/periurban areas.

Identification rates of host-derived blood-meals were not consistent with Sella's scale, since we did not observe a marked reduction in the amplification success as the degree of digestion in the blood increased (Martínez-de la Puente et al., 2013; Brugman et al., 2017; Santos et al., 2019). These discrepancies may be attributable to the technique implemented which has been improved for amplification of degraded DNA. In addition, the technique used did not allow the identification of host-derived blood meals from gravid females, thus limiting the determination of host-feeding patterns exclusively to specimens containing signs of blood on their abdomens.

Our study has demonstrated the effectiveness of the sampling performance of the Prokopack aspirator for collection of outdoor-mosquitoes in comparison to other superior models (i.e., CDC-BP aspirator) (Vazquez-Prokopec et al., 2009; Maia et al., 2011). The Prokopack aspirator is simple to use, easier to maneuver, cheap, and lightweight. In addition, aspiration yielded larger numbers of blood-engorged specimens when compared to other standard methods (Maia et al., 2011; Brugman et al., 2017; Santos et al., 2019), which makes this technique suitable for studies of host-feeding preferences and/or pathogen detection. Thus, captures of blood-fed females are usually scarce (0.5–3.0%) using CDC traps (Roiz et al., 2012; Radrova et al., 2013). Moreover, direct aspiration from mosquito resting sites provides a more representative information and estimation of physiological condition, richness, abundance, sex ratio, and age structure (Silver, 2008) in comparison to other trapping methods. Conversely, the main constraint is the damage inflicted on the specimens after being aspired and retained in the trap mesh for a long time, which frequently hampers proper identification of females. The differences in the gonotrophic stage frequencies (i.e., proportion of blood meals taken by mosquitoes) of female mosquitoes among habitats may be influenced by multiple factors difficult to be analyzed, such as the composition, abundance, and availability of vertebrate hosts, which in turn is linked to the

landscape anthropization level and ecological conditions. Other variables such as collection methods (e.g., CDC traps baited with CO_2 capture preferentially host-seeking females) and availability of breeding and resting places with different accessibility (e.g., in the urban setting lower resting places were available compared to the wild) (Maia et al., 2011; Barrera et al., 2012; Muñoz et al., 2012; Ferraguti et al., 2016), could explain the high proportion of blood-fed and unfed mosquitoes in urban and wild habitats, respectively. For example, in our study collections might be biased toward mosquitoes fed on avian hosts as most sampling sites were bird resting sites (bushes, tree hedges, etc.). A more controlled design of the sampling sites selection and a more accurate host counting method might help to solve these issues.

Faunistic studies focused on mosquito communities in northern Spain are scarce and mostly devoted to the detection of those species with relevant medical interest (Ruiz-Arrondo et al., 2019; Goiri et al., 2020). Here, we report for the first time the presence of six new species in the region, increasing from 14 to 20 the total number of species present in the Basque Country (Bueno-Marí et al., 2012; González et al., 2015, 2020; Goiri et al., 2020), including new records of *Anopheles maculipennis* s.s., a major vector of several pathogens in Europe (Kampen et al., 2016). Interestingly, in nearby territories, only *An. atroparvus* had been identified within this species complex (Ruiz-Arrondo et al., 2019). This study also contributes to the differentiation of *Culiseta* sibling species by barcoding. The separation of some female species of *Culiseta* remains challenging by both DNA barcoding and morphological features (Ruiz-Arrondo et al., 2020). The female sequences obtained had enough genetic divergence to be separated into individual OTUs (data not shown).

In conclusion, *Cx. pipiens* was the most widespread species along the studied habitats according to the aspiration-based sampling methodology. *Cx. pipiens*, *Cs. fumipennis,* and *Cs. morsitans* exhibited an overwhelming affinity for avian hosts. These results on mosquito blood meals and community composition contribute to a better understanding on the transmission risk of pathogenic agents of medical and veterinary importance. The study also highlights the importance of implementing monitoring programs that include mosquito species and host-feeding surveillance but also vector ecology.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material.**

AUTHOR CONTRIBUTIONS

MG and AG-P conceived and planned the experiments. MG carried out the field experiments and morphological identification of mosquitoes. SL performed statistical analyses. SP, PH, and LH-T carried out the molecular analysis. FG made molecular identification of sibling species and forms. MG, PA-E, SL, and IR-A wrote the manuscript. AG-P and FG reviewed and edited the final version of the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.568835/full#supplementary-material

Supplementary Table 1 | Species of mosquitoes collected during the five sampling periods in each habitat (urban, periurban, rural, and wild).

Supplementary Table 2 | Gonotrophic stages (blood-engorged = B, gravid = G and unfed = U) of female mosquito species along the habitats sampled.

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frontiers in Ecology and Evolution

MINI REVIEW
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Does *Plasmodium* Infection Affect Mosquito Attraction?

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Vector-host interactions play a central role in the transmission of vector-borne diseases. Determining the factors that affect vector attraction to vertebrate hosts is critical to understand disease ecological dynamics. Using malaria as the primary model, we reviewed studies that examined whether pathogen infection and host traits affect mosquito attraction. We found contradictory results examining mosquito attraction to birds infected with *Plasmodium* parasites, presumably because of widely variable experimental approaches. We summarize current experimental findings and propose standardized experimental approaches for future studies.

Keywords: behavioral manipulation, vector attraction, experimental parasitology, arboviruses, avian malaria, Culicidae, Diptera

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INTRODUCTION

Insect vectors have often been considered passive vessels that carry pathogens without exerting much selection. However, vectors may suffer high pathogen-related mortalities (Valkiūnas et al., 2014; Gutiérrez-López et al., 2019a,b), and vectors selection of vertebrate hosts to obtain blood meals is not random or solely based on host availability, they follow general and specific host cues such as carbon dioxide and volatile body chemicals (Takken and Verhulst, 2013). Interestingly, both host choice and host attractiveness may be manipulated by the parasite in order to increase its transmission success (i.e., manipulation hypothesis; Heil, 2016).

The process by which pathogens manipulate vectors' behavior can be indirect during the development in a vertebrate host (e.g., increased attractiveness of infected hosts to uninfected vectors), and/or direct during the cycle inside the vectors (e.g., increase in attraction or feeding rates toward uninfected hosts; Gandon, 2018). These changes have profound implications for pathogen transmission. For instance, uninfected vectors highly attracted toward infected hosts would initially increase transmission in local populations (Gandon, 2018). Generalist mosquito species feeding on birds and mammals (depending on host availability; Abella-Medrano et al., 2018) play important roles in the transmission of zoonotic pathogens (e.g., *Culex pipiens* transmitting West Nile virus (WNV) and Usutu (USUV) virus among birds, equids, and humans; Brugman et al., 2018; Rochlin et al., 2019). Thus, investigating drivers of mosquito host selection is essential to understand the dynamics of vertebrate-vector-pathogen systems.

In recent years avian haemosporidian parasites have received attention as model systems to understand the ecology and evolution of vector-borne parasites (Rivero and Gandon, 2018). In particular because they are widespread, infect a large proportion of bird species worldwide, and affect bird fitness and survival (Atkinson and Van Riper, 1991; Valkiūnas, 2005;

Palinauskas et al., 2020). Thus, by focusing on avian malaria, here we provide an overview of the factors affecting host selection by mosquitoes concentrating on *Culex* mosquitoes, which transmit human pathogens, bird pathogens, and zoonotic pathogens that use birds as reservoirs (WNV, USUV). Although *Culex* mosquitoes have a broad range of hosts, some are predominantly mammophilic and others are ornithophilic (e.g., *Culex theileri* vs. *Cx. pipiens*), yet their feeding behaviors depend on the species and population of origin, which also affects the parasites they transmit (Santa-Ana et al., 2006; Farajollahi et al., 2011; Rochlin et al., 2019).

Birds are used as models to analyze factors within species that affect host attractiveness to mosquitoes, which include mosquito and bird pathogen infection status, differences in the production of natural chemicals by birds (uropygial gland secretion and volatile compounds), host sex, and interactions among some of these factors (**Table 1**). We took advantage of studies on human and mice malaria systems that also addressed some of these factors to suggest next steps for studies on bird attractiveness to mosquitoes. We found discrepancies among studies, which may be due to differences in study design (**Table 1**). Therefore, we propose standardized experimental approaches to help identify generalities and contingencies in pathogen-vector-host interactions using avian malaria as a model (**Figure 1**).

CURRENT EXPERIMENTAL APPROACHES

In general, laboratory experiments assess mosquito attraction using dual-choice olfactometers, or they assess feeding choice by placing mosquitoes inside cages containing target host groups. Olfactometers expose mosquitoes to olfactory host cues (CO_2 and volatile compounds) and prevent mosquitoes from using visual cues or differences in body temperature for host detection (Lalubin et al., 2012; De Moraes et al., 2014; Díez-Fernández et al., 2020a). This approach is useful for studies using chemical stimuli collected from hosts and has the advantage to protect hosts from being bitten by mosquitoes. Host attractiveness in single-choice system can be measured as the number of mosquitoes approaching a host in one end of an enclosure or in a tunnel in relation to the number of mosquitoes released at the opposite end (Cator et al., 2013; Batista et al., 2014).

Feeding rates can be measured by comparing the proportion of mosquitoes taking a blood meal from single-housed (Cornet et al., 2019) or pair-housed hosts. In the latter, two hosts from different groups (e.g., control vs. experimental group) are placed inside the same cage where mosquitoes are released and host choice is assessed via microsatellite testing (Yan et al., 2018b) or by molecular sexing methods (Cornet et al., 2013a; Yan et al., 2018a) using engorged mosquitoes to determine blood source. Overall, studies varied in duration and type (attraction assays using olfactometers vs. feeding choice inside cages), with feeding experiments used either immobilized or non-immobilized birds (**Table 1**). These differences, therefore, may explain discrepancies in the general findings.

INDIRECT AND DIRECT MANIPULATION OF VECTOR'S BEHAVIOR BY PATHOGENS

Pathogens can modify conditions within vertebrate hosts or within vectors to increase transmission rates from infected to uninfected hosts. These indirect and direct vector manipulations, respectively, may affect host-seeking behaviors, feeding behaviors, or both (Gandon, 2018).

Infected Hosts and Mosquito Attraction

Most studies tested whether hosts infected with *Plasmodium* parasites are more attractive to uninfected mosquitoes or whether uninfected mosquitoes feed more on infected hosts (indirect vector manipulation). Some of these studies also tested whether hosts with higher number of circulating gametocytes (sexual parasite stage that is infective to mosquitoes) were more attractive to mosquitoes even when compared with hosts with low levels of gametocytes (**Table 1**).

Avian Malaria

Cornet et al. (2013a,b) found that laboratory-reared *Cx. pipiens* fed more on domestic canaries (*Serinus canaria*) infected with a laboratory strain of *Plasmodium relictum* (SGS1) when compared to uninfected birds. This effect was detected in birds undergoing chronic infections (24–26 days post infection – dpi), but not in birds undergoing the acute phase of the infection (10 dpi; Cornet et al., 2013b). Mosquitoes fed more on birds with a higher hematocrit, a measure that can be interpreted as a higher blood quality (Cornet et al., 2013a).

Four avian malaria studies used wild caught *Cx. pipiens* and free-living birds infected with *Plasmodium* spp. and uninfected birds to assess vector manipulation in more natural systems (**Table 1**). One set of experiments conducted by Yan et al. (2018b) compared infected birds with their counterparts treated with primaquine to reduce parasitemia (the number of circulating parasites). They found mosquitoes fed more on infected birds than on treated birds, which provided experimental support for the vector manipulation hypothesis. Similarly, mosquitoes were more attracted to body odors isolated from infected birds when compared to uninfected birds (Díez-Fernández et al., 2020b). However, this effect was not observed when using uropygial gland secretion collected from the same bird groups. Other experiments found that mosquitoes were either more attracted to uninfected birds (Lalubin et al., 2012) or that infection status did not change mosquito feeding rates when compared to control groups (Yan et al., 2018b; Gutiérrez-López et al., 2019a).

From the discussed studies, two used dual-choice olfactometers to assess mosquito attraction to birds or to chemical compounds collected from birds, while three studies released mosquitoes inside cages containing single birds or pairs of infected and uninfected birds that were immobilized in most cases (except Yan et al., 2018b; **Table 1**). Observed contrasting results addressing the host manipulation hypothesis may be the result of differences in host, vector and parasites used and/or due to differences in experimental settings.

TABLE 1 | Studies conducted on mosquito attraction toward bird, humans and mice.

***Plasmodium* Infections**						
Indirect manipulation						
Host sp.	**Vector sp.***	**Parasite species**	**Factor**	**Main Results**	**Experimental details**	**Source****
Avian hosts						
Great tit – *Parus major*	*Culex pipiens* (wild caught). Not tested for pipiens and molestus forms and hybrids.	*Plasmodium* sp. – natural infections	Mosquito attraction as a function of bird infection.	Infected birds less attractive than uninfected birds. Females more attractive to mosquitoes than males.	Dual-choice olfactometer. Twenty-eight paired experiments combining six infected and five uninfected birds. Birds kept out of sight of the mosquitoes. Parasitemia not assessed. Experiments lasted 15 min.	Lalubin et al. (2012)[1]
Domestic canary – *Serinus canaria*	*Cx. pipiens* (S-LAB strain)	*Plasmodium relictum* (SGS1) – experimental infection	Feeding choice as a function of bird infection and infection stage (acute or chronic).	Mosquitoes fed more on chronically infected birds than on either uninfected or acutely infected birds. Mosquito preference for birds with higher hematocrit. No difference between uninfected birds and birds during acute infection.	Paired birds in 25 independent cages with 70 mosquitoes each. Feeding experiments lasted 2 h. Birds were immobilized.	Cornet et al. (2013b)[2]
House sparrow – *Passer domesticus*	*Cx. pipiens* – wild caught. Not tested for pipiens and molestus forms and hybrids.	*Plasmodium* sp. – natural infections	Feeding choice as a function of bird infection.	No difference in feeding rates between infected and uninfected birds.	Twenty pairs of infected and uninfected birds exposed to mosquitoes. Birds were not immobilized. Experiments lasted 12 h.	Yan et al. (2018b)[3]
House sparrow – *Passer domesticus*	*Cx. pipiens* – wild caught. Not tested for pipiens and molestus forms and hybrids.	*Plasmodium* sp. – natural infections	Feeding choice as a function of parasite levels.	Higher feeding rates on non-treated (higher parasite levels) than on treated birds. Higher feeding rates on females.	Parasitemia reduced via primaquine treatment. Nineteen pairs of infected non-treated birds and infected treated birds exposed to an average of 151 mosquitoes. Birds were not immobilized. Experiments lasted 12 h.	Yan et al. (2018b)[4]
Jackdaw – *Corvus monedula*, and house sparrow – *Passer domesticus*	Wild caught *Ochlerotatus* (*Aedes*) *caspius* and *Cx. pipiens* (not tested for pipiens and molestus forms and hybrids).	*Plasmodium* sp. – natural infections	Feeding choice as a function of bird infection, sex and body mass	No difference in feeding rates for both mosquito species according to *Plasmodium* infection status and body mass for both bird species. *Ochlerotatus caspius* fed more on female than on male jackdaws.	Birds single-housed. Parasitemia not assessed. Experiments lasted 30 min. Birds were immobilized.	Gutiérrez-López et al. (2019a)[5]

(Continued)

TABLE 1 | Continued

***Plasmodium* Infections**						
Indirect manipulation						
Host sp.	**Vector sp.***	**Parasite species**	**Factor**	**Main Results**	**Experimental details**	**Source****
Mammal hosts						
Mice – *Mus musculus*	*Anopheles stephensi*	*Plasmodium chabaudi* – clones CR and ER	Feeding choice as a function of host infection.	Higher rates of mosquito feeding on infected hosts when compared to uninfected hosts. Positive relationship between parasitemia and feeding rates.	Groups of six mice uninfected, infected with either CR or ER or infected with a 1:1 mix of both *Plasmodium* clones. Mosquito exposure at 14 dpi. Asexual stages and gametocyte ratios calculated by microscopy. Anesthetized single-house mice exposed to 20–40 mosquitoes. Experiments lasted 20 min.	Ferguson et al. (2003)
Mice – *Mus musculus*	*Anopheles stephensi*	*Plasmodium chabaudi* – clone AS	Mosquito attraction as a function of host infection and infection stage.	Gametocyte-positive mice after acute stage more attractive (vs. negative and vs. chronically infected gametocyte-negative mice). Attractiveness associated to changes in volatile profiles due to infection.	Dual-choice assay in wind tunnel. Six pairs of infected and uninfected mice exposed to 20 mosquitoes for 15 min.	De Moraes et al. (2014)
Humans – *Homo sapiens* (children)	*Anopheles gambiae* – ICIPE lab strain	*Plasmodium falciparum* – natural infections	Mosquito attraction as a function of infection and gametocyte presence.	Children harboring gametocytes more attractive when compared to uninfected and to infected children harboring asexual parasite stages only. No difference in attractiveness after antimalarial treatment.	Children divided into three categories: uninfected, infected presenting asexual parasite forms only, and infected presenting gametocytes. One hundred mosquitoes released at the central chamber of three-way olfactometers in 12 experiments containing one child of each category. Experiments lasted 30 min.	Lacroix et al. (2005)
Humans – *Homo sapiens* (adults)	*Anopheles darlingi*	*Plasmodium vivax* – natural infections	Mosquito attraction as a function of gametocyte presence in infected humans.	Patients harboring gametocytes more attractive when compared to patients with no visible gametocytes and when compared to patients during and after antimalarial treatment.	One foot of *P. vivax*-infected patients exposed to 10 F_1 mosquitoes in single-choice olfactometers (three replicates each time) for 10 min. A subset of these patients were exposed to mosquitoes during and after antimalarial therapy (7 and 14 days after diagnosis, respectively). Gametocyte presence evaluated by microscopy only.	Batista et al. (2014)

(Continued)

TABLE 1 | Continued

***Plasmodium* Infections**						
Indirect manipulation						
Host sp.	**Vector sp.***	**Parasite species**	**Factor**	**Main Results**	**Experimental details**	**Source****
Mammal hosts						
Humans – *Homo sapiens* (children)	*Anopheles gambiae* – Mbita strain	*Plasmodium falciparum* – natural infections	Mosquito attraction as a function of gametocyte presence and intensity in infected humans.	Children with gametocytes detected by microscopy (higher intensity) more attractive than the other three groups (parasite free, asexual stages only and submicroscopic gametocyte levels). Similar attractiveness among all groups after treatment.	Children divided into four groups: Uninfected, asexual parasite stage only, gametocyte detected by qPCR only, and gametocyte detected by microscopy and by qPCR. One hundred mosquitoes in a dual-choice system. Experiments lasted 30 min and were repeated 3 weeks after antimalarial treatment.	Busula et al. (2017)
Humans – *Homo sapiens* (adults)	*Anopheles coluzzii* – lab colony	*Plasmodium falciparum* NF54 strain – experimental infections	Mosquito attraction as a function of host infection.	Odor samples from infected individuals less attractive when compared to treated and to uninfected individuals in one trial. No difference observed in the second trial.	Groups of 30 mosquitoes exposed to cotton pads with odor from infected humans and with ammonia (negative control) in a dual-choice system for 15 min. Odor samples from infected humans collected before, during (6–8 dpi) and after (34 dpi) treatment with antimalarial.	de Boer et al. (2017)
Humans – *Homo sapiens* (children – body odor)	*Anopheles gambiae* – Mbita strain	*Plasmodium falciparum* – natural infection	Mosquito attractiveness of foot odor as a function of host infection.	Odors from infected children more attractive when compared to odor from the same children after parasite clearance with antimalarials. No effect of gametocyte presence or absence.	Groups of 10 mosquitoes placed in a dual-choice system containing socks worn by the same child before and after antimalarial treatment (six replicates). A total 23 children presented circulating gametocytes (qPCR testing), 10 did not present circulating gametocytes (microscopy testing) and 12 were not infected (qPCR testing). Experiments lasted 15 min.	Robinson et al. (2018)
Direct manipulation						
Host sp.	**Vector sp.***	**Parasite species**	**Factor**	**Main Results**	**Experimental details**	**Source****
Avian hosts						
Domestic canary – *Serinus canaria*	*Cx. pipiens* (S-LAB strain)	*Plasmodium relictum* (SGS1) – experimental infection	Feeding rate of sporozoite-infected and uninfected mosquitoes as function of bird infection	Both infective and uninfected mosquitoes feed more on chronically infected birds when compared with uninfected birds. Positive correlation between hematocrit level and feeding rate in infected birds for both infected and uninfected mosquitoes	Birds from different groups exposed to mosquitoes in different cages. Ten cages with uninfected birds and 10 cages with infected birds exposed to 40 infected and 40 uninfected mosquitoes each. Behavioral assay at peak of sporozoite presence in mosquitoes, 12–14 dpbm. Behavioral assay 53–55 dpi of bird host. Immobilized birds. Feeding experiment lasted 2 h.	Cornet et al. (2013a)[6]
Domestic canary – *Serinus canaria*	*Cx. pipiens* (S-LAB strain)	*Plasmodium relictum* (SGS1) – experimental infection	Feeding rate as a function of mosquito infectivity (harboring sporozoites).	No difference in feeding rate between infective and uninfected mosquitoes at the end of the experiments (3 h). Uninfected mosquitoes feed faster than infective mosquitoes.	Six cages, each with an uninfected bird exposed to 45 uninfected and 45 sporozoite-infected mosquitoes (13 dpbm). Immobilized birds.	Cornet et al. (2019)[7]

(Continued)

TABLE 1 | Continued

***Plasmodium* Infections**						
Direct manipulation						
Host sp.	**Vector sp.***	**Parasite species**	**Factor**	**Main Results**	**Experimental details**	**Source****
Mammal hosts						
Humans – *Homo sapiens* (adults)	*Anopheles stephensi* (NIH strain)	*Plasmodium yoelii* (clone 17XNL)	Host choice as a function of mosquito infection, parasite stage and immune challenge.	Mosquitoes feeding on infected blood less likely to fly toward a human during oocyst stage and more likely to fly toward a human during sporozoites stage when compared to negative controls. Same changes were observed in mosquitoes infected with heat-killed *E. coli.*	Host-seeking was tested at short and long ranges. Behavioral challenges in mosquitoes injected with heat-killed *E. coli.* Trials conducted over a 1 h period.	Cator et al. (2013)
Human – *Homo sapiens* (odor)	*Anopheles gambiae* – Ngousso strain	*P. falciparum* – NF54 strain	Landing behavior in matrixes with human odor as a function of sporozoite infection in mosquitoes.	Infective mosquitoes more attracted to human odor when compared to uninfected mosquitoes.	Twenty mosquitoes of each group were provided with a choice of matrixes with and without human odor. Average landing rate calculated for each matrix group over 3 min.	Smallegange et al. (2013)
Humans – *Homo sapiens*	*Anopheles gambiae* and *An. coluzzii* – lab colonies replenished with wild-caught mosquitoes. *An. arabiensis* – wild-caught.	*Plasmodium falciparum* – natural infections.	Host choice as a factor of mosquito infection and parasite developmental stages (non-infectious vs. infectious).	No difference in host choice between infected and uninfected mosquitoes and between mosquitoes harboring either oocysts or sporozoites.	Mosquitoes fed on blood containing active and inactivated gametocytes from the same human host. Mosquito host-seeking tested in dual-choice system using humans, calf and outdoor air (control) lasting 30 min. Twenty infected and 20 uninfected mosquitoes tested simultaneously. Eight experimental replicates using 9 different gametocyte carriers.	Nguyen et al. (2017)
Arboviruses infections						
Host sp.	**Vector sp.**	**Virus**	**Factor**	**Main Results**	**Experimental details**	**Source**
House sparrow – *Passer domesticus*	*Culex quinquefasciatus* and *Culex tarsalis* (lab colony)	St. Louis encephalitis virus and Western equine encephalitis virus – laboratory strains	Mosquito attraction as a function of Arbovirus infection in birds	No difference in *C. quinquefasciatus* attraction toward SLEV-infected birds. No difference in *C. tarsalis* attraction toward WEEV-infected birds. *Culex quinquefasciatus* more attracted to adults than to nestlings.	Host attractiveness measured with 10 mosquitoes in each trial in a 50 cm single-choice vertical olfactometer (birds placed at the bottom) for 10 min.	Scott et al. (1990)[8]
Human – *Homo sapiens* and chicken – *Gallus gallus domesticus* (odors)	*Cx. pipiens* form pipiens (wild caught).	West Nile virus lineage 2 (originated from Greece)	Host-seeking response and host choice as a function of mosquito infection	WNV-infected mosquitoes had lower host-seeking response when compared to controls. No changes in flight duration and blood-feeding propensity.	Host-seeking response measured in an olfactometer. Between 53 and 57 mosquitoes from each group exposed to human and chicken odors for 7 min.	Vogels et al. (2017)

(Continued)

TABLE 1 | Continued

Uropygial secretion, volatile compounds and other attributes					
Host sp.	**Vector sp.**	**Factor**	**Main Results**	**Experimental details**	**Source**
Crow – *Corvus brachyrhynchus*	*Cx. pipiens/Cx. restuans* and *Aedes vexans* (experiment performed in the wild)	Mosquito attraction toward CO_2 baited traps as a function of trap height and presence of uropygial gland secretion.	*Cx. pipiens/Cx. restuans* more attracted to traps at 5 m high with uropygial gland secretion and dry ice (source of CO_2) when compared to traps with dry ice only (control group). No difference when traps were placed at 1.5 m high and in the attraction of *Ae. vexans* at both heights.	Study performed in Ontario, Canada. Uropygial secretion from frozen glands applied to cotton swabs. Swabs with and without uropygial gland secretion were attached to CDC traps baited with dry ice. Traps run at heights of 1.5 and 5 m in a total of 280 trap catches.	Russell and Hunter (2005)
American robin – *Turdus migratorius*	Several species observed during filming, including *Cx. pipiens*, *Cx. restuans*, and *Aedes albopictus* as the most common	Mosquito landing rates on either adults or nestlings of American robins at nests.	Mosquitoes land more frequently on nesting adults than on nestlings, and parental brooding activities reduces mosquitos landing rates on nestlings.	Natural experiment using direct observations via filming.	Griffing et al. (2007)
Blue birds – *Sialia sialis*	*Culex quinquefasciatus* (wild caught)	Feeding choice as a function of bird's age (mother vs. nestlings).	No feeding preference between mother and nestlings. Feeding upon mothers decreased as age of the nestlings increased.	Mosquitoes introduced into nest boxes containing mother and nestlings. Around 22 mosquitoes per nest box. Exposure run overnight.	Burkett-Cadena et al. (2010)
Zebra finch – *Taeniopygia guttata*	*Culex quinquefasciatus* – laboratory colony	Feeding choice as a function of corticosterone level in birds	Higher feeding rates on birds with artificially elevated levels of corticosterone.	Birds received one or two implants of corticosterone and were assigned to CORT+ and CORT++ groups, respectively. Negative control and birds from both CORT groups exposed simultaneously to 50 mosquitoes for 13 h. Birds were not immobilized.	Gervasi et al. (2016)[11]
House sparrows – *Passer domesticus*	*Cx. pipiens* (Laboratory colony)	Mosquito attraction toward chemicals from uropygial gland secretions as a function of bird sex and age	Mosquitoes more attracted to uropygial gland secretions from adults over nestlings. No preference for adults over fledglings. No difference in preference for adults, nestlings or fledglings when using semi-volatile components isolated from secretions.	Use of dual olfactometer. Thirty mosquitoes for each trial, which lasted 45 min.	Garvin et al. (2018a)
House sparrow – *Passer domesticus* European starling – *Sturnus vulgaris* American robin – *Turdus migratorius*	*Cx. pipiens* (laboratory colony)	Mosquito attraction to chemicals from uropygial gland secretions as a function of bird species.	Chemical composition of robins' uropygial gland secretions was different to those of sparrows and starlings. No differences on *Cx. pipiens* preference for secretions of robins over those of sparrows. However, *Cx. pipiens* more attracted to live starlings and starlings secretions than to either live robins or their secretions.	Adult House sparrows, European starlings and American robins. Thirty mosquitoes for each trials, which lasted 2 h.	Garvin et al. (2018b)

(Continued)

TABLE 1 | Continued

Uropygial secretion, volatile compounds and other attributes					
Host sp.	**Vector sp.**	**Factor**	**Main Results**	**Experimental details**	**Source**
House sparrow – *Passer domesticus*	*Cx. pipiens* – wild caught (not distinguished for pipiens and molestus forms).	Feeding choice as a function of bird metabolic rate.	Mosquito feeding rate similar between control and treatment group. Negative association between mosquito feeding preference and bird resting metabolic rate. Positive association between mosquito feeding preference and bird body mass.	Use of 2,4-dinitrophenol to induce higher metabolic rate. Thirty juvenile House sparrows, 21 males and nine females. Trials included a pair of birds exposed to an average of 190 mosquitoes for 12 h. Birds were not immobilized.	Yan et al. (2018a)[12]
Great tit – *Parus major*	*Cx. pipiens* (laboratory colony established with local mosquitoes	Feeding choice as a function of host sex.	No difference in host choice due to nestling sex	Eighteen independent cages with a pair of 14 days old siblings, one male and on female. Twenty-five uninfected mosquitoes in each cage allowed to feed for 1 h. Host sex determined by PCR.	Cozzarolo et al. (2019)
House sparrow – *Passer domesticus* (juveniles)	Wild caught *Ochlerotatus* (*Aedes*) *caspius* and *Cx. pipiens* (not tested for pipiens and molestus forms and hybrids)	Mosquito attraction toward uropygial gland secretion.	No difference in mosquito attraction between uropygial gland secretion and negative controls.	Dual choice (Y-shaped) olfactometer. Negative controls consisted on air flow with no stimuli. Each trial run with 20 mosquitoes (6–15 days old) for 15 min. *Cx. pipiens* assayed in 21 trials and *O. caspius* assayed in 20 trials.	Díez-Fernández et al. (2020a)[10]
House sparrow – *Passer domesticus* (juveniles)	*Cx. pipiens* – wild caught. Not tested for pipiens and molestus forms and hybrids.	Mosquito attraction toward uropygial gland secretions and whole-body odor as a function of *Plasmodium* infection.	Mosquitoes more attracted to whole-body odors of infected sparrows regardless of sex. No effect when using uropygial secretions. The volatile lipophilic fraction of the uropygial secretion was not different between infected and uninfected sparrows.	Dual choice (Y-shaped) olfactometer. Experimental pairs were always of the same sex. Each trial run with 20 mosquitoes (6–15 days old) for 15 min in complete darkness. Analyzed whether *Plasmodium* spp. infections alter chemical composition of uropygial secretions.	Díez-Fernández et al. (2020b)[9]
Chicken (*Gallus gallus domesticus*), pigeon (*Columba livia*), and magpie (*Pica pica*)	*Culex quinquefasciatus* (Thai strain) and *Cx. pipiens molestus* (laboratory colony)	Mosquito attraction toward whole-body odor in the presence or absence of an extra source of CO_2.	*Cx. quinquefasciatus* more attracted to both chicken sexes and to female pigeons. No difference toward male pigeons and magpies. Addition of CO_2 increased attraction toward chickens and pigeons, but not toward magpies. *Culex p. molestus* more attracted only to chickens (both sexes). No effect on the addition of CO_2.	Single-choice and dual choice olfactometers used for *Cx. quinquefasciatus* and for *Cx. p. molestus*, respectively. Bird sex determined only for chickens and pigeons. Ten trials performed with 30 mosquitoes lasting 4 min for *Cx. quinquefasciatus* and 2 min for *Cx. p. molestus*.	Spanoudis et al. (2020)

** Cx. pipiens for Diptera taxonomists is still under debate, and sometimes is used as a synonym of Cx. quinquefasciatus. Some authors even write is as Cx. pipiens quinquefasciatus.*
*** Superscript numbers, whenever present, are related to the experiments described in **Figure 1**.*
From the perspective of the vector, here we consider an indirect manipulation when the parasite is infecting the vertebrate host (i.e., the parasitic infection changes some trait of the host that makes it more attractive to the vector), and a direct manipulation when the parasite is inside the vector.

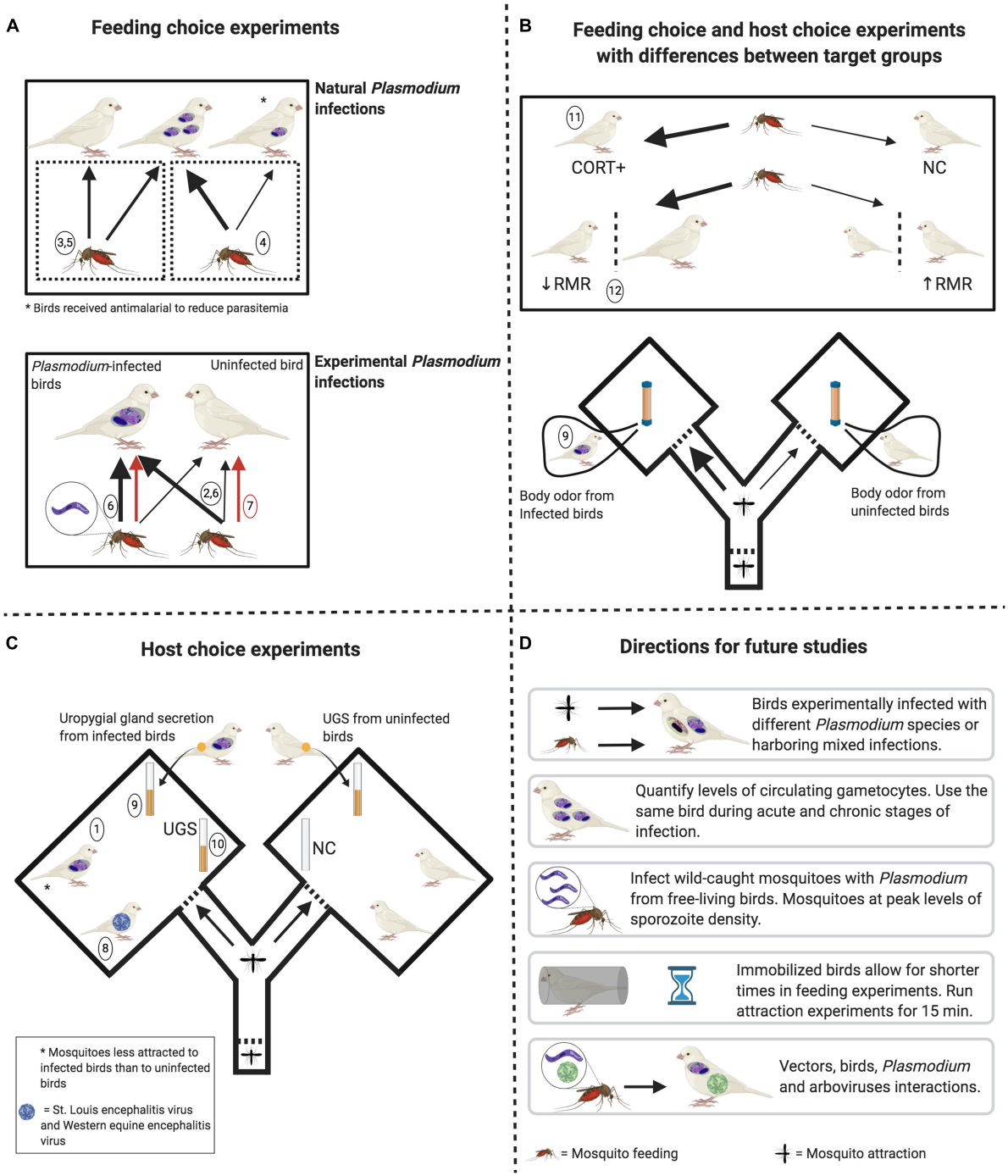

FIGURE 1 | Experimental designs to determine the factors potentially affecting mosquito feeding behavior and attraction toward birds. **(A) Upper** pane: mosquitoes do not feed more on naturally infected birds than on uninfected birds. However, feeding rates are higher on birds with higher levels of circulating parasites (thick black arrow) when compared to birds with reduced levels of parasites after antimalarial treatment (thin black arrow). **Lower** pane: uninfected mosquitoes feed more on birds experimentally infected with *Plasmodium relictum* (thick black arrow) when compared to uninfected birds. Infective mosquitoes (harboring sporozoites) may (thick black arrow) or may not (red arrows) feed more on infected birds than on uninfected ones. **(B) Upper** pane: Mosquitoes feed more (thick black arrows) on birds with higher levels of corticosterone (CORT+) and feed more on birds with lower resting metabolic rate (RMR) and with higher body mass. **Lower** pane: Mosquitoes are more attracted toward whole-body odor from birds naturally infected with *Plasmodium* parasites. **(C)** No mosquito preference in relation to bird arboviral infection and to uropygial gland secretion (UGS) from *Plasmodium*-infected birds when compared to UGS from uninfected birds. No mosquito preference toward UGS when compared to vials containing no stimuli. Mosquitos were less attracted to birds naturally infected with *Plasmodium*. **(D)** Open questions that can be addressed in future studies, always keeping in mind to standardize bird and mosquito cohorts in terms of age and sex. Numbers in circles refer to specific studies indicated in **Table 1** (see Source column). Figure created with BioRender.com.

Human and Mice Malaria

Most studies on human malaria found that natural *Plasmodium* infections are associated with higher host attractiveness to vectors. Three out of four studies found that *Anopheles* mosquitoes were more attracted to humans harboring microscopically detectable gametocytes when compared to uninfected people and to people with only asexual stages detectable in the blood stream (Lacroix et al., 2005; Batista et al., 2014; Busula et al., 2017). The fourth study showed that socks worn by children parasitized by *Plasmodium falciparum* were more attractive to mosquitoes when compared with socks worn by the same children after the completion of antimalarial treatment (Robinson et al., 2018). A study during its first trial using feet odors found that samples from individuals infected with *P. falciparum* were less attractive to mosquitoes when compared to cotton pads from the same individuals after antimalaria treatment and compared to odors from uninfected people; the second trial did not find any difference in mosquito attraction (de Boer et al., 2017).

Two studies using mice malaria found that *Plasmodium chabaudi* infection increased *Anopheles stephensi* feeding rates (Ferguson et al., 2003) and attraction (De Moraes et al., 2014) when compared to uninfected hosts. This latter study found this effect only during the initial chronic stage, which was associated to microscopically observable levels of circulating gametocytes. These studies on mammals also show contrasting results, precluding generalizations and strengthening the fact of having a standard initial experimental design.

Infected Mosquitoes and Host-Feeding

Pathogens can influence host-seeking and feeding behaviors during their development inside mosquitoes. Because mosquitoes only become infective a few days or even weeks after blood feeding on an *Plasmodium*-infected host (i.e., once sporozoites are found in salivary glands; Valkiūnas et al., 2013; Palinauskas et al., 2016), changes in host-seeking and feeding behaviors may be displayed differentially according to vector infective stage rather than infection status only.

When mosquitoes were simultaneously exposed to both *Plasmodium*-infected and uninfected birds, both infective and uninfected mosquitoes fed more frequently on *Plasmodium*-infected birds (Cornet et al., 2013a). When exposed to uninfected birds, uninfected mosquitoes fed faster than infective mosquitoes, but the final feeding rate after the 3 h the experiment lasted was identical between both groups (Cornet et al., 2019). This demonstrates that experiment duration can affect results, because the authors would have observed higher feeding rates of uninfected mosquitoes if experiments had lasted only 1 h.

Mosquitoes that fed on *Plasmodium yoelii*-infected mice were less attracted to hosts during the oocyst stage (non-infective) than during the sporozoite stage, when they engaged in higher rates of host-seeking behavior when compared to negative controls (Cator et al., 2013). However, these changes were also observed in mosquitoes that were not infected after feeding on infected mice. Therefore, the observed behavioral changes may be due to *Plasmodium* infections and to unspecific immune stimuli in the challenged mosquitoes.

Laboratory reared *Anopheles gambiae* harboring sporozoites of a laboratory strain of *P. falciparum* were more attracted to human odor when compared to infective mosquitoes exposed to negative-controls (Smallegange et al., 2013). In contrast, three different species of wild caught *Anopheles* infected with *P. falciparum* from natural infections had similar attraction rates to humans when compared with uninfected mosquitoes (Nguyen et al., 2017).

CHEMICAL COMPOUNDS STUDIED IN RELATION TO VECTOR ATTRACTION

Differences in mosquito attraction are related to dissimilarities in chemical volatiles produced by each host, which can be modified by pathogen infections. Díez-Fernández et al. (2020b) were the first to show that whole-body odor from *Plasmodium*-infected birds are more attractive to mosquitoes when compared to uninfected birds. De Moraes et al. (2014) demonstrated that mice displayed different odor profiles when infected with *Plasmodium chabaudi* and became more attractive to mosquitoes after the acute phase of infection. Mice produced fewer volatile chemicals during the acute phase of infection and attracted less mosquitoes. In humans, increased mosquito attraction toward *Plasmodium*-infected children was associated to changes in skin odor profile driven by increases in the production of aldehydes (Robinson et al., 2018). Children infected with *P. falciparum* in Malawi produced a different chemical breath composition than uninfected children (Schaber et al., 2018), which mosquitos can use as a cue to find their hosts (Kelly et al., 2015). Thus, the chemical volatiles emitted by hosts and how they change during infection likely constitute the most important factor determining vector attraction.

Birds' uropygial (or preen) gland produces some antimicrobial and antifungal chemicals that protect the plumage and skin of birds; they also provide defense against ectoparasites and haemosporidian infection (Salibian and Montalti, 2009; Magallanes et al., 2016). Some studies suggest that uropygial gland secretions can attract vectors (Russell and Hunter, 2005), but others suggest no increased attraction toward such secretions (Díez-Fernández et al., 2020a) or toward common chemicals from uropygial glands (Allan et al., 2006) when compared to negative controls (no stimuli). The amount of volatile compounds of uropygial glands differ among adult, fledging and nestling house sparrows (*Passer domesticus*), but these differences did not modify mosquito attraction in dual-choice olfactometer assays (Garvin et al., 2018a). *Plasmodium* infection did not change the composition of the volatile fraction of uropygial gland secretion in house sparrows when compared to the uninfected group, and this may explain the similar mosquito attraction toward the secretion from both bird groups (Díez-Fernández et al., 2020b). However, other study showed that exposure to *Plasmodium* altered the wax ester composition of the secretion in song sparrows (*Melospiza melodia*), regardless of whether infections were cleared or not (Grieves et al., 2018).

Therefore, more research is needed to cover the whole array of chemicals produced by the uropygial gland and their effect on different vector species and families (Martínez-de la Puente et al., 2020). Particularly considering that parasites from different Haemosporida genera (e.g., *Haemoproteus*, *Leucocytozoon*) are transmitted by different Diptera families, which have both different life cycles and ecologies (Ibáñez-Bernal et al., 2020).

STUDIES FOCUSED ON OTHER HOST ATTRIBUTES

Physiological differences (e.g., hormones) and differential exposure between males and females due to sex-specific physiology (e.g., immunosuppression due to breeding activities), behavior (e.g., nesting), morphology (e.g., body size), and pathogen infections can influence mosquito attraction (Darbro et al., 2007; Cozzarolo et al., 2019). For instance, *Cx. pipiens* were more attracted to female Great tits (*Parus major*; Lalubin et al., 2012). *Ochlerotatus caspius* fed more on female than on male jackdaws (*Corvus monedula*), but no difference was observed when using *Cx. pipiens*. In addition, these two mosquito species did not show sex-biased feeding preference toward house sparrows (Gutiérrez-López et al., 2019a). Cozzarolo et al. (2019) did not find any mosquito feeding preference between sexes, but they used nestlings instead of adults (see also Simpson et al., 2009). Higher feeding rate on female birds by wild-caught *Culex restuans* has been reported during the nesting period, suggesting the role of seasonality in this process (Egizi et al., 2014), but this has yet to be investigated under laboratory conditions. Yan et al. (2018a) found that mosquitos fed less on birds with higher resting metabolic rate, which authors interpreted as related to bird activity, where birds with higher metabolic rates are more active and thus display more defensive behaviors than more lethargic individuals. Finally, elevated levels of corticosterone increased bird attractiveness to mosquitoes despite the fact that these birds displayed increased anti-mosquito behaviors when compared to the control group (Gervasi et al., 2016). Increases in corticosterone levels may lead to *Plasmodium* resurgence in the blood stream in chronically infected birds with an increase in parasitemia (Applegate and Beaudoin, 1970). Therefore, the interaction between elevated stress hormone levels and parasitemia may synergistically increase bird attractiveness to mosquitoes, but this has yet to be tested. Different results across studies may be due to differences in life history traits of bird species, which may in turn influence exposure to vectors and species-specific physiological trade-offs. Hence, we suggest that fair comparisons and generalizations must be done across species (both hosts and vectors) with similar life history traits.

SUMMARY OF CURRENT EVIDENCE AND IMPLICATIONS FOR FUTURE STUDIES

We found that fewer studies assessed direct effects of avian and mammalian *Plasmodium* on mosquito host choice (infective mosquitoes) when compared to the number of studies on indirect effects on mosquitoes (infected vertebrate hosts). Rearing mosquitoes under laboratory conditions over many generations may alter some genetic traits involved in their responsiveness to host cues (Takken and Verhulst, 2013), but nothing is known about whether genetic bottlenecks would change as a function of host-vector-parasite lineage assemblages (Gutiérrez-López et al., 2020). Although using hosts (birds and mice), mosquitoes and pathogens maintained in laboratories has provided fundamental tests of hypothesis related to the vector manipulation by pathogens, experiments mimicking natural conditions using wild caught mosquitoes are essential to fill in gaps for real world situations.

Free-living birds are infected with genetic lineages that vary in their pathological effects as a function of host species and parasite lineage (Palinauskas et al., 2011; Himmel et al., 2020). Assays using experimental infections have focused on domestic canaries infected with a *P. relictum* strain (SGS1) kept in the laboratory over dozens of passages, while assays using free-living birds have used three avian species naturally infected with a high diversity of parasites. In Gutiérrez-López et al. (2019a), 21 of 23 jackdaws exposed to wild-caught *Cx. pipiens* were naturally infected with *P. relictum* (SGS1), and yet these authors did not find differences in mosquito feeding rates between infected and uninfected birds. On the other hand, laboratory-reared mosquitoes fed more on canaries experimentally infected with a laboratory strain of *P. relictum* SGS1 (Cornet et al., 2013a,b).

The use of naturally infected birds has the limitation of confounding the bird's infection stage during mosquito exposure. This is because in birds the *Plasmodium* acute stage of infection usually finishes within a month post infection. After this period surviving birds enter the chronic stage of infection, in which they display low parasitemia for long periods or even for their entire life (Valkiūnas, 2005). In many of the studies, free-living birds were undergoing chronic infections when exposed to mosquitoes and parasitemia was not evaluated. Interestingly, mosquitoes were more attracted to chronically infected birds when compared to acute infection stages in experimental settings (Cornet et al., 2013b), which may be interpreted as a parasite avoidance strategy because high parasitemia in the host is associated to high vector mortality (Gutiérrez-López et al., 2019b).

Birds and mosquitoes can be co-infected by different genetically related or unrelated parasites (e.g., co-infection between *Plasmodium* parasites and WNV; Hughes et al., 2010; Medeiros et al., 2014). Co-infections by different *Plasmodium* spp. and other related haemosporidians are often found in wild birds (e.g., Valkiūnas et al., 2006). Yet, evidence for the effects of multiple infections on vector feeding behavior and on fitness of both bird hosts (e.g., Marzal et al., 2008) and vectors (e.g., Gutiérrez-López et al., 2020) is still very limited (see Martínez-de la Puente et al., 2020). To complicate matters further, if mosquitoes are more attracted to birds co-infected with *Plasmodium* and arboviruses than to birds infected with arboviruses only, avian malaria may indirectly increase the transmission of zoonotic pathogens. Future studies, therefore, should perform feeding or attraction assays using birds

experimentally infected with strains of different *Plasmodium* species with as few laboratory passages as possible, and also using different parasite groups (e.g., malaria and arbovirus). In addition, stage of infection (acute vs. chronic) and gametocyte levels (parasitemia) should be taken into consideration in studies using both natural and experimental infections. Furthermore, mosquitoes captured from the wild should be favored over populations kept in the laboratory for many generations. This would provide more real settings to understand host attractiveness to free-moving mosquitoes in nature.

Important issues to consider when planning experiments are:

(1) will birds be free or immobilized? Free birds may reflect natural conditions, but immobilized birds will increase vector sample size and reduce vector mortality by birds' behavioral defenses. (2) Will birds be visible to vectors or just odor cues will be investigated? Our opinion is that light levels should reflect those from the natural conditions during which vector activity peaks, which depends on vector species. (3) What would be the duration of the experiment? Immobilizing birds allows for shorter feeding experiments (1–3 h), while 15 min seems to be a common and reasonable time for attraction experiments. Ideally, researchers must know the natural feeding ecology of the studied vector in order to make an informed decision on the duration of experiments. Finally, (4) co-infections by different *Plasmodium* lineages or different parasite genera (e.g., *Plasmodium*/*Haemoproteus*) that are ubiquitous in nature, or even by Haemosporidians and arboviruses (e.g., WNV and USUV) should be considered. Understanding factors that change mosquito attraction to birds may have implications for human health as well, since highly ornithophilic mosquitoes such as *Cx. pipiens*, *Cx. perexiguus*, and *Cx. restuans* are vectors of some arboviruses that may affect humans, such as WNV and USUV (e.g., Kilpatrick et al., 2006; Vázquez et al., 2011; Brugman et al., 2018). Thus, further studies are necessary to understand the range of effects that multiple infections may have on host-vector contact rates and, hence, on the dynamics of parasite transmission. Our review highlights the need to design and implement general standard experimental procedures to obtain comparable results, which can help to generalize findings across studies and to identify contingencies (**Figure 1**) that later will allow drawing generalizations by, for instance, conducting meta-analyses.

AUTHOR CONTRIBUTIONS

Both authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Mosquito Behavior and Vertebrate Microbiota Interaction: Implications for Pathogen Transmission

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The microbiota is increasingly recognized for its ability to influence host health and individual fitness through multiple pathways, such as nutrient synthesis, immune system development, and even behavioral processes. Most of these studies though focus on the direct effects microbiota has on its host, but they do not consider possible interactions with other individuals. However, host microbiota can change not only host behavior but also the behavior of other individuals or species toward the host. For example, microbes can have an effect on animal chemistry, influencing animal behaviors mediated by chemical communication, such as mosquito attraction. We know that host skin microbes play a major role in odor production and thus can affect the behavior of mosquitoes leading to differences in attraction to their hosts. Ultimately, the vector feeding preference of mosquitoes conditions the risk of vertebrates of coming into contact with a vector-borne pathogen, affecting its transmission, and thus epidemiology of vector-borne diseases. In this mini review, I provide an overview of the current status of research on the interaction between mosquito behavior and host skin microbiota, both in humans and other vertebrates. I consider as well the factors that influence vertebrate skin microbiota composition, such as sex, genetic makeup, and infection status, and discuss the implications for pathogen transmission.

Keywords: microbiota, chemical communication, host preference, mosquitoes, vector-borne disease, pathogen transmission

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INTRODUCTION

In a world dominated by microorganisms, animals host diverse microbial communities on different body parts. These communities of symbiotic microorganisms consist of bacteria, archaea, fungi, and viruses, and are of significant importance to host health. In addition to their involvement in important host physiological processes like digestion and nutrient synthesis (Cummings and Macfarlane, 1997), they modulate immune system development (Round and Mazmanian, 2009; Bengmark, 2013) and offer protection against pathogens. This protection is achieved by limiting pathogen adhesion to host cells (Buffie and Pamer, 2013), competing for resources (Kamada et al., 2012), or producing antimicrobial compounds (Fukuda et al., 2011). Changes in composition of the microbiota can lead to physiological changes that will increase the risk of infection by opportunistic pathogens or impair the immune response (Croswell et al., 2009).

The skin is the largest organ in the body and the first barrier of interaction with the environment. The direct effect of skin microbiota on host health has been studied both in humans (Cogen et al., 2008) and wildlife (Williams et al., 2019). For instance, susceptibility to chytridiomycosis in amphibians is associated with differences in skin microbiota composition (Bates et al., 2018). Research also shows that certain skin-associated bacteria inhibit the fungal pathogens that cause chytridiomycosis (Kueneman et al., 2016) and white nose syndrome in bats (Hoyt et al., 2015). In addition, skin microbiota generates odors that act as chemical cues to attract vectors that use olfaction as their main sense to choose their host. This is the case of mosquitoes (Rudolfs, 1922), which are known vectors of many life-threatening diseases, such as malaria, West Nile virus (WNV), or Dengue (Chen et al., 1993; Shin et al., 2002; Balenghien et al., 2008). The transmission of vector-borne diseases depends significantly on how the vector selects its feeding host (Gandon, 2018). Those individuals that are more attractive have a higher risk of coming into contact with a pathogen. Therefore, the specific odor profile of individuals and species that lead to differences in the attraction will profoundly affect the epidemiology of vector-borne diseases. The present paper provides an overview of the current status of research on the interaction between mosquito behavior and skin microbiota both in humans and other vertebrates. The focus of this mini review is mammals and birds because they are the main zoonosis reservoirs. I will discuss what we know about: (i) the role that host skin microbiota plays in odor production; (ii) how changes in skin microbiota composition can lead to differences in mosquito attraction to their hosts both at the intraspecies and interspecies level; and (iii) what factors may affect the skin microbiota composition and thus mediate disease transmission. I will identify both the knowledge gaps and potential future research lines that would help to understand the interaction between mosquito behavior and vertebrate skin microbiota and its impact on health and disease spread.

THE VERTEBRATE SKIN MICROBIOTA AND VOLATILE PRODUCTION

The microbiota of the human skin is highly complex (Grice et al., 2008), and overall microbial composition varies strongly between individuals depending on factors, such as age, sex, or habits (Fierer et al., 2008). Studies in other mammals have revealed that different species have distinct skin microbial communities, which are in general more diverse than the microbial communities in humans (Council et al., 2016; Ross et al., 2018). Some of these differences were driven by changes in the abundance of certain groups of bacteria (A decrease in *Actinobacteria* and an increase of *Chloroflexi* and *Bacteroidetes*; Ross et al., 2018). For some mammals, including humans, skin sites vary in their microbial composition and skin microorganisms tend to be more abundant around glands and skin pouches (Verhulst et al., 2010a; Leclaire et al., 2014; Kamus et al., 2018).

Studies in birds show that like mammal skin, feathers harbor their own microbial community with a composition that varies between individuals and species (Engel et al., 2018). *Staphilococcus*, *Bacillus*, *Pseudomonas*, and *Stenotrophomona* are some of the most common bacteria genera found on the plumage of different bird species (Whittaker et al., 2019). Birds' main holocrine gland is the uropygial gland, which produces an oily secretion that birds preen onto their feathers. A significant amount of this secretion is formed by lipids that may control the growth of feather-degrading bacteria living on the plumage or be used as nutrients by feather microbiota that produce body odors (Purton, 1986). In addition, it has been shown in several species that uropygial gland area and secretion produced also contain bacteria (Martín-Vivaldi et al., 2010; Whittaker et al., 2019).

Symbiotic microbial communities in the skin of both mammals and birds play a significant role in odor production through the generation of volatile compounds like linear alcohols, methyl ketones, and carboxylic acids (Madigan et al., 2010). Different species of skin bacteria have distinct metabolic routes that produce a variety of compounds. For example, in humans, the odor associated with the axillary glands has been linked to *Corynebacteria* (Leyden et al., 1981), which generate volatile fatty acids (James et al., 2004). *Staphylococcus* species, common in humans and birds, can metabolize branched-chain amino acids into short-chain amino acids that are volatile and highly odorous (James et al., 2004). In birds, volatile compounds from the uropygial gland secretion were linked with several bacteria genera, including *Pseudomonas* and *Staphylococcus* (Whittaker et al., 2019). Therefore, skin/feather microbiota that differs not only in composition but also in the abundance of certain bacteria species will generate a characteristic odor profile for each individual (Theis et al., 2012; Leclaire et al., 2014). These olfactory cues are associated with host sex, age, and social interactions, indicating the potential for chemical communication (Fierer et al., 2008; Theis et al., 2012; Leclaire et al., 2014).

MOSQUITO BEHAVIORAL RESPONSE TO SKIN MICROBIOTA VOLATILES IN VERTEBRATES

The idea that the volatile compounds produced by skin microbiota could attract mosquitoes was first proposed by Schreck and James (1968). They tested whether the volatiles of a broth where they had grown bacteria (*Bacillus cereus*) was attractive to *Aedes aegypti*. The results showed that the volatiles produced were attractive. Since then, several studies have shown that human skin bacteria produce volatiles that are attractive to mosquitoes (De Jong and Knols, 1995; Braks et al., 2000; Verhulst et al., 2009). These studies confirm that skin microbiota influences host seeking behavior in mosquitoes. But skin microbiota composition is unique for each individual, contributing to the generation of distinct odor profiles, which drive mosquito host seeking behavior and selection (Verhulst et al., 2018; **Figure 1**). In fact, it is

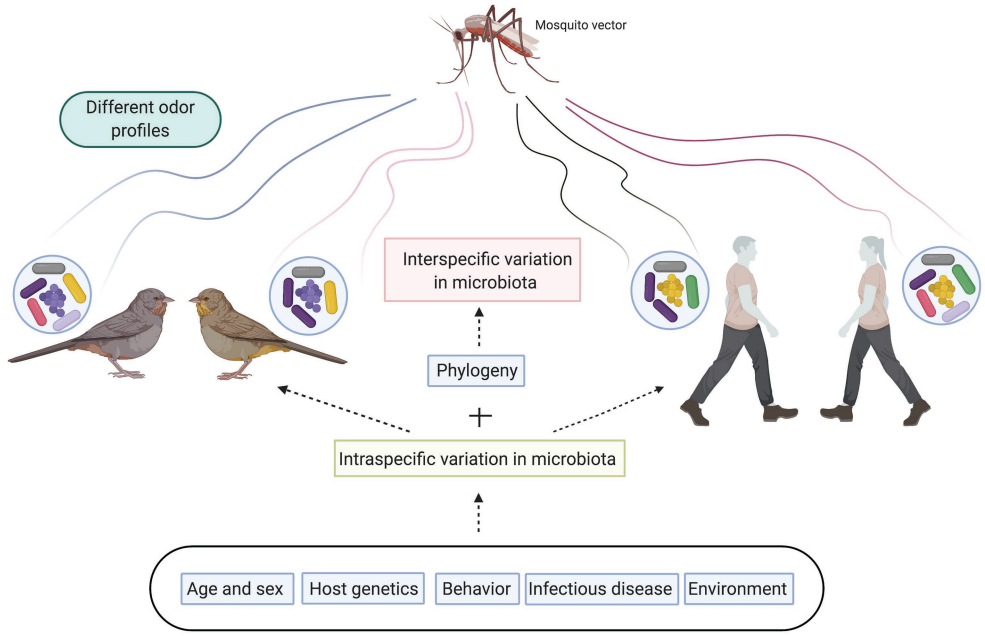

FIGURE 1 | Graphical outline of factors affecting skin and plumage microbiota composition leading to intraspecific and interspecific differences in microbiota profiles. These different microbiota profiles will produce distinct odor profiles that mosquitos use as cue to select host they will feed on.

clear that mosquitoes show a preference to bite certain individuals, demonstrating that there are intraspecific differences in the attractiveness of individuals to mosquitoes (see Bernier et al., 2002 and Mukabana et al., 2002 for examples in humans). Several studies in humans have shown that these differences in attractiveness are mediated by the microbiota. Two species of bacteria were identified as key in determining attractiveness, *Pseudomonas aeruginosa*, and *Staphilococcus epidermis*. Mosquito species of the *Anopheles gambiae* complex were not attracted to the odor produced by *P. aeruginosa*, while the odor of *S. epidermis* was attractive (Verhulst et al., 2010b, 2011). In addition, Verhulst et al. (2011) demonstrated that the more attractive individuals had less diverse microbiota, but hosted a higher abundance of some bacteria including *Staphylococcus*. In contrast, *Pseudomonas* was more abundant in poorly attractive individuals. This pattern of intraspecific differences in attractiveness to mosquitoes plays a significant role in pathogen exposure and pathogen transmission dynamics. The more attractive individuals have more likelihood of being bitten, increasing their chances of being infected by mosquito-borne pathogens and driving pathogen transmission.

Although most studies linking bacteria, volatile production and mosquito attraction have been carried out in humans and anthropophilic mosquitoes, studies in other species show similar patterns. For example, in some species of birds, both the uropygial gland secretion and volatile compounds of skin and plumage have been shown to attract mosquitoes (Russell and Hunter, 2005; Bernier et al., 2008; but see Douglas et al., 2005). However, despite the evidence that bacteria in the uropygial gland and plumage produce volatile compounds that can be used in olfactory communication (Maraci et al., 2018), there is no study yet linking the production of volatile compounds that specifically attract mosquitos with bacteria species in birds.

Mosquito species differ widely in their host preference, defined as "the trait to preferentially select certain host species above others" (Takken and Verhulst, 2013). While many mosquito species are opportunistic and their host preference is mainly driven by host abundance and availability, other species show strong preferences, feeding mainly on mammals (mammophilic), birds (ornithophilic), or other vertebrate groups such as reptiles (Chaves et al., 2010; Martínez-de la Puente et al., 2015). Some mosquito species even exhibit a marked preference to feed on a single host species, no matter the circumstances, like *An. gambiae* sensu stricto that is highly anthropophilic (Pates, 2002). Host preference is one of the key determinants of the vectorial competence for disease transmission because it determines the risk that a vertebrate species will come into contact with a pathogen (Gandon, 2018). Those species of mosquitoes with plastic behavior that adapt to feed on available vertebrate species play a key role in pathogen transmission between species and may drive zoonosis emergence (Takken and Verhulst, 2013; Yakob et al., 2018). One of the classic examples illustrating this situation is the transmission of WNV to humans. This virus is maintained in nature in an enzootic cycle involving ornithophilic mosquitoes, which are the transmission vector. *Culex pipiens* is one of the primary vectors of WNV and typically feeds on birds. However, under certain circumstances like migration events when the abundance of birds changes, they switch host to humans contributing to the spread of WNV in humans (Farajollahi et al., 2011). To further understand host selection in mosquitoes that act as vectors of WNV, Spanoudis et al. (2020) compared

the responses of *Culex quinquefasciatus* and *Cx. pipiens molestus* to volatiles of different bird species. *Culex quinquefasciatus* shows phenotypic plasticity and its feeding preference varies in different ecotypes (Mboera and Takken, 1999; Molaei et al., 2006). *Culex pipiens molestus* is an anthropophilic form of *Cx. pipiens* (Vinogradova, 2000) adapted to urban environments. Both species of mosquitoes responded to bird volatiles but differed in their preference (Spanoudis et al., 2020). *Culex quinquefasciatus* responded to the volatiles of both sexes of chickens (*Gallus Gallus domesticus*), and female pigeons (*Columba livia*). *Culex pipiens molestus* responded to the volatiles of chickens and magpies (*Pica pica*). These differences in feeding preference are critical for disease transmission. Out of the species included in the study, magpies are the most susceptible to WNV (Jiménez de Oya et al., 2018) allowing its circulation in wild populations (Napp et al., 2019). Mosquitoes that prefer feeding on both magpies and humans facilitate transmission of WNV between the bird reservoir and humans. This example shows that to determine the potential of mosquitoes as disease vectors it is crucial to understand their feeding preferences, which involves studying how mosquitoes select their host, and how odor profiles and microbiota are influencing this choice. In this sense, several studies in different taxa have analyzed if the odor of different species was equally attractive to a set of species of mosquitoes. Bakker et al. (2020) set traps with odors from chimpanzees (*Pan troglodytes*), humans, and cows (*Bos taurus*) and identified the mosquitoes captured. Most of the mosquito species trapped during this study were equally attracted to all mammal species tested showing a generalistic host preference. Another study carried out with house sparrows (*Passer domesticus*) showed no differences in the response of the ornitophilic mosquitoes (*Cx. pipiens*) and mammophilic mosquitoes [*Aedes* (*Ochlerotatus*) *caspius*] when exposed to the uropygial gland secretions (Díez-Fernández et al., 2020a). Therefore, it seems that the preference of ornithophilic mosquitoes for avian hosts is not associated with attraction to the uropygial gland secretion odor. However, it is possible that mosquitoes respond to specific volatile compound released when the secretion is deposited on the feathers and metabolized by the bacterial community (Díez-Fernández et al., 2020a). In humans, it has been shown that this variation in mosquito response is in fact mediated by the composition of skin microbiota volatiles (Busula et al., 2017). In this study, two species of mosquitoes with different host preferences (*An. gambiae* and *An. arabiensis*), exhibited a different response to the volatiles released from skin bacteria from three different mammal species (human, cow, and chicken). *Anopheles gambiae* showed higher attraction to bacteria volatiles of human origin, and displayed a specialized response to four species of bacteria preferring volatiles from *Corinebacterium minutissimum*, one of the most abundant microbes in human skin. In contrast, *An. arabiensis* showed more attraction to bacterial volatiles from chickens responding equally to all species of bacteria tested. More studies like this one, including different vertebrate and mosquito species, will help to understand how skin microbiota drives interspecies differences in mosquito attraction and mediates potential pathogen transmission.

FACTORS AFFECTING SKIN MICROBIOTA COMPOSITION: POTENTIAL IMPACT ON MOSQUITO ATTRACTION AND PATHOGEN TRANSMISSION

Skin microbiota composition is influenced by multiple factors. In addition to the phylogeny of the host discussed above, age, sex, behavior, genetic makeup of the host, and environmental variables, also impact skin microbiota composition (Fierer et al., 2008; Theis et al., 2012; Leclaire et al., 2014; **Figure 1**). Therefore, these factors will indirectly affect how mosquitoes select their host leading to differential exposure to vector-borne pathogens among individuals. Some studies have analyzed the effect of sex on attractiveness to mosquitoes. One study found no differences in exposure from males and females of great tit nestlings (*Parus major*) to bites of *Cx. pipiens* (Cozzarolo et al., 2019). In contrast, Spanoudis et al. (2020) showed that female pigeons were attractive to *Cx. quinquefasciatus* while males were not attractive. Regarding genetic makeup, it is recognized that there is a genetic component determining susceptibility to being bitten (Fernández-Grandon et al., 2015) and odor profile is partially genetically based (Kuhn and Natsch, 2009). Interestingly, the genes of the major histocompatibility complex (MHC), the most important cluster of immune genes, are also some of the most important candidate genes for explaining body odor (Penn and Potts, 1998) and have been linked in humans with differences in mosquito attraction (Verhulst et al., 2013). Two combined hypotheses explain the potential effect of MHC on odor. First, it has been proposed that degraded MHC molecules directly influence odor (Pearse-Pratt et al., 1992). Second, it is known that MHC variation correlates with gut microbiota composition and diversity (Bolnick et al., 2014), and that skin microbiota triggers immune responses associated with the MHC (Dillon et al., 2000). Furthermore, in two seabird species, there is an association between MHC genotypes and microbiota composition in the uropygial gland (Pearce et al., 2017) and plumage (Leclaire et al., 2019). Thus, MHC genes may also influence the odor of individuals by shaping the microbiota composition of skin/feathers and adjacent glands, leading to differences in exposure to vector-borne pathogens among individuals.

A factor that can impact microbiota composition and has not been studied so far is infectious disease. It is known that mosquito-borne pathogens, like malaria (Taniguchi et al., 2015), are associated with changes in gut microbiota. Little is known however, about potential changes in skin microbiota. But changes in skin microbiota composition might be critical in determining disease transmission, if the new profiles make the infected individuals more attractive to mosquitoes. Although so far there are no studies associating changes in skin microbiota due to infection with attractiveness to mosquitoes, research in several vertebrate species shows that individuals infected with *Plasmodium* spp. are more attractive to mosquitoes [rodents (Ferguson and Read, 2004; De Moraes et al., 2014), canaries (*Serinus canaria*; Cornet et al., 2013a,b), house sparrows (Díez-Fernández et al., 2020b), and humans (Lacroix et al., 2005; Batista et al., 2014; Robinson et al., 2018)]. These studies show that pathogen infection,

in this case malaria, can change an individual's odor profile, making the infected individuals more attractive to mosquitoes. If infected individuals are then more frequently bitten, this would enhance the transmission of the pathogen in the population. Although all of these studies identify odor profiles, it seems reasonable to think that potential changes in skin microbiota composition may play a substantial role and it should be studied if the skin and plumage microbiota also change during different infections caused by mosquito-borne pathogens.

CONCLUSION AND FUTURE DIRECTIONS

Vector-borne pathogens are the causative agents of some of the most harmful diseases in humans, like malaria, dengue, and yellow fever. In addition, they are also the cause of enormous economic losses in domestic animals and population declines among wildlife. Understanding how vectors select their feeding host is critical to understand parasite transmission. For some vectors, like mosquitoes, odor is the main cue to select their hosts. Multiple studies confirm that the microbiota on skin and plumage plays a critical role in body odor production. Therefore, the differences in attractiveness of individuals and species to mosquitoes may be explained by variation in the microbiota composition. However, most of these studies are associative and do not demonstrate causal relationships. One of the challenges is linking bacteria species with volatiles and odor production that will cause differences in the attractiveness to mosquitoes. To bridge this gap, a potential avenue would be to stablish gnotobiotic animal models colonized by known microbes. These animal models in combination with the use of "omic" tools, like metatranscriptomics and metabolomics, will help to link bacteria species with metabolic pathways that are responsible for the volatile by-products. In addition, to further comprehend the mechanisms underlying the interaction between mosquito behavior and host microbiota, more studies including different species of vertebrates and mosquitoes are necessary. This will help to understand which are the cues generalists and specialized mosquitoes are responding to and what are the common patterns of host-seeking behavior. For example, mosquitoes with different host feeding preferences may be responding to different odor profiles generated by different microbiota composition or specific bacteria. In contrast, mosquitoes with a generalist feeding preference might be responding to common odor profiles across species generated by bacteria that are found across different host species. It will also be important to gain a better understanding of how the factors that affect microbiota affect host selection by mosquitoes. Some examples of what can be done include looking at the genetic basis of skin microbiota composition (Davenport, 2016) using genome wide association studies, or linking infectious disease caused by mosquito-borne pathogens with changes in skin microbiota that can lead to differences in attractiveness to mosquitoes. Overall, future research should include a combination of laboratory and ecological studies on the interaction between skin microbiota and host seeking behavior of mosquitoes that will help to reveal some of the most important factors underlying pathogen amplification and transmission.

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MR-L designed the mini review and wrote the manuscript.

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An Integrated Molecular Approach to Untangling Host–Vector–Pathogen Interactions in Mosquitoes (Diptera: Culicidae) From Sylvan Communities in Mexico

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There are ∼240 species of Culicidae in Mexico, of which some are vectors of arthropod-borne viruses such as Zika virus, dengue virus, chikungunya virus, and West Nile virus. Thus, the identification of mosquito feeding preferences is paramount to understanding of vector–host–pathogen interactions that, in turn, can aid the control of disease outbreaks. Typically, DNA and RNA are extracted separately for animal (insects and blood meal hosts) and viral identification, but this study demonstrates that multiple organisms can be analyzed from a single RNA extract. For the first time, residual DNA present in standard RNA extracts was analyzed by DNA barcoding in concert with Sanger and next-generation sequencing (NGS) to identify both the mosquito species and the source of their meals in blood-fed females caught in seven sylvan communities in Chiapas State, Mexico. While mosquito molecular identification involved standard barcoding methods, the sensitivity of blood meal identification was maximized by employing short primers with NGS. In total, we collected 1,634 specimens belonging to 14 genera, 25 subgenera, and 61 morphospecies of mosquitoes. Of these, four species were new records for Mexico (*Aedes guatemala*, *Ae. insolitus*, *Limatus asulleptus*, *Trichoprosopon pallidiventer*), and nine were new records for Chiapas State. DNA barcode sequences for >300 bp of the COI gene were obtained from 291 specimens, whereas 130 bp sequences were recovered from another 179 specimens. High intraspecific divergence values (>2%) suggesting cryptic species complexes were observed in nine taxa: *Anopheles eiseni* (5.39%), *An. pseudopunctipennis* (2.79%), *Ae. podographicus* (4.05%), *Culex eastor* (4.88%), *Cx. erraticus* (2.28%), *Toxorhynchites haemorrhoidalis* (4.30%), *Tr. pallidiventer* (4.95%), *Wyeomyia adelpha/Wy. guatemala* (7.30%), and *Wy. pseudopecten* (4.04%). The study increased the number of mosquito species known from 128 species to 138

species for Chiapas State, and 239 for Mexico as a whole. Blood meal analysis showed that *Aedes angustivittatus* fed on ducks and chicken, whereas *Psorophora albipes* fed on humans. *Culex quinquefasciatus* fed on diverse hosts including chicken, human, turkey, and Mexican grackle. No arbovirus RNA was detected by reverse transcriptase–polymerase chain reaction in the surveyed specimens. This study demonstrated, for the first time, that residual DNA present in RNA blood meal extracts can be used to identify host vectors, highlighting the important role of molecular approaches in both vector identification and revealing host–vector–pathogen interactions.

Keywords: bloodmeals, mosquitoes, cytochrome c oxidase I, DNA barcoding, chiapas state, Mexico

INTRODUCTION

The family Culicidae is medically important because of the large number of pathogens that some species transmit to animals and humans, and it is also a driver of numerous emerging infectious diseases around the world (1, 2). Knowledge of the blood-feeding preferences of a mosquito species provides important insight into the dynamics of virus transmission, allowing public health authorities to design and implement efficient strategies for vector control (3). Mosquito-vectored pathogens contribute to the greatest diversity of neglected tropical diseases that significantly impact human and animal health (4). There are 3,574 recognized species of Culicidae worldwide (5), so correct identification of the species that act as vectors is critical for characterizing pathogen transmission pathways.

Host selection and feeding preference studies of mosquitoes and other hematophagous arthropods, in combination with pathogen screening play a major role in understanding the dynamics of vector–host–pathogen interactions (6–16). Once the feeding preferences are known, and host species at risk of transmitting arthropod-borne pathogens are identified, the mechanisms of disease transmission can be elucidated (17–19). Systematic characterization of bird and mammalian host genetics has increased the specificity of studies. Driven by the use of molecular techniques, genetic analysis has largely replaced serological methods for blood meal identification (9). Several genetic markers have been used for this purpose, including mitochondrial (e.g., cytB, COI) and nuclear (e.g., ITS2) (20, 21) markers.

While genetic analysis has largely replaced serological methods, host-preference studies face challenges. First, the accurate identification of arthropod vectors is complicated by the morphological similarity of species, by decreasing taxonomic expertise, and by the presence of species complexes (22–25). Second, the capacity to recover a sequence for the host is affected by the degree of digestion of the blood meal within the mosquito, as well as the method of preservation after capture (15, 16, 26). Third, the potential presence of pathogens within the blood meal increases biosafety issues. To overcome the first barrier, analysis of the COI mtDNA barcode region (27, 28) is now widely used for mosquito identifications worldwide (29–34). To mitigate the second challenge, researchers now employ high-throughput sequencing in combination with vertebrate-specific primer cocktails (35, 36). Thirdly, the use of FTA cards, and their analysis in facilities with high containment operating under strict biosecurity regulations have lessened biosafety concerns. Collectively, these advances now enable researchers to extend their understanding of host–vector–pathogen interactions.

In Mexico, 234 mosquito species have been recorded (37). As some (*Aedes aegypti*. *Ae. albopictus*, *Culex quinquefasciatus*) are key vector species, Mexico is experiencing ongoing circulation of arboviruses such as chikungunya (CHIKV), dengue (DENV), Zika (ZIKV), and West Nile (WNV) (38). Sylvatic settings in Chiapas such as the Lancadon Jungle represent much of the tropical forests in Mexico (39). Although it is one of the most biodiverse regions in Central America, it faces imminent destruction due to human activities (40). There is little information about mosquito diversity or the arboviruses circulating in the Lancadon Jungle or in other reserves in Mexico with the exception of one previous study (41). In addition, only a few epidemiological studies have investigated blood meal identification in Mexican mosquitoes. For example, (42) studied the host feeding preference of *Cx. quinquefasciatus* in Monterrey, whereas (3), (43), and (44) examined cities in the Yucatán Peninsula, or (45) within a montane forest. In this study, an integrated approach including mosquito identification using morphology and DNA barcoding, blood meal identification using high-throughput sequencing, and arbovirus screening using reverse transcriptase–polymerase chain reaction (RT-PCR) was used to characterize the mosquito fauna and unravel the host–vector–pathogen interactions in sylvan communities in Chiapas State. Furthermore, this study employs a novel method of identifying vertebrate host DNA from residual traces within arthropod RNA extracts.

METHODOLOGY

Study Area, Collection, and Morphological Identification of Mosquitoes

Located in southeastern Mexico, Chiapas State has an area of 73,311 km^2 and is bordered to the north by the States of

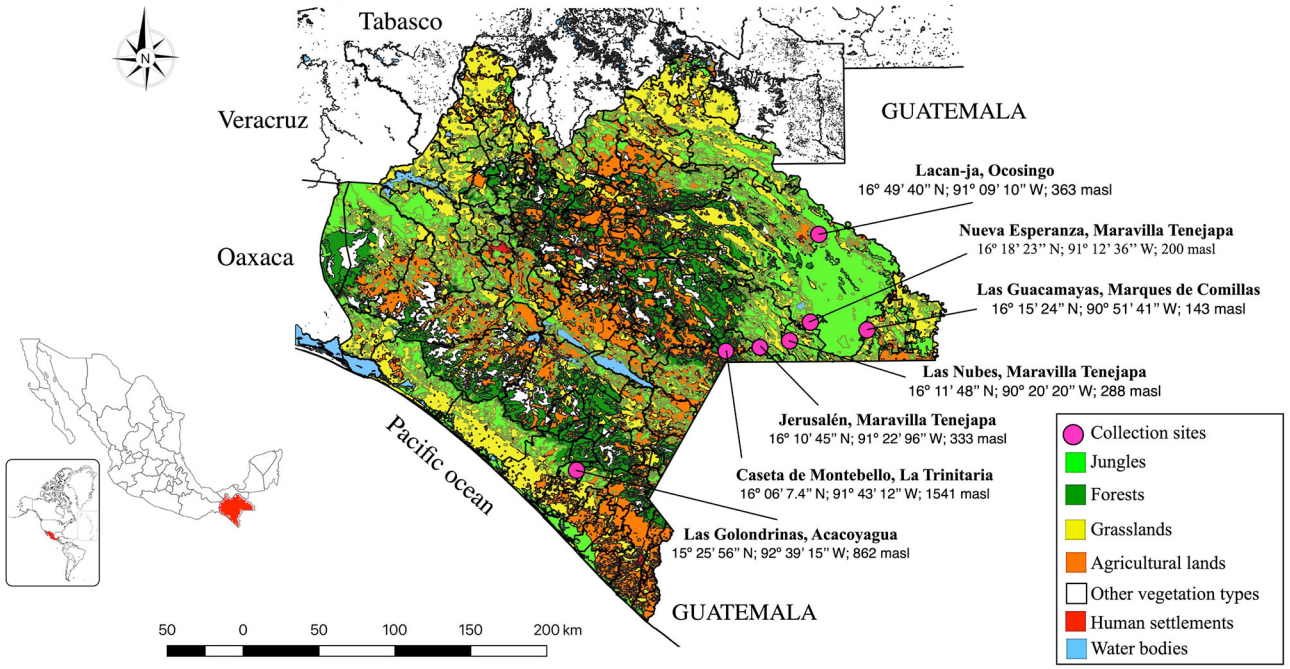

FIGURE 1 | Study area showing the mosquito's collection sites of sylvan communities in Chiapas State, Mexico.

Tabasco, to the east by Guatemala, to the west by the States of Oaxaca and Veracruz, and to the south by the Pacific Ocean. The weather is tropical or subtropical and Chiapas is divided into 11 physiographic regions, seven Biosphere Reserves (BR), and three National Parks (NP). One NP ("Lagos de Montebello") and two BR ("El Triunfo" and "Montes Azules") were sampled in this study (**Figure 1**). In total, seven sylvan communities were sampled during the rainy season of July–August 2016, from the NP Lagos de Montebello (Caseta de Montebello in La Trinitaria municipality 16°06′7.4″N−91°43′12″W, 1,541 masl), from BR El Triunfo (Las Golondrinas in Acacoyagua municipality 15°25′56″N−92°39′15″W, 862 masl), and from BR Montes Azules (Las Nubes 16°11′48″N−90°20′20″W, 288 masl; Jerusalén 16°11′34.3″N−91°22′47.3″W; 333 masl; and Nueva Esperanza 16°18′23″N−91°12′36″W, 200 masl in Maravilla Tenejapa municipality; Las Guacamayas 16°15′24″N−90°51′41″W, 143 masl in Marqués de Comillas municipality, and Lacanjá 16°49′40″N−91°09′10″W, 363 masl in Ocosingo municipality) (**Figure 1**, **Table 1**). Mosquitoes were collected from inside homes and from resting places in close proximity to them. In each locality, collections were made using 10 octanol-baited CDC light traps that were deployed every 30 m following a transect at 1–1.5 m above ground level at night (18:00–22:00); the collecting effort per site was similar. Shannon traps baited with humans were also used at night (20:00–3:00), and mosquitoes were also collected from resting places using two Insectzookas (BioQuip No. 2888A) during the day between 9:00 and 17:00. In addition, immatures were collected from aquatic habitats and held alive in individual tubes to obtain adults and associated exuviae. Adults were killed using triethylamine vapors, stored in vials, and preserved in liquid nitrogen vapors. All material was transported to the Molecular Biology Laboratory, Parasitology Department Universidad Autónoma Agraria Antonio Narro, Unidad Laguna (UAAAN-UL) for taxonomic identification. In the laboratory, representatives of each species (unfed females and males when available) were pinned and identified using taxonomic keys. The classification system proposed by Wilkerson et al. (46) for the Aedini tribe and (47) for the rest of tribes and Anophelinae was followed.

Fully engorged females of identified specimens were individually placed in 1.5 Eppendorf® tubes for blood meal host detection, whereas pools of the remaining unfed adults (2–15 females and males in each pool) were placed in 1.5 mL Eppendorf® tubes for virus detection and DNA barcoding. The mounted specimens, adults on insect pins and immature stages, and exuviae mounted on microscope slides were deposited in the Culicidae Collection of the UAAAN-UL, whereas the remaining specimens in tubes were preserved on dry ice and sent to the Animal and Plant Health Agency, UK (APHA), for molecular analysis.

DNA Extraction and Sanger Sequencing for Mosquito Molecular Identification

Standard DNA barcoding protocols (i.e., sequencing of 658 bp barcode region of COI) were used to identify unfed specimens of the morphospecies. For DNA extraction, a modified Hotshot technique (44, 48) was employed. Briefly, one to two legs from single specimens were placed directly into 50 μL of alkaline

TABLE 1 | Checklist of mosquito species collected in seven sylvan communities in Chiapas State, Mexico.

Species	Las Golondrinas	Caseta de Montebello	Las Nubes	Jerusalén	Nueva Esperanza	Las Guacamayas	Lacanjá
Anophelinae							
1. *Anopheles* (*Anopheles*) *eiseni*	X	X			X		
2. *An.* (*Anopheles*) *pseudopunctipennis*					X	X	
3. *An.* (*Kerteszia*) *neivai*		X					
4. *An.* (*Nyssorhynchus*) *albimanus*					X		
Culicinae							
5. *Aedes* (*Georgecraigius*) *fluviatilis*			X				
6. *Ae.* (*Howardina*) *allotecnon*		X					
7. ***Ae.* (*Howardina*) *guatemala****		X					
8. *Ae.* (*Howardina*) *quadrivittatus*	X	X					X
9. *Ae.* (*Ochlerotatus*) *angustivittatus*	X				X	X	
10. *Ae* (*Ochlerotatus*) *euplocamus*					X	X	
11. *Ae.* (*Ochlerotatus*) *fulvus*					X		
12. *Ae.* (*Ochlerotatus*) *serratus*					X	X	X
13. *Ae.* (*Ochlerotatus*) *trivitatus*					X		
14. ***Ae.* (*Protomacleaya*) *insolitus****	X				X		
15. *Ae.* (*Protomacleaya*) *podographicus*	X	X					
16. *Ae.* (*Stegomyia*) *aegypti*			X				X
17. *Ae.* (*Stegomyia*) *albopictus*	X		X			X	
18. *Haemagogus* (*Haemagogus*) *equinus*		X					
19. *Hg.* (*Haemagogus*) *mesodentatus*					X	X	X
20. *Psorophora* (*Grabhamia*) *cingulata*					X		
21. *Ps.* (*Grabhamia*) *columbiae*					X	X	
22. *Ps.* (*Janthinosoma*) *albipes*					X	X	
23. *Ps.* (*Janthinosoma*) *champerico*					X	X	
24. *Ps.* (*Janthinosoma*) *ferox*					X	X	X
25. *Ps.* (*Psorophora*) *ciliata*						X	
26. *Culex* (*Anoedioporpa*) *restrictor*							X
27. *Cx.* (*Culex*) *coronator s.l.*					X	X	
28. *Cx.* (*Culex*) *mollis*						X	
29. *Cx.* (*Culex*) *nigripalpus*	X		X		X	X	
30. *Cx.* (*Culex*) *pinarocampa*			X				
31. *Cx.* (*Culex*) *quinquefasciatus*					X	X	X
32. *Cx.* (*Culex*) *usquatus***			X				
33. *Cx.* (*Melanoconion*) *bastagarius*						X	
34. *Cx.* (*Melanoconion*) *eastor***			X				
35. *Cx.* (*Melanoconion*) *erraticus*						X	
36. *Cx.* (*Melanoconion*) *pedroi***					X		
37. *Cx.* (*Melanoconion*) *pilosus*			X			X	X
38. *Cx.* (*Melanoconion*) *spissipes*						X	
39. *Cx.* (*Microculex*) *daumastocampa*				X			
40. *Cx.* (*Microculex*) *rejector*		X	X	X			
41. *Cx.* (*Phenacomyia*) *corniger*							
42. *Mansonia* (*Mansonia*) *titillans*						X	
43. *Johnbelkinia ulopus*					X		
44. ***Limatus asulleptus****					X		
45. *Li. durhamii*			X		X	X	
46. *Sabethes* (*Sabethes*) *cyaneus*					X		
47. *Sa.* (*Sabethoides*) *chloropterus*		X					
48. *Shannoniana moralesi*	X	X			X		X

(Continued)

TABLE 1 | Continued

Species	Las Golondrinas	Caseta de Montebello	Las Nubes	Jerusalén	Nueva Esperanza	Las Guacamayas	Lacanjá
49. *Trichoprosopon digitatum*					X		
50. ***Tr. pallidiventer****					X		
51. *Tr.* nr. *brevipes*					X		
52. *Trichoprosopon* sp. nr. spG							
53. *Wyeomyia* (*Decamyia*) *pseudopecten*					X		
54. *Wy.* (*Triamyia*) *aporonoma***	X	X			X		
55. *Wy.* (*Wyeomyia*) *abebela*	X	X	X	X	X		
56. *Wy.* (*Wyeomyia*) *adelpha/Wy. guatemala* groups I, II, III, IV	X	X	X		X	X	
57. *Wy.* (*Wyeomyia*) *melanopus*	X	X	X	X			
58. *Wy.* (*Wyeomyia*) *stonei*		X					
59. *Wy*. (*Wyeomyia*) sp. nr. *Wy. complosa*					X		
60. *Toxorhynchites* (*Lynchiella*) *haemorrhoidalis***			X				
61. *Uranotaenia* (*Uranotaenia*) *lowii*				X			

*(In bold) New national records for Mexico. **New records for Chiapas State.

lysis buffer in a 96-well plate, which was then sonicated in a water bath for 20 min. The plate was subsequently incubated in a thermocycler for 30 min at 94°C and cooled for 5 min at 4°C, after which 50 μL of the neutralizing buffer was added to each well. PCR amplification of the full-length COI barcode region (27, 28) was performed using a protocol and primers developed by Montero-Pau et al. (LCO1490 and HCO2198) and a QIAgen PCR system with the following reaction mix, final volume 50 μL: 2 μL of DNA template, 25 μL H_2O, 5 μL NH_4, 5 μL of dNTPs (2 mM/μL), 2.5 μL of $MgCl_2$ (25 mM/μL), 0.1 μL Bioline Taq Polymerase (Bioline Reagents Ltd., London, UK), 5 μL of each primer (each at 10 pmol/μL), and 0.38 μL of bovine serum albumin (20 mg/mL) (48, 49). The thermal profile consisted of the following: an initial denaturation step at 94°C for 1 min, 5 cycles of preamplification of 94°C for 1 min, 45°C for 1.5 min, 72°C for 1.5 min, followed by 35 cycles of amplification of 94°C for 1 min, 57°C for 1.5 min, and 72°C for 1 min, followed by a final elongation step of 72°C for 5 min. All PCR products were visualized with a 1.5% agarose gel, and samples showing bands of the correct size were bidirectionally sequenced using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) at the Sequencing Unit, APHA.

RNA Extraction and Sanger Sequencing for Blood-Fed Mosquito Molecular Identification

Blood-fed females were subjected to more extensive analysis than their unfed counterparts. Because of the potential presence of pathogens in the blood meals, RNA extraction was performed in a high-biosecurity facility at the APHA. Engorged abdomens were individually transferred from their Eppendorf storage tubes into 2 mL Qiagen flat-cap disruption tubes containing two pretreated 5-mm stainless-steel beads and 500 μL of tissue cell culture media (E-MEM/10%FBS). Each microtube was homogenized for 3 min at 25 Hz in a TissueLyser (Qiagen) and then centrifuged for 3 min at 14,000 *g*. One hundred microliters of the supernatant was removed and stored at −80°C for potential virus isolation, whereas the remainder was used for RNA purification using TRIzol following the recommended protocol (www.thermofisherscientific.com). Contrary to most RNA extraction protocols, residual co-purified DNA was not removed via DNase treatment. The RNA extracts therefore contained trace amounts of DNA from both the blood meal host and the mosquito, allowing its identification via standard barcoding.

To that end, 50 μL of RNA extract was sent to the Center for Biodiversity Genomics, at the University of Guelph for further analysis. Because of accidental loss of the cold chain during courier transportation from Mexico to APHA, which compromised DNA preservation, mosquitoes were identified using the primers AncientLepF3 (TTATAATTGGDGGWTTTGGWAATTG) and AncientLepR3 (CCTCCATGRGCRATATTWGADG), which amplify a short fragment (120–180 bp) of the COI barcode region (50). Sanger sequencing was performed following standard protocols (27, 28, 36).

Phylogenetic Analysis of Mosquito COI Sanger Sequences

The resulting Sanger trace files from both unfed and blood-fed mosquitoes were edited and analyzed in the same manner. Paired bidirectional traces were combined to produce a single consensus sequence for the full 658-bp barcode sequence for the unfed mosquitoes and a shorter 130-bp barcode sequence for the blood-fed mosquitoes. For species recorded in the collecting sites, but from which we could not obtain a DNA barcode sequence, we employed sequences from the Barcode of Life Database (BOLD-www.barcodingoflife.org) or NCBI

TABLE 2 | Vertebrate-specific primers used for first-round PCR from blood-fed mosquito's species collected in sylvan communities in Chiapas State, Mexico.

Primer Name	Sequence (5′->3′)	Direction	References
BloodmealF1_t1	**TGTAAAACGACGGCCAGT**ACCACWATTATTAAYATAAARCCMC	Forward	(55, 56), This study.
BloodmealF2_t1	**TGTAAAACGACGGCCAGT**ACTACAGCAATTAACATAAAACCMC	Forward	(55, 56), This study.
VR1_t1	**CAGGAAACAGCTATGAC**TAGACTTCTGGGTGGCCAAAGAATCA	Forward	(24)
VR1d_t1	**CAGGAAACAGCTATGAC**TAGACTTCTGGGTGGCCRAARAAYCA	Reverse	(24)
VR1i_t1	**CAGGAAACAGCTATGAC**TAGACTTCTGGGTGICCIAAIAAICA	Reverse	(24)

The sequences of the M13F and M13R tails are in bold, whereas the COI-specific sequences are in regular font. The M13 tails served as second-round PCR primer binding sites, on which Ion Torrent sequencing adapters and UMIs (IonXpress 1–96) were fused.

(www.ncbi.nlm.nih.gov/genbank/). In total, 20 species and 139 sequences were added to the dataset (**Supplementary Table 1**); no sequences of *An. neivai*, *Cx. bastagarius*, *Cx. daumastocampa, Cx. spissipes*, or *Wy. stonei* were included in the analysis.

Genetic relationships between species were analyzed using three methods: neighbor joining (NJ), maximum likelihood (ML), and maximum parsimony (MP). For the NJ and MP, the dataset was analyzed in MEGA v.6 (51). NJ analysis employed the K2P distance metric. Bootstrap values to test the robustness of the tree were obtained by conducting 1,000 pseudoreplicates; only groups with more than 80% bootstrap support are shown (19, 52). The MP tree was obtained using the subtree–pruning–regrafting algorithm with the initial trees obtained by the random addition of sequences (10 replicates). ML analysis was implemented in PhyML 3.0 (52); branch support was calculated using approximate likelihood ratio tests (53). For the phylogenetic analyses, a COI DNA barcode sequence of a black fly, *Simulium weji* Takaoka (accession no. KF289451) was used as an outgroup. NJ, MP, and ML trees were exported as JPG files in Acrobat 8.Professional, and then Adobe Photoshop CS3 (v. 10.0.1) was used to edit them.

After sequences were uploaded to BOLD, most barcode sequences longer than 500 bp were assigned a Barcode Index Number (BIN), a taxonomic system that assigns similar barcode sequences into species proxies without the need for Linnaean nomenclature (54). An NJ tree composed of BINs was generated on BOLD, and each morphospecies was mapped to BINs in the tree. Taxonomic discordance in our dataset was analyzed using BOLD tools, one of which provides a means of confirming the concordance between barcode sequence clusters and species designations.

Next-Generation Sequencing of Blood-Fed Female Mosquitoes for Host Identification

The same RNA extracts employed for mosquito identification were used for blood meal identification via next-generation sequencing (NGS). As mentioned previously, following RNA extraction, residual DNA was not removed by DNase treatment.

Instead, the residual DNA was used as a template for PCR. A two-step PCR protocol was used to amplify blood meal (host) DNA and to prepare it for sequencing on an Ion Torrent platform. The first PCR reaction consisted of 6.25 μL of 10% D-(+)-trehalose dihydrate (Fluka Analytical), 2.0 μL of Hyclone ultra-pure water (Thermo Scientific), 1.25 μL of 10X PlatinumTaq buffer (Invitrogen), 0.625 μL of 50 mM $MgCl^2$ (Invitrogen), 0.125 μL of each 10 μM primer cocktail, 0.0625 μL of 10 mM dNTP (KAPA Biosystems), 0.060 μL of 5U/lL PlatinumTaq DNA Polymerase (Invitrogen), and 2 μL of RNA, for a total reaction volume of 12.5 μL. The primers (BloodmealF1_t1, BloodmealF2_t1, VR1_t1, VR1d_t1, and VR1i_t1; **Table 2**) were designed to amplify a 185-bp region of the COI barcode from diverse birds and mammals and were tailed with M13F and M13R sequences that provided universal primer binding sites during the second round of PCR. Thermocycling consisted of an initial denaturation at 95°C for 2 min, 60 cycles of 95°C for 40 s, 56°C for 40 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. After PCR, the products were visualized on a 2% E-gel (Invitrogen) to confirm amplification and were then diluted 2-fold with sterile water.

The diluted products were then used as template for a second round of PCR using M13F primers tailed with IonXpress universal molecular identifiers (UMIs) tags and the Ion Torrent "A" sequencing adapter, and M13R primers tailed with the Ion Torrent trP1 sequencing adapter (**Table 1** for primer sequences). Reaction components for the second round of PCR were identical to the first; the thermocycling regimen consisted of an initial denaturation at 95°C for 2 min, 5 cycles of 95°C for 40 s, 45°C for 40 s, and 72°C for 30 s, 35 cycles of 95°C for 40 s, 51°C for 40 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The products of the second round of PCR were pooled in equal volumes and purified by mixing 400 μL of pooled product with 200 μL of purification beads (Aline Biosciences, Woburn, MA, USA) for a ratio of 0.5X beads: DNA (vol:vol). The mixture was incubated at room temperature for 8 min to allow the DNA to bind to the beads, after which the beads were pelleted on a magnetic rack. The supernatant (550 μL) was transferred to a clean 1.5-mL tube and mixed with 113 μL of sterile water and 417 μL of fresh purification beads for a final ratio of 1.2X beads: DNA (vol:vol). The mixture was incubated at room temperature for 8 min and then pelleted on a magnet. The supernatant was carefully discarded, and the pellet was washed three times with 1 mL of freshly prepared 80% ethanol and then air-dried. The purified product was eluted from the beads by resuspending them in 200 μL of sterile water, pelleting the beads, and then carefully transferring 180 μL of the supernatant to a clean tube. The purified product was quantified using a Qubit 2.0 fluorometer

and adjusted to 22 pM with sterile water. The 22 pM library was then sequenced on an Ion Torrent PGM following the manufacturer's instructions using a 316v.2 chip. Each sequence was automatically assigned to its source sample via the UMI tags by the Torrent Browser suite.

The raw reads for each sample were then processed through a custom analytical pipeline that first filtered the reads based on a minimum quality value (PHRED = 20) and a minimum read length of 100 bp. All adapter and primer sequences were identified and removed using CutAdapt (57). As the forward primer should be readily visible in the reads, those lacking it were discarded, so only high-quality reads were included in the final dataset. The trimmed reads were collapsed into unique haplotypes (http://hannonlab.cshl.edu/fastx_toolkit/index.html) while retaining the original read counts. Each sequence was used to query (BLAST) a custom database composed of global vertebrate COI sequences downloaded from BOLD. The resulting BLAST hits were filtered to retain only those with a minimum match of 95% identity and 100 bp of coverage between the queried sequence and a reference sequence. Furthermore, identifications were retained only if supported by at least 50 original reads.

Virus Testing

Virus screening was performed on all blood-fed specimens that were analyzed for host DNA, as well as on pools of adult mosquitoes that were not previously analyzed (the former provided a detailed screen at the individual level, whereas the latter screened at the population level). In the case of the pools, each morphospecies was separated into subsets containing 2–15 specimens per tube, and the same methodology employed for homogenizing the engorged abdomens was followed. Again, 100 μL of the homogenate was stored at −80°C for potential virus isolation, whereas the remainder was used for RNA extraction using TRIzol (www.thermofisherscientific.com).

The RNA samples were screened for the presence of common viruses using a one-step semiquantitative SYBR Green RT-PCR employing generic primers that target a broad range of *Flavivirus* and *Alphavirus* species. For *Flavivirus* detection, we used the following primers of Johnson et al. (58): Flavi Forward (GTRTCCCAKCCDGCNGTRTC) and Flavi Reverse (GCMATHTGGTWCATGTGG). The primers of Johnson et al. (58) were used for *Alphavirus* detection: VIR2052 Forward (TGGCGCTAGATGAAATCTGGAATGTT) and VIR2052 reverse (TACGTGTTGTCGTCGCCG ATGAA). The RT-PCR reactions included 6.25 μL of molecular grade water, 12.5 μL of QuantiTect SYBR Green RT-PCR kit (Qiagen), 0.25 μL of Quantitec RT Mix (Qiagen), 2 μL of each primer at 10 pmol/μL, and 2 μL of RNA. Thermocycling consisted of one cycle of reverse transcription at 50°C for 30 min, one cycle of initial denaturation at 95°C for 15 min, 45 cycles of amplification at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, and a final cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. RNA from the WNV (Goose Israel strain) and the Sindbis virus (SINDV) (Germany 5.3 strain) was used as positive controls for flaviviruses and alphaviruses, respectively. All positive controls were passaged two or three times in Vero cells.

RESULTS

Faunistic Survey and Mosquito Species Identification Using COI DNA Barcoding

The 1,634 collected specimens included representatives of two subfamilies, 14 genera, 25 subgenera, and 61 named species, as well as two taxa that could only be assigned to a genus (*Trichoprosopon*, *Wyeomyia*) (**Table 2**). The genera *Aedes* and *Culex* were the most diverse with 13 and 16 species, respectively, followed by *Psorophora* with six species. *Aedes guatemala*, *Ae. insolitus, Limatus asulleptus*, and *Tr. pallidiventer* represent new records for Mexico, whereas two apparently undescribed species of *Trichoprosopon* were discovered. As well, four species (*Culex usquatus*, *Cx. eastor*, *Cx. pedroi*, *Wy. aporonoma*, and *Tx. haemorrhoidalis*) are new records for Chiapas State. The largest number of species was collected at Nueva Esperanza (32) followed by Las Guacamayas (21), Caseta de Montebello (5), Las Nubes (14), Las Golondrinas (12), Lacanja (9), and Jerusalén (5).

In total, 570 specimens were DNA barcoded. Among these, 285 non-engorged specimens were analyzed using DNA extracted from a single leg, whereas 285 blood-fed females were analyzed using a modified protocol that employed RNA extracts as the template for DNA barcoding. The overall sequencing success was 76% (436/570) with barcodes recovered from five morphospecies, but sequences were recovered from 96% (273/285) of the DNA extracts from a single leg with most (235) >300 bp in length. By contrast, only 38% (108/285) of the blood-fed specimens yielded a sequence >130 bp in length (**Supplementary Data Sheet 1**).

The 291 sequences of >300 bp in length were combined with 78 publicly available sequences from BOLD from Mexico and other countries in the Americas (representing 20 species) to create a final dataset with 369 sequences. Intraspecific sequence divergences were variable across taxa, ranging from zero to 7.30% with an average of 1.56% (**Table 3**). Because the NJ, ML, and MP trees had similar topology and strong support values, only the NJ tree (**Figure 2**) is shown (see **Supplementary Figures 1**, **2** for ML and MP trees, respectively). High intraspecific K2P distance (above 2%) was observed for nine taxa: *Anopheles eiseni*—average of 5.39% (maximum of 7.76% among three specimens), *An. pseudopunctipennis*—average of 2.79% (maximum of 5.4% among seven specimens), *Ae. podographicus*—average of 4.05% (maximum of 11.45% among 11 specimens), *Cx. eastor*—average 4.88% (maximum of 15.8% among six specimens), *Cx. erraticus*–average 2.28% (maximum of 2.28% between two specimens), *Tr. pallidiventer*—average of 4.95% (maximum of 8.2% among four specimens), *Wy. adelpha*/*Wy. guatemala*—average of 7.30% (maximum of 12.14% among 27 specimens), *Wy. pseudopecten*—average 4.05% (maximum of 11.96% among five specimens), and *Tx. haemorrhoidalis*—average of 4.30% (maximum 12.71% among 11 specimens). Interspecific divergence values were low for a few species such as *Cx. nigripalpus/Cx mollis* (1.84%) and *Cx. nigripalpus/Cx. quinquefasciatus* (6.7%), but much higher between species in different genera such as *Tx. haemorroidalis/Cx. quinquefasciatus* (20.62%) and *An. pseudopunctipennis/Sa. cyaneus* (21.81%) (**Supplementary Table 2**).

TABLE 3 | List of mosquito species and number of specimens (*n*) from which DNA barcodes (>400 bp) were obtained collected at sylvan communities in Chiapas State, Mexico.

Species	Average genetic diversity (%)	Country	*n*	BOLD BIN
Anophelinae				
Anopheles albimanus	1.47%	Colombia, Mexico	16	BOLD:ADU8918
Anopheles eiseni	5.39%	French Guiana, Mexico	3	BOLD:ACZ3766, BOLD:ADE7573
*Anopheles pseudopunctipennis**	2.79%	Colombia, Mexico	7	BOLD:ABX5930, BOLD:AAF5940
Culicinae				
Aedes albopictus	0.10%	Mexico	5	BOLD:AAA5870
Aedes alloctenon	0.06%	Mexico	3	BOLD:ACT1072
Aedes angustivitatus	0.88%	Mexico	5	BOLD:AAX5452
Aedes aegypti	1.48%	Mexico, Puerto Rico, USA	15	BOLD:AAF5940
Aedes fluviatilis	0.10%	Mexico	1	BOLD:ABW1628
Aedes fulvus	n/a	Mexico	1	BOLD:ACN9154
Aedes guatemala	n/a	Mexico	1	BOLD:ACT1072
Aedes insolitus	n/a	Mexico	1	BOLD:ADE8493
*Aedes podographicus**	4.05%	Mexico	11	BOLD:ADE6045, BOLD:ADE8493
Aedes quadrivittatus	0.32%	Mexico	2	BOLD:ADL5199
Aedes serratus	1.68%	French Guiana, Mexico	4	BOLD:AAN3110, BOLD:ACN3711
Aedes trivittatus	0.46%	Canada	5	BOLD:AAC9486
Culex corniger	0.12%	Colombia	10	BOLD:ABU8489
Culex coronator s.l. **	0.60%	Mexico	6	BOLD:AAN3636
*Culex eastor**	4.88%	Brazil, Mexico	5	BOLD:AAG3857, BOLD:ADJ7929
Culex erraticus	2.28%	Mexico	2	BOLD:AAG3848
Culex quinquefasciatus	0.12%	Brazil, French Guiana, USA	10	BOLD:AAA4751
Culex mollis	0.08%	Brazil	4	BOLD:AAF1735
Culex nigripalpus	0.17%	Mexico	15	BOLD:AAF1735
Culex pedroi	0.67%	Brazil	7	BOLD:ADK4497
Culex pinarocampa	0%	Mexico	3	BOLD:AAF1735
Culex pilosus	0%	Mexico	2	BOLD:ACU4075
Culex restrictor	0.25%	Mexico	4	BOLD:ADT6223
*Culex usquatus***	0.48%	Mexico	2	BOLD:AAN3636
Haemagogus equinus	1.13%	Mexico	11	BOLD:ADE6727
Haemagogus mesodentatus	2.11%	Mexico	2	BOLD:ACN9157
Johnbelkinia ulopus	0%	Mexico	3	BOLD:ADE8406
Limatus asulleptus	0.21%	Mexico	3	BOD:AAW1293
Limatus durhamii	0.11%	Mexico	3	BOLD:AAU2690
Mansonia titillans	0.03%	Mexico	10	BOLD:AAC3206
Psorophora albipes	0.19%	Mexico	5	BOLD:ADE0378
Psorophora champerico	n/a	Mexico	1	BOLD:ADE2950
Psorophora ciliata	0%	Mexico	4	BOLD:AAG3849
Psorophora cingulata	0.46%	Mexico	14	BOLD:ADE8647
Psorophora columbiae	0.38%	Mexico, USA	7	BOLD:AAG3850
Psorophora ferox	1.49%	Mexico, USA	4	BOLD:ADQ2015, BOLD:ACC4707
Sabethes chloropterus	0.55%	Mexico	5	BOLD:ACX6560
Sabethes cyaneus	0.16%	Colombia, USA	3	BOLD:AAX9629
Shannoniana moralesi	0.39%	Mexico	8	BOLD:ADE5529
*Toxorhynchites haemorrhoidalis (sub. haemorrhoidalis, sub. superbus)**	4.35%	French Guiana, Mexico	11	BOLD:ADE6036, BOLD:ACZ4120/BOLD:ACZ3996, BOLD:ACZ3913
Trichoprosopron nr. *brevipes*	0%	Mexico	1	BOLD:ADE5656
Trichoprosopron digitatum	0.21%	Mexico	3	BOLD:ADE7783

(Continued)

TABLE 3 | Continued

Species	Average genetic diversity (%)	Country	*n*	BOLD BIN
*Trichoprosopron pallidiventer**	4.95%	Mexico	5	BOLD:ADE8543, BOLD:ADE8544
Trichoprosopon sp. nr. *Tr.* stG	0.16%	Mexico	2	BOLD:ADL4862
Uranotaenia lowii	1.53	Mexico, Puerto Rico, USA	11	BOLD:AAA7620
Wyeomyia abebela	0.24%	Mexico	4	BOLD:ACA1022
Wyeomyia adelpha/Wy. guatemala groups I, II, III, IV*	7.30%	Mexico	27	BOLD:ACA0979 (group I), BOLD:AAW5415 (group II), BOLD:ADE:8349 (groups III, IV)
Wyeomyia aponoroma	0.29%	Mexico	25	BOLD:ACA1021
Wyeomyia melanopus	1.42%	Mexico	35	BOLD:ACM7671
*Wyeomyia pseudopecten**	4.04	French Guiana, Mexico	5	BOLD:ADL2623, BOLD:ACZ4104, BOLD:AAG3839
Wyeomyia nr. *complosa*	0%	Mexico	2	BOLD:ACA09978

*Taxa with > 2% genetic divergence. **Taxa with same BIN.
Mean (%) intraspecific values of sequence divergence (Kimura 2-parameter distance) are shown with missing entries, indicating that less than two barcode sequences were obtained.

NJ analysis showed that most conspecific specimens formed a single cluster in the tree with high bootstrap support value (**Figure 2**), but there were exceptions. *Ae. podographicus* split into two groups that were assigned to different BINs (BOLD:ADE8493, BOLD:ADE6045). Likewise, specimens of *Cx. eastor* were assigned to two BINs (BOLD:AAG3857, BOLD:ADJ7929). *Trichoprosopon pallidiventer* was similarly divided into two BINs (BOLD:ADE8543, BOLD:ACA0979), whereas *Wy. adelpha*/*Wy. guatemala* showed a deep division in the NJ tree forming four groups, here designated as group I (BOLD:ACA0979), group II (BOLD:AAW545), group III, and group IV both with BINs number (BOLD:ADE:8349), each supported with 100% bootstrap values (**Figure 2**). Interestingly, specimens of *Tr. haemorrhoidalis* from Mexico (BOLD:ADE6036) clustered separately from their French Guiana counterparts: *Tx. haemorrhoidalis haemorrhoidalis* (BOLD:ACZ4120) and *Tx. haemorrhoidalis superbus* (BOLD: ACZ3966). By contrast, two pairs of morphologically identified species (*Ae. alloctenon* + *Ae. guatemala*, *Cx. coronator* + *Cx. usquatus*) showed intermingling of their barcodes (**Figure 2**). The BOLD ID engine was used to identify specimens that lacked a species assignment based on morphological study. Two sequences assigned to *Wyeomyia* sp. 98.3% similarity to Costa Rican *Wy. complosa* (BOLD:ACA0978), so they were assigned to this species.

The 369 barcode sequences generated in this study represented 64 BINs deriving from 55 morphologically identified species. Of these, 42 were represented by a single BIN, seven were represented by two, whereas three BINs were recognized in *Wy. adelpha*/*Wy. guatemala* and *Wy. pseudopecten*, and four within *Tx. haemorrhoidalis* (**Figure 2**, **Table 3**). Eight species shared a BIN with at least one other species in its genus. Most of these cases involved species of *Aedes* (*Ae. allotecnon*, *Ae. guatemala*, *Ae. insolitus*, and *Ae. podographicus*) or *Culex* (*Cx. coronator*, *Cx. mollis*, *Cx. nigripalpus*, *Cx. pinarocampa*, and *Cx. usquatus*) (**Figure 2**, **Table 3**).

Identification of Vertebrate Hosts From Mosquito Blood Meals

The 285 females collected with varying degrees of blood engorgement in the Sella scale represented 22 morphospecies (**Table 4**). The source of the blood meal was ascertained for 30% (59) of these mosquitoes. They included representatives from three genera and eight species: *Ae. angustivittatus*, *Ae. podographicus*, *Ae. trivittatus*, *Cx. quinquefasciatus*, *Cx. nigripalpus, Culex* sp., *Ps. albipes*, and *Ps. ferox* (**Table 4**). The others failed to generate host information despite repeated attempts at PCR.

Analysis of the 80 vertebrate sequences recovered from blood meals revealed that most mosquito species fed on birds, primarily chicken (*Gallus gallus*), followed by mammals such as the Virginia opossum (*Didelphis virginiana*) and human (*Homo sapiens*) (**Table 5**). *Culex quinquefasciatus* showed the highest diversity in host use as it fed on chicken, turkey (*Meleagris gallopavo*), Muscovy duck (*Cairina mochata*), Great-tailed grackle (*Quiscalus mexicanus*), horse (*Equus ferus*), and cow (*Bos taurus*). To the best of our knowledge, the hosts of four species (*Ae. angustivittatus*, *Ae. podographicus, Ae. insolitus*, *Ae. trivittatus*) were previously unknown in Mexico.

Virus Testing

In total, 270 blood-fed specimens and 204 pools of mosquitoes (1,064 specimens) were screened for flavivirus and alphavirus RNA (**Tables 4**, **6**) spanning across all seven sylvan communities. No *Flavivirus* or *Alphavirus* RNA was detected in any sample. Positive controls generated expected results indicating that the assays were effective.

DISCUSSION

The elucidation of vector–host–pathogen interactions typically require separate analytical pathways: DNA for the insect vector and the vertebrate host(s), and RNA for alphaviral and flaviviral pathogens. In this study, we used

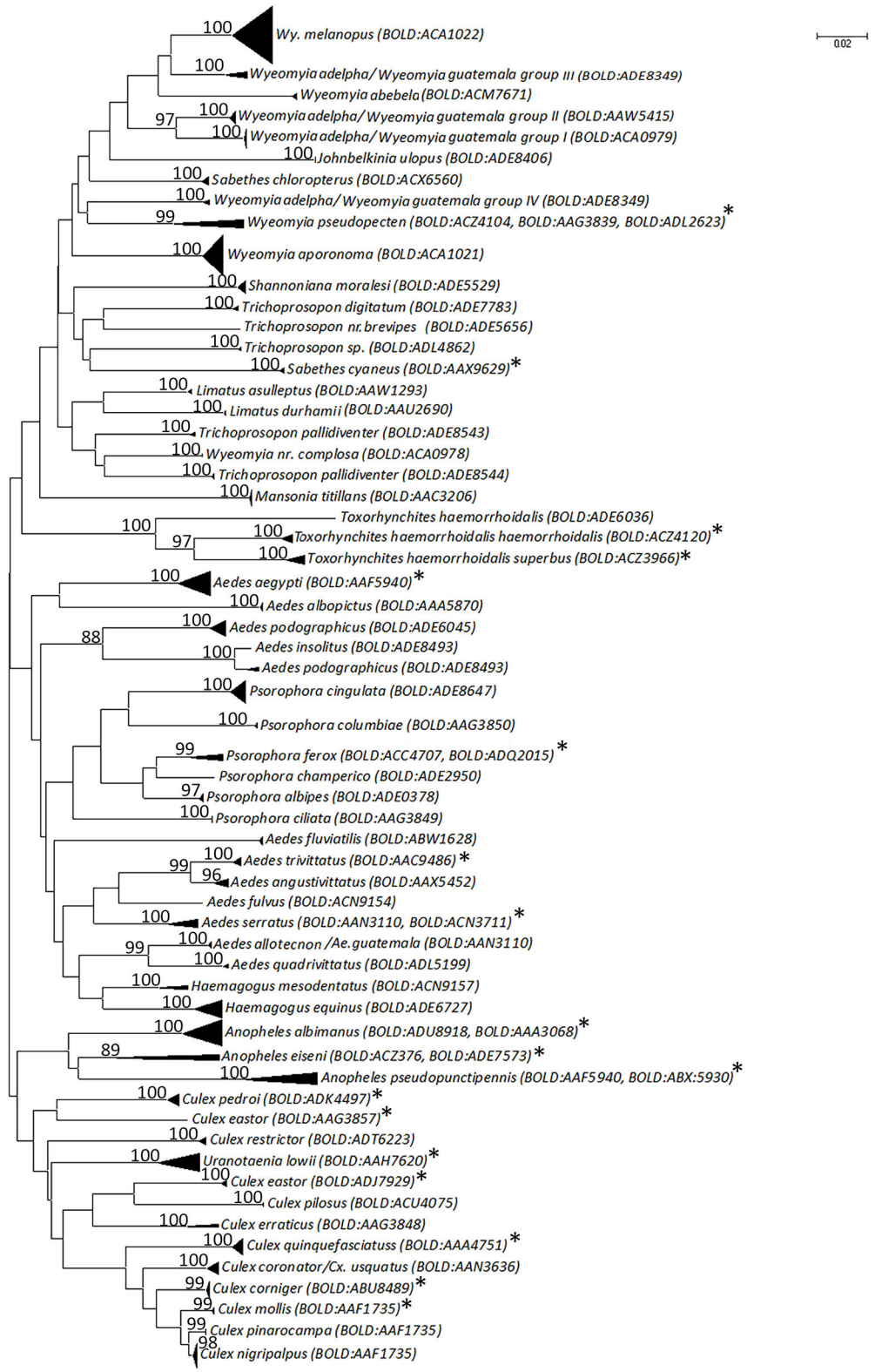

FIGURE 2 | Neighbor-joining tree based on the the Kimura two-parameter distances of *COI* DNA barcodes (>300 bp) for mosquito species recorded in sylvan communities in Chiapas State, Mexico. A divergence > 2% may be indicative of separate operational taxonomic units. Only bootstrap support values > 80% are shown. An asterisk (*) relates to species from which sequences have been downloaded from BOLD and NCBI databases.

TABLE 4 | Checklist of blood-fed mosquito's species and total number of specimens (*n*) collected in sylvan communities in Chiapas State, Mexico.

Species	*n*
Anophelinae	
Anopheles pseudopunctipennis	3
Culicinae	
Aedes aegypti	7
Aedes albopictus	4
Aedes angustivittatus	14
Aedes podographicus	1
Aedes serratus	3
Aedes sp.	1
Aedes trivittatus	2
Culex bastagarius	2
Culex corniger	1
Culex coronator s.l.	1
Culex erraticus	1
Culex nigripalpus	5
Culex pedroi	1
Culex pilosus	4
Culex quinquefasciatus	201
Culex sp.	5
Culex spissipes	1
Limatus durhamii	5
Mansonia titillans	1
Psorophora albipes	6
Psorophora champerico	3
Psorophora columbiae	1
Psorophora ferox	6
Sabethes chloropterus	1
Uranotaenia lowii	1
Wyeomyia adelpha/Wy. guatemala	4

TABLE 5 | Vertebrate hosts species, host scientific and common name, and number of specimens (*n*) identified for each mosquito species collected in sylvan communities in Chiapas State, Mexico.

Mosquito species	Host	Host common name	Host group	*n*
Aedes angustivittatus	*Anas platyrhynchos*	Mallard	Birds	1
	Gallus gallus	Chicken	Birds	1
Aedes podographicus	*Bos taurus*	Cow	Mammals	1
Aedes trivittatus	*Equus ferus caballus*	Horse	Mammals	1
Culex quinquefasciatus	*Bos taurus*	Cow	Mammals	
	Didelphis virginiana	Virginia opossum	Mammals	2
	Equus ferus caballus	Horse	Mammals	1
	Meleagris gallopavo	Wild turkey	Birds	1
	Quiscalus mexicanus	Great-tailed grackle	Birds	1
	Gallus gallus	Chicken	Birds	64
	Cairina mochata	Muscovy duck	Birds	1
Culex nigripalpus	*Sus scrofa*	Wild boar	Mammals	1
Culex sp.	*Gallus gallus*	Chicken	Birds	1
	Turdus grayi	Clay-colored thrush	Birds	1
Psorophora albipes	*Home sapiens*	Human	Mammals	2
Psorophora ferox	*Bos taurus*	Cow	Mammals	1

morphologically identified mosquitoes from communities in Chiapas State to demonstrate that these interactions can be revealed by analyzing DNA recovered by a standard RNA extraction protocol. By eliminating the need for a separate DNA extraction, vector–host–pathogen interactions can be ascertained in a simpler, cost-effective manner, an important consideration for areas where mosquitoes vector and viral diseases occur. Among the 61 mosquito taxa detected in this study, at least 10 (*An. albimanus*, *An. pseudopunctipennis*, *Ae. albopictus*, *Ae. angustivittatus*, *Ae. aegypti*, *Cx. nigripalpus*, *Cx. quinquefasciatus*, *Cx. restuans*, *Cs. inornata*, *Ps. ferox*) are pathogen vectors in Mexico and other countries in the neotropics. Given the medical importance of the viruses that they transmit (60–63), the need for regular vector surveillance to aid disease control is essential in Mexico and throughout Central America.

DNA barcoding proved effective at identifying mosquito species in Quintana Roo State, Mexico (60, 61, 64), and 96% for mosquitoes processed with standard barcoding methods in this study showed similar performance. Success in barcode recovery was substantially lower (38%) for blood-fed females using residual DNA in RNA extracts as template. Residual DNA is typically removed during conventional RNA extraction, but the quantity of residual DNA likely varies with different RNA extraction methods, but this matter has not been investigated in detail so further studies should examine multiple RNA extraction methods and vector taxa to optimize DNA retention. Second, during transfer from Mexico to the UK, the blood-fed specimens were exposed to room temperatures for 48 h. Because nucleic acid degradation likely occurred, a shorter than normal barcode sequence was targeted (130 bp) for amplification. While this approach likely resulted in a higher success rate than if standard primers (e.g., 658 bp) were used, sequence recovery would certainly have increased if the specimens were frozen during transfer. Methods are available to recover longer sequences from highly degraded samples (50), but this study aimed to develop a simple approach to delineate vector–host–pathogen interactions. Despite the lower sequence recovery from blood-fed specimens, the barcodes that were recovered did confirm morphological identifications in all cases (**Supplementary Data Sheet 1**).

This study did not aim to examine phylogeny relationships, but the dataset was analyzed using ML and MP phylogenetic methods. Analysis of the barcode sequences with NJ (**Figure 2**) in comparison with ML and MP phylogenetic algorithms (**Supplementary Figures 1**, **2**) showed fair concordance with phylogenetic relationships proposed for Anophelinae, Aeidini, Culicini, and Sabethini (5, 50, 65), confirming the phylogenetic signal present in COI (23). Intraspecific genetic divergences for most species were within the 2% limit standard for insects and the Culicidae [e.g., (27, 28, 32, 66–68)], with the exception of *An. eiseni*, *An. pseudopunctipennis*, *Ae. podographicus*, *Cx. eastor*, *Cx. erraticus*, *Tr. pallidiventer*, *Tx. haemorrhoidalis*, *Wy.*

TABLE 6 | Mosquito species, number of pools, and total number of specimens per pool (*n*) per community processed for the detection of *Flavivirus* and *Alphavirus* RNA in pools of unfed mosquitoes collected in sylvan communities in Chiapas State, Mexico.

Species	Locality/no. of pools (*n*)				
	Las Golondrinas	**Las Guacamayas**	**Las Nubes**	**Nueva Esperanza**	**Lacan-ja**
Culicinae					
Aedes aegypti			4 (10)		1 (2)
Aedes albopictus		1 (2)			
Aedes angustivittatus	1 (6)		1 (10)		23 (195)
Aedes podographicus	1 (8)				
Aedes serratus					4 (33)
Aedes trivittatus			1 (2)		
Aedes sp.	1 (5)				
Culex quinquefasciatus		46 (311)	14 (93)	28 (196)	19 (73)
Culex nigripalpus	14 (133)		1 (2)		3 (18)
Culex pilosus		1 (2)			
Haemagogus sp.	1 (6)				
Limatus asulleptus					1 (10)
Limatus durhami	1 (2)			1 (2)	
Psorophora albipes					1 (3)
Psorophora champerico		1 (2)			2 (8)
Psorophora ferox	1 (96)				8 (60)
Psorophora cingulata					5 (24)
Sabethes sp.	1 (8)				
Shannoniana moralesi					1(3)
Wyeomyia adelpha/Wy. guatemala	14 (117)				2 (12)

adelpha/Wy. guatemala, and *Wy. pseudopecten* (**Table 3**), whose higher intraspecific divergences and deep splits in the NJ tree (**Figure 2**) suggest cryptic diversity. However, future studies should use rapid mutating markers (microsatellites, Single Nucleotide Polymorphisms) to analyse more thoroughly such intraspecific diversity.

The genus *Anopheles* includes many vector species for malaria of which several are species complexes (5). Indeed, the separation of both *An. eiseni* and *An. pseudopunctipennis* into multiple BINs reveals the likely presence of cryptic lineages within them. The deep genetic divergence observed in *An. pseudopunctipennis* reinforces earlier reports that it is a species complex (69, 70). Similar cases have been documented in *An. apicimacula* and in the *An. crucians s.l.* and *An. lindesayi s.l.* complexes using DNA barcoding (71, 72). *Aedes podographicus* is a member of the Terrens group in the subgenus *Protomacleaya*, which includes some 28 nominal species and two forms with neotropical distributions (72, 73). Among these, 10 species are found in Mexico and three in Chiapas State. The adults of several of these species are so morphologically similar that their discrimination is difficult. Further morphological and zoogeographical evidence discussed in Schick (73) and Schick (74) supports the hypothesis that *Ae. podographicus* is a species complex. Another member of the Terrens group encountered in Chiapas is *Ae. insolitus*, which is also a suspected species complex related to the *Ae. podographicus* complex (73, 74).

The subgenus *Melanoconion* of *Culex* includes ∼160 described species (5), making it one of the most species-rich subgenera within the Culicidae. Further taxonomic clarity is important as its members are vectors for viruses such as Venezuelan Equine Encephalitis (VEE) (74). The usefulness of DNA barcodes for discriminating species in this subgenus has been reported, as well as the discovery of cryptic species or new species within *Culex* (71, 75, 76). In this study, six species of *Melanoconion* were detected (**Table 2**). One of these species, *Cx. eastor*, was separated into two groups with an average genetic divergence of 4.88%, one from Mexico (BIN:AAG3857) and the other from Brazil (BIN:ADJ7929), supporting the presence of two cryptic species.

The genus *Trichoprosopon* includes 13 species in Central and South America, but their importance as disease vectors is poorly known (77). Two of these species (*Tr. digitatum*, *Tr. soaresi*) have been reported from Mexico (78). The present study extends this list by three species: *Tr. pallidiventer*, and a species that is close to *Tr. brevipes* from Brazil based upon morphological features (79, 80), and another undescribed taxon close to the *Trichoprosopon* spG of Talaga et al. (34). An average intraspecific diversity of 4.95% was obtained for *Tr. pallidiventer* and this group separated from other specimens identified *Tr. digitatum*, *Tr.* nr. *brevipes*, and *Trichoprosopon* sp. in the NJ tree (**Figure 2**) with high support. This suggests that the specimens identified as *Tr. pallidiventer* include two lineages, a result also noted by studies in French Guiana (34). These results support (77)

conclusions that species of the genera *Runchomyia*, *Shannoniana*, and *Trichoprosopon* are difficult to identify because of lack of adequate descriptions. A single sequence was obtained for a specimen identified as *Trichoprosopon* nr. *brevipes*, but any final assessment of its taxonomic status requires more specimens.

Although taxonomic revision is required, the genus *Wyeomyia* includes 139 species with neotropical and Nearctic distributions (5, 81), and 10 of these species occur in Mexico (59). *Wyeomyia pseudopecten*, a member of the subgenus *Decamyia*, includes records from Guatemala, Honduras, and the Caribbean to Brazil (82). Little is known about its biology (34), but the presence of two BINs suggests it is a species complex. Specimens identified as *Wy. adelpha*/*Wy. guatemala* showed high intraspecific divergence (7.30%, $n = 10$), and barcode analysis revealed four groups, named here groups I, II, III, and IV (**Figure 2**), again suggesting cryptic species. Taxonomy uncertainty surrounds three species: *Wy. adelpha*, *Wy. guatemala*, and *Wy. mitchellii*. *Wyeomyia guatemala* was described from Guatemala [(83), p. 139], *Wy. adelpha* from Costa Rica [(82), p. 140]] and *Wy. mitchellii* from Jamaica (84). *Wyeomyia guatemala* was separated from *Wy. mitchellii* by Theobald (84) based on the morphology of the larva and the male genitalia, but the females were separated based on their geographical distribution restricting the name *Wy. guatemala* for Central America and *Wy. mitchellii* for Florida, USA, and the West Indies. However, (85, 86) placed *Wy. guatemala* as a synonym of *Wy. mitchellii*, but (87) stated that specimens from Central America identified as *Wy. guatemala* or *Wy. mitchellii* should be named as *Wy. adelpha*. This was confirmed by Belkin et al. (88) in their review of mosquitoes in Jamaica, where they concluded that supposed records of *Wy. mitchellii* from Mexico to Panama were likely to represent another species. Currently, *Wy. mitchellii* is only applied to populations from the United States, but all aforementioned names remain as valid species in Harbach (5). Because of the lack of COI DNA barcode sequences from correctly identified specimens of *Wyeomyia* in Central America, we have identified Mexican specimens as *Wy. adelpha*/*Wy. guatemala*. This fact highlights yet again the need for expansion of the DNA barcode reference library in combination with revisionary taxonomy.

Although members of the genus *Toxorhynchites* are not of medical importance, their predatory larvae have been employed for biological control with some success (5). We compared the single barcode sequence from *Tx. haemorrhoidalis haemorrhoidalis* (BOLD:ADE6036) obtained in this study with sequences from French Guiana that were identified as this subspecies (BOLD:ACZ4120), as well as to *Tx. haemorrhoidalis superbus* (BOLD:ACZ3966). This comparison revealed a deep split in the NJ tree with average genetic divergence value of 4.35%. Some authors (34) have suggested the presence of several lineages within this species, and the present results support this conclusion.

In contrast to the cases where the DNA barcode results suggested cryptic species, incomplete separation was apparent between *Ae. insolitus* and *Ae. podographicus* (BOLD:ADE8493) and between *Cx. coronator* and *Cx. usquatus* (BOLD:AAN3636). In these cases, interspecific divergence between the species pairs were < 1%, so each pair of species was assigned to the same BIN. As expected from their barcode similarity, *Ae. insolitus* and *Ae. podographicus* both belong to the Podographicus complex of *Aedes*, Similarly, *Cx. coronator* and *Cx. usquatus* belong to the Coronator complex of *Culex*. A few other species pairs were assigned to the same BIN, but they can be separated in the NJ tree. For example, *Cx. mollis*, *Cx. nigripalpus*, and *Cx. pinarocampa* all share a BIN assignment (BOLD:AAF1735), but they form monophyletic clusters in the NJ tree. The close similarity in their sequences suggests that these species are recently diverged or that there has been recent introgression (71). Despite such complexities, the COI barcodes were always useful in narrowing the taxonomic identity of specimens. This was particularly useful in cases where morphological study only allowed a generic assignment, as in *Wyeomyia* sp. (=*Wy*. nr. *complosa*). When resources permit, it is worth supplementing COI DNA barcodes with a nuclear marker such as ITS2 to help clarify cases of uncertainty (32). With the new addition of several mosquito species to its fauna, Chiapas State is now known to host 148 mosquito species, the greatest diversity of any Mexican state, while the Mexican fauna increases to 238 species.

The use of NGS was essential to identify the vertebrate species that served as the source of the blood meals, as a single female can feed on several hosts, creating amplicon pools that cannot be analyzed by Sanger sequencing. Although it is a common practice to employ a separate DNA extraction for blood meal analysis (16, 19), the single RNA extraction performed conformed with protocols established at APHA for the detection of viral pathogens. By omitting DNase treatment, this approach circumvented the need for a separate DNA extraction to allow vector and host identification, saving time, and resources.

A broad range of host species were identified from blood-fed females, including both birds and mammals. *Aedes angustivittatus*, *Ae. podographicus*, *Ae. trivittatus*, *Culex* sp., *Cx. nigripalpus*, *Ps. albipes*, and *Ps. ferox* each fed on only one or two hosts (**Table 5**), but collectively fed on a wide diversity of large mammals, birds, and humans. The females of these species are highly anthropophilic, so they can maintain arbovirus circulation in rural or sylvatic settings. For example, the importance of *Ps. albipes* and *Ps. ferox* in the circulation of Venezuelan equine encephalitis virus (VEEV), WNV, and LaCrosse virus in tropical regions has been well-established (62). By contrast to the focused host use of other species, *Cx. quinquefasciatus* fed on a wide range of hosts such as cow, horse, chicken, human, turkey, great-tailed grackle, Virginia opossum, and Muscovy duck, all species common in farmland settings. This result contrasts with other studies; Janssen et al. (44) found humans were its primary host food (63–77%), whereas Estrada-Franco et al. (56) found it fed largely on dogs. Interestingly, (89) found it used diverse hosts in Nevada, USA. Our results suggest that *Cx. quinquefasciatus* is mainly ornithophilic across sylvan communities in Chiapas State, but also feeds on mammals, confirming that it could have an important bridge role in arbovirus transmission (3, 42, 56, 90–93).

There is known circulation of VEEV and St, Louis encephalitis virus in southern Mexico, and WNV antibodies have also

been reported in chicken, turkey, and cattle in Chiapas (39, 94–96). Despite these observations, we failed to detect *Flavivirus* or *Alphavirus* RNA using generic primers on both pools of unfed mosquitoes (**Table 6**) or individual blood-fed specimens (**Table 4**). It needs emphasis that in regions with high circulation of arboviruses, many thousands of mosquitoes are typically pooled must routinely for effective detection. Viewed from this perspective, the number of samples tested in this study was small involving only 204 pools (**Table 4**), so we may not have collected a statistically significant number of mosquitoes infected with an arbovirus. As well, loss of the cold chain during the transport of specimens to APHA undoubtedly had a negative effect on any viral RNA that may have been present. As a result, additional collecting should be undertaken in Chiapas to assess viruses that are in circulation.

In conclusion, this study has established that residual DNA in standard RNA extracts can be employed as a template for DNA barcoding to enable vector and host identification. However, we acknowledge that their suggested procedure is still not proven to be effective at detecting RNA based viruses because many samples were not maintained at low temperatures during transport, and we have not tested in detail how DNA in an RNA sample can interfere with the PCR assay in a varied set of samples. In addition, we are aware that usually viral RNA is very low in wild samples originating either from mosquitoes or vertebrates; thus, we advocate for further studies to analyze the effectiveness of this methodology in detecting RNA viruses across a broader range of taxa. Nonetheless, this approach will help to clarify the interactions between insect vectors and both their vertebrate hosts and viral pathogens more efficiently by avoiding the DNA and RNA coextraction from each sample. This, in turn, will provide the essential information needed in order to manage and establish the relevant control strategies against vector borne diseases.

This study has extended understanding of the mosquito fauna in the sylvatic areas of Chiapas State and suggests the presence of cryptic species in nine morphospecies. A broad range of host species was used as a blood meal source by *Cx. quinquefasciatus*, supporting its likely role as a bridge vector for arbovirus transmission. Finally, this study highlights the need to develop a comprehensive DNA barcode molecular library for the mosquito fauna in Mexico and other countries in Central America.

DATA AVAILABILITY STATEMENT

Detailed specimen records and sequence information (including trace files) were uploaded to the Barcode of Life Database (BOLD—http://www.boldsystems.org) within datasets (projects): DS-MQLC "DNA Barcoding mosquitoes sylvan communities in Mexico (records more than 300 bp) Lacandon Jungle (records < 300 bp)"; DS-MQLCJ "DNA Barcoding mosquitoes sylvan communities in Mexico (13- bp shorter sequences)." The Digital Object Identifier (DOI) for the publicly available projects in BOLD is dx.doi.org/10.5883/DS-MQJLC and dx.doi.org/10.5883/DS-MQLCJ. All generated sequences of more than 300 bp have been submitted to GenBank (accession numbers: MT552364—MT552598).200526.

ETHICS STATEMENT

The Animal and Plant Health Agency received permits to carry out surveillance studies on potential infected samples.

AUTHOR CONTRIBUTIONS

LH-T, JG-H, AO, SP, PH, AF, and MR-P contribution to the study conception and design. JG-H, AO, EL-S, VG-A, and RM-L material preparation, specimens' collection, and morphological identification of specimens, interpretation for the work. LH-T, SP, PH, NN, EB, and RC-C molecular identification and analysis of sequences. LH-T, AF, PH, and MR-P funding acquisition. LH-T, JG-H, AO, SP, PH, NN, EB, EL-S, VG-A, RM-L, RC-C, AF, and MR-P drafting the manuscript or revising it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.564791/full#supplementary-material

Supplementary Data Sheet 1 | Neighbor-joining tree based on the Kimura two-parameter (K2P) distances showing the arrangement of COI DNA barcodes of 130 bp in length in relation to barcodes of >300 bp for mosquito species collected in sylvan communities in Chiapas State, Mexico. A divergence > 2%

may be indicative of separate operational taxonomic units. All barcodes of 130 bp are highlighted in red.

Supplementary Figure 1 | Maximum Likelihood tree based on COI DNA barcodes (>300 bp) for mosquito species recorded in sylvan communities in Chiapas State, Mexico. A divergence > 2% may be indicative of separate operational taxonomic units. Values over each node indicate support values. An asterisk (*) relates to species from which sequences have been downloaded from BOLD and NCBI databases.

Supplementary Figure 2 | Maximum parsimony tree based on COI DNA barcodes (>300 bp) for mosquito species recorded in sylvan communities in Chiapas State, Mexico. A divergence > 2% may be indicative of separate operational taxonomic units. Values over each node indicate support values. An asterisk (*) relates to species from which sequences have been downloaded from BOLD and NCBI databases.

Supplementary Table 1 | Species, number of sequences, sample ID, process ID, BIN, and country of sequences downloaded from BOLD or NCBI and added to the dataset of COI DNA barcodes (>300 bp) obtained from mosquitoes collected in sylvan communities in Chiapas State, Mexico.

Supplementary Table 2 | Percentage of interspecific (between groups) pairwise Kimura two-parameter (K2P) genetic divergence of unique DNA barcodes (658 bp) for 55 species of mosquitoes collected in sylvan communities in Chiapas State, Mexico. Highest pairwise distances (most divergent taxa) and lowest pairwise distances (most closely related taxa) are highlighted in orange and yellow, respectively.

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Evidence of Wing Shape Sexual Dimorphism in *Aedes* (Stegomyia) *albopictus* in Mallorca, Spain

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The Asian tiger mosquito *Aedes albopictus* (Skuse, 1894) is a highly invasive species widely distributed on the Spanish Mediterranean coast and the Balearic archipelago. Most studies involving this species in Spain have been focused on surveillance and control methods. However, micro-evolutionary studies for *Ae. albopictus* in Spain have been traditionally neglected. Morphological diversity could be the result of long-term evolutionary diversification in responses to selective pressures such as temperature, precipitation, food availability, predation, or competition that may influence flight activity, host-seeking, and blood-feeding behavior. Wing geometric morphometric have been used not only to study micro- and macro-evolution in mosquitoes but also in studies of population structuring and sexual dimorphism. Therefore, the main goal of this study was to investigate the wing shape patterns of *Ae. albopictus* populations to unveil sexual dimorphism that could provide information about their ecology and behavior. Mosquito eggs were collected using oviposition traps at the main campus of the University of the Balearic Islands (Palma de Mallorca, Spain) and reared under laboratory conditions. In order to study wing shape variation patterns in *Ae. albopictus* males and females, the left wing of each adult mosquito was removed and analyzed based on 18 landmarks. Our results indicated strong levels of sexual dimorphism between *Ae. albopictus* males and females. Furthermore, according to the cross-validated reclassification test, males were correctly distinguished from females with an accuracy of 84% and females from males 75%. We observed a significant sexual dimorphism in the wing shape patterns of *Ae. albopictus* when considering different seasonal patterns (spring vs. autumn). Our results suggested that selective pressures may affect males differently to females. Host-seeking, blood-feeding, and oviposition behavior of females may act as a major driver for wing shape sexual dimorphism. These results should be considered for the development of more effective and targeted mosquito control strategies.

Keywords: *Aedes albopictus*, sexual dimorphism, wing shape, host-seeking, mosquito

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INTRODUCTION

The Asian tiger mosquito *Aedes albopictus* (Skuse, 1894) is a highly invasive species currently distributed throughout the Mediterranean area including the Balearic archipelago. This mosquito species is native to south-west tropical and subtropical forests in Asia and it has spread through all the continents except Antarctica (Kraemer et al., 2015; Cunze et al., 2016). Due to its ecological

plasticity, *Ae. albopictus* is well adapted to exploit the resources available in urban and peri-urban areas (Bonizzoni et al., 2013).

The introduction of *Ae. albopictus* to new geographic regions has been facilitated by globalization processes such as transport of commodities and by anthropogenic modifications of natural environments generating suitable breeding sites (Schaffner et al., 2013; Wilke et al., 2020). Worldwide trade of tires and lucky bamboo (*Dracaena* spp.) have been pointed out as the main pathways for its spreading (Eritja et al., 2017; Sánchez et al., 2017; Wilke et al., 2020) and recent studies have also shown that cars and commercial flights are major drivers in the expansion of *Ae. albopictus* to new areas (Eritja et al., 2017; Ibañez-Justicia et al., 2017).

Aedes albopictus was first introduced in Europe in Albania in 1979. Since then, it has spread throughout the continent (Gratz, 2004) and it has been recorded in at least 31 European countries (Collantes et al., 2015; Petrić et al., 2018). The most recent recordings include the south of England (Medlock et al., 2017), the north of Portugal (Osório et al., 2018), the region of Bohemia in the Czech Republic (Rettich and Kulma, 2018), and the southern Italian islands of Lampedusa, Linosa and Pantelleria (Di Luca et al., 2017).

In Spain, it was first recorded in Sant Cugat del Vallès in 2004 (Aranda et al., 2006) ten years later *Ae. albopictus* has spread out along all the east coast of the Iberian Peninsula and the Balearic Islands (Collantes et al., 2015). Nowadays, it can be found in Catalonia, Castellón, Valencia, Alicante, Murcia, Málaga, Andalusia, Basque Country, Extremadura, and the Balearic Islands (Roiz et al., 2008; Bueno-Marí et al., 2009, 2010, 2013, 2014; Delacour-Estrella et al., 2010; Collantes and Delgado, 2011; Miquel et al., 2013; Delacour-estrella et al., 2014; Delacour et al., 2015; Bravo-Barriga et al., 2019). The first detection of *Ae. albopictus* in the Balearic Islands was in 2012 in Mallorca (Miquel et al., 2013), then in Ibiza in 2014 (Barceló et al., 2015) and in Minorca in 2015 (Bengoa et al., 2016).

Aedes albopictus alongside with *Aedes aegypti* (L.) is considered the main vector of dengue and chikungunya viruses causing major disease outbreaks in the Southwestern Indian Ocean, India, and Central Africa (Paupy et al., 2009). This species is also a competent vector for other arboviruses such as Zika, yellow fever, and filarial worms (Paupy et al., 2009; Grard et al., 2014; Gutiérrez-López et al., 2019). In Europe, *Ae. albopictus* is currently responsible for local outbreaks of chikungunya (Rezza et al., 2007) and dengue (Gould et al., 2010; Lazzarini et al., 2020) due to virus introduction by travelers arriving from endemic areas (Emmanouil et al., 2020). Similarly, local transmission cases of the dengue virus were reported from Italy, Croatia, and France since 2010 (La Ruche et al., 2010; Gjenero-Margan et al., 2011; ECDC, 2020). More recently and for the first time, six dengue autochthonous cases were reported in 2018 and one in 2019 in Spain (ECDC, 2019; Monge et al., 2020) in areas where *Ae. albopictus* is considered the primary vector.

In the Balearics, *Ae. albopictus* is considered established but increasing its distribution area every year (Tavecchia et al., 2017; European Centre for Disease Prevention and Control and European Food Safety Authority, 2020). This species breeds preferably in small containers, mostly human-made, that are present in urban and peri-urban areas. *Aedes albopictus* is known for being anthropophilic and a nuisance species. However, despite its current importance as vector and nuisance species, little information is available about the main drivers responsible for modulating its ecology, behavior, and genetics. Consequently, identifying the possible microevolutionary patterns that may affect the behavior of *Ae. albopictus* (Suesdek, 2019) is crucial. Environmental factors can affect mosquito bionomy traits such as longevity, body size, reproduction, larval development, and fecundity, among others (Chandrasegaran et al., 2020). Those factors vary across the mosquito spatial distribution and may change its behavior related to its flight activity, host-seeking, or feeding frequency (Bara et al., 2015; Carvajal et al., 2016; Chandrasegaran et al., 2020).

Wing geometric morphometrics (WGM) is a practical tool for describing phenotypic variation in organisms representing individuality by the relative position of morphological landmarks that define the shape of the morphological trait studied (Klingenberg, 2010, 2011, 2016; Sánchez et al., 2017; Multini et al., 2019). In insects, the wing is the preferred structure for morphometric analyses due to its bi-dimensional composition reducing digitizing error (Dujardin, 2008).

This methodology has been proven effective to study micro- and macro-evolution in mosquitoes and also in studies of population structuring and sexual dimorphism (Dujardin, 2011; Louise et al., 2015; Virginio et al., 2015; de Oliveira et al., 2016; Wilke et al., 2016; Lorenz et al., 2017; Multini et al., 2019). The population structuring may be affected by exogenous or endogenous pressures that could affect males and females differentially (Carvajal et al., 2016; de Oliveira et al., 2016). Morphological diversity could be the reflection of biological differences such as shape, that result from long-term evolutionary diversification (Zelditch et al., 2012). This dissimilarity in shape would mean different functional roles or responses to selective pressures (Zelditch et al., 2012).

According to Lorenz and Suesdek (2020), the wing is an important structure for sexual signaling and flight, but it is still unknown if variation in wing patterns have some ecological or behavioral role that directly influences the flight or mating. Hence, the study of sexual dimorphism patterns can provide answers to questions about mating behavior, genetic drift, and how populations react to selective pressures, including host-seeking (Chandrasegaran et al., 2020). Comprehensive knowledge of the morphometrical characteristics of the vector may help in describing the population diversity, morpho-ecological traits and could then be useful for success in control campaigns (Jirakanjanakit et al., 2005; Sendaydiego et al., 2013; Chaiphongpachara et al., 2019).

The goal of this study is to investigate the sexual dimorphism based on wing shape variations in *Ae. albopictus* populations from the Balearic Islands and to explore if *Ae. albopictus* male and female populations are driven differently by selective pressures. In addition, the study also assessed the wing shape variation between different seasons.

MATERIALS AND METHODS

Mosquito Sampling

The sampled area was located in the municipality of Palma (Mallorca, Spain), the most urbanized and populated city on the island (**Figure 1**). Mallorca has a Mediterranean climate represented by mild wet winters and hot dry summers with mean annual precipitation of about 500 mm and a mean annual temperature of 17°C (Gelabert et al., 2003).

A total of 72 black plastic container ovitraps (Ø 9 cm, h = 15 cm, 950 mL) were placed to collect *Ae. albopictus* eggs at the main campus in an experimental plot of the University of the Balearic Islands (UTM 31 S 469347.93 m E 4387387.76 m N). This suburban area is characterized by having residential houses, usually provided with gardens and human-made small containers (plant pots, vases, etc.) which are favorable for *Ae. albopictus* development. Ovitraps were filled with 500 mL of tap water and 2.5 g of hay as an attraction lure. Wood tongue depressors were placed inside ovitraps as oviposition substrate (ECDC, 2012). Thirteen samplings were performed during autumn 2017 and spring - summer 2018, with six samplings between October 2017 and December 2017, and seven between April 2018 and July 2018. From 21st September to 20th December was considered as Autumn, from 21st December to 20th March was Winter, from 21st March to 20th June was Spring and from 21st June to 20th September was Summer.

Collected eggs were hatched in the laboratory under controlled conditions (27 ± 1°C, 70% relative humidity, 12 h photoperiod), flooding them into tap water in containers until the emergence of adults. The emerged adults were maintained *ad libitum* in 10% sucrose solution, morphologically identified, sexed, and preserved at −20°C when dead.

In addition to ovitraps, five mosquito adults' samplings were performed using one BG-Sentinel trap (BG-1 Sentinel, Biogents, Germany) baited with BG-lure (Biogents, Germany) and CO_2 (2 Kg of dry ice per day). Adult traps were set for 24 h per sampling between 23/10/2017 and 07/11/2017. By conducting a large sampling effort and different collection methods we greatly reduced the probability of collecting sibling specimens. For the analysis, a total of 50 adults from autumn were collected with the BG-Sentinel trap. The remaining mosquitoes (191) were reared from the egg batches collected by the oviposition traps deployed during spring and summer. To further reduce the probability of analyzing sibling specimens, when the sampling sufficiency was reached (more than 30 mosquitoes per population) adult mosquitoes were chosen randomly for the analyses.

Geometric Data Acquisition

A total of 241 left wings, 123 from females and 118 from males emerged in the laboratory and collected from the field in different seasons (**Table 1**) were dissected and mounted between a slide and a coverslip (without mounting medium) fixing the coverslip with adhesive tape (Vidal et al., 2012; Beriotto et al., 2021). Wings were photographed at 40x magnification using a Zeiss Scope A.1 stereoscopic microscope attached to a camera AxioCamICc1 (Zeiss, Germany) by one of the authors (JLM) to minimize error measurements (Fruciano, 2016). The images were processed using ZEN 2.3 lite program (Blue edition). Eighteen landmarks (LMs) were selected to study the geometric morphometry of *Ae. albopictus* wings according to Bookstein (1997); Vidal et al. (2011), and Vidal and Suesdek (2012). The coordinates of the 18 landmarks represented by vein intersections (**Figure 2**) were obtained using tpsDig2 2.30 (Rohlf, 2005; Vidal and Suesdek, 2012; Sendaydiego et al., 2013). These landmarks were selected for being homologous and found in all representatives of the Culicidae family (Lorenz et al., 2017; Beriotto et al., 2021).

Morphometric Analysis

The wing geometric morphometric analysis was conducted using MorphoJ 1.06d software (Oracle Corporation) that provides a wide range of tests for two- or three-dimensional landmark data (Klingenberg, 2011). Classifiers about sex and season information were generated for each specimen to examine morphometric differences. Firstly, allometric effect of the wing size on the wing shape was estimated using multivariate regression analysis of the Procrustes coordinates on the centroid size with a permutation test of 10,000 randomizations, and, finally, removed from the subsequent analyses (Klingenberg, 2016). Due to the different sample methods, we focused on wing shape variation patterns disregarding size variations, coordinates, and orientation of the landmarks (Klingenberg, 2010; Börstler et al., 2014; Virginio et al., 2015). After that, to extract shape information, landmarks were subjected to the generalized least-squares Procrustes superimposition algorithm aligning by the principal axes in order to standardize the shape of wings and determine the wing shape coordinates (partial warps) for sex (Virginio et al., 2015; Carvajal et al., 2016) and then for season classifiers (Vidal et al., 2012). After that, a covariance matrix was performed to assess the linear relationship between the independent and the dependent variables. Principal Component Analysis (PCA) was carried out using a covariance matrix for all populations. Then, a Canonical Variate Analysis (CVA) with a permutation of 10,000 randomizations was carried out to test the pairwise distances between males and females, and between seasons (Klingenberg, 2010; Carvajal et al., 2016). In the visualization of the CVA, every possible shape corresponds to a shape point in the morphospace and every direction in the morphospace

TABLE 1 | Season and sampling period, number of samplings, sampling method and number of males and females of *Ae. albopictus* that were collected for wing geometric morphometrics.

Season	Sampling period	Sampling method	Number of samplings	Adult males	Adult females
Autumn	23/10/2017 – 04/12/2017	BG trap and oviposition trap	6	16	41
Spring	20/04/2018 – 13/06/2018	Oviposition trap	5	78	58
Summer	09/07/2018	Oviposition trap	2	24	24
Total			13	118	123

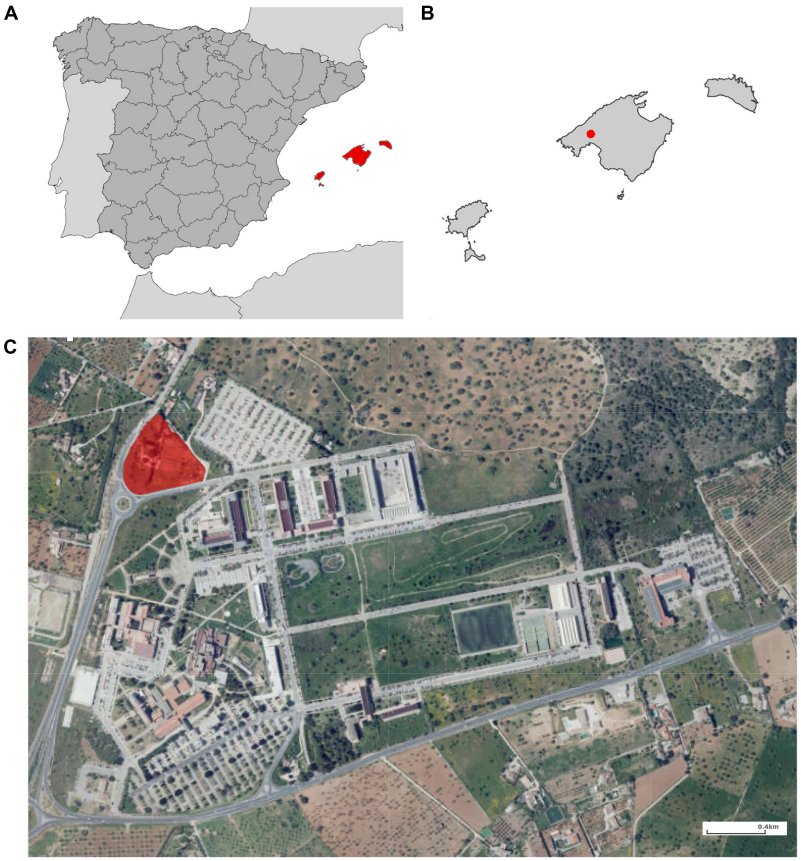

FIGURE 1 | Map of the study area. **(A)** Spain; **(B)** Balearic Islands; and **(C)** Campus of the University of the Balearic Islands. The collection area is highlighted in red.

corresponds to a specific shape change (Klingenberg, 2010). Subsequently, thin-plate splines were obtained by the regression analysis of CVA scores against wing shape variation (Bookstein, 1989; Wilke et al., 2016). Each specimen was then reclassified using the cross-validated classification test of the discriminant analysis based on Mahalanobis distances to determine the degree of wing shape dissimilarity between sex and seasons (Vidal et al., 2012; Wilke et al., 2016). Finally, the Mahalanobis distances resulted from the CVA analysis with season classifier were used to group clusters defined by unweighted pair group method with arithmetic mean (UPGMA, Sneath and Sokal, 1973) to assess the similarity of the wing shape patterns (Carvajal et al., 2016). UPGMA was achieved using PAST software.

RESULTS

The allometry test predicted 0.81% ($P = 0.0790$) of the wing shape variation. The allometric effect was not considered as it only explained a negligible proportion of the variance.

The Procrustes superimposition analysis using the classifier sex resulted in significant differences in the wing shape patterns of males and females. In the wireframe, it was observed that male wings were narrow and lengthened, whereas females were wider and shorter. The CVA showed significant differences in the wing shape patterns between males and females (**Figure 3A**) and, in the principal-partial warps from the PCA analysis, it was observed that the most variable anatomical landmarks

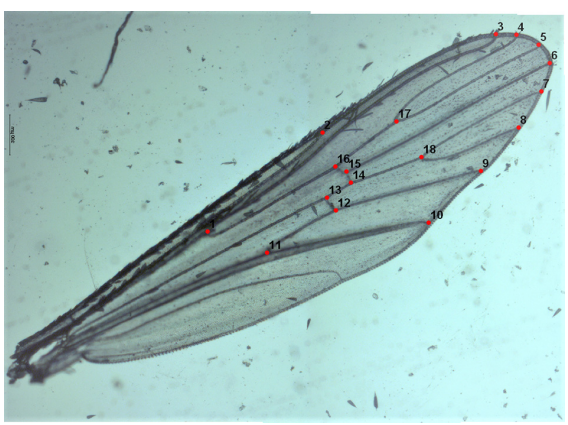

FIGURE 2 | Left wing of *Ae. albopictus* (female) mounted between a microscope slide and coverslip showing the 18 landmarks selected for the geometric morphometrics analysis.

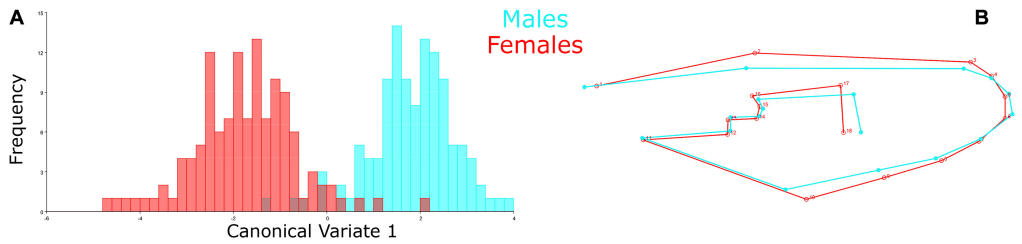

FIGURE 3 | Comparison between male and female specimens. **(A)** CVA represented in the morphospace to compare the wing shape variation patterns between males (blue) and females (red). *X*-axis: First canonical variate; *Y*-axis: Frequency of each wing shape. **(B)** Wireframe graph from the PCA displaying the mean value of wing shape variation in the male (blue) and female (red) populations.

were landmarks 2, 12, 17, and 18 (**Figure 3B**). The Procrustes distance of discriminant function showed a value of 0.0288 and a Mahalanobis distance of 3.5673 ($P < 0.05$) (**Table 2**).

Female specimens yielded high values in the cross-validated classification test from the discriminant function, yielding 94.31% of accuracy when compared to male specimens (**Table 2**). Conversely, males also yielded high cross-validated classification values, resulting in 93.22% accuracy when compared to females (**Figure 3** and **Table 2**).

The CVA considering seasonality showed significant differences in wing shape between seasons of the year for both sexes (**Figure 4**). Discriminant analysis revealed that the dissimilarity in wing shape separately for males and females over the seasons was statistically significant ($P < 0.05$) except between these groups: Females-Spring vs. Females-Summer ($P = 0.2275$), Males-Autumn vs. Males-Spring ($P = 0.1542$), Males-Autumn vs. Males-Summer ($P = 0.2900$) and Males-Spring vs. Males-Summer ($P = 0.1151$). From the 482 total comparisons carried out with the 241 wings, 350 of them resulted in the cross-validated classification values based on Mahalanobis distances yielding scores higher than 70%, indicating significant differences in wing shape (**Table 2**). In general, *Ae. albopictus* collected during the summer yielded lower reclassification values (38–79%) suggesting a higher overlap in wing shape patterns between males and females in this specific season.

Constructed UPGMA tree based on Mahalanobis distances highlighted the segregation between *Ae. albopictus* sex and seasons, showing a Cophenic correlation of 0.9767 (**Figure 5**). Male and female groups were segregated into two separate clusters. In males, spring and summer samples were clustered together, whereas Autumn was clustered apart from the others. Results indicate that the Autumn male population has a higher dissimilarity when compared to the other populations. Conversely, Autumn and Spring were clustered together in female population, and summer was clustered apart.

DISCUSSION

Since the introduction of *Ae. albopictus* in the Balearic Islands in 2012 (Miquel et al., 2013), it has increased its abundance (Tavecchia et al., 2017) and has become a public health concern in many places of the Mediterranean coast of Spain (Collantes et al., 2015). In Europe, emerging and re-emerging outbreaks of diseases transmitted by mosquito vectors are increasing (Martinet et al., 2019; Emmanouil et al., 2020). For that reason, it is essential to know the bioecology of vector species as well as the preferred breeding sites, host preference, and dispersal capacity among other characteristics.

Our results showed significant wing sexual dimorphism between male and female *Ae. albopictus* specimens collected either as eggs or adults in a suburban area of Palma city. Male

TABLE 2 | Pairwise cross-validated reclassification rates, Mahalanobis distances and *P*-value of males and females *Ae. albopictus* specimens.

Group 1 vs. Group 2		Reclassification score (%)	Mahalanobis distance	*P*-value
Female vs. Male	Female	94	3.5673	<0.0001
	Male	93		
Female Autumn vs. Female Spring	Autumn	63	2.4270	<0.0001
	Spring	74		
Female Autumn vs. Female Summer	Autumn	68	2.8859	0.0269
	Summer	67		
Female Spring vs. Female Summer	Spring	66	1.9708	0.2275
	Summer	54		
Male Autumn vs. Male Spring	Autumn	38	2.2173	0.1542
	Spring	71		
Male Autumn vs. Male Summer	Autumn	50	5.2681	0.2900
	Summer	58		
Male Spring vs. Male Summer	Spring	71	1.8908	0.1151
	Summer	46		
Female Autumn vs. Male Autumn	Female	100	7.5777	<0.0001
	Male	88		
Female Spring vs. Male Spring	Female	88	3.8023	<0.0001
	Male	96		
Female Summer vs. Male Summer	Female	79	6.6439	0.0006
	Male	79		

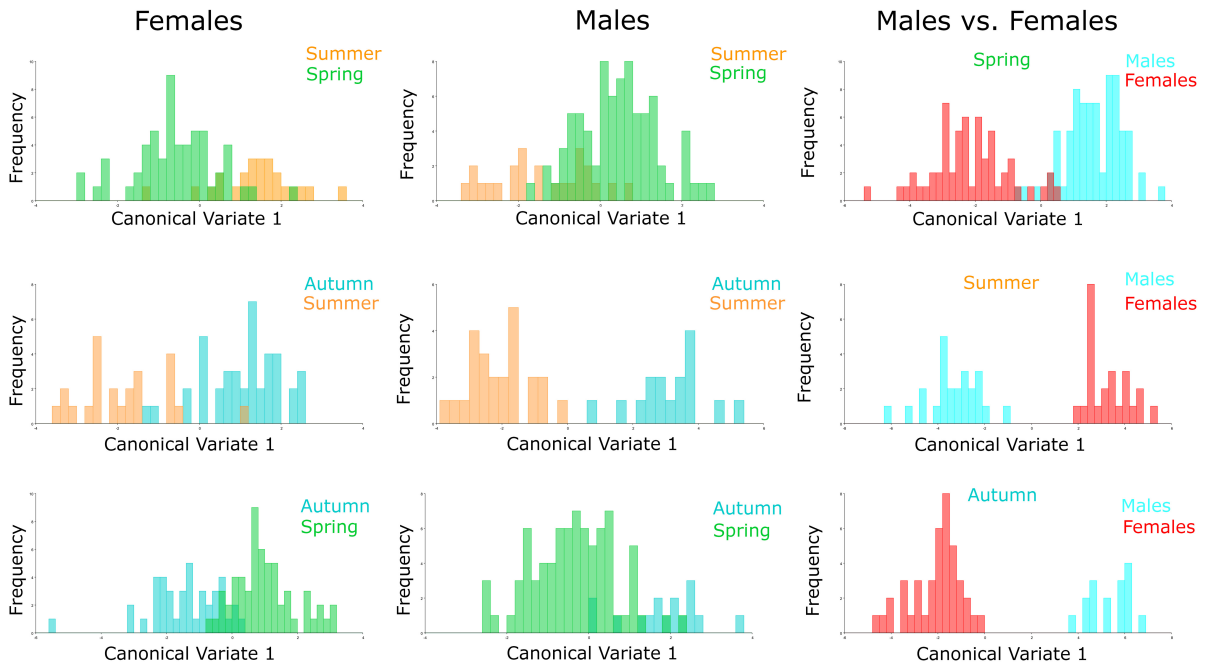

FIGURE 4 | Comparison of specimens by season. Morphological space produced by CVA of *Ae. albopictus* males, females and males vs. females collected in autumn 2017 (red), spring 2018 (green), and summer 2018 (blue). Females: Summer-Spring ($P = 0.2275$), Autumn-Summer ($P = 0.0269$), Autumn-Spring ($P = 0.0002$); Males: Summer-Spring ($P = 0.1151$), Autumn-Summer ($P = 0.2900$), Autumn-Spring ($P = 0.1542$); Males vs. Females: Spring ($P < 0.001$), Summer ($P = 0.0006$), Autumn ($P < 0.001$).

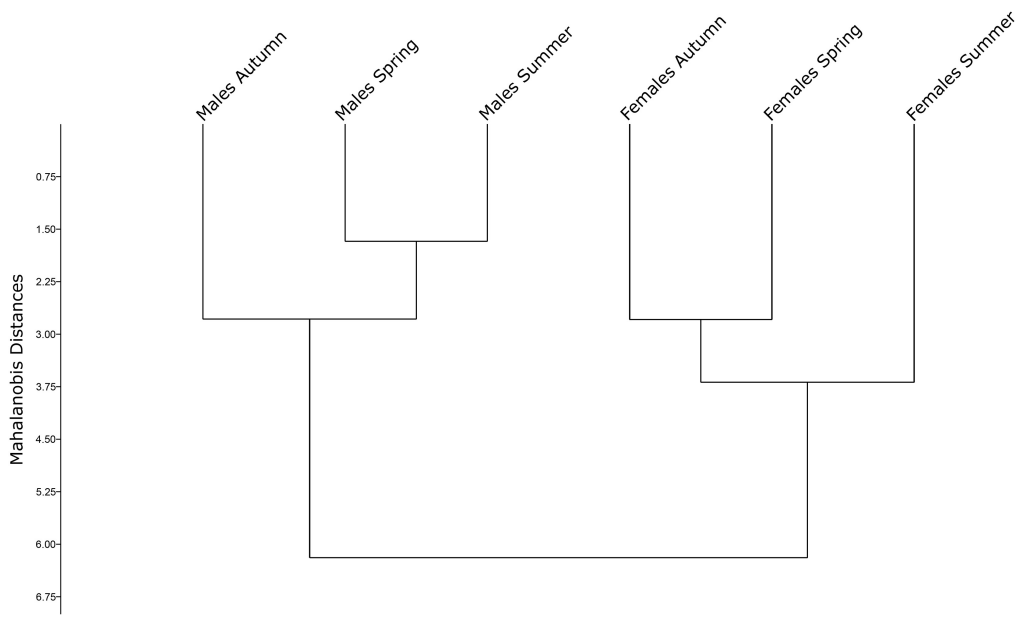

FIGURE 5 | Mahalanobis distances of seasonal mosquito groups (Spring, Summer, and Autumn) represented in an unweighted pair group method with arithmetic mean.

wings were narrower and lengthened, whereas females were wider and shorter. In our study, the highest differences observed in the wireframe were found at the central contraction zones of the wing (LM 2, 10, 17, and 18) most likely due to the robust and mechanical stability of the wings. These wing morphometrics differences could be a consequence of different selective pressures such as sex-specific behaviors (i.e., host-seeking or mating) (Vidal et al., 2012; Chandrasegaran et al., 2020). In fact, shape variation

in sexual dimorphism studies is mainly concentrated in the landmarks located between the middle and distal regions of the wing (Lorenz et al., 2017). Similar results of sexual dimorphism based on wing shape using WGM were observed in *Ae. aegypti* species collected in urban areas in the Philippines (Sendaydiego et al., 2013; Carvajal et al., 2016). Similarly, such sex-specific variation in wing shape patterns has been also confirmed within ten mosquito species belonging to the genus *Culex, Aedes,* and *Anopheles* in southeastern Brazil (Virginio et al., 2015).

Furthermore, our results indicated that the wing pattern variation was associated with seasonality, finding that shape overlapping occurred on mosquitoes of both sexes collected in spring. On the contrary, female mosquitoes collected during autumn showed lower levels of wing shape pattern similarity in comparison to summer and spring. Our findings indicate that different conditions (extrinsic or intrinsic) of each season may significantly affect *Ae. albopictus* wing shape in male and female specimens and possibly other morphological and physiological traits (Virginio et al., 2015). Vidal et al. (2012) and Louise et al. (2015) used the same methodology to demonstrate that short periods of time (less than a year) are enough to result in detectable wing shape changes (Suesdek, 2019).

Our results suggest that microevolutionary changes affected the wing shape patterns of the *Ae. albopictus* populations analyzed in this study. Wing shape pattern evolution may express flight activity changing behavior, intrinsic to the host-seeking process, host preference, vectorial capacity, and habitat suitability, among others (Morales Vargas et al., 2013; Suesdek, 2019; Chandrasegaran et al., 2020).

The evolution of populations within species and how this process impacts vector biology and epidemiology is not the only consequence of biotic factors (Suesdek, 2019; Chandrasegaran et al., 2020). Abiotic local factors, such as temperature, humidity, landscape, and presence of breeding sites could influence microevolution (Chandrasegaran et al., 2020).

There are several ecological differences between males and females of Culicidae that may explain the difference in wing shape of both sexes (Virginio et al., 2015; Christe et al., 2019). Usually, males remain near breeding sites, feeding on sugar sources, such as flowers, or resting in vegetation waiting for females to mate. In addition, females must seek hosts to obtain blood to perform oogenesis and look for aquatic habitats for oviposition. These ecological selective pressures could be responsible for the morphological variations between sex. Other studies such as de Oliveira Christe et al. (2016) and Christe et al. (2019) also demonstrated that males and females of *Aedes fluviatilis* (Lutz, 1904) react to selective pressures such as anthropogenic changes in the environment differently. Further, Nasci (1986) studied the wing length between host-seeking and non-host-seeking females of *Ae. aegypti* and found differences. These results concur with our results, indicating that wing shape patterns are sex-specific and reflect the mechanisms employed by each sex to cope with selective pressures. Landscape heterogeneity was not measured in this study; however, wing shape variations may be attributed to host and habitat availability causing local population changes (Carvajal et al., 2016; Christe et al., 2019). In fact, urbanization can act as a driver of microevolution events that can be observed in the wing shape of *Culex quinquefasciatus* in São Paulo (Brazil) (Wilke et al., 2017). Multini et al. (2019) studied the population structure of *Anopheles cruzii* (Dyar and Knab, 1908) populations for 3 years from three locations with different urbanization levels (urban, peri-urban and sylvatic), finding different wing morphometrics patterns between years and collection sites. Their results suggested that urban disturbances may affect mosquito biology that is reflected with a phenotypic change in the wing. In this sense, evidence of habitat-related morphometric variation was found in urban parks in Sao Paulo, Brazil, where De Carvalho et al. (2017) observed heterogeneity in the wing shape of *Culex nigripalpus* (Theobald, 1901) females. In the case of *Ae. albopictus,* habitat disturbances may lead to the creation of new breeding sites (mostly human-made) compared to natural breeding sites, allowing the rapid spread of the population increasing the contact between mosquito vectors and human hosts. However, this important evolutionary force remains mostly unexplored (Medlock et al., 2015).

To the best of our knowledge, this is the first geometric morphometric work with *Ae. albopictus* conducted in Spain. We have observed a significant sexual dimorphism variation in the wing shape patterns of *Ae. albopictus*. Furthermore, we have detected microevolutionary changes in wing shape between female populations from autumn in comparison to spring and summer. However, its impact on *Ae. albopictus* ecology and behavior remain unknown. Nevertheless, we can only assume that the morphometric results show us morphological differences between groups being a limitation when compared with the entire population and address functional and control strategies of the species (Richtsmeier et al., 2002). Further research increasing the number of samples per season or even include different populations must be considered to address these functional implications.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JL-M designed and performed the experiment. MM designed and directed the experiment. CB assisted with the wing mounting. AB contributed to the design of the figures and the performance of the analysis. JL-M, AB, CB, and MM wrote the manuscript. All authors agreed to be accountable for the content of the work.

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