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# Lizards Are Important Hosts for Zoonotic Flavivirus Vectors, Subgenus *Culex*, in the Southern USA

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Host association is among the most important factors that drive the transmission dynamics of mosquito-vectored pathogens. Competent vectors that feed exclusively upon non-competent hosts will not transmit pathogens, and highly competent hosts that are not bitten by competent vectors will not contribute to pathogen amplification. Therefore, characterizing the blood-feeding ecology of vector mosquitoes is critical to understanding how zoonotic pathogens amplify within ecosystems and spillover to humans and domesticated animals. In North America, mosquito species of the subgenus *Culex* are considered the most important vectors of zoonotic Flaviviruses, particularly West Nile virus (WNV), St. Louis encephalitis virus (SLEV), and western equine encephalitis virus. Many species of the *Culex* subgenus *Culex* are thought to feed predominantly upon birds and mammals, a behavior that facilitates the amplification and spillover of these zoonotic pathogens. Much of our understanding of the host associations of *Culex* vectors is based on research conducted in the 1960s and 1970s that used serological methods to infer host group(s). Here we reevaluate host associations of six *Culex* species from the southern US (Florida and Arizona) using DNA barcoding-based blood meal analysis. Our results demonstrate that reptiles, particularly lizards, constitute an important, and previously underappreciated, group of vertebrate hosts for several subgenus *Culex* mosquitoes. In Florida, >25% of *Culex nigripalpus* blood meals were derived from lizards (mainly *Anolis* spp.), and reptile host use generally increased from north to south with ~10%, ~25% and ~60% of *Cx. nigripalpus* blood meals derived from reptiles in northern, central, and southern Florida, respectively. In southern Arizona, lizards (mainly *Sceloporus* spp. and *Urosaurus ornatus*) constituted 40-45% of blood meals of *Culex tarsalis*, *Culex thriambus*, and *Culex stigmatosoma*. Other species of the subgenus *Culex*, including *Culex quinquefasciatus*, were not found to feed upon reptiles at

the same sites, suggesting host association variation within *Culex* subgenus *Culex*. Whether or not lizards contribute to or dilute amplification of zoonotic Flaviviruses depends upon host competency of the lizard species bitten for WNV and SLEV. To date, very few studies have evaluated host competence of lizards for these viruses, so their roles in transmission cycles of zoonotic Flaviviruses remains obscure.

## INTRODUCTION

West Nile virus (WNV) and St. Louis encephalitis virus (SLEV) are pathogenic mosquito-borne zoonotic Flaviviruses that are primarily transmitted by subgenus *Culex* mosquitoes in North America. Passeriform (WNV) and columbiform birds (SLEV) are considered to be the major amplifying hosts of these zoonotic viruses (1). Several major vectors of WNV and SLEV, including *Culex nigripalpus* (2), *Culex quinquefasciatus* (3), and *Culex tarsalis* (4), feed heavily upon birds in the amplification phase of the virus' transmission cycle (typically spring and early summer), then shift to feeding more heavily upon mammals in the epidemic/epizootic phase (late summer and fall). These seasonal shifts from avian to mammalian hosts by the vector mosquitoes, are thought to drive the amplification, as well as the spillover of these viruses to humans and other mammals (5). Therefore, the host associations of these *Culex* mosquitoes are critical determinants of WNV and SLEV transmission, and, by extension, the risk these viruses pose to public health in the US.

Our understanding of mosquito host associations has been heavily influenced by early (1960s – 1970s) large-scale studies using serology-based blood meal analysis. Working mainly in Florida and California, respectively, John D. Edman and Constantine H. Tempelis, analyzed tens of thousands of mosquito blood meals of numerous species to determine their vertebrate sources, typically identifying mosquito hosts to class or family. Their methodology for blood meal analyses relied largely on the precipitin test, in which blood from a vertebrate animal was injected into a laboratory animal, typically rabbit or game bird, to produce "anti-sera" that reacts when combined with blood from the original vertebrate animal and related species (6, 7). In this way, blood-engorged mosquito homogenates could be diluted and aliquoted into antisera from diverse vertebrate groups with serological reactions indicating an association. Importantly, the sensitivity of the assays was dependent upon the relatedness of organisms, such that cross reactivity was desired within members of a related taxonomic group (family or order, for example) but not between classes. These differences in antisera reactiveness have influenced our understanding of the host associations of important mosquito vectors, including the *Culex* vectors of WNV and SLEV.

Reptiles have been historically overlooked as vertebrate hosts for vector mosquitoes. In their 25-page review of mosquito host blood meal identification, Washino and Tempelis (8) mention reptiles just once, in reference to the difficulty obtaining "sufficient animal sera to prepare the necessary antibody … that will react with small reptiles, birds, or mammals". The importance of this statement is reinforced by Edman (2, 9) and Tempelis et al. (4) in their papers on host feeding patterns of *Culex* spp. mosquitoes. In Florida and California, respectively, Edman (2) and Tempelis et al. (4), found that *Cx. quinquefasciatus, Cx. nigripalpus* and *Cx. tarsalis* feed almost exclusively from birds and mammals. Less than 1% of blood meals from any of these three major vectors of WNV and SLEV were determined to originate from reptiles or amphibians. Importantly, both Edman (2) and Tempelis et al. (4) used *Iguana iguana* (green iguana) as the source species for producing anti-sera (6, 7), a species that was not breeding on mainland USA at that time (10). In his ultimate paper on the blood-feeding patterns of Florida mosquitoes (9), Edman revealed that *Iguana* antisera "reacted poorly with the blood of local lizards", presumably *Anolis* spp. lizards. *Iguana iguana* was likely chosen for their availability in the pet trade and large size, as the local lizards "were too small to obtain sufficient blood for antibody production". It is therefore unclear to what extent major vectors of zoonotic Flaviviruses feed upon the blood of reptiles, and what implications this may have for the transmission of WNV and SLEV in the US.

Recent research in Lyme disease transmission dynamics has demonstrated that lizards can be consequential organisms for the transmission systems of arthropod-transmitted pathogens. *Borrelia burgdorferi* is a tick-borne pathogen that causes Lyme disease. In the US, Lyme disease is most prevalent in northern states, while rare in the southeastern states, despite the presence of *Borrelia burgdorferi*, and its tick vectors and rodent amplifying hosts. This pattern in the geographic prevalence of Lyme is linked, in part, to the host associations of tick vectors: in the southern US, ticks selectively attach to lizards, which are poor amplifiers of *B. burgdorferi*, leading to fewer bites on competent rodent amplifying hosts, fewer ticks infected with *B. burgdorferi* and thus, reduced incidence of human disease (11). Lizards could exert a similarly consequential effect within the transmission systems of mosquito-vectored pathogens, particularly WNV and SLEV, if the host associations of their primary vectors encompass lizards, and the lizard species that are fed upon are poor amplifiers of these viruses.

Here we re-investigate the host associations of *Culex* (*Culex*) spp. mosquitoes in two southern US states (Florida and Arizona) using polymerase chain reaction (PCR)- and sequencing-based blood meal analysis. We show that lizards are important hosts for several *Culex* spp., including major zoonotic Flavivirus vectors. We investigate the potential effects of location and season on host use and discuss how determining host competence of a few preferred lizard species could shed light on the potential suppressive effect of lizards on arbovirus transmission.

# METHODS

Female mosquitoes for these analyses were collected from numerous localities, using several methods, over a seven-year period (**Table 1** and **Supplemental Table 1**). Our intent was not to intensively sample at any one location, but to gather samples from diverse settings, minimizing biases that occur due to method, habitat, or season. In Florida, mosquitoes were collected from five counties, spanning northern (Alachua, Levy), central (Indian River, Pinellas) and southern (Miami-Dade) portions of the state. In Arizona, mosquitoes were collected in the Sky Islands Region from the three southeasternmost contiguous counties (Cochise, Pima, Santa Cruz), in or near the Huachuca, Pajarito and Santa Rita Mountain Ranges. Mosquitoes were sampled primarily with a large-diameter aspirator (12), resting shelters (13), or carbon dioxide-baited light traps. In Florida, mosquitoes were generally sampled during all seasons, while in Arizona sampling only occurred during summer months (July and August), corresponding with the onset of the North American monsoon.

Collected mosquitoes were killed by exposure to low temperature, either by placing collection chambers containing mosquitoes into -20°C freezers for >10 minutes, or into dry ice-filled coolers. Samples were examined under a stereoscope and blood fed females were visually identified and separated from the sample. Blood fed female mosquitoes were identified morphologically with dichotomous keys (14, 15). To ensure correct identification of the morphologically similar *Cx. stigmatosoma* and *Cx. thriambus*, each specimen morphologically identified as these species was verified using molecular identification following the protocol described in Reeves et al. (16) for sequence-based mosquito identification using the DNA barcoding region of the cytochrome c oxidase subunit I gene (COI). All other *Culex* specimens were identified using morphological characters. After morphological identification of the mosquito species, each blood meal was individually preserved by smearing the contents of the mosquito abdomen onto the surface of a Whatman Flinders Technology Associates (FTA) Classic Card with a sterile pipette tip (17). Extent of digestion was visually estimated (18). The cards were stored at room temperature until molecular processing.

We used the Hot Sodium Hydroxide and Tris (HotSHOT) method (19) to extract DNA from the FTA Card-preserved blood meals, following the protocol described in Reeves et al. (18), with some adjustments to reagent volumes and incubation times. We removed two 1 mm (diameter) round punches from each blood meal using a hole-punch. Both punches were transferred to the same 0.2 ml tube. Thirty μl of lysis buffer were added to each tube, and the samples, with lysis buffer, were incubated in a thermocycler for three hours at 95°C followed by 4°C for five minutes. After incubation, 30 μl of neutralization buffer were added to each tube, and the tubes were stored at -20°C until PCR.

Extracted DNA was used as template in PCRs intended to amplify a fragment of the DNA barcoding region of the vertebrate COI gene. We applied a hierarchical approach to amplification of vertebrate templates using the Reeves et al. (18) primer combinations. Under this approach, extracted DNA from each blood meal was used in an initial PCR using the VertCOI_7194_F and Mod_RepCOI_R primer combination. The PCR products were electrophoresed on a 1.5% agarose gel and visualized under a transilluminator. Reaction products exhibiting an amplicon of the expected size were sent to Eurofins Genomics (Louisville, Kentucky) for sequencing using the Sanger method (20). In cases in which a DNA template failed to produce an amplicon of the expected size, the DNA template was used in a second PCR with the Mod_RepCOI_F and VertCOI_7216_R primer combination. Of these, templates from any failed reaction were used in a third reaction using the Mod_RepCOI_F and Mod_RepCOI_R primer combination. If a template failed to produce an amplicon in all three reactions, no further steps were taken, and the blood meal was not identified. All reactions were performed in a final volume of 20 μl consisting of 10 μl 2.0X Apex Taq RED Master Mix (Genesee Scientific Corp., San Diego, CA), 0.75 μl forward primer (10μM), 0.75 μl reverse primer (10μM), 7.5 μl ultra-pure water, and 1 μl extracted DNA. Thermal cycling conditions for all reactions consisted of 95°C for 3 minutes, followed by 40 cycles of 95°C for 40 seconds, 45°C for 30 seconds, and 72°C for 3 minutes, with a final extension step of 72°C for 7 minutes.

Resulting vertebrate COI sequence chromatograms were examined and edited for quality using the bioinformatic software Geneious Prime Version 11.0.3. Unambiguous stretches of sequences were submitted to the Barcode of Life Datasystem (BOLD) v. 4 Identification Engine for species-level identification by alignment to reference sequences (21).

**TABLE 1** | Summary of locations in Florida and Arizona where *Culex* (*Culex*) mosquitoes were collected.

| State | County | Years | Seasons | Sites (n) | Blood meals (n) | Methods |
|---|---|---|---|---|---|---|
| Florida | Alachua | 2015-2019 | All | 5 | 145 | RS, Asp |
| | Indian River | 2019-2021 | All | 3 | 129 | LT, RS, Asp |
| | Levy | 2019-2021 | Summer | 1 | 5 | LT |
| | Miami-Dade | 2015-2021 | All | 16 | 94 | LT, RS, Asp |
| | Pinellas | 2020 | Fall | 4 | 6 | RS |
| Arizona | Cochise | 2018-2021 | Summer | 4 | 97 | Asp |
| | Pima | 2018-2021 | Summer | 5 | 77 | LT, RS, Asp |
| | Santa Cruz | 2019-2021 | Summer | 3 | 88 | LT, Asp |

*Methods: LT, CO$_2$-baited CDC miniature light trap; RS, Resting shelter; Asp, large-diameter aspirator.*
*See **Supplemental Table 1** for detailed information on each site.*

Sequences that were 98-100% similar to reference sequences were considered conspecific with the species from which the reference sequences were derived. In a few cases, host COI sequences were poor matches (<98% similar) to reference sequences. Of these, nearly all were matched (98-100%) to independently collected reference sequences (18) for *Sylvilagus palustris* (marsh rabbit) or *Anolis carolinensis* (green anole). For *S. palustris*, reference sequences are not yet available on BOLD, while *A. carolinensis* has high levels of intraspecific variation with some geographic structuring (22) and introgression *via* hybridization with introduced *Anolis porcatus* (Cuban green anole) from Cuba (23). Sequences that were 98-100% similar to independently collected *S. palustris* or *A. carolinensis* reference sequences were considered conspecific with the corresponding species. *Odocoileus virginianus* (white-tailed deer) and *Odocoileus hemionus* (mule deer) could not be distinguished molecularly, but all sequences derived from these hosts were assumed to be *O. virginianus* based on geographic distribution and the absence of *O. hemionus* occuring naturally in Florida.

We compiled lists of species of birds, mammals, reptiles, and amphibians recorded from Florida and Arizona to explore patterns of diversity and host use as it relates to availability of vertebrate hosts, the number of species utilized by *Culex* mosquitoes, and status (exotic, domesticated or native) from published sources (24–31). We used chi-square tests of independence to test for differences in the distributions of available hosts and blood meals between location and season. Rank abundance curves (32) were plotted to visualize the relative abundance of host species detected for *Cx. nigripalpus*, *Cx. quinquefasciatus*, *Cx. thriambus*, the three mosquito species for which more than 100 blood meals were successfully identified. Rank abundance curves were plotted using the Vegan (33) and BiodiversityR (34) packages in R.

## RESULTS

A total of 1,166 blood-engorged female *Culex* (*Culex*) mosquitoes were analyzed by PCR, of which, 88.1% produced an identifiable sequence. The overall rate of PCR and sequencing success was high (85.3% on average), but varied by species and location, the lowest being *Culex coronator* from Indian River County (47.1%). *Culex* (*Culex*) spp. mosquitoes in Florida and Arizona were found to feed upon diverse species of birds, reptiles and mammals, but, somewhat surprisingly, not amphibians (**Table 2**). Across *Culex* (*Culex*) species, birds were a major host group in both Florida and Arizona (**Table 2**), constituting 69.2% and 44.2% of total identified blood meals, respectively. Mammals were also important hosts in both states (19.0 and 35.7%, in Florida and Arizona respectively) as were reptiles (11.8 and 20.1%, in Florida and Arizona respectively). The overall distribution of blood meals between the vertebrate classes differed significantly between Arizona and Florida ($X^2 = 109.96$, df=2, $P<0.001$).

When vertebrates are examined according to ecological groupings, songbirds (order Passeriformes) and lizards (suborder Lacertilia) are notable groups that were important to our samples of *Culex* mosquitoes from both Florida and Arizona (**Figure 1**). In both states, songbirds outnumber all other vertebrate groups in terms of species available (species

**TABLE 2** | Class-level host associations of *Culex* (*Culex*) spp. in Florida and Arizona.

| State | County | Mosquito species | Bird N (%) | Mammal N (%) | Reptile N (%) | No result N (%) | Total N |
|---|---|---|---|---|---|---|---|
| Florida | Alachua | *Culex interrogator* | 4 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 4 |
| | | *Culex nigripalpus* | 85 (58.6) | 37 (25.5) | 13 (9.0) | 10 (6.9) | 145 |
| | | *Culex quinquefasciatus* | 367 (80.0) | 63 (13.7) | 6 (1.3) | 23 (5.0) | 459 |
| | | *Culex restuans* | 2 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 |
| | | *Culex coronator* | 1 (6.3) | 7 (43.8) | 0 (0.0) | 8 (50.0) | 16 |
| | Indian River | *Culex declarator* | 6 (54.5) | 1 (9.1) | 1 (9.1) | 3 (27.3) | 11 |
| | | *Culex interrogator* | 1 (50.0) | 1 (50.0) | 0 (0.0) | 0 (0.0) | 2 |
| | | *Culex nigripalpus* | 58 (45.0) | 25 (19.4) | 20 (15.5) | 26 (20.2) | 129 |
| | | *Culex quinquefasciatus* | 6 (75.0) | 0 (0.0) | 0 (0.0) | 2 (25.0) | 8 |
| | | *Culex coronator* | 0 (0.0) | 8 (47.1) | 0 (0.0) | 9 (52.9) | 17 |
| | Levy | *Culex nigripalpus* | 2 (40.0) | 1 (20.0) | 0 (0.0) | 2 (40.0) | 5 |
| | Miami-Dade | *Culex nigripalpus* | 27 (28.7) | 9 (9.6) | 52 (55.3) | 6 (6.4) | 94 |
| | | *Culex coronator* | 1 (33.3) | 2 (66.7) | 0 (0.0) | 0 (0.0) | 3 |
| | Pinellas | *Culex nigripalpus* | 2 (33.3) | 1 (16.7) | 3 (50.0) | 0 (0.0) | 6 |
| | | *Culex quinquefasciatus* | 2 (66.7) | 0 (0.0) | 1 (33.3) | 0 (0.0) | 3 |
| Arizona | Cochise | *Culex erythrothorax* | 1 (7.7) | 10 (76.9) | 0 (0.0) | 2 (15.4) | 13 |
| | | *Culex stigmatosoma* | 2 (40.0) | 0 (0.0) | 3 (60.0) | 0 (0.0) | 5 |
| | | *Culex thriambus* | 28 (35.4) | 0 (0.0) | 40 (50.6) | 11 (13.9) | 79 |
| | Pima | *Culex erythrothorax* | 0 (0) | 2 (100.0) | 0 (0.0) | 0 (0.0) | 2 |
| | | *Culex stigmatosoma* | 3 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3 |
| | | *Culex tarsalis* | 2 (22.2) | 0 (0.0) | 4 (44.4) | 3 (33.3) | 9 |
| | | *Culex thriambus* | 19 (30.2) | 1 (1.6) | 29 (46.0) | 14 (22.2) | 63 |
| | Santa Cruz | *Culex stigmatosoma* | 6 (54.5) | 0 (0.0) | 2 (18.2) | 3 (27.3) | 11 |
| | | *Culex tarsalis* | 1 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 |
| | | *Culex thriambus* | 48 (63.2) | 0 (0.0) | 11 (14.5) | 17 (22.4) | 76 |
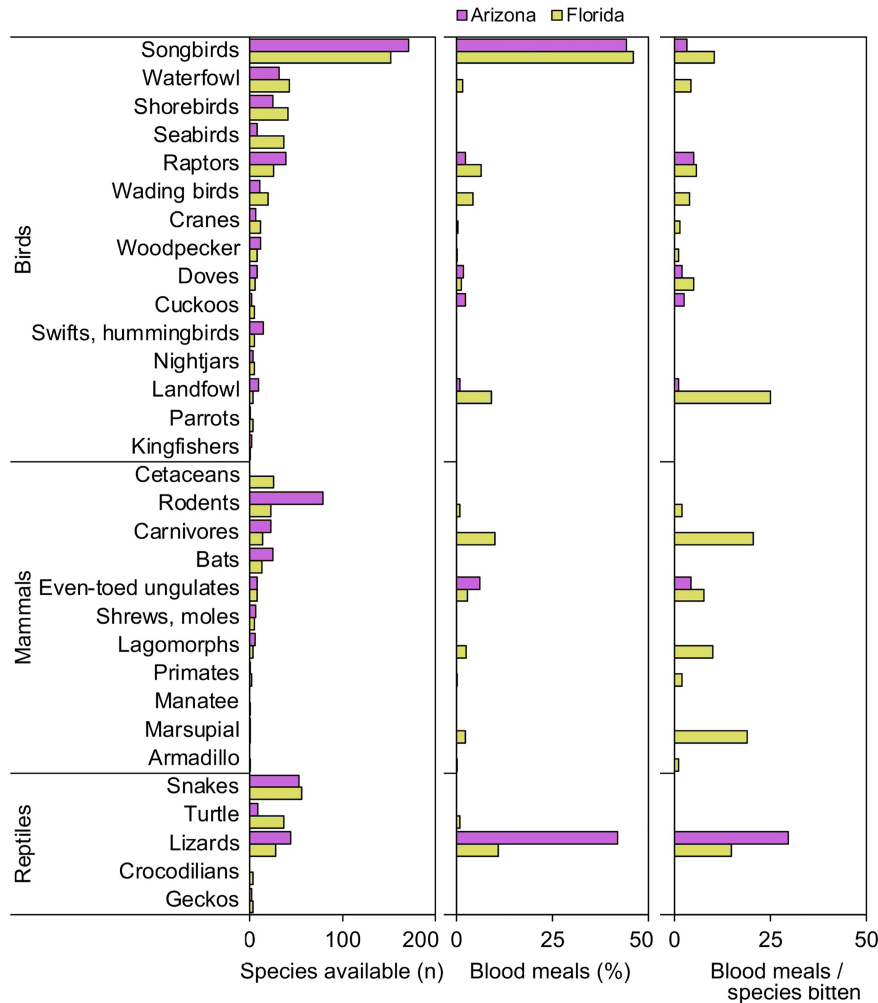
**FIGURE 1** | Availability and host use of vertebrate groups for *Culex* mosquitoes in Arizona and Florida, USA. Numbers of host species available in each ecological group were derived from published sources. Blood meal numbers were combined for 4-6 *Culex* (*Culex*) spp. in each state. Vertebrate host species were determined by PCR and Sanger sequencing of field-collected blood-engorged females. Data were combined from three counties (Arizona) and five counties (Florida).

richness) and fraction of total blood meals (**Figure 1**). However, the number of blood meals per songbird species bitten is relatively low, a function of blood meals being spread over a large number of species. Lizards, while not the most species rich group of reptiles in either state (**Figure 1**), contributed relatively large fractions of total blood meals in Arizona (41.9%) and Florida (10.9%) across *Culex* (*Culex*) species. The numbers of blood meals per lizard species bitten were relatively high, a function of blood meals being focused on a relatively small number of species (**Figure 1**). Other vertebrate groups for which the number of blood meals per species bitten was relatively high included landfowl (mainly domestic chicken), carnivores (mainly raccoon), and marsupials (North American opossum).

In Florida, class-level host use varied between *Culex* species, location and, in some cases, season. *Culex coronator* and *Culex interrogator* fed exclusively upon birds and mammals (**Table 2**).

*Culex restuans* blood meals were from birds, though sample size was small (n=2). *Culex declarator, Cx. nigripalpus* and *Cx. quinquefasciatus* fed upon birds, mammals, and reptiles to varying degrees (**Table 2**). Class-level host use of *Cx. nigripalpus* varied by season and location. The proportion of *Cx. nigripalpus* blood meals from reptiles generally increased from north to south in Florida (**Figure 2**), which corresponded with decreasing use of avian and mammalian hosts with decreasing latitude. Reptiles constituted just 9.0% (n=13/145) of total *Cx. nigripalpus* blood meals in northern Florida (Alachua County), for example, but 55.3% (n=52/94) in southern Florida (Miami-Dade County). In Alachua, Indian River and Miami-Dade Counties, *Cx. nigripalpus* fed predominantly on birds in winter and spring (**Figure 3**), but predominantly fed upon other host classes in other seasons. The distribution of *Cx. nigripalpus* blood meals between the vertebrate classes differed significantly between fall and winter in Alachua County ($X^2$ = 7.92, df=2,
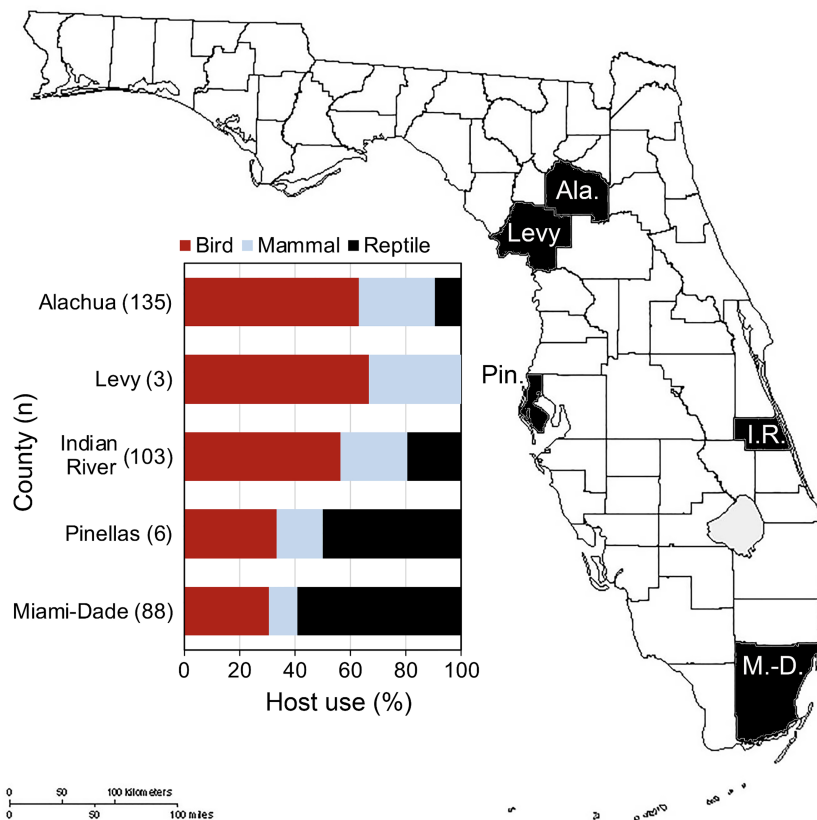
**FIGURE 2** | Class-level host use of *Culex nigripalpus* in Florida. Vertebrate host species were determined by PCR and Sanger sequencing of field-collected blood-engorged females from five counties.

$P$=0.019), in Indian River County and in Florida, across all three counties ($X^2$ = 12.09, df=2, $P$=0.002), and between spring and summer ($X^2$ = 14.25, df=2, $P$<0.001) and summer and fall ($X^2$ = 17.34, df=2, $P$<0.001) in Miami-Dade County. *Culex quinquefasciatus* fed predominantly upon birds in Alachua, Indian River and Pinellas Counties, constituting 66.7 – 80% of all blood meals from these counties (**Table 2**). No seasonal shifts in host use were observed for *Cx. quinquefasciatus* however the distribution of class-level host use differed significantly between spring and summer ($X^2$ = 10.57, df=2, $P$=0.005) and winter and spring ($X^2$ = 8.73, df=2, $P$=0.013) in Alachua County (**Figure 3**). Relatively small sample sizes for some species in Florida (2 *Cx. restuans*, 11 *Cx. declarator*, 6 *Cx. interrogator*) likely affect the probability of detecting blood meals from other vertebrate classes and limit the ability to draw conclusions about the host associations of these species.

In Arizona, class-level host use did not vary significantly between counties for mosquito species, with the exception of *Cx. thriambus* ($X^2$ = 28.98, df=4, $P$<0.001), which took more total blood meals from reptiles in Cochise County (50.6%) and Pima County (46.0%) than in Santa Cruz County (14.5%). *Culex thriambus* took a substantial fraction of blood meals from birds in all three counties, ranging from 30.2 to 63.2% of total

blood meals (**Table 2**), while mammals contributed <2% of blood meals. *Culex tarsalis* and *Cx. stigmatosoma* were only found to feed upon birds and reptiles (**Table 2**), although sample sizes for these species were relatively low (19 *Cx. stigmatosoma*, 10 *Cx. tarsalis*). *Culex erythrothorax* fed mainly upon mammals (12 of 13 identified blood meals), with a single avian blood meal detected (**Table 1**).

Rank abundance curves of the host species identified from *Cx. nigripalpus*, *Cx. quinquefasciatus*, and *Cx. thriambus*, the three species for which sample size of identified blood meals was >100, indicated that these mosquitoes have strong hosts associations: a small number of host species contributed a majority of blood meals while a large number of host species were bitten occasionally (**Figure 4**). For all three *Culex* species, a single host species dominated the blood meal set and was fed upon to a greater extent than all others (1.8 – 4.6 times more frequently than the second most frequently fed upon host species). For *Cx. nigripalpus* and *Cx. thriambus*, species of diurnal, tree-roosting lizards (*Anolis sagrei*, brown anole, and *Sceloporus clarkii*, Clark's spiny lizard, respectively) were the most frequently detected hosts, constituting 1.8 – 2.3 times more blood meals than the second most frequently detected host (birds in both cases) for each mosquito species. The three most frequently fed upon hosts
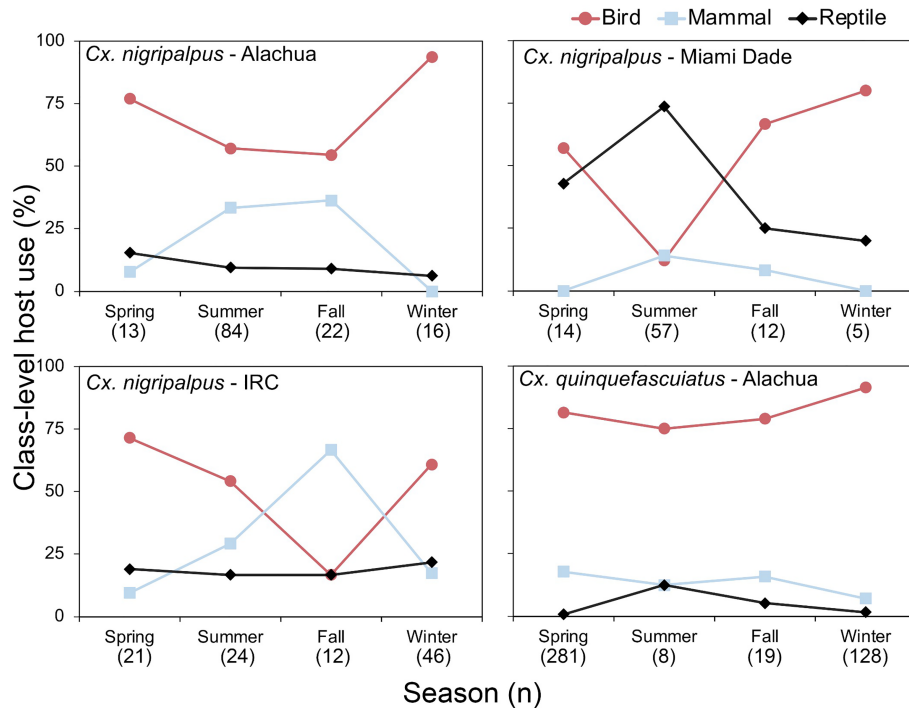
**FIGURE 3** | Seasonal patterns of class-level host use of zoonotic Flavivirus vector mosquitoes in Florida. Vertebrate host species were determined by PCR and Sanger sequencing of field-collected blood-engorged females of *Culex nigripalpus* and *Culex quinquefasciatus*. Season dates correspond to astronomical calendar.

for *Cx. nigripalpus* were a lizard (*Anolis sagrei*, 60 detections), *Gallus gallus* (domestic chicken, 34 detections), and *Procyon lotor* (raccoon, 20 detections). For *Cx. thriambus*, lizard species were the first (*Sceloporus clarkii*, 56 detections) and third (*Urosaurus ornatus*, ornate tree lizard, 21 detections) most frequently detected species, while a songbird (*Thryomanes bewickii*, Bewick's wren, 24 detections) was the second most frequently bitten host. Lizards were not among the most frequently bitten hosts for *Cx. quinquefasciatus*, and out of 445 identified blood meals, only three were derived from lizards (two from *A. carolinensis*, one from *A. sagrei*). *Cardinalis cardinalis* (northern cardinal), was by far the most frequently detected host species for *Cx. quinquefasciatus* (167 detections), followed by *G. gallus* and *P. lotor* (36 and 25 detections, respectively).

Exotic (nonnative and domesticated) animals contributed a substantial fraction of blood meals, particularly in Florida (**Figure 5**). Compared to the relative number of exotic species available, the fraction of blood meals from exotic birds and reptiles was greater than from the native species of their respective classes (**Figure 5A**). Exotic reptiles, in particular, constituted 75% of the total reptile blood meals in Florida, but make up 39% of available reptile species (**Figure 5A**; $X^2$ = 37.91, df=1, *P*<0.001). *Anolis sagrei* was the most commonly bitten reptile, with 64.6% (n=62/96) of reptile blood meals arising from this nonnative lizard. Other nonnative reptiles bitten (three or less blood meals) include *Anolis equestris* (knight anole), *I. iguana*, *Basiliscus vittatus* (brown basilisk), *Anolis cristatellus*

(Puerto Rican crested anole), and *Python bivittatus* (Burmese python). Domestic birds (mainly chickens) and domestic mammals (mainly house cat) contributed a relatively large proportion of blood meals in Florida, 15.4% and 26.9% of avian and mammalian blood meals, respectively. In contrast, domestic mammals (cow) contributed a relatively large percentage of blood meals derived from mammals (77.0%), whereas few blood meals were detected from exotic birds (0.9%) or reptiles (0%) in Arizona (**Figure 5B**).

## DISCUSSION

The class-level patterns of host use of some *Culex* (*Culex*) spp. mosquitoes observed in this study are contradictory to findings of previous studies of their host associations. While birds and mammals are acknowledged as important host groups for well-studied vector species such as *Cx. quinquefasciatus, Cx. nigripalpus* and *Cx. tarsalis*, few studies have documented significant utilization of reptilian hosts by these same species (**Table 2**). The potential reasons for this disparity include inadequacy of assays (serological or molecular) to recognize or amplify reptile blood proteins or DNA targets, respectively (9), geographical variation in the US in reptile diversity and abundance, and/or shifts in host association due to recent introductions, establishments and geographic expansions of reptile species that colonized the US in recent decades. On
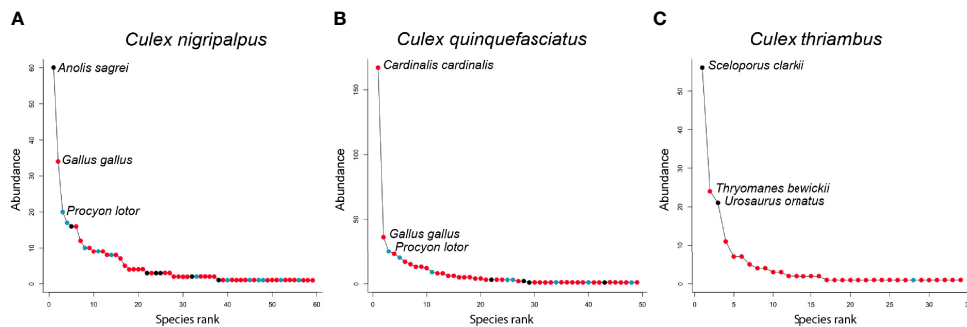
**FIGURE 4** | Rank abundance curves of host species detected by *Culex nigripalpus* **(A)**, *Culex quinquefasciatus* **(B)** and *Culex thriambus* **(C)**. Points on each curve indicate the abundance of a host species among the blood meal sample for each species, with coloring indicating vertebrate class of the host species: Aves (red), Mammalia (blue), Reptilia (black). Note that the y-axis scale differs between panels.

average, studies that used PCR and sequencing-based blood meal analysis (including this study) found much higher reptile host use (20.0%) by *Cx. nigripalpus* than studies which employed serological methods (0.5%). This suggests that methodological limitations contribute to the disparity. Our own blood meal results (**Figure 3**) suggest that latitude and its inverse relationship with reptile diversity and abundance (35) may also contribute to the high level of reptile-biting observed in our own study, compared to most prior studies of subgenus *Culex* host use, which have largely focused on more northerly regions of the continental US. Interestingly, in Puerto Rico half (five of 10) of *Cx. nigripalpus* blood meals were from reptiles (36). Similarly, reptiles constituted a surprisingly large proportion of blood meals in Arizona (**Table 1**). *Culex thriambus*, for example, took 36.7% of 218 total blood meals from reptiles in southern Arizona. Previous studies of host use by this species found 96-100% of blood meals were derived from birds (37, 38) in two California counties. Similarly, *Cx. tarsalis*, an important arbovirus vector species, for which numerous blood meal studies have been published, took 40% (four of 10 blood meals) from reptiles in Arizona, while prior studies had reported between 1 and 5% of blood meals from reptiles (39–43). Fewer studies have been published on the host associations of *Cx. stigmatosoma*, but in California, McPhatter et al. (44) reported that 98.6%, 1.2% and 0.2% of 647 blood meals were derived from birds, mammals, and reptiles (*Sceloporus occidentalis*), respectively.

Our findings that lizards were one of the two most bitten host groups, in terms of percentage of total blood meals and blood meals per available species (**Figure 1**), indicates that lizards are important hosts for some vector mosquitoes, relative to other reptiles, and even most other vertebrate groups. The finding that the blood meals were focused on a relatively small number of lizard species indicates that a few species of lizard (*A. sagrei* in Florida, *S. clarkii* and *U. ornatus* in Arizona) have a disproportionately strong influence on host use patterns at locations where these species occur (**Figure 1**). For songbirds, however, blood meals were distributed across a large number of species (**Figure 1**), although *C. cardinalis* was an especially

important host to *Cx. quinquefasciatus* (**Figure 4**). Therefore, determining host competence of songbirds in a region requires evaluating many species in laboratory experiments, sensu Komar et al. (45). For reptiles however, understanding the host competence of these three lizard species (*A. sagrei, S. clarkii, U. ornatus*) would provide valuable information on their role in amplifying or diluting arboviruses in nature and could help explain geographical patterns of zoonotic Flavivirus prevalence in North America, i.e., why incidence of WNV and SLEV have been historically lower in tropical regions than in temperate regions. As discussed above, Lyme disease prevalence is higher in northern states than in southern states, due in part to tick vectors selectively attaching to lizards in the southern US (11). Lizards are poor amplifiers of *B. burgdorferi*, leading to fewer bites on competent rodent amplifying hosts. If lizards are poor amplifiers of WNV and SLEV, they should have a similar suppressive impact on arbovirus transmission.

In Florida and Arizona, blood meals from lizards were derived from only a subset of the available lizard species in both states. In Florida (**Figure 6**), *Culex* mosquitoes fed from *A. sagrei, A. carolinensis, A. equestris, A. cristatellus, B. vittatus,* and *I. iguana*, while in Arizona, they fed only from *S. clarkii, Sceloporus cowlesii* (southwestern fence lizard), and *U. ornatus*. These lizards are all diurnal and, to varying extents, arboreal, associated with trees or vegetation (25, 27). Conversely, diurnal lizard species that are abundant but associated with the ground, rocks, or crevices were absent from our blood meal samples. In both states, there are abundant and relatively diverse terrestrial lizard communities that include *Agama agama* (African rock agama), *Leiocephalus carinatus* (northern curly-tailed lizard), *Sceloporus undulatus* (eastern fence lizard), *Aspidoscelis sexlineata* (six-lined racerunner) and various species of *Eumeces* skinks in Florida, and various species of *Aspidoscelis* whiptails, *Phrynosoma* horned lizards, and other *Sceloporus* species, among many others, in Arizona (25, 27). Similarly, blood meals derived from nocturnally active lizards (e.g., *Heloderma suspectum, Coleonyx* banded geckos, *Hemidactylus* house geckos) were not found. A possible explanation for the assemblage of lizard species detected in *Culex* blood meals, and
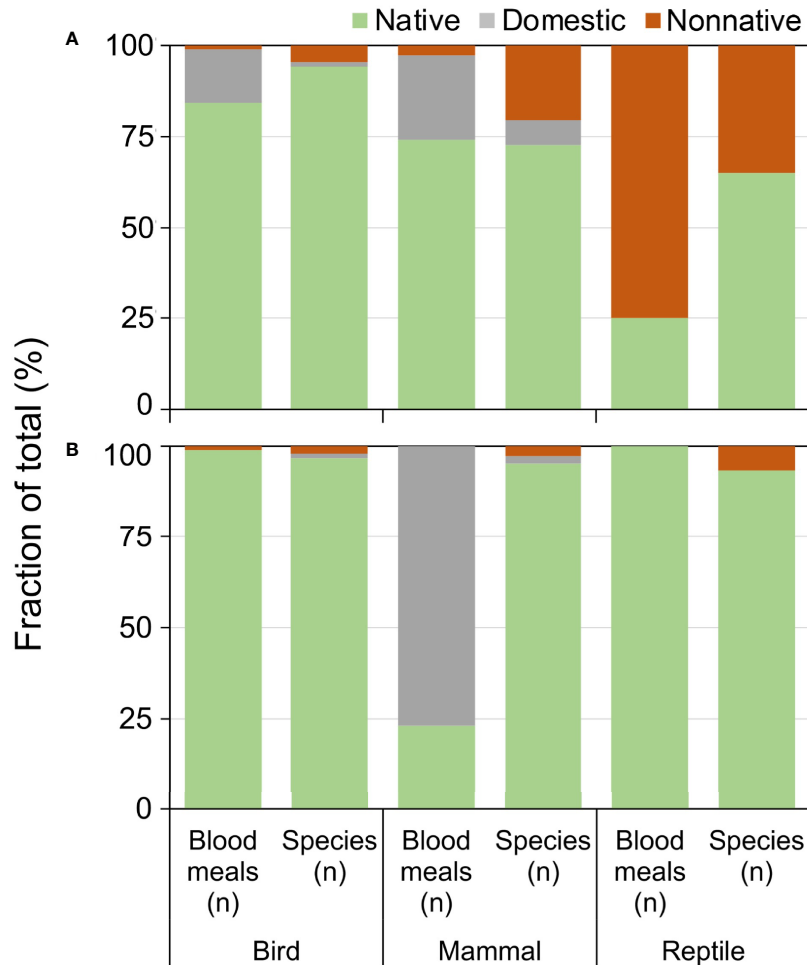
**FIGURE 5** | Relationship between availability and host use of exotic and native vertebrate classes for *Culex* (*Culex*) spp. mosquitoes collected in Florida **(A)** and Arizona **(B)**. Numbers of host species available in each class were derived from published sources. Blood meal numbers were combined for 4-6 *Culex* (*Culex*) spp. in each state. Vertebrate host blood meal species were determined by PCR and Sanger sequencing of field-collected blood-engorged females. Data were combined from three counties (Arizona) and 5 counties (Florida).

the absence of ground-associated and nocturnal species, is that nocturnally active, host-seeking *Culex* females forage in trees or vegetation, where they encounter and feed from lizard species that roost on exposed branches or leaves, while they do not encounter those that rest underground or in crevices, or those that are active by night.

Seasonal shifts in bird-to-mammal host use observed for *Cx. nigripalpus* in Indian River County, Florida (**Figure 3**) confirms findings of Edman and Taylor (46). Interestingly, this same vector species shifted seasonally between birds and reptiles in Miami-Dade County (**Figure 3**), suggesting that class-level host shifts are labile within a given species. Edman and Taylor (46) postulated that *Cx. nigripalpus* bird-to-mammal host shifts were driven by seasonal weather-driven changes in foraging behavior of the mosquitoes, i.e., that females forage in open fields in the late summer and fall, where they are more likely to encounter mammals. Our prior work with *Culiseta melanura* indicated that

seasonal bird-to-reptile host shifts were due to weather-induced changes in host behavior, in that *Anolis* lizards were more likely to sleep on exposed surfaces such as vegetation or terminal branches of trees or shrubs when temperatures were higher (47), which is in agreement with the summertime peak of lizard biting in Miami-Dade County observed here (**Figure 3**). The county-level differences in class-level host use observed for several species is not unexpected, as several studies have reported these differences. For example, Thiemann et al. (42) found that avian host use by *Cx. tarsalis* varied from 51.5 to 96.0% between four California counties.

Our results demonstrate that introductions of vertebrates outside of their native distributions can affect the species-level host associations of mosquitoes, and may even affect the class-level host associations when a favored exotic species establishes in a new area. The disproportionately high fraction of blood meals derived from exotic vertebrates in Florida is reflective of
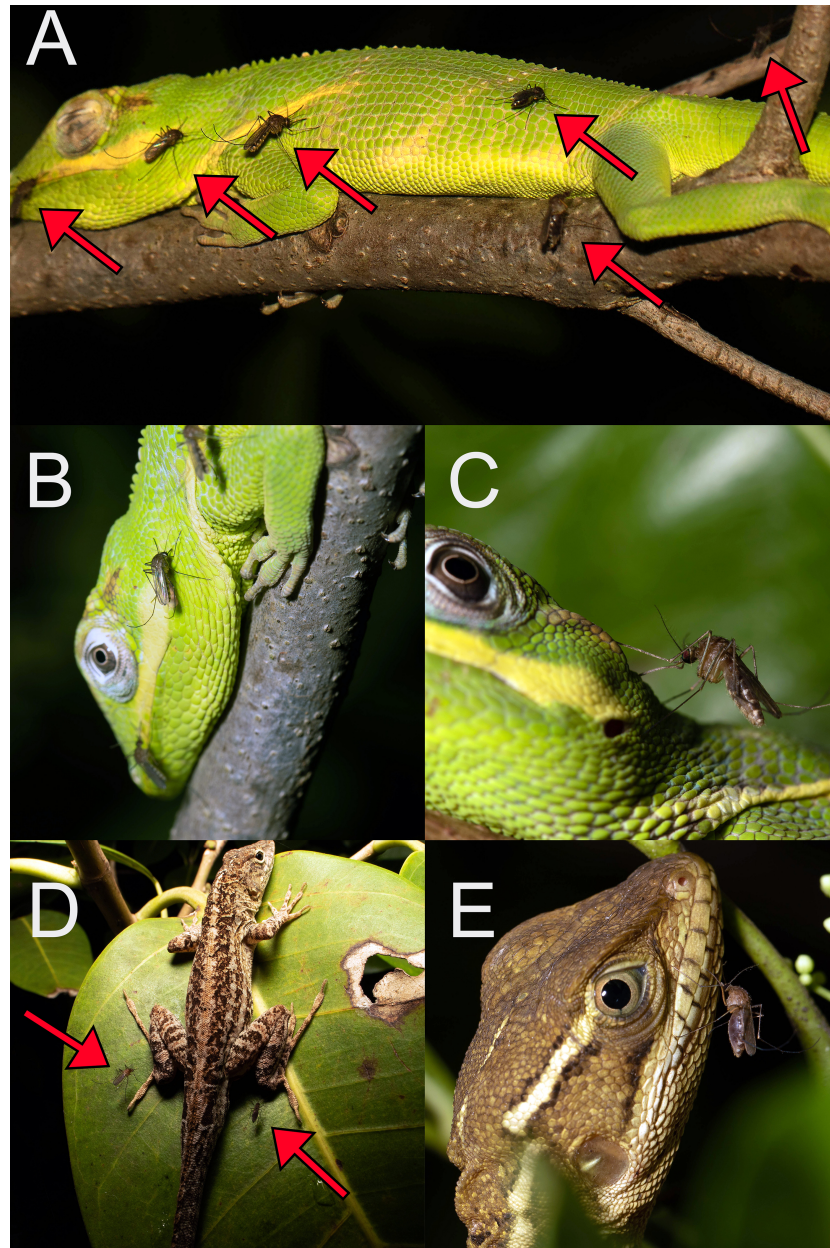
**FIGURE 6** | Adult female *Culex nigripalpus* mosquitoes blood feeding from non-native, diurnal lizards in Miami-Dade County, Florida, USA: *Anolis equestris* **(A, B)**, 11 October 2015; *Anolis equestris* **(C)**, 10 October 2015; *Anolis sagrei* **(D)**, 9 July 2016; *Basiliscus vittatus* **(E)**, 11 October 2015. Red arrows indicate position of *Culex nigripalpus* females on or near lizards.

the large number of invasive species in the state (48). The very large fraction of reptile blood meals from nonnative reptile species (75%) is due primarily to blood meals from *A. sagrei*. *Anolis sagrei* was the most important host in our sample of *Cx. nigripalpus* blood meals, and was fed upon nearly twice as often as the next most abundant host (**Figure 4**). This small lizard, native to Caribbean islands, has been in Florida since the 1880s, when they were first reported in the lower Florida Keys (49). By 2002, *A. sagrei* had been recorded from every Florida county,

across a largely contiguous distribution (50) and has become one of the most abundant lizards in the state. Previous work, based on blood meals collected in the 1960s (7, 46) at a time when *A. sagrei* was still rare in Florida and had not yet become widespread and abundant in the state (25), forms the basis of our current understanding of the host associations of *Cx. nigripalpus* in the state. The timeline of invasion and dispersal of this preferred reptile species could explain the increase in reptilian host use by species of *Culex* (this study) and *Culiseta*

(51) observed in Florida over the past 40-50 years. Not only has the colonization of Florida by *A. sagrei* provided mosquitoes with an omnipresent new vertebrate host, but the presence of this anole impacts the behavior of the native *A. carolinensis*, pushing them to higher perches within vegetation (52), which may affect their susceptibility to mosquito feeding.

The lizard species fed upon by *Culex* mosquitoes in Arizona were all native species. The relatively lower importance of exotic host species, overall, in Arizona is representative of the low numbers of invasive vertebrates in that state, compared to Florida. While the changing vertebrate host community of Florida with the colonization of the state by nonnative lizards may, in part, explain the incongruence of host associations between previous studies and this study, in Arizona, *Culex* host associations have not been thoroughly assessed. Blood meal data are available only for *Cx. quinquefasciatus* (n=143) and *Cx. tarsalis* (n=3) collected in residential and metropolitan areas around Phoenix, Maricopa Co. and Tucson, Pima Co. (53, 54). For *Cx. quinquefasciatus*, the results of these studies paralleled our results for Florida, with most blood meals derived from birds and, to a lesser extent, mammals, and less than 1% derived from lizards (*U. ornatus*), while all three *Cx. tarsalis* had fed from birds. Our sampling efforts for blood fed *Culex* in Arizona were exclusively focused on undeveloped areas of largely natural habitat in the Sky Islands Region, which, coupled with the paucity of information on *Culex* host associations in Arizona, may contribute to the novel finding that *Cx. thriambus*, *Cx. stigmatosoma*, and *Cx. tarsalis* feed from tree-associated lizards in these areas. Distribution and abundance patterns of diurnal and arboreal lizard species could further explain the high levels of lizard feeding we found across multiple sites in southern Arizona. *Sceloporus clarkii* has a limited distribution in the southwestern US (55), and *U. ornatus*, although more widespread, is largely absent from California (56), where much of the host association work has been focused for *Cx. tarsalis*, *Cx. thriambus*, and *Cx. stigmatosoma* (4, 37–40, 42, 44).

We found that host association varied among the *Culex* species we assessed, and not all *Culex* species fed from lizards to the same extent. For *Cx. nigripalpus*, *Cx. stigmatosoma*, *Cx. tarsalis*, and *Cx. thriambus*, lizards made up a large proportion (24–57%) of the identified blood meals, and diurnal/arboreal lizard species were the most important hosts for *Cx. nigripalpus* and *Cx. thriambus*. Lizard-derived blood meals were not found in *Cx. interrogator*, *Cx. restuans*, *Cx. coronator*, and *Cx. erythrothorax*, though for all, sample size was relatively small. While the former two species fed predominantly from birds, *Cx. coronator* and *Cx. erythrothorax* fed almost exclusively from mammals, findings that are in line with the results of previous studies (57, 58). Together, this suggests that *Cx. coronator* and *Cx. erythrothorax* are largely associated with mammalian hosts, with occasional use of avian hosts. For *Cx. quinquefasciatus*, an important Flavivirus vector in the US and elsewhere, our sample included 445 identified blood meals, derived almost entirely from birds, particularly *C. cardinalis*, and mammals. The majority of this sample (434/445 blood meals) was collected at the same site in Alachua Co., Florida where 13/139 (9%) *Cx. nigripalpus* blood

meals were derived from lizards. At this site, only 3/434 (0.7%) identified *Cx. quinquefasciatus* blood meals were derived from lizards, suggesting that *Cx. quinquefasciatus* does not feed from lizards to the same extent as *Cx. nigripalpus*. This discrepancy could be due to innate preferences for certain host classes over others, to differences in foraging strategies or foraging times, or to differences in the importance of host-seeking cues between these species.

## CONCLUSIONS

Recognizing the potential for nonnative vertebrate hosts to impact mosquito host associations and vector-borne disease transmission is important, as climate change is likely to increasingly facilitate establishments of nonnative species (59). Shift in vector distribution is typically considered the major implication of climate change for vector-borne disease [e.g., (60)]. However, as exotic mosquito vectors can change the transmission dynamics and public health risks of mosquito-vectored pathogens, so, too, can introductions of nonindigenous vertebrate hosts (61). Since Edman's work on *Culex* host associations in the 1960s, Florida's vertebrate community has transformed. Notably, there have been numerous establishments of nonindigenous lizard and other reptile species, to the extent that Florida ranks first in the world in number of nonnative reptile species (28). We found that in Florida many of these nonnative species, the diurnal and arboreal lizards, are important hosts for *Cx. nigripalpus*, but not for *Cx. quinquefasciatus*, both primary vectors of WNV and SLEV. In southern Arizona, an unexpectedly large proportion of *Cx. thriambus*, *Cx. tarsalis*, and *Cx. stigmatosoma* blood meals were derived from native lizard species, possibly due to the availability of these hosts in the ecosystems of our study sites. If lizards are found to be dilution hosts for WNV and SLEV, it may be possible to encourage or support populations of native diurnal/arboreal lizards to serve as dilution effect biological controls, particularly in metropolitan areas, with the intent of dampening circulation of these Flaviviruses among competent hosts.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

LER and NDB-C designed studies, performed fieldwork. LER performed molecular analysis. NDB-C and LER performed statistical analysis. Both authors drafted the manuscript and approved the final 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/fitd.2022.842523/full#supplementary-material

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