View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by K-State Research Exchange

First Report of Phlebotomine Sand Flies (Diptera: Psychodidae) in Kansas and Missouri, and a PCR Method to Distinguish *Lutzomyia shannoni* From *Lutzomyia vexator*

JU-LIN WENG,[1] SAMANTHA L. YOUNG,[2] DAVID M. GORDON,[2] DAVID CLABORN,[3] CHRISTINE PETERSEN,[4] AND MARCELO RAMALHO-ORTIGAO[1,5]

J. Med. Entomol. 49(6): 1460–1465 (2012); DOI: http://dx.doi.org/10.1603/ME12105

ABSTRACT Sand flies *Lutzomyia* (Psathyromyia) *shannoni* (Dyar) and *Lu.* (Helcocyrtomyia) *vexator* (Coquillet) were collected for the first time in southwest Missouri and southeast Kansas, expanding the known range of these species in North America. Altogether, 680 sand flies (356 males and 324 females) were collected during trapping from May through October 2011 and identified using morphological characters. Of the total sand flies collected, 315 were identified as *Lu. shannoni*, with 181 individuals (or 26.6% of all sand flies) trapped in Missouri and 134 individuals (or 19.7%) trapped in Kansas. Whereas 358 *Lu. vexator* were identified from southwest Missouri, only a single specimen was trapped in southeast Kansas. One male *Lu. vexator* with asymmetric gonostyli was trapped in Missouri. We also developed a polymerase chain reaction protocol to consistently and accurately distinguish *Lu. shannoni* from *Lu. vexator* based on presence or absence of a 416 bp fragment from the cytochrome oxidase c subunit 1 gene.

KEY WORDS sand fly, *Lutzomyia shannoni*, *Lutzomyia vexator*

Phlebotomine sand flies are blood-feeding dipterans known for their role as vectors for *Leishmania,* certain phleboviruses and bacteria. Leishmaniasis is prevalent in over 88 countries with 1.5–2 million cases of cutaneous leishmaniasis (CL) and 500 thousand cases of visceral leishmaniasis (VL) per year (World Health Organization [WHO] 2010). Sand flies of the genus *Lutzomyia* are present in the New World, with 14 species considered native to North America (Young and Perkins 1984). Among these, *Lu.* (*Psathyromyia*) *shannoni* (Dyar) and *Lu.* (*Helcocyrtomyia*) *vexator* (Coquillet) are frequently reported in sand fly surveys conducted in the eastern United States (Price et al. 2011).

In North America *Lu. shannoni* is commonly associated with hardwood forest habitats, and recent reports have pointed to an expansion of its historical range. Currently, reports on the presence of *Lu. shannoni* include 14 U.S. states (Alabama, Arkansas, Delaware, Florida, Georgia, Louisiana, Maryland, Mississippi, North Carolina, South Carolina, New Jersey, Kentucky, Ohio, Tennessee, and Texas) (Young and Perkins 1984, Comer et al. 1990, Haddow et al. 2008, Claborn et al. 2009, Minter et al. 2009, Price et al. 2011).

Lu. vexator is commonly found in sympatry with *Lu. shannoni* and has been reported in 21 states within the United States, including eastern and western portions of the country (Young and Perkins 1984, Ostfeld et al. 2004, Haddow et al. 2008, Minter et al. 2009). *Lu. shannoni* is of particular interest because of its role as a vector of vesicular stomatitis virus (Comer et al. 1990) and as a potential vector of leishmaniasis, including VL (Ferro et al. 1998, Travi et al. 2002). Cases of canine VL were previously identified in foxhounds in Missouri and Kansas (Duprey et al. 2006, Petersen and Barr 2009), without any previous reports of sand flies.

Here, we report for the first time the presence of sand flies (*Lu. shannoni* and *Lu. vexator*) in Kansas and Missouri based on trapping in one location in Kansas and two in Missouri from May to October 2011. We also describe a polymerase chain reaction (PCR) protocol to molecularly distinguish *Lu. shannoni* from *Lu. vexator,* and report on a *Lu. vexator* specimen displaying asymmetric gonostyli.

Materials and Methods

Sand Fly Trapping and Sites. Sand flies were trapped from dusk to dawn using CO_2-baited Centers for Disease Control and Prevention (CDC) miniature light traps (model #512, John W. Hock). Sand flies were microscopically identified using the morpholog-

[1] Department of Entomology, Kansas State University, 123 Waters Hall, Manhattan, KS 66506.
[2] Department of Biology, Pittsburg State University, 1701 S Broadway, Pittsburg, KS 66762.
[3] Master of Public Health Program and Center for Homeland Security, Missouri State University, 901 South National Avenue, Springfield, MO 65897.
[4] Department of Veterinary Pathology, Iowa State University, 2714 Veterinary Medicine, Ames, IA 50011.
[5] Corresponding author, e-mail: mortigao@ksu.edu.

0022-2585/12/1460–1465$04.00/0 © 2012 Entomological Society of America

ical characteristics of external genitalia for males and of the spermatheca for females (Young and Perkins 1984, Young and Duncan 1994).

Trapping locations were in southeastern Kansas and southwestern Missouri. Wilderness Park (WP; 37° 27′14″ N 94° 42′50″ W), on the northern boundary of Pittsburg, KS (population density of 611/km^2 within 32.4 km^2; U.S. Census Bureau 2012), is a roughly 0.4 km^2 public access area on an unreclaimed coal strip mine that has become an oak-hickory forest containing numerous water bodies formed in strip pits and a creek running from the north to the south. It is surrounded by forested land, residential development and farmland. In Missouri, two locations were sampled: Springfield with a population density of 754/km^2 within an area of 212 km^2 (U.S. Census Bureau 2012), and the Bull Shoals Field Station (BSFS; 36° 34′ N, 93° 4′ W), a preserve located in the Drury–Mincy Conservation Area southeast of Branson, MO. Springfield was further subdivided into three focal sites located on the outskirts of town, with Focal Site 1 located in an area of hardwood and hickory adjacent to a pasture for a horse farm (37° 6′33″ N, 93° 19′12″ W); Focal Site 2 was a wood and pasture habitat in a residential area (37° 6′24″ N, 93° 17′52″ W); and Focal Site 3 was borderline between a secondary forest patch and a surrounding pasture (37° 5′54″ N, 93° 19′51″ W). Distances between focal sites ranged from 1.5 to 3.0 km.

The number of light traps set at each location varied because of logistics and environmental factors. At WP, as few as three and as many as 11 light traps were placed when sampling the location. For the focal sites in Springfield the number of light traps used varied from three to eight traps per night at the Focal Site 1, one to three traps per night for Focal Sites 2 and 3. A single trapping event took place in site 3, on 12 June. At the BSFS location, between 7 and 11 light traps were used per night. Generally, the distance between traps placed at any given location or focal site (in the case of Springfield) ranged from 10 to 100 m. The distances between the three locations used for this study were: ≈138 km between WP (Kansas) and any of the three focal sites in Springfield (Missouri); 192 km between WP and the BSFS (Missouri); and 65 km between Springfield and the BSFS (http://www.gpsvisualizer.com/calculators#distance_address).

Effect of Weather on Flight Activity. Because of variations in trapping frequency and trap density among the three locations, data were pooled and the mean number of sand flies trapped within a 2-wk period (referred to as trapping periods below) was analyzed (the total number of sand flies trapped at any given location divided by the total number of traps used during a 2-wk period). Trapping results from the three focal sites in Springfield were pooled and treated as a single location.

The effects of weather on sand fly activity at WP were assessed by performing linear regressions of the proportion of sand flies per trap captured during each 2-wk interval against 1) the average daily temperature for those 2-wk intervals, 2) the average daily humidity for those 2-wk intervals, and 3) total precipitation for those 2-wk intervals. Weather data were obtained from the weather station KKSFRONT2 located in Frontenac, KS, and 1.21 km (or 0.75 miles) from WP (http://www.wunderground.com/weatherstation/WXDailyHistory.asp?ID = KKSFRONT2&day = 4&year = 2011&month = 6&graphspan = year). Linear regressions were performed using R programming for Statistical Language v2.14 (http://www.r-project.org).

Sex Ratios. We assessed the sex ratios using the binomial tests (R programming for Statistical Language v2.14) for sand flies trapped during each 2-wk period for sample sizes greater than five individuals.

DNA Extraction and PCR. Genomic DNA from 47 *Lu. shannoni* and 23 *Lu. vexator* females was extracted using 10% Chelex 100 resin beads (Bio-Rad Laboratories, Hercules, CA). Sand flies were homogenized individually in 20 μl of molecular grade water, heated in 120 μl of Chelex 10% solution at 95°C for 30 min, centrifuged briefly (6 s) at 4,000 $\times$ g, and the supernatant transferred to a new tube. DNA extraction was confirmed by amplification of a 285 bp fragment of the internal transcribed spacer region 2 (ITS-2) using GoTaq Colorless Master Mix (Promega, Madison, WI), 2 μM each forward ACTGCATGGACCACGTATGG and reverse CACATATGAGTTGAGATCGC primers, in 10 μl reaction. ITS2 PCR conditions were: 94°C for 2 min, two cycles of 94°C for 30 s and 72°C for 45 s, two cycles of 94°C for 30 s and 68°C for 45 s, 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s; with final extension at 72°C for 10 min.

A *Lu. shannoni*-specific PCR was developed to amplify a 416 bp fragment of the mitochondrial cytochrome oxydase c subunit 1 (CO1) in GeneBank. Forward and reverse primers were ATTTGGAAATTGATTGGTCC and TAAAAGTATGGTAATTGCAC, and PCR conditions done as indicated above were set at 94°C 2 min, 30 cycles of 94°C 1 min, 54°C for 1 min, 72°C for 1 min, with a final extension of 72°C for 10 min. PCR products were visualized following electrophoresis on ethidium bromide-stained 1.2% agarose gel. To verify that the 416 bp PCR product was indeed that of the CO1 gene, amplified fragments from several individuals were sequenced with 100% sequence identity to *Lu. shannoni* CO1 (GenBank Accession GU597891.1). In addition, the blood source from the single engorged *Lu. shannoni* trapped at WP in late July was determined by amplification of an 850 bp fragment of the cytB gene followed by DNA sequencing. All DNA sequencing was carried out using an ABI3730 DNA Analyzer at the Sequencing and Genotyping Facility at Kansas State University.

Results

Sand Fly Trappings. Phlebotomine (*Lu. shannoni* and *Lu. vexator*) sand flies were trapped for the first time in Kansas and Missouri. In total, 680 sand flies comprising 315 *Lu. shannoni* and 359 *Lu. vexator* were identified. Among the *Lu. shannoni*, 42.5% were trapped in southeast Kansas, with 39.4 and 18.1% trapped in Springfield and BSFS, respectively. Of the

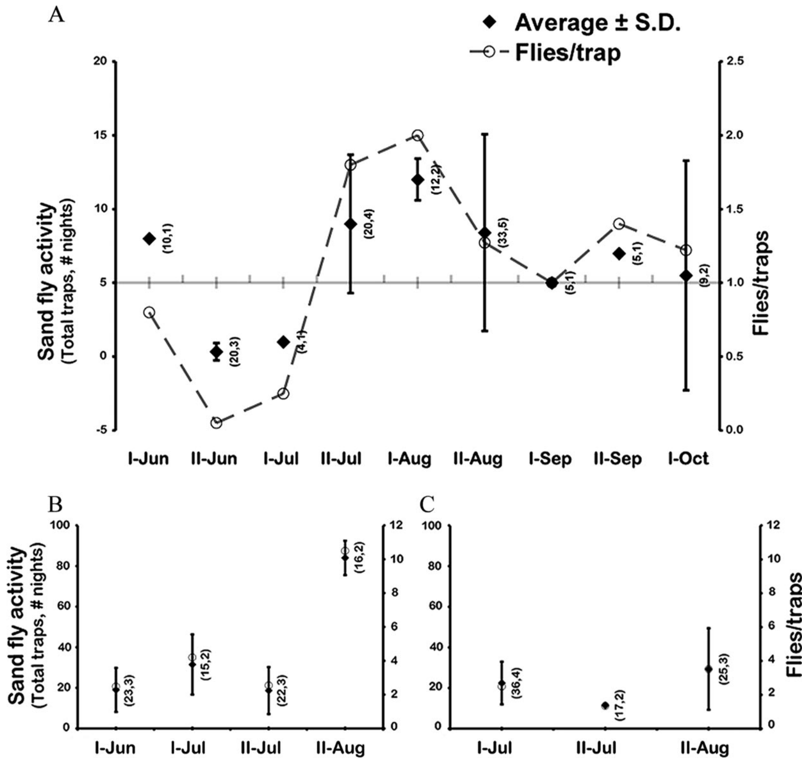

Fig. 1. Sand flies trapped. Sand fly activity according to sites, trapping period, trap nights, and number of traps used. (A) Wilderness Park, Pittsburg, KS; (B) Springfield, MO; and (C) Bull Shoals Field Station, MO. The average number of sand flies trapped per night (diamond; ±SD) was calculated for each 2-wk period. Total number of traps used and number of nights are listed in parenthesis. Number of sand flies per trap night (open circles) is shown on the right Y axis. A trend line was added in (A) for apparent activity of *Lu. shannoni* at WP.

Lu. vexator, 59.9% were trapped in Springfield and 39.8% in BSFS. A single specimen (0.3%) was trapped in southeast Kansas. Specimens are deposited as voucher number 222 in the KSU Museum of Entomological and Prairie Arthropod Research.

At WP, 135 sand flies were trapped between mid-June and mid-October using 118 trap nights. The sand fly activity increased from 0.08 flies per trap night in June to 1.8 flies per trap night in July and 2.0 flies per trap night in August. By the first half of September it fell to one fly per trap (Fig. 1A). There was no significant correlation between the abundance of sand flies trapped at WP and the environmental variables tested (temperature: $F_{1,7} = 0.05$, $P = 0.82$; precipitation: $F_{1,7} = 0.89$, $P = 0.37$; relative humidity: $F_{1,7} = 0.13$, $P = 0.73$). In addition, a *Lu. shannoni* engorged with blood from white-tailed deer (*Odocoileus virginianus* Zimmermann) was collected in late August.

In Springfield, MO, 344 sand flies were collected during 10 nights (76 trap nights in total) for all three focal sites, with 124 identified as *Lu. shannoni*, 215 identified as *Lu. vexator*, and five unidentified individuals (either no DNA was obtained or identification via morphological features was not possible). During trappings in June and July, 2.5–4.2 flies per trap night were observed. In late August, rates averaged 10.5 flies per trap night with a total of 168 sand flies collected in two nights (Fig. 1B). At the BSFS, 201 sand flies (57 *Lu. shannoni*, 143 *Lu. vexator*, and 1 unidentified female) were trapped during nine nights (Fig. 1C). Trapping at BSFS occurred three times, twice in July for a total of 113 flies with an average of 2.13 flies/trap/night (six nights), and 88 flies trapped in August for an average of 3.52 flies/trap/night over three nights. Overall, more *Lu. shannoni* males than females were trapped at WP ($P = 0.046$; Table 1, Supp. Fig. [online only]), but this difference was not significant when individually considering each of the six 2-wk trapping periods analyzed (Table 1).

Lu. shannoni sex ratio for Springfield alternated according to trapping periods, with significantly more females trapped in June ($P = 0.001$) and significantly more males trapped in July and August ($P = 0.036$ and $P = 0.001$, respectively) (Table 2; Supp. Fig. [online only]). At the BSFS, the *Lu. shannoni* sex ratio based on three trapping periods was evenly distributed (Table 2; Supp. Fig. [online only]). For *Lu. vexator*, no significant differences in the sex ratio were observed for any of the trapping periods at any of the locations (Table 2). In addition, 28 gravid females (6 *Lu. shannoni* and 22 *Lu. vexator*) with fully developed eggs were collected during the months of June through September (Table 3).

Table 1. *Lu. shannoni* sex ratio and no. of individuals trapped in the WP

Period	*Lu. shannoni*		
	♀	♂	*P*
I-Jun	3	5	0.727
II-Jun	0	1	N/A
I-Jul	1	0	N/A
II-Jul	13	23	0.133
I-Aug	7	17	0.064
II-Aug	21	20	1
I-Sep	5	0	N/A
II-Sep	2	5	0.453
I-Oct	3	8	0.227
Total	55	79	***0.046***

Two-week trapping periods shown on left column. Statistical significance difference was found only in the total of all trapped individuals (*P* value in bold and italic). Probability according to Binomial test ($\alpha = 0.05$) is shown for $n > 5$; N/A, analysis was not performed, $n \leq 5$.

Table 3. Distribution of gravid females trapped

	Lu. Shannoni [N (%)]			*Lu. vexator* [N (%)]		
	WP	Springfield	BSFS	WP	Springfield	BSFS
July	0 (0)	0 (0)	1 (7)	0	4 (8)	6 (14)
Aug.	1 (3)	2 (18)	1 (7)	0	11 (21)	1 (4)
Sept.	1 (14)	N/T	N/T	0	N/T	N/T

N indicates no. of gravid flies trapped; % of gravid per total females trapped each month is shown in parenthesis. N/T, trapping for sand flies did not occur at these locations.

Three peaks of *Lu. shannoni* activity were observed in the WP, beginning with the first trapping period in June (Fig. 1A). The last successful trapping at that location was on 8 October, with a total of 11 flies collected in five traps. In Springfield, both *Lu. shannoni* and *Lu. vexator* were trapped in all four trapping periods in this location. In Springfield, however, 97% of all sand flies were trapped in late August (Fig. 1B). Moreover, twice as many sand flies per trap were collected in Focal site 1 than the other two focal sites combined.

Morphological variations in *Lu. shannoni* male genitalia were reported by Florin (Florin et al. 2010, 2011) that can lead to misidentification of this species as *Lu. vexator.* One individual *Lu. shannoni* from Springfield matching the variation reported by Florin (the presence of five spines in one of the gonostylus) (Florin et al. 2010) was observed and one *Lu. vexator* male displaying asymmetric gonostyles (Fig. 2) also was identified.

***Lu. shannoni* Specific CO1 PCR Amplification.** The PCR protocol designed to specifically amplify the 416 bp fragment from the CO1 gene was effective in distinguishing *Lu. shannoni* from *Lu. vexator.* Specimens from all three sites were identified using morphological characters and DNA isolated. In addition, DNA samples from 20 male sand flies of each species and from each of the three locations (with the exception of WP, where a single *Lu. vexator* was captured) were PCR amplified with the specific primers. Only the DNA obtained from *Lu. shannoni* was amplified in any of these samples. In addition, no amplification of the CO1 gene was observed with DNA isolated from colonized *Lu. longipalpis* and *Phlebotomus papatasi* (from our laboratory colonies), using the PCR conditions described. After amplification, the 416 bp fragment was matched to 100% nucleotide sequence identity to *Lu. shannoni* CO1.

Twenty three sand fly specimens preserved in 70% ethanol were identified based solely on the PCR described above. These included 8 *Lu. shannoni* females (6 from Springfield and 2 from BSFS), 11 *Lu. vexator* females (9 from Springfield and 2 from the BSFS site), 2 *Lu. vexator* males (one each from Springfield and BSFS), and 2 male *Lu. shannoni* from Springfield (Fig. 3).

Discussion

Here, we report for the first time the presence of *Lu. shannoni* and *Lu. vexator* in Kansas and Missouri, adding to the known distribution of sand flies in North America. We did not observe a significant statistical correlation between weather and sand fly abundance for the analyses performed for the sand flies trapped at WP. However, this might be due in part to the fact that our results were based on a single sand fly trapping season. Our data suggest at least two generations of *Lu. shannoni* occurring per year in Pittsburg and Springfield, matching previous reports (Minter 2010). In contrast to what has been reported for *Lu. shannoni* in Florida (Mann and Kaufman 2010), no sand flies were trapped after 8 October in Kansas, suggesting that they undergo diapause to survive the winter

Table 2. Sex ratio and total sand flies trapped in Springfield and at the BSFS

Period	Springfield						BSFS					
	Lu. shannoni			*Lu. vexator*			*Lu. shannoni*			*Lu. vexator*		
	♀	♂	*P*	♀	♂	*P*	♀	♂	*P*	♀	♂	*P*
II-June	35	12	***0.001***	6	2	0.289	—	—	—	—	—	—
I-July	2	2	N/A	31	25	0.504	6	5	1.000	40	39	1.000
II-July	8	20	***0.036***	18	10	0.185	7	9	0.804	4	1	N/A
II-Aug	11	34	***0.001***	52	71	0.104	14	15	1.000	28	31	0.795
Total	56	68	0.323	107	108	0.576	27	29	0.894	72	71	1.000

Sex ratio and numbers of sand flies collected during 2-wk trapping periods showing in the left column. Significant statistical difference was found between no. of females and males trapped in Springfield (*P* value in bold and italic). Probability according to Binomial test ($\alpha = 0.05$) is shown for $n > 5$; N/A, analysis not performed, $n \leq 5$.

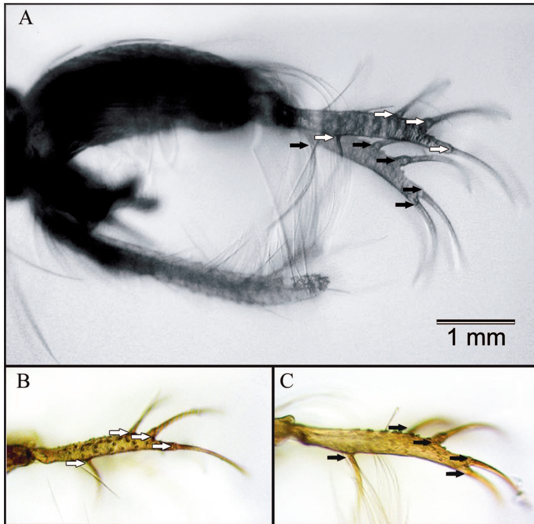

Fig. 2. *Lu. vexator* male with asymmetric gonostylus. (A) shows a superimposed image of (B) and (C), with white arrows pointing to the four spines in one gonostylus, and the black arrows pointing the five spines characteristics of the species. Note one missing spine in the end of gonostylus in (A) and (B). (Online figure in color.)

months. We expect this to also be the case in Missouri. Likely, *Lu. shannoni* undergoes facultative diapause during the winter months as reported for *Lu. diabolica* (Lawyer and Young 1991).

We also established a PCR protocol that specifically amplifies a 416 bp fragment of the CO1 gene in *Lu. shannoni* and not *Lu. vexator.* This protocol can be applied as a molecular tool in situations where confounding morphological differences may be present in *Lu. shannoni,* such as the ones described by Florin et al. (2010).

Because of the introduction of *Leishmania infantum* through importation of dogs from endemic areas, the potential exists for the parasite to become endemic in North America. The role of sand flies in the current canine outbreak is unknown; however, the presence of a vector species that feeds on mammals may be significant. Fourteen sand fly species are native to North America, including two, *Lu. authophora* (Addis) and *Lu. diabolica* (Hall), that are proven vectors of *Le. mexicana* (Endris et al. 1987, Lawyer and Young 1987, Lawyer et al. 1987). *Lu. shannoni,* also native to North America, is a suspected vector of *Le. mexicana* in Mexico (Pech–May et al. 2010, González et al. 2011), was shown to be susceptible to infection with Old World parasites, such as *Le. major* (Claborn et al. 2009), and has also been incriminated in the transmission of pathogens, including *Le. infantum* (Travi et al. 2002). If this sand fly is indeed able to transmit *Le. infantum* to and from domestic dogs, it may also transmit to wild canids (e.g., coyotes and foxes) as well as other animals, creating a scenario for the establishment of the parasite in North America. Further studies on the bionomics and current distribution of *Lu. shannoni,* as well as its potential as vector for canine VL in North America, will be important for assessing disease risk because of sand fly-borne disease in North America.

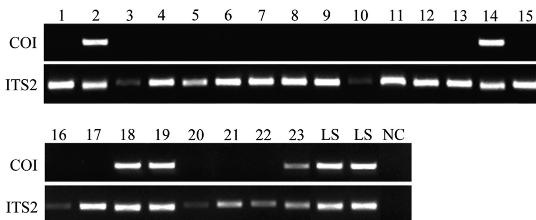

Fig. 3. *Lu. shannoni* PCR. Twenty three sand flies not identified by morphological characters were identified by presence (*Lu. shannoni*) or absence (*Lu. vexator*) of the 416 bp fragment of the CO1 gene. ITS2 amplification was used to confirm DNA isolation. Lanes 1, 3–13, 15–17, and 20–22 were identified as *Lu. vexator*; lanes 2, 14, 18,19, and 23 were identified as *Lu. shannoni.* LS, DNA from *Lu. shannoni* females identified via morphological characters. NC, negative control (no DNA).

Acknowledgments

We thank Dr. Janice Greene, Director of the Bull Shoals Field Station of the Missouri State University for use of facilities and logistics. We also thank Sarah Mull and Nathan Elliot for assistance with trappings. This work was funded by grant AI088051 from the National Institutes of Allergy and Infectious Diseases, National Institutes of Health. Samantha Young was partially supported by a Student Scholar Award from the Kansas IDeA Network of Biomedical Research Excellence (K-INBRE). National Institutes of Health (NIH) grant number P20 RR016475 from the National Center for Research Resources. This manuscript is Kansas State Research and Extension contribution number 12-406-J.

References Cited

Claborn, D. M., E. D. Rowton, P. G. Lawyer, G. C. Brown, and L. W. Keep. 2009. Species diversity and relative abundance of phlebotomine sand flies (Diptera: Psychodidae) on three Army installations in the southern United States and susceptibility of a domestic sand fly to infection with Old World *Leishmania major.* Mil. Med. 174: 1203–1208.

Comer, J. A., R. B. Tesh, G. B. Modi, J. L. Corn, and V. F. Nettles. 1990. Vesicular stomatitis virus, New Jersey serotype: replication in and transmission by *Lutzomyia shannoni* (Diptera: Psychodidae). Am. J. Trop. Med. Hyg. 42: 483–490.

Duprey, Z. H., F. J. Steuer, J. A. Rooney, L. V. Kirchhoff, J. E. Jackson, E. D. Rowton, and P. M. Schantz. 2006. Canine visceral leishmaniasis, United States and Canada, 2000–2003. Emerg. Infect Dis. 12: 440–446.

Endris, R. G., D. G. Young, and P. V. Perkins. 1987. Experimental transmission of *Leishmania mexicana* by a North American sand fly, *Lutzomyia anthophora* (Diptera: Psychodidae). J. Med. Entomol. 24: 243–247.

Ferro, C., E. Cárdenas, D. Corredor, A. Morales, and L. E. Munstermann. 1998. Life cycle and fecundity analysis of *Lutzomyia shannoni* (Dyar) (Diptera: Psychodidae). Mem. Inst. Oswaldo Cruz 93: 195–199.

Florin, D. A., P. Lawyer, E. Rowton, G. Schultz, R. Wilkerson, S. J. Davies, R. Lipnick, and L. Keep. 2010. Morphological anomalies in two *Lutzomyia* (*Psathyromyia*) *shannoni* (Diptera: Psychodidae: Phlebotominae) specimens collected from Fort Rucker, Alabama, and Fort Campbell, Kentucky. J. Med. Entomol. 47: 952–956.

Florin, D. A., S. J. Davies, C. Olsen, P. Lawyer, R. Lipnick, G. Schultz, E. Rowton, R. Wilkerson, and L. Keep. 2011. Morphometric and molecular analyses of the sand fly species *Lutzomyia shannoni* (Diptera: Psychodidae: Phlebotominae) collected from seven different geographical areas in the southeastern United States. J. Med. Entomol. 48: 154–166.

González, C., E. A. Rebollar–Téllez, S. Ibáñez–Bernal, I. Becker–Fauser, E. Martínez-Meyer, A. T. Peterson, and V. Sánchez-Cordero. 2011. Current knowledge of *Leishmania* vectors in Mexico: how geographic distributions of species relate to transmission areas. Am. J. Trop. Med. Hyg. 85: 839–846.

Haddow, A., G. Curler, and J. Moulton. 2008. New records of *Lutzomyia shannoni* and *Lutzomyia vexator* (Diptera: Psychodidae) in eastern Tennessee. J. Vector Ecol. 33: 393–396.

Lawyer, P., and D. Young. 1991. Diapause and quiescence in *Lutzomyia diabolica* (Diptera:Psychodidae). Parassitologia 33(Suppl): 353–360.

Lawyer, P. G., and D. G. Young. 1987. Experimental transmission of *Leishmania mexicana* to hamsters by bites of phlebotomine sand flies (Diptera: Psychodidae) from the United States. J. Med. Entomol. 24: 458–462.

Lawyer, P. G., D. G. Young, J. F. Butler, and D. E. Akin. 1987. Development of *Leishmania mexicana* in *Lutzomyia diabolica* and *Lutzomyia shannoni* (Diptera: Psychodidae). J. Med. Entomol. 24: 347–355.

Mann, R. S., and P. E. Kaufman. 2010. The seasonal abundance of phlebotomine sand flies, Lutzomyia species in Florida. J. Am. Mosq. Control Assoc. 26: 10–17.

Minter, L., B. Kovacic, D. M. Claborn, P. Lawyer, D. Florin, and G. C. Brown. 2009. New State Records for *Lutzomyia shannoni* (Dyar) and *Lutzomyia vexator* (Coquillett). J. Med. Entomol. 46: 965–968.

Minter, L. M. 2010. Mesoscale spatial and temporal distribution of *Lutzomyia spp.* (Diptera: Psychodidae) in deciduous habitats of the eastern United States. M.S. thesis dissertation, University of Kentucky, Lexington, KY.

Ostfeld, R. S., P. Roy, W. Haumaier, L. Canter, F. Keesing, and E. D. Rowton. 2004. Sand fly (*Lutzomyia vexator*) (Diptera: Psychodidae) populations in upstate New York: abundance, microhabitat, and phenology. J. Med. Entomol. 41: 774–778.

Pech–May, A., F. J. Escobedo–Ortegón, M. Berzunza–Cruz, and E. A. Rebolla–Téllez. 2010. Incrimination of four sandfly species previously unrecognized as vectors of *Leishmania* parasites in Mexico. Med. Vet. Entomol. 24: 150–161.

Petersen, C. A., and S. C. Barr. 2009. Canine leishmaniasis in North America: emerging or newly recognized? Vet. Clin. North Am. Small Anim. Pract. 39: 1065–1074.

Price, D. C., D. E. Gunther, and R. Gaugler. 2011. First collection records of phlebotomine sand flies (Diptera: Psychodidae) from New Jersey. J. Med. Entomol. 48: 476–478.

Travi, B. L., C. Ferro, H. Cadena, J. Montoya–Lerma, and G. H. Adler. 2002. Canine visceral leishmaniasis: dog infectivity to sand flies from non-endemic areas. Res. Vet. Sci. 72: 83–86.

U.S. Census Bureau. 2012. Census 2012. U.S. Department of Commerce. (http://quickfacts.census.gov/qfd/state/29/2970000.html).

(WHO) World Health Organization. 2010. World Health Organization Technical Report Series no. 949: control of the leishmaniases. Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, 22–26 March 2010, Geneva, Switzerland.

Young, D., and M. Duncan. 1994. Guide to the identification and geographic distribution of *Lutzomyia* sand flies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). Mem. Am. Entomol. Inst. 54: 1–881.

Young, D. G., and P. V. Perkins. 1984. Phlebotomine sand flies of North America (Diptera:Psychodidae). Mosq. News. 44: 263–304.

Received 3 May 2012; accepted 29 June 2012.