Prevalence of *Trypanosoma cruzi*, the Etiologic Agent of Chagas Disease, Infection in Texas Skunks (Mammalia: Mephitidae)

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Abstract

Chagas disease is one of the world’s most neglected tropical diseases, infecting over six million people across the Americas. The hemoparasite *Trypanosoma cruzi* is the etiological agent for the disease, circulating in domestic, peridomestic, and sylvatic transmission cycles that are maintained by triatomine vectors and a diversity of wild and synanthropic hosts. Public health and wildlife management interventions targeting the interruption of *T. cruzi* transmission rely on an understanding of the dynamics driving the ecology of this zoonotic pathogen. One wildlife host that purportedly plays a role in the transmission of Chagas disease within the southern United States is the striped skunk (*Mephitis mephitis*), though infection prevalence in this species is poorly understood. To this end, we conducted a PCR-based surveillance of *T. cruzi* in 235 wild skunks, representing four species, across 76 counties and ten ecoregions in Texas, USA, along with an evaluation of risk factors associated with infection. We recovered an overall *T. cruzi* prevalence of 17.9% for all mephitid taxa aggregated, ranging between 6.7% for plains spotted skunks (*Spilogale putorius interrupta*) and 42.9% for western spotted skunks (*S. gracilis*). We report the first cases of *T. cruzi* infection in plains spotted and American hog-nosed skunks (*Conepatus leuconotus*), of important note for conservation medicine since populations of both species are declining within Texas. Although not statistically significant, we also detected trends for juveniles to exhibit greater infection risk than adults and for differential sex biases in *T. cruzi* prevalence between taxa, which align with variations in species-specific seasonal activity patterns. No geographic or taxonomic risk factors were identified. Our study contributes key data for population viability analyses and epidemiologic models in addition to providing a baseline for future *T. cruzi* surveillance among skunks and other wildlife species.

Keywords: Chagas disease, *Conepatus*, *Mephitis*, skunk, *Spilogale*, *Trypanosoma cruzi*

Introduction
The zoonotic hemoflagellate parasite *Trypanosoma cruzi* is the etiologic agent of Chagas disease (American trypanosomiasis), which is estimated to infect six to seven million people across the Americas (WHO 2020). Although *T. cruzi* has historically inflicted the greatest social and economic costs on poor, rural populations throughout Latin America, a growing number of autochthonous cases of Chagas disease have been reported in the United States in recent years (Bern 2015; Garcia et al 2015, 2017; Beatty and Klotz 2020, Irish et al 2022). In light of the underreporting of locally acquired infections and the globalized spread of infection via human migrations, the disease burden in the continental United States alone is estimated to be as high as 300,000 cases (Schmunis and Yadon 2010, Bern 2015, Montgomery et al 2016, Irish et al 2022). To mitigate the risk of Chagas disease in North America, effective vector control and disease surveillance strategies must be developed using a holistic understanding of the complex adaptive system that underlays *T. cruzi*’s transmission cycle (Silveira and Vinhaes 1999, Levin 2005, Jansen et al 2015, Moo-Millan et al 2019).


Only McKeever et al (1958) have attempted to conduct *T. cruzi* surveillance among a large sample size of skunks across an extensive geographic area in Georgia and Florida. Skunk surveys for *T. cruzi* in Texas have remained limited in both size and scope (Table 1). The risk for *T. cruzi* transmission is especially high in Texas, which encompasses impoverished and vulnerable communities with a history of neglected tropical diseases (Hanford et al 2007, Sarkar et al 2010, Hotez et al 2012a,b). Texas also overlaps the endemic range of five skunk species, which are distributed across a wide diversity of the state’s eleven ecoregions and a variety of anthropogenically influenced habitats (Gould et al 1960, Schmidly and Bradley 2016). Therefore, we aimed to investigate skunk involvement in Chagas disease ecology by: (1) evaluating the prevalence of circulating *T. cruzi* infections in Texas mephitids through polymerase chain reaction (PCR)-based surveillance and (2) exploring factors contributing to their disease risk (i.e., sex, taxonomy, age, and location). Risk factors that we identify can inform ecological niche models to increase the precision and accuracy of predictions related to vector-host interactions and the transmission dynamics of Chagas disease across geographic and temporal scales, which can ultimately be employed in public health strategy development (Peterson 2006, Peterson et al 2002; Gurgel-Gonçalves et al 2012). Moreover, an understanding of skunk disease ecology based on this field
survey of *T. cruzi* infections can assist in wildlife conservation and management planning, such as creating baseline data for population viability analyses incorporating parasite-induced morbidity and mortality estimates (Wilber et al 2020).

### Materials and Methods

#### Ethical approval

This study was classified as exempt by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University because our methodology did not constitute “use of animals” as defined by IACUC.

#### Sample collection

Whole blood (WB) specimens were opportunistically collected from living and deceased mephitids within Texas, USA and stored on Type I Nobuto blood sampling filter paper (ADVANTEC, Tokyo, Japan) prior to deoxyribonucleic acid (DNA) extraction. Vehicle-killed carcasses were sampled between March 2017 and January 2019. Additional samples consisted of scavenged WB derived from excess WB collected for independent purposes (e.g., genetic analyses, diagnostic procedures) by: (1) biologists researching skunk species in Texas with animal use protocols approved by the IACUC of their home institutions, (2) wildlife rehabilitators licensed by Texas Parks and Wildlife Department (TPWD), and (3) personnel at Angelo State University for Angelo State Natural History Collections’ skunk biobanking project. Information related to the following factors were recorded for each individual sampled: county of origination, collection date, species, sex, and age (non-sexually-mature juveniles less than one year of age; sexually-mature adults one year of age or older as determined by morphological indicators per Crabb [1944] and Verts [1967]).

#### DNA extraction and PCR

When compared to serologic and culturing methods, PCR is the optimal means for detecting circulating *T. cruzi* during the acute phase of infection, even when immune response is low or absent (Gürtler et al 1993; Ferreira and Borges 2002; Picka et al 2007; Braz 2008; Jiménez-Coello et al 2008, 2010, 2015; Kramm et al 2019). PCR can also identify *T. cruzi* with high specificity in the more chronic phases of infection if parasites have been liberated and are present in peripheral blood and/or if reinfection has occurred (Braz 2008, Jiménez-Coello et al 2008).

DNA was extracted from WB samples stored on Nobuto strips using the DNeasy Blood & Tissue Kit (QIAGEN, MD, USA) with the optimized DNA extraction methodology outlined in Gulas-Wroblewski et al (2021). Extracted DNA was added to a 20 µl reaction with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, MA, USA), then analyzed on a ViiA 7 Real Time (RT) PCR System (Thermo Fisher Scientific). To detect *T. cruzi* DNA, Piron et al (2007)’s Cruzi 1/2/3 assay was used, while the Actin f/r/p assay described by Piorkowski et al (2014) was employed to detect β-actin. An extraction negative control, no template control (5 µl of laboratory grade H₂O), and a positive control (5 µl of *T. cruzi*/mouse DNA sample) was included in each RT-PCR run. DNA samples were tested in triplicate for *T. cruzi* DNA detection and singly for β-actin detection.

Samples with cycle threshold (Ct) values below 40 were interpreted as positive in the Cruzi 1/2/3 assay, and those with Ct values below 38 were identified as positive in the Act.f/r/p assay. If all
samples for an individual were negative based on these measures, but at least one sample was within 1 Ct unit of the cut-off point, the WB specimen was re-extracted, re-tested, and reanalyzed per the protocols detailed above. Individuals were defined as positive for *T. cruzi* if at least one of their three DNA samples was positive in the Cruzi 1/2/3 assay. A skunk was identified as negative for *T. cruzi* if all three DNA samples were negative in the Cruzi 1/2/3 assay but the sample was positive in the Act.f/r/p assay.

*Statistical analysis*

County information for each sampled skunk was used to delineate the ecoregion and level of Chagas disease hotspot in which the individual was located. Since the ecoregions of Texas cross county lines, counties sampled for skunks were assigned to ten ecoregions following the TPWD’s deer management ecoregion assessments (TPWD 2011). Each county was also appointed a value between 0 and 3 to represent the degree to which positive cases of Chagas disease were previously recorded in the jurisdiction. A value of one was added to a county’s “Chagas disease hotspot” sum for the presence of each of the following: *T. cruzi*-positive triatomine vectors (2013-2018), *T. cruzi*-positive canine sentinels (2013-2015), and autochthonous human Chagas disease cases (2013-2018) as reported by the Texas Department of State Health Services (TDSHS 2019).

Pearson’s Chi-squared tests (α = 0.05) were used to evaluate *T. cruzi* prevalence within each species and with all mephitid species pooled for deviations between species, sexes, ages, ecoregions, and Chagas disease hotspots. When 20% or more of the cells in the Chi-squared tests had expected counts below 5, likelihood ratio values (α = 0.05) were assessed. Odds ratios were also calculated for sex and age based on all skunk species and within each mephitid taxon. All statistical analyses were performed on STATA 16.1 software (StataCorp, TX, USA).

*Results*

*Prevalence of T. cruzi in Texas skunks*

WB samples were collected and successfully extracted for DNA from a total of 235 individual skunks sampled between March 2004 and June 2019, representing 42 *Conopatus leuconotus* (American hog-nosed skunk), 171 *M. mephitis* (striped skunk), seven *Spilogale gracilis* (western spotted skunk), and fifteen *Spilogale putorius interrupta* (plains spotted skunk). No hooded skunk (*Mephitis macroura*) specimens were available for analysis. Overall, 42 mephitids (17.9%) tested positive via PCR for *T. cruzi* infection. Of these positive individuals, 30 were striped skunks, eight were American hog-nosed skunks, three were western spotted skunks, and one was an eastern spotted skunk. Within skunk species, the resulting prevalence of Chagas disease ranged from 6.7% (*S. putorius interrupta*) to 42.9% (*S. gracilis*) (Figure 1). The variation in *T. cruzi* infection between species was not statistically significant, a pattern that was consistent when both species of *Spilogale* were pooled for analysis and across paired species comparisons (Supplementary Table S1).

*Age variation in T. cruzi infections*

All sampled skunks were definitively classed according to age (juveniles <1 year old; adults ≥1 year old) with 226 adults and nine juveniles identified in total. Six striped skunks, two American hog-nosed skunks, and one plains spotted skunk were juveniles, of which 33.3%, 50%, and 0%, respectively, tested positive for *T. cruzi* infection. In comparison, adult striped skunks, American hog-nosed skunks,
and plains spotted skunks exhibited Chagas disease prevalence of 17%, 17.5%, and 7.1%, respectively. With all mephitid species aggregated for analysis, juvenile skunks were approximately 2.4 times more likely to test positive for T. cruzi than were adults, though without statistically significant support (Table 2). Similarly, Chi-squared tests and likelihood ratio values failed to recover any significant relationship between age and T. cruzi infection in skunks overall or across species (Table S1).

Sex variation in T. cruzi infections

Extensive degradation and/or damage of road-killed and otherwise deceased skunks precluded the determination of sex for 115 sampled individuals (49% of the total tested). However, 41 females and 79 males were incontrovertibly identified. No western spotted skunk females were sampled, and only one plains spotted skunk female was sampled. Overall, the prevalence of T. cruzi infections in female skunks (24.4%) was slightly higher than in male skunks (21.5%), a variation that is not statistically significant (Table 2, S1). When evaluated at the species level, female striped skunks exhibited higher T. cruzi prevalence (28.1%) than males (17.6%), whereas prevalence was greater in male American hog-nosed skunks (28%) than in females (12.5%) (Table 2, S2).

Geographic variation in T. cruzi infections

Only one individual, an adult male American hog-nosed skunk that tested negative for T. cruzi, was unable to be identified to the county-level. At least one skunk was collected from 76 counties, which cover all ten of Gould’s ecoregions of Texas (Figure 2; Table 3). T. cruzi infections were identified in skunks collected in 24 counties (31.6% of the total counties surveyed) ranging from 7.7% (Val Verde county) to 100% (Coryell, Guadalupe, and Webb counties) prevalence with an average of 44% and a median of 37% prevalence (Figure 1; Table S2). When counties were clustered by ecoregion, T. cruzi-positive skunks were recorded in six of the ten ecoregions with prevalence ranging from 7.1% (Rolling Plains) to 27.3% (Blackland Prairies) with a median of 20% (Table 3). We noted a weak statistical association between T. cruzi prevalence for striped skunks and location within a “Chagas disease hotspot” (likelihood ratio value of 6.75, $P = 0.08$). Otherwise, we did not recover any statistically significant relationship between skunk infections and county, ecoregion, or “Chagas disease hotspot” (Table S1).

Discussion

Our sampling of 235 individual skunks from four species across Texas is the most extensive T. cruzi surveillance of mephitid taxa across a wide geographic region to date. All prior records of T. cruzi infections in North American skunks have been reported from striped skunks, with prevalence ranging between 0 and 100% and a median incidence of infection of 32%, 38%, and 100% based on PCR, culture, and serology, respectively (Table 1). Our value of 18% PCR positivity based on 171 striped skunks is substantially lower than the pooled average (48%) for the total 112 striped skunks tested prior to our investigation, demonstrating the imprecision of low sampling sizes for Chagas disease surveillance in this species (Table 1).

Previous evaluations of T. cruzi in members of the genus Spilogale recovered negative results for two Mexican southern spotted skunks (S. angustifrons) and seven eastern spotted skunks (McKeever et al 1958, Zavala-Velázquez et al 1996). As such, ours are the first records of T. cruzi infections in the genus Spilogale as well as for the taxa S. gracilis and S. putorius interrupta. We also report the first

Our detection of circulating *T. cruzi* infections in four species representing all three New World mephitid genera provided a unique opportunity to evaluate risk factors associated with Chagas disease in skunks. However, sampling bias hindered our capacity to assess variations between age classes with statistical confidence (Table 2, Table S1). Although not statistically significant, the trend of higher *T. cruzi* risk for juveniles of all skunk species pooled, striped skunks, and American hog-nosed skunks aligns with previous findings for a smaller sample of striped skunks in El Paso county, Texas as well as for domestic dogs (*Canis lupus familiaris*) and suburban common opossums (*Didelphis marsupialis*) in Mexico and laboratory rats (*Rattus norvegicus*) (Pérez et al 2011, Matamoros 2016, Arce-Fonseca et al 2017, Galaviz-Silva et al 2017) (Table 2). Increased disease risk in juveniles may be a function of detrimental immune-endocrine response, which promotes elevated parasitemias in younger animals (Pérez et al 2011). Chagas disease in neonates can also be exacerbated by vertical transmission of parasites as evidenced in bats, laboratory rodents, domestic dogs, and humans (Andrade 1982, Moreno et al 2003, Sánchez Negrette et al 2005, Añez et al 2009, Rodríguez-Morales et al 2011, Alkmim-Oliveira et al 2013, Howard et al 2014). Since other studies have reported more severe disease manifestations and elevated mortality in *T. cruzi*-positive younger animals when compared to adults, a trend towards higher infection risk in juvenile skunks holds significance for the population ecology of these species, especially concerning for taxa of conservation interest (Moreno et al 2003, Kjos et al 2008, Rodríguez-Morales et al 2011).

In contrast to the age-related prevalence patterns we observed, no difference was found between juvenile and adult *T. cruzi* infection incidence for striped skunks in Uvalde County, Texas and in Neua León, Mexico (Charles et al 2013, Galaviz-Silva et al 2017). Age bias in *T. cruzi* infections was also absent in populations of domestic dogs in Panama and raccoons and rodent species in south Texas (Pineda et al 2011, Charles et al 2013). Therefore, our inability to find a statistically significant correlation between age and *T. cruzi* infection risk among Texas skunks may represent the absence of any relationship between these conditions.

Similarly, our surveillance did not find any statistically significant difference in *T. cruzi* circulating infections between male and female skunk taxa overall, with the prevalence for both sexes varying by only 2.9% when all species were aggregated (Table 2, S1). Previous studies found no sex bias in *T. cruzi* prevalence among striped skunks in Bexar and Uvalde counties, Texas and in Mexico (Charles et al 2013, Galaviz-Silva et al 2017, Soria 2018). The independence of host sex and *T. cruzi* infection dynamics has been supported by other studies in relation to parasite loads in laboratory mice, susceptibility in laboratory rats, and prevalence in populations of white-eared opossums (*Didelphis albiventris*) in Argentina, Virginia opossums (*Didelphis virginiana*) in south Texas, and domestic dogs in Panama (Wisnivesky-Colli et al 1992, Pérez et al 2011, Pineda et al 2011, Soares et al 2012, Zecca et al 2020).

The highest total daily movement (in terms of both activity level and distance covered) corresponds with the period(s) of greatest energetic requirements in skunks and varies between sexes (Zhang et al 2019). For female skunks, total activity levels are greatest during the stages of lactation and young at heel, while males experience these during the mate-searching and intrasexual competitions of the breeding season (Larivière and Messier 1997, Ellsworth 2016, Zhang et al 2019). These time periods correspond to a higher risk of exposure to *T. cruzi* vectors due to both elevated activity across a greater area and increased consumption of prey, including triatomines, to meet increased energetic demand. In Texas, the peak breeding season for male striped skunks ranges from late February through the end of March, while females are lactating with dependent young primarily between May through August and into early fall in the case of late March (Patton 1974, Schmidly and Bradley 2016) (Figure 3). Male American hog-nosed skunks in Texas are lactating with young from April through late June (Bailey 1905, Taylor and Davis 1947, Patton 1974, Ellsworth 2016, Schmidly and Bradley 2016) (Figure 3). When these seasonal behavioral patterns are aligned with *T. cruzi*-prevalence values for Texas triatomines (Curtis-Robles et al 2018), the periods of greatest potential vector exposure for striped skunk females and American hog-nosed skunk males coincide with peak parasite prevalence within triatomines. In contrast, when compared to the peak activity periods for the opposite sex, peak activity periods for male striped skunks and female American hog-nosed skunks overlap seasons of lower triatomin *T. cruzi*-positivity (Figure 3). The effects of reproductive ecology and behavior on the prevalence of *T. cruzi* infection within and between skunk species deserves more attention in future research, particularly among western and eastern spotted skunk species. These spotted skunk taxa share similar timing of lactation and young-rearing by females but exhibit a significantly offset breeding season due to the presence of delayed implantation in western spotted skunks (Mead 1968, Greensides 1973, Kaplan and Mead 1994, Kinlaw 1995).

**Conclusions**

We performed the first known wide-scale and taxonomically diverse survey for circulating *T. cruzi* infections in skunks across Texas, reporting the first positive cases for plains spotted and American hog-nosed skunks. Although sampling biases precluded our incontrovertible assessment of the risk factors associated with *T. cruzi* prevalence, several trends were discernable related to age and sex biases, which can inform population and host-parasite models supporting conservation, wildlife
management, and public health strategies. Future research should extend the geographic scope and number of individuals sampled, particularly in the case of spotted skunks, to further investigate the risk factors for *T. cruzi* in skunks and the roles these mesocarnivores play within Chagas disease transmission cycles. The present study highlights the value in surveying multiple taxa of closely related wildlife species across a wide expanse of habitats to investigate the complex adaptive systems that underpin the ecology of vector-borne zoonotic diseases.

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**Author Contributions**

Author contributions are as follows: writing—original draft preparation, B.E.G.-W.; writing—review and editing, B.E.G.-W., R.G., R.B.K., R.C.D., and K.O.M.; conceptualization, B.E.G.-W. and K.O.M.; methodology, investigation, and analysis, B.E.G.-W., R.G., and R.B.K.; resources and project administration, K.O.M.

**Author Disclosure Statement**

No conflicting financial interests exist.

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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>RT-PCR</td>
<td>Real Time polymerase chain reaction</td>
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Supplementary Material

Supplementary Table S1
Supplementary Table S2

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### Tables

Table 1. Previous reports of *Trypanosoma cruzi* prevalence in North American striped skunks (*Mephitis mephitis*). Counties are noted for locations within Texas, USA.

<table>
<thead>
<tr>
<th>Location</th>
<th>Method(s) of diagnosis</th>
<th>Prevalence of <em>T. cruzi</em> infection (number positive individuals/total number tested)</th>
<th>References</th>
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<tbody>
<tr>
<td>Georgia and Florida, USA</td>
<td>Culture grown from kidney tissue</td>
<td>3/306</td>
<td>McKeever et al (1958)</td>
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<tr>
<td>California, USA</td>
<td>Indirect hemagglutination and histology</td>
<td>1/1</td>
<td>Ryan et al (1985)</td>
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<tr>
<td>El Paso county, Texas, USA</td>
<td>PCR</td>
<td>3/24</td>
<td>Matamoros (2016)</td>
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<tr>
<td>Location, Country</td>
<td>Methodology</td>
<td>Positive/Pooled</td>
<td>Prevalence (%)</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Nuevo León, Mexico</td>
<td>PCR, blood smears, and histopathology</td>
<td>11/34</td>
<td>32.4</td>
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<tr>
<td>Bexar County, Texas, USA</td>
<td>PCR</td>
<td>9/33</td>
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<tr>
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Table 2. Prevalence and odds ratio values for variables associated with *Trypanosoma cruzi* infection in pooled and individual species of Texas skunk. *n* = total number of individuals tested.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Risk factor</th>
<th>n</th>
<th>Positive</th>
<th>Prevalence (%)</th>
<th>Odds ratio</th>
<th>95% Confidence Interval</th>
<th>P-value</th>
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<td>Sex</td>
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<tr>
<td>Female</td>
<td></td>
<td>41</td>
<td>10</td>
<td>24.4</td>
<td>1.18</td>
<td>0.43-3.1</td>
<td>1-sided: Fisher’s exact: 0.44; 2-sided: 0.82</td>
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<tr>
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<td></td>
<td>79</td>
<td>17</td>
<td>21.5</td>
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<tr>
<td>Age (years)</td>
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<tr>
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<td>9</td>
<td>3</td>
<td>33.3</td>
<td>2.4</td>
<td>0.37-11.76</td>
<td>1-sided: Fisher’s exact: 0.2; 2-sided: 0.2</td>
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<td>≥1</td>
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<td>226</td>
<td>39</td>
<td>17.3</td>
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<td>Conepatus leuconotus</td>
<td>Sex</td>
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<tr>
<td>Female</td>
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<td>7</td>
<td>28.0</td>
<td>0.37</td>
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<tr>
<td>Age (years)</td>
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<td>2</td>
<td>1</td>
<td>50</td>
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<td>0.05-380.9</td>
<td>1-sided: Fisher’s exact: 0.35; 2-sided: 0.35</td>
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Table 3. *Trypanosoma cruzi* prevalence values for each Gould ecoregion of Texas. Skunk species were aggregated within each ecoregion. \( n = \) total number of individuals tested.
Figure 1. Prevalence values for circulating *Trypanosoma cruzi* infections in Texas skunk species.
Figure 2. Geographic distribution of sampling locations and *Trypanosoma cruzi* prevalence values for skunks across Texas. (A) Distribution of counties where at least one skunk was sampled across the Gould ecoregions of Texas. (B) County-level *Trypanosoma cruzi* prevalence values for sampled skunks. Values were calculated by aggregating skunk species within each county. Only counties in which at least one skunk was sampled are shown.
Figure 3. Temporal intersection of *Trypanosoma cruzi* prevalence of triatomine vectors (bold black line) and sexually divergent activity patterns of American hog-nosed (*Conopatus leuconotus*) and striped skunks (*Mephitis mephitis*) in Texas. Prevalence values of triatomine vectors per Curtis-Robles et al (2018). For American hog-nosed skunks, note the overlap of the males’ peak activity periods in August and October-December with higher *T. cruzi* prevalence in triatomines when compared to *T. cruzi* prevalence in triatomines during females’ peak activity period from April-June. For striped skunks, note the overlap of the females’ peak activity periods in the summer through early fall with higher *T. cruzi* prevalence in triatomines when compared to *T. cruzi* prevalence in triatomines during males’ peak activity period in the spring.