Infection, Genetics and Evolution 74 (2019) 103998

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research Paper

Protein mass spectrometry detects multiple bloodmeals for enhanced Chagas disease vector ecology

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ARTICLE INFO

Keywords:
Chagas
Ecohealth
LC-MS/MS
Bloodmeal
Mass spectrometry
Triatoma dimidiata

ABSTRACT

Chagas disease, a neglected tropical disease endemic in Latin America, is caused by the protozoan parasite *Trypanosoma cruzi* and is responsible for significant health impacts, especially in rural communities. The parasite is transmitted by insect vectors in the Triatominae subfamily and due to lack of vaccines and limited treatment options, vector control is the main way of controlling the disease. Knowing what vectors are feeding on directly enhances our understanding of the ecology and biology of the different vector species and can potentially aid in engaging communities in active disease control, a concept known as Ecohealth management. We evaluated bloodmeals in rural community, house-caught insect vectors previously evaluated for bloodmeals via DNA analysis as part of a larger collaborative project from three countries in Central America, including Guatemala. In addition to identifying bloodmeals in 100% of all samples using liquid chromatography tandem mass spectrometry (LC-MS/MS) (n = 50), strikingly for 53% of these samples there was no evidence of a recent bloodmeal by DNA-PCR. As individual vectors often feed on multiple sources, we developed an enhanced detection pipeline, and showed the ability to quantify a bloodmeal using stable-isotope-containing synthetic references peptides, a first step in further exploration of species-specific bloodmeal composition. Furthermore, we show that a lower resolution mass spectrometer is sufficient to correctly identify taxa from bloodmeals, an important and strong attribute of our LC-MS/MS-based method, opening the door to using proteomics in countries where Chagas disease is endemic.

1. Introduction

Chagas disease is a neglected tropical disease of great importance in Latin America, affecting millions of people (World Health Organization and UNICEF, 2017). While the acute phase of this disease usually only causes mild symptoms of malaise, chronic disease complications, with cardiomyopathy being the most prevalent, have significant impacts on health and well-being in local communities. As the *Trypansosoma cruzi* parasite, the etiological agent of Chagas disease, is transmitted mainly by an insect vector, this disease involving multiple players can be challenging to control: the Triatominae subfamily of vectors, colloquially known as 'kissing bugs', contains over 150 species (Justi and Galvão, 2017), and as far as is known all with the potential of carrying the Chagas parasite, and with the ability to infect any mammal.

For many rural communities in Guatemala, local government resources are often minimal making it challenging to implement large-scale disease management strategies. Although vector management through large-scale insecticide spraying has occurred in the past and is still happening today, the effectiveness of this single strategy in controlling the vector is limited to introduced species outside their native ranges (e.g., *Rhodnius prolixus*, *Triatoma infestans*) (Cecere et al., 2006; Nakagawa et al., 2003). Native vector management, however, such as that of *Triatoma dimidiata* which is abundant in Central America and especially Guatemala, faces different challenges, such as a rapid re-colonization of houses from sylvatic habitats following insecticide spraying (Hashimoto et al., 2006; Peterson et al., 2019).

In recent years, the concept of implementation science using Ecohealth-based approaches has been recognized as a sustainable method of Chagas disease control. Engaging affected communities in Central and South America, (Pellecer et al., 2013; Waleckx et al., 2015),

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https://doi.org/10.1016/j.meegid.2019.103998
Received 22 May 2019; Received in revised form 22 July 2019; Accepted 7 August 2019
Available online 08 August 2019

and using education coupled with scientific data, have had an impact at various scales in controlling disease burdens. In addition, bloodmeal data and vector prevalence of various hosts fosters an understanding of local vector ecology and behavior, and ultimately allows communities to make sustainable decisions for not only managing Chagas disease, but also decreasing helminth infections and increasing nutrition (Dorn et al., 2019).

Various methods to detect feeding profiles have been applied to Chagas disease vectors. While protein-based methods were commonly used in early studies of bloodmeals (i.e., precipitin and antisera tests, recently evaluated in (Rabinovich et al., 2011)), DNA-based detection has come to the forefront. Many DNA methods are based on the relatively abundant mitochondrial DNA or repetitive nuclear DNA regions (Kent, 2009). The polymerase chain reaction (PCR), sometimes coupled with DNA sequencing, using species-specific or general target primers (i.e., vertebrate mitochondrial 12 S ribosomal gene) is common (Klotz et al., 2014; Lima-Cordón et al., 2018; Lucero et al., 2013; Lucero et al., 2014; Pizarro et al., 2007; Pizarro and Stevens, 2008). Indeed, we have previously evaluated if various locations in Central America differ in their role of sylvatic, synanthropic, and domestic animals in the local Chagas transmission cycles as part of an Ecohealth management approach in these areas using taxa specific primers to detect bloodmeal prevalence (Lima-Cordón et al., 2018).

However, DNA-based methods come with the challenge of needing uncontaminated, high-quality DNA, often in ample quantity from recently-fed vectors (Stevens et al., 2012). Proteomics studies are becoming more frequent in determining bloodmeal sources from various arthropod disease vectors (Laskay et al., 2013; Niare et al., 2016; Önder et al., 2013), and we have previously shown a liquid chromatography tandem mass spectrometry (LC-MS/MS)-based approach that identified a single bloodmeals in insect vectors (Keller et al., 2017; Keller et al., 2018). Proteomics resources are available in most Chagas endemic areas (Padrón and Domont, 2014) and previous cost analysis demonstrates LC-MS/MS for bloodmeal identification is very realistic for endemic countries in Latin America (Keller et al., 2017; Önder et al., 2013). In addition, LC-MS/MS targeting hemoglobin protein peptides found in the bloodmeal have allowed us to identify bloodmeals over long temporal scales and even post-molting to the limits of our experimental replicates (4 and 12 weeks, respectively) (Keller et al., 2018). Hemoglobin peptide signatures lasted longer than DNA when compared to short interspersed nuclear element (SINE)-DNA PCR, and LC-MS/MS always detected bloodmeals samples in instances where no bloodmeal was evident using DNA-based methods (Keller et al., 2017; Keller et al., 2018)

Coupling 12 S PCR with cloning and sequencing, multiple bloodmeals are often detected in Triatominae vectors (Georgieva et al., 2017; Lucero et al., 2014; Stevens et al., 2014; Waleckx et al., 2014). Although genomics-based approaches are emerging (Collini et al., 2015; Dumonteil et al., 2018; Kieran et al., 2017; Logue et al., 2016; Orantes et al., 2018), cost, sample processing time, the window of time to detect multiple bloodmeals, and contamination are challenges. Another strong advantage of mass spectrometry-based methods addressed in this current study is the ability of a single LC-MS/MS run to identify all/multiple bloodmeals in a single run, rather than with a single PCR reaction for each taxa, or cloning and sequencing reactions.

In this study we (1) enhance our hemoglobin-based LC-MS/MS pipeline to detect multiple bloodmeals and validate our pipeline using stable-isotope-containing synthetic reference peptides (SRPs). We then (2) detect and identify bloodmeals in field-collected *Triatoma dimidiata* and *Triatoma nitida* from Chiquimula, Guatemala that are part of a large-scale Ecohealth intervention project. As the bloodmeal profiles of the field-collected *T. dimidiata* and *T. nitida* specimens used in this study have been previously investigated using taxa-specific primers by PCR, we compare the detection efficiency of LC-MS/MS for samples where DNA-based methods both detected and did not detect a bloodmeal. Lastly, we also develop the ability to quantify the bloodmeal using general SRPs.

2. Methods

2.1. Ethics statement

A comprehensive statement of ethical practices used for the previously investigated samples are contained within Lima-Cordón et al., 2018. In short, ethical clearance was granted from the Ministry of Health in Guatemala and the PanAmerican Health Organization. In addition, a single mouse blood sample used for this study was obtained in accordance with an IACUC-approved protocol encouraging post-mortem “tissue sharing”. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Vermont in accordance with the requirements of the Office of Laboratory Animal Welfare (IACUC protocol 12–045). The mouse blood used in this study was previously described in Keller et al., 2017.

2.2. Insect vector collection and storage

In this study we compared DNA and LC-MS/MS-detected bloodmeal sources for 43 rural community, house-caught *Triatoma dimidiata* collected and analyzed previously as part of a larger collaborative project “Ecohealth interventions for the prevention of Chagas Disease in Central America” (https://www.idrc.ca/en/project/ecohealth-interventions-chagas-disease-prevention-central-america-0) (for pre-Ecohealth intervention baseline survey data see Bustamante-Zamora et al., 2015; Lima-Cordón et al., 2018.) An additional 7 *Triatoma nitida* specimens collected at the same time, but not reported on previously, were also included. For 45 of the 50 specimens pre-intervention survey data were available regarding numbers of vertebrate animals in each household (Table 1) (Bustamante-Zamora et al., 2015; Lima-Cordón et al., 2018).

The 50 insects examined were a subset of those collected from in (intradomicile) and around (peridomicile) houses and transported to the Laboratory of Applied Entomology and Parasitology (LENAP), Universidad the San Carlos de Guatemala, Guatemala as previously described (Lima-Cordón et al., 2018) in August–October 2011, stored in 95% ethanol and 5% glycerol at room temperature, and insect abdomens were transported to the University of Vermont in June 2016 and March 2017. Samples were subsequently stored at 4 °C for up to 9 months before processing for mass spectrometry analysis.

2.3. Bloodmeal detection by DNA

A subset of specimens used in Lima-Cordón et al. (2018) was used in this study and analyzed for bloodmeal sources. Of 568 specimens previously examined from Guatemala, 30% (n = 170) were found to have evidence of a recent bloodmeal by PCR and of these, 7% (n = 12) had evidence of two bloodmeals per specimen by DNA (Lima-Cordón et al., 2018). In the study reported here, we further examined 23 samples with a previous bloodmeal (4 samples had 2 bloodmeals detected) and 27 samples for which no bloodmeal was detected by PCR. Two of the samples with no previous bloodmeal detected had also been re-examined by PCR and sequencing targeting the mitochondrial 12 S genes.

Specimens that were positive for a bloodmeal by DNA were chosen randomly, while we chose some engorged and some starved specimens for samples that were negative for a bloodmeal with DNA (Table 1). A few specimens were not reported on in Lima-Cordón et al. (2018) for various reasons (i.e. different vector species, no insect age recorded, etc.) but were included in this study since bloodmeal species identification by DNA data were readily available. For DNA and protein extractions from adult specimens, the last three segments of the abdomen were used for bloodmeal analysis.

We briefly summarize the previously reported DNA methods here. A

Table 1
Triatoma dimidiata and *T. nitida* sample information of specimens collected in Chiquimula, Guatemala in 2011 as part of a large-scale Ecohealth project.

Sample ID		Species of *Triatoma*	Country	Department	Village (Location)	Subvillage	Ecotope	Stage	Visible bloodmeal	Survey responses for household where specimen collected. =not data available					
T. nitida and † samples not included in Lima-Cordón et al., 2018							Place where vector was collected	Age of vector at collection	Present/ absent/ unknown	# humans living in house	# dogs	# birds	# pigs	# beasts/ livestock	Traces/presence of rodents in house 1 = yes 0 = no
†Jkop_001	TPG_007	*dimidiata*	Guatemala	Chiquimula	Olopa	Amatillo	Intradomicile	V	unknown	.	.	.	.	.	.
†Jkop_002	TPG_011	*dimidiata*	Guatemala	Chiquimula	Olopa	Amatillo	Intradomicile	V	unknown	.	.	.	.	.	.
†Jkop_003	TPG_012	*dimidiata*	Guatemala	Chiquimula	Olopa	Amatillo	Intradomicile	V	unknown	.	.	.	.	.	.
Jkop_004	TPG0028[e]	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	female	unknown	8	4	0	0	0	1
Jkop_005	TPG0030[e]	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	III	unknown	8	4	0	0	0	1
Jkop_006	TPG0038	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	male	unknown	.	.	.	.	.	.
Jkop_007	TPG0044	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	V	present	3	0	2	0	0	0
†Jkop_008	TPG_067	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	unknown	unknown	.	.	.	.	.	.
Jkop_009	TPG0075	*dimidiata*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	V	present	2	12	5	0	0	1
Jkop_011	TPG_086	*nitida*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	III	unknown	4	2	12	0	0	1
Jkop_012	TPG_087	*nitida*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	male	unknown	6	4	5	0	1	1
Jkop_013	TPG_088	*nitida*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	male	unknown	8	1	4	0	0	1
Jkop_015	TPG_092	*nitida*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	female	unknown	6	0	6	0	0	1
Jkop_016	TPG_097[b]	*dimidiata*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	V	present	11	3	4	0	0	1
Jkop_017	TPG_100[a]	*dimidiata*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	V	present	4	6	2	0	1	1
Jkop_018	TPG_101[a]	*nitida*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	V	present	4	6	2	0	1	1
Jkop_019	TPG_104[a]	*dimidiata*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	V	present	4	6	2	0	1	1
Jkop_020	TPG_178	*dimidiata*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	V	present	5	1	15	0	0	0
Jkop_021	TPG_158[d]	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	V	present	5	1	18	0	0	1
Jkop_022	TPG_168	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Peridomicile	male	present	5	0	8	0	0	1
Jkop_023	TPG_186	*nitida*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	male	unknown	3	5	35	0	0	1
Jkop_024	TPG_238[a]	*dimidiata*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	III	unknown	4	6	2	0	1	1
Jkop_025	TPG_336	*dimidiata*	Guatemala	Chiquimula	Olopa	El Cerrón	Intradomicile	V	unknown	6	2	8	0	0	1
Jkop_027	TPG_630	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	female	present	7	2	8	0	0	1
Jkop_028	TPG_670	*nitida*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	male	unknown	9	2	12	0	0	1
†Jkop_029	TPG_016	*dimidiata*	Guatemala	Chiquimula	Olopa	Amatillo	Intradomicile	III	present	10	0	11	1	0	1
Jkop_030	TPG0045	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	male	absent	3	3	1	0	0	0
Jkop_031	TPG0102	*dimidiata*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	V	present	4	6	2	0	1	1
Jkop_032	TPG0159[d]	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	V	present	5	1	18	0	0	1
Jkop_033	TPG0244[b]	*dimidiata*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	III	absent	11	3	4	0	0	1
Jkop_034	TPG0263	*dimidiata*	Guatemala	Chiquimula	Olopa	El Guayabo	Intradomicile	III	present	.	.	.	.	.	.
Jkop_035	TPG0288	*dimidiata*	Guatemala	Chiquimula	Olopa	El Cerrón	Intradomicile	III	absent	10	1	1	0	0	1
Jkop_036	TPG0302	*dimidiata*	Guatemala	Chiquimula	Olopa	El Cerrón	Intradomicile	III	present	2	0	0	0	0	1
Jkop_037	TPG0334	*dimidiata*	Guatemala	Chiquimula	Olopa	El Cerrón	Intradomicile	III	present	6	2	8	0	0	1
Jkop_038	TPG0381	*dimidiata*	Guatemala	Chiquimula	Olopa	Paternito	Peridomicile	female	absent	7	2	8	0	0	1

(*continued on next page*)

Table 1 (*continued*)

Sample ID	Species of *Triatoma*	Country	Department	Village (Location)	Subvillage	Ecotope	Stage	Visible bloodmeal	Survey responses for household where specimen collected. = not data					
						Place where vector was collected	Age of vector at collection	Present/ absent/ unknown	# humans living in house available	# dogs	# birds	# pigs	# beasts/ livestock	Traces/presence of rodents in house 1 = yes 0 = no
T. nitida and † samples not included in Lima-Cordón et al., 2018														
Jkop_039	TPG0385	*dimidiata*	Guatemala	Chiquimula	Olopa	Paternito	Intradomicile	III	absent	4	3	10	0	1	1
Jkop_040	TPG0412[c]	*dimidiata*	Guatemala	Chiquimula	Olopa	Paternito	Intradomicile	III	present	7	2	8	0	0	1
Jkop_041	TPG0415[c]	*dimidiata*	Guatemala	Chiquimula	Olopa	Paternito	Intradomicile	IV	present	7	2	8	0	0	1
Jkop_042	TPG0471	*dimidiata*	Guatemala	Chiquimula	Olopa	Paternito	Intradomicile	IV	present	9	2	10	0	0	1
Jkop_043	TPG0585	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	unknown	V	present	4	0	6	0	0	0
Jkop_044	TPG0596	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	IV	present	4	2	22	0	0	1
Jkop_045	TPG0603	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	IV	present	6	1	21	0	0	1
Jkop_046	TPG0635	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	V	present	.	.	.	.	.	.
Jkop_047	TPG0679	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Peridomicile	male	absent	6	1	0	0	0	1
Jkop_048	TPG0703	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	V	present	5	2	0	0	0	1
Jkop_049	TPG0749	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	IV	present	8	2	0	0	0	1
Jkop_050	TPG0771	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	male	present	6	0	0	0	0	1
Jkop_051	TPG0807	*dimidiata*	Guatemala	Chiquimula	Olopa	El Cerrón	Peridomicile	III	present	4	2	0	2	0	1
Jkop_052	TPG0814	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	male	absent	6	2	0	0	0	1
Jkop_053	TPG0717	*dimidiata*	Guatemala	Chiquimula	Olopa	La Prensa	Intradomicile	IV	present	4	1	0	0	0	1

[a-e] designates specimens collected from the same house. All other vectors were collected from individual houses. † samples were not included in Lima-Cordón et al., 2018.

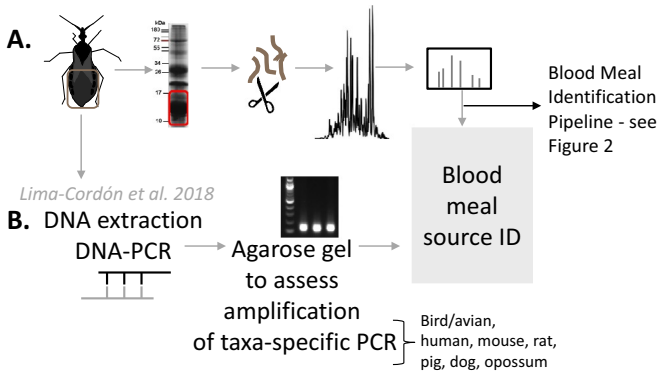

Fig. 1. General workflow describing comparison between LC-MS/MS and DNA-based bloodmeal identification methods. Specimen abdomens were dissected and used for (A) hemoglobin protein-based LC-MS/MS analysis, and (B) DNA-based bloodmeal identification as previously described in Keller et al., 2017, Lima-Cordón et al., 2018, respectively.

separate PCR reactions was done using taxa specific primers for human, dog, bird, mouse, rat, opossum, and pig with results scored from agarose gel electrophoresis and transillumination based on appropriately sized bands (Lima-Cordón et al., 2018). Most of the taxa-specific PCR assays targeted highly repetitive short interspersed nuclear element (SINE) regions (Supplementary Table 1).

2.4. Hemoglobin peptide detection by LC-MS/MS and taxonomic indexing

We extracted protein from the 50 specimens as previously described (Fig. 1) (Keller et al., 2018). Briefly, abdominal insect tissue was ground in 95 °C denaturing buffer, boiled and centrifuged. Denatured proteins were separated by 15% SDS-PAGE and cut from gel regions surrounding the molecular weight of hemoglobin (~16 kDa). Following in-gel digestions with trypsin, dried samples were reconstituted with 20 µL 2.5% acetonitrile/2.5% formic acid (Solvent A) and separated on a reverse-phase high performance liquid chromatography (HPLC) column (length = 12 cm, diameter = 100 µm) packed in house with 5-µm C18 beads (pore size = 200 Å). Samples were not reduced or alkylated. Our assays used a linear ion trap-orbitrap to identify peptides where all spectra were collected in the orbitrap (Ballif et al., 2008; Keller et al., 2018). Briefly, samples were analyzed on the LTQ (linear trap quadrupole)-Orbitrap Discovery mass spectrometer fitted with a Finnigan Surveyor Pump Plus and Micro AS autosampler (Thermo Electron; San Jose, CA, USA) and controlled with Xcalibur™ 2.1 Software (Thermo Fisher Scientific, Inc.; Waltham, MA, USA). Following a 15-min loading phase (flow rate = 100 µL/min) in Solvent A, peptides were eluted with a 0–50% gradient of Solvent B (99.85% MeCN, 0.15% FA) over 45 min and electrosprayed (1.9 kV) into the mass spectrometer. This gradient was followed by 10 min at 100% Solvent B prior to a 10-min equilibration in 100% Solvent A. The precursor scan (360–2000 m/z) was followed by ten low energy collision-induced dissociation (CID) tandem mass spectra (normalized collision energy (NCE) was 35%). CID spectra were acquired for the top ten most abundant ions in the precursor scan in a data-dependent acquisition mode (dynamic exclusion settings: repeat count = 2, repeat duration = 30 s, exclusion list size = 500, exclusion duration = 60 s, isolation width = ± 2 m/z). All mass spectra were obtained in centroid, either in the orbitrap for high resolution experiments or in the LTQ for low resolution experiments in positive ion mode. In order to examine the ability of lower resolution mass spectrometry instruments for bloodmeal identification, three samples were subjected to an additional run with all spectra collected in the LTQ.

As described previously, we searched mass spectra using the SEQUEST algorithm (Thermo Electron V26.12) against a custom forward and reverse concatenated database containing vertebrate

hemoglobin sequences (17 Jan 2018, 18,000+ entries) extracted from GenBank using 'hemoglobin' as a keyword in any curated field and 'vertebrate' as a filter (Keller et al., 2017; Keller et al., 2018). Peptide filter parameters in SEQUEST were also as described previously, except for the lower resolution LTQ instrument where the different features of the instruments required changing the measured precursor mass tolerance from ± 5 ppm to 2 Da.

Some trypsin digested hemoglobin peptides are variable within species, while others are conserved within species but variable between species. The unique peptide sequences identified for each specimen were indexed with the corresponding taxonomic information extracted from GenBank as previously described using Pyteomics python tools and library (Keller et al., 2017). Prior to indexing, three aspects of the database were further curated. First, we BLAST searched each unique peptide identified, confirming it was a hemoglobin sequence because we include peptides with hemoglobin in any field from GenBank, not just fully curated entries. Non-hemoglobin peptides were not considered for downstream analysis. Second, taxonomic categories vary among vertebrates, and thus, we added taxonomic information where necessary (e.g., it is debated whether the monophyletic clades artiodactyla and cetacea are more likely comprised of the non-monophyletic clade cetartiodactyla, therefore 'order' is left blank in GenBank) (Supplementary Table 2). Finally, a few peptides (SAVTAXWGK, (K)VLNSFSDGXK) included an "X" amino acid, signifying either leucine or isoleucine in the sequence and for these we individually adjusted the "X" amino acid after determining they were leucine (L) through a BLAST search.

We indexed the unique peptides of each sample against the associated taxonomy using JMP, Version 14 (SAS Institute Inc., Cary, NC, 1989–2018). Below we describe our enhanced pipeline that addresses the determination of multiple blood sources (Fig. 2).

2.5. Pipeline development to identify multiple bloodmeals from a single specimen

While we only had evidence for a single bloodmeal source in each specimen examined in our previous LC-MS/MS studies (Keller et al., 2017; Keller et al., 2018), the DNA-based study of the specimens examined here had reported evidence of two bloodmeal sources in four of the specimens (Lima-Cordón et al., 2018).

Our previous pipeline demonstrated that evaluating the unique hemoglobin peptides in a sample adequately differentiates bloodmeal species (Keller et al., 2017; Keller et al., 2018). Thus, using our list of unique peptides from each specimen with associated taxonomic data, we examined the number of classes, orders, families, genera, and species represented in each sample. Finding that taxonomic order was the most informative for the first step in determining multiple bloodmeals, we then made a histogram with the possible orders represented in a sample along with the number of unique peptides in each of the orders. If sets of peptides for any order were an exact subset of an order with more unique peptides, that order was no longer considered a possible bloodmeal source (e.g., Fig. 2B). Similarly, if a set of peptides was a subset of two or more orders, that order was no longer considered a possible bloodmeal source (Fig. 2 D.1).

To obtain high stringency and minimize both the false discovery rate and incorrectly inferring an additional bloodmeal, we developed the "top-50%" approach (Fig. 2) to identify major bloodmeals. For a bloodmeal source to be inferred for a particular specimen, it has to be either the most supported order (maximum number of unique peptides N_{max}, Fig. 1 D) or contain more than $N_{50\%max}$ unique peptides (Fig. 2 E).

Our analysis becomes problematic when there are unique taxonomic orders and their associated peptides that do not fall within the determined major bloodmeal orders as this can arise from two causes: unreported polymorphisms (Otte and Schlötterer, 2017) or misidentification of the peptide sequence by the SEQUEST program. For example, our database of all hemoglobin sequences in GenBank includes the tryptic peptide VADALTTAVSHIDDLPGALSALSDLHAYK found in seal, and our previous work determined this to be an unreported polymorphism for dog (Keller et al., 2017). The peptide VNADEVGGEALGR is often misidentified by SEQUEST as NVADEVGGEALGR (Keller et al., 2018). If a peptide is not identified as containing unreported polymorphisms or as a misidentified peptide based on our previously reported criteria, we infer its origin is a low abundance "minor" bloodmeal (Fig. 2 E.1).

2.6. Multiple bloodmeal validation with SRPs

We have previously demonstrated our ability to profile Triatomine bloodmeal sources using stable isotope-containing synthetic hemoglobin reference peptides (SRPs) (Keller et al., 2017). In this study we expand this concept by validating our ability to detect multiple bloodmeals using SRPs that differ among the previously reported most common bloodmeals (dog, chicken/turkey, birds, human, Table 2) (Lima-Cordón et al., 2018).

A subset of 11 samples was run with SRPs using previously described methods (Keller et al., 2017; Keller et al., 2018), except that for the SRPs a top-2 approach, collecting the top two most abundant ions, was followed by a targeted approach where we targeted the dominant charge state of each native peptide for which we had spiked in a corresponding SRP (Table 2). Concentrations (in pmol/µl) were provided for each SRP by the manufacturer, and a 10-SRP mix was optimized to run with insect vector samples. Due to overlapping species matches, only 6 SRPs were included in the analysis presented here.

The SRPs were synthesized by Cell Signaling Technology (Danvers, Massachusetts, USA). Specifically, since the vast majority of DNA-identified bloodmeals were human, bird (chicken), and dog, SRPs distinguishing these species were synthesized (dog, chicken/turkey, human, Table 2). We also included a more general bird SRP that is common throughout 15 avian orders (bird, Table 2). Each SRP contained one heavy-labeled amino acid (Table 2). Presence/absence data of native vs. SRP MS1 peaks for these SRPs was used to validate our bloodmeal identification pipeline.

2.7. General quantification of a bloodmeal with SRPs

We have previously shown our ability to quantitatively profile an insect vector bloodmeal and we expanded this concept to quantify total amount of hemoglobin using highly conserved "general" hemoglobin peptides (Table 2) as a proxy for the total amount of hemoglobin present in a sample. The peptide chosen showed the lowest decay rate over time in ticks fed on mouse as nymphs and could still be found 308 days after molting to adults (Laskay et al., 2013), while the species-specific peptides were detected up to 120 days (dog, chicken/turkey), 176 days (bird), and 183 days (human) (Table 2). Quantification of a bloodmeal does not necessarily correspond to when an insect vector fed, as varying amounts of blood can be ingested during a bloodmeal. However, this is the first step toward using SRP quantification for profiling vector bloodmeals and perhaps identifying bloodmeals not present in GenBank (Keller et al., 2017).

Methods and protocols for the use SRPs in quantifying a range of proteins and their modifications have been developed (Kettenbach et al., 2011; Matsumoto et al., 2018). To generate an appropriate method using a given instrument for any given SRP, it is important to determine the linear range for quantification and the lower and upper limits of quantification. To do this, a dilution series for each SRP can be generated within a complex peptide mixture, such as yeast digested with trypsin.

To quantify bloodmeals, absolute amounts (in fmol) of SRP and native peptides were measured and relative abundances calculated based on the heavy-to-light ratio of SRP to native peptide comparing monoisotopic peak intensities for the general peptides (Table 2). Since we and others (Keller et al., 2018; Lundgren et al., 2010), have used

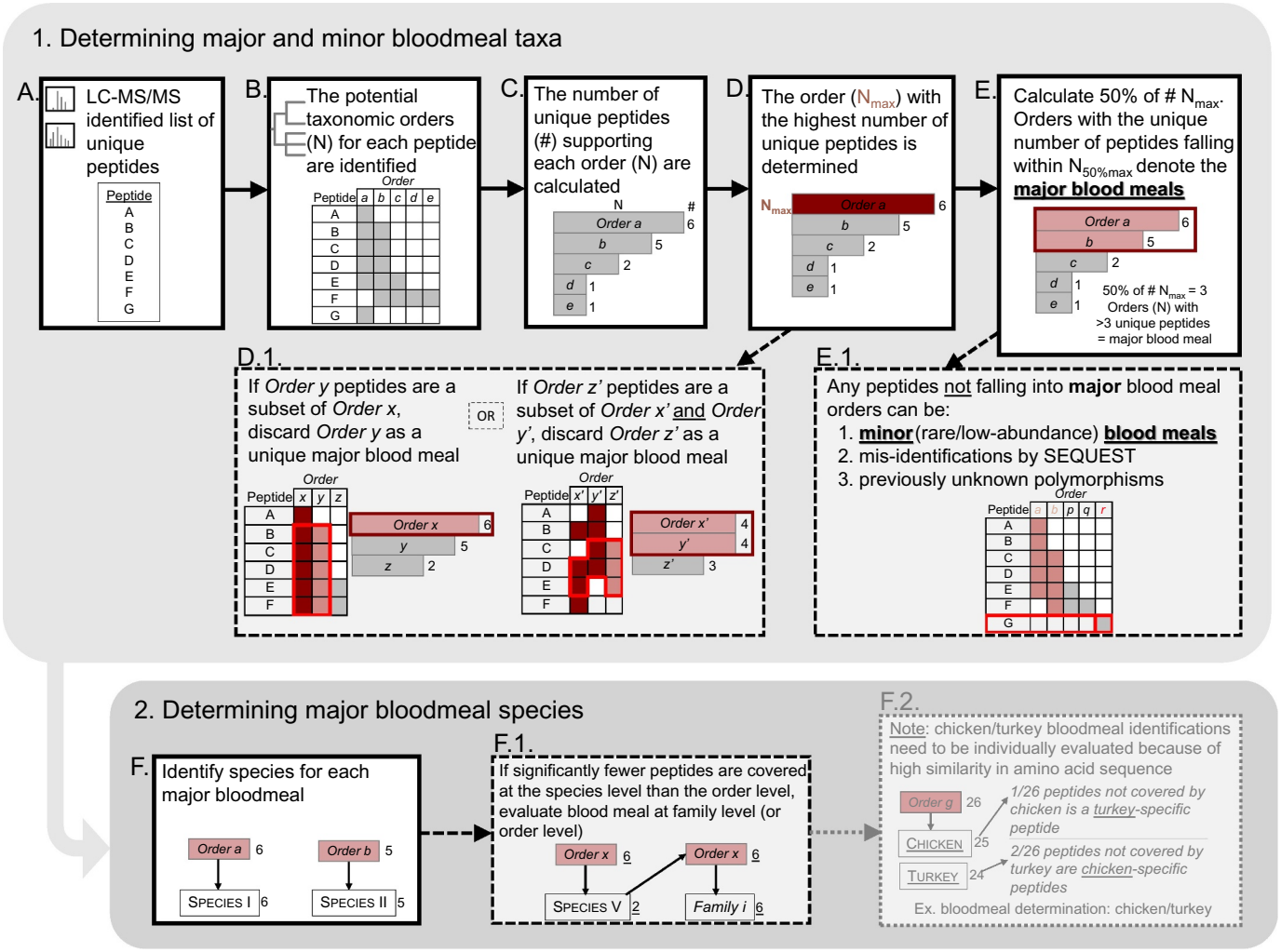

Fig. 2. Workflow of enhance pipeline for taxonomic evaluation of single and multiple bloodmeals identified by LC-MS/MS. (A) LC-MS/MS-identified hemoglobin peptides, from which a list of unique peptides is created. (B) Using taxonomy information from GenBank, the potential taxonomic orders are identified for each peptide, and (C) subsequently indexed- the number of unique peptides matching each order is recorded. The order with the highest number of unique peptides matching it (N_{max}) is identified (D) and (E) divided by 2 in order to determine the major bloodmeal cut-off. All orders with the number of unique peptides falling within 50% of N_{max} are designated as major bloodmeals. Orders are only considered as unique major bloodmeals if unique peptides are not subsets of another major bloodmeal (D.1). If any peptides do not fall into the major bloodmeal orders, they can be classified into the categories found in (E.1). Each major bloodmeal set is then identified to species (F), and to (F.1) family level if considerably less peptides are identified between order and species, or the order level if considerably less peptides are identified at the family level. (F.2) refers to a special case for chicken/turkey bloodmeals.

spectral counts, or the number of peptides identified in a sample as a proxy for protein quantification, we compared the fmol amount of the general peptides (a representation for total amount of hemoglobin identified in a sample) to the spectral count of all hemoglobin peptides identified in a sample. We previously used highly conservative peptide filters which served for very accurate bloodmeal species identification. However, we found that lowering the XCorr filters to ≥1.8 (z = 1), 2 (z = 2), 2.2 (z = 3), 2.4 (z = 4), 2.5 (z = 5) expanded the repertoire of identified hemoglobin peptides without significantly increasing false discovery rates. We also examined two specimens where we did not identify a bloodmeal species and five additional specimens with the enhanced pipeline using these lower stringency filters.

3. Results

In this study we showed the superior ability of hemoglobin peptide-based LC-MS/MS to detect and identify Chagas disease vector bloodmeal sources of field-collected Triatomine vectors from Guatemala compared to classical DNA-based PCR. First, we developed an enhanced pipeline to identify multiple bloodmeals, which we then validated using synthetic reference peptides as positive controls. Our enhanced LC-MS/MS pipeline identified bloodmeals from all 50 samples which we directly compared with DNA-identified vector bloodmeals. Lastly, we quantified bloodmeals using general SRPs, and showed a direct correlation between hemoglobin peptide amount (fmol) and peptide spectral count, the number of hemoglobin peptides identified in a sample.

3.1. SRP MS1 spectra confirm single and multiple bloodmeals

To evaluate our ability to correctly identify multiple vector bloodmeals using our enhanced pipeline, we re-ran a subset of 11 samples spiked with SRPs. Based on the previously published DNA data (Lima-Cordón et al., 2018), the most common bloodmeals were human, bird (i.e. chicken), and dog. As we were expecting a similar distribution of bloodmeal sources with LC-MS/MS, our SRPs correspond to these common bloodmeals (Fig. 3, Table 2, Table 3).

The SRPs validated our pipeline for multiple bloodmeal sources. The species-specific peptides differed in the duration of detection post-feeding (120 days for dog, chicken/turkey; 176 days for bird; 183 days for human), and as expected, for the seven samples where the LC-MS/

Table 2
Synthetic reference peptides designed for validation of multiple bloodmeals and quantification. Synthetically modified heavier amino acids are marked as bold and underlined. Average LC (liquid chromatography) retention times are shown of 11 runs done with SRPs. Two known polymorphisms of the hemoglobin beta position 31 peptide exist, LLIVYPWTQR and LLVVYPWTQR. One of these two variants occur in over 639 species (17 Jan 2018).

Name	ID	Sequence	Species	Monoisotopic mass (m/z)	Dominant charge state (m/z)	(2 +) Light sequence (m/z)	Average LC retention time (min)	Days peptide detected in ticks post-molt (Laskay et al., 2013)
M14–28011	"Dog"	FFAAVSTV**I**TSK	*Canis lupus familiaris* (dog), 5 lemur species	1276.715	638.865	635.856	43.78	120
M14–28012	"Chicken/ turkey"	FLSAVSAV**I**AEK	*Gallus gallus* (chicken), *Meleagris gallopavo* (turkey), 4 other galliformes	1240.715	620.865	617.856	44.51	120
M14–28013	"Birds"	VLTSFGDA**v**K	15 Avian orders, incl. Duck, songbird, chicken, turkey, 7 reptiles, 31 mammals	1041.574	521.295	518.787	38.52	176
M14–28016	"Human"	FFESFGDLSTPDAVMGN**p**K	*Homo sapiens* (human), 7 other primates	2063.955	1032.485	1029.978	46.16	183
M14–3888	General	LLIVY**p**WTQR	General peptides of highly conserved regions	1293.748	647.882	644.875	45.86	308
M14–3889		LLVVY**p**WTQR	of hemoglobin matching over 639 species	1279.733	640.874	637.867	45.03	

MS pipeline identified bird (chicken/turkey or duck) (Table 3), native peptide peaks were present for the bird SRPs. However only five of the six samples where chicken/turkey was detected had peaks for the chicken/turkey SRP. Lack of detection could be because this SRP had relatively short duration of detection post-feeding, or because the peptide has an unreported polymorphism. For example, sample JKop23_TPG186 contained peptide (FLSAVSAVLSEK) differing by one amino acid from the SRP (FLSAVSAVLAEK). For sample JKop30_TPG045 which identified as a duck, the general avian native peptide was present while the chicken/turkey peptide was absent. For the four samples where the pipeline identified human, only two had native peptide peaks for the human SRP. For the two samples (JKop19_TPG104 and JKop09_TPG075) that did not have a native human peptide identified, there were fewer human peptides identified than the primary bloodmeal, dog, (20:10 and 15:10). This suggests the human bloodmeal was either smaller or less recent. The three samples where the pipeline identified dog, also had native peptide peaks for the dog SRP. In samples where bloodmeals were identified that did not have a corresponding SRP, as expected, no native peptide peaks were detected for dog, chicken/turkey, bird, or human.

3.2. LC-MS/MS and DNA-identified vector bloodmeals

The enhanced detection ability of our LC-MS/MS pipeline is demonstrated by the identification of at least one bloodmeal source in all 50 Triatomine vectors (Fig. 4, Supplementary Table 3, Supplementary Table 4), including the 27 where no bloodmeal was detected by DNA. Almost twice as many human bloodmeals were detected by LC-MS/MS than by DNA (10 vs 22) and 70% of specimens where only LC-MS/MS identified a bloodmeal had fed on human. This means that DNA-based studies could underestimate Chagas disease risk.

We detected significantly more bloodmeals by LC-MS/MS than by DNA ($\bar{x} = 1.6$ vs. 0.5, $t = 7.66$, $P < .0001$). Multiple major bloodmeals were detected in 46% of specimens (23 of 50), including 28% with two major bloodmeals and 18% with three. In comparison, DNA only identified multiple bloodmeals in four samples (8%), each with two.

For the two most common bloodmeal sources by DNA in our subset of the data (bird 35% and dog 26%), there was significant agreement (Table 4), while for human and rodent there were significantly more bloodmeals detected by LC-MS/MS. LC-MS/MS-identified bloodmeals generally agreed with DNA except for 4 samples among the 23 samples with DNA-identified bloodmeals. Among these samples, four differences (opossum, mouse, human, cow) occurred only once and with a single sample we cannot speculate on the cause. However, all three samples where pig was identified by PCR were not confirmed by LC-MS/MS, suggesting that this DNA-based assay be further evaluated before future use.

3.3. Taxonomic level of resolution

For some samples where a human bloodmeal was inferred, the pipeline identified multiple Hominidae species. For example, JKop045_TPG603 had equal support for *Pan paniscus* (bonobo) and *Homo sapiens* (human). As bonobos are native to Africa, we infer human (Supplementary Table 3). For specimen JKop33_TPG244, 7 primate species were equally supported, yet again the only species likely to occur in our study location is human.

Some rodent bloodmeals (Rodentia/Cricetidae or Sciuridae) were not resolved to species (Supplementary Table 3). Interestingly these likely represent sylvatic bloodmeals from species not represented in GenBank, possibly indicating a more prominent role for the sylvatic cycle in disease transmission.

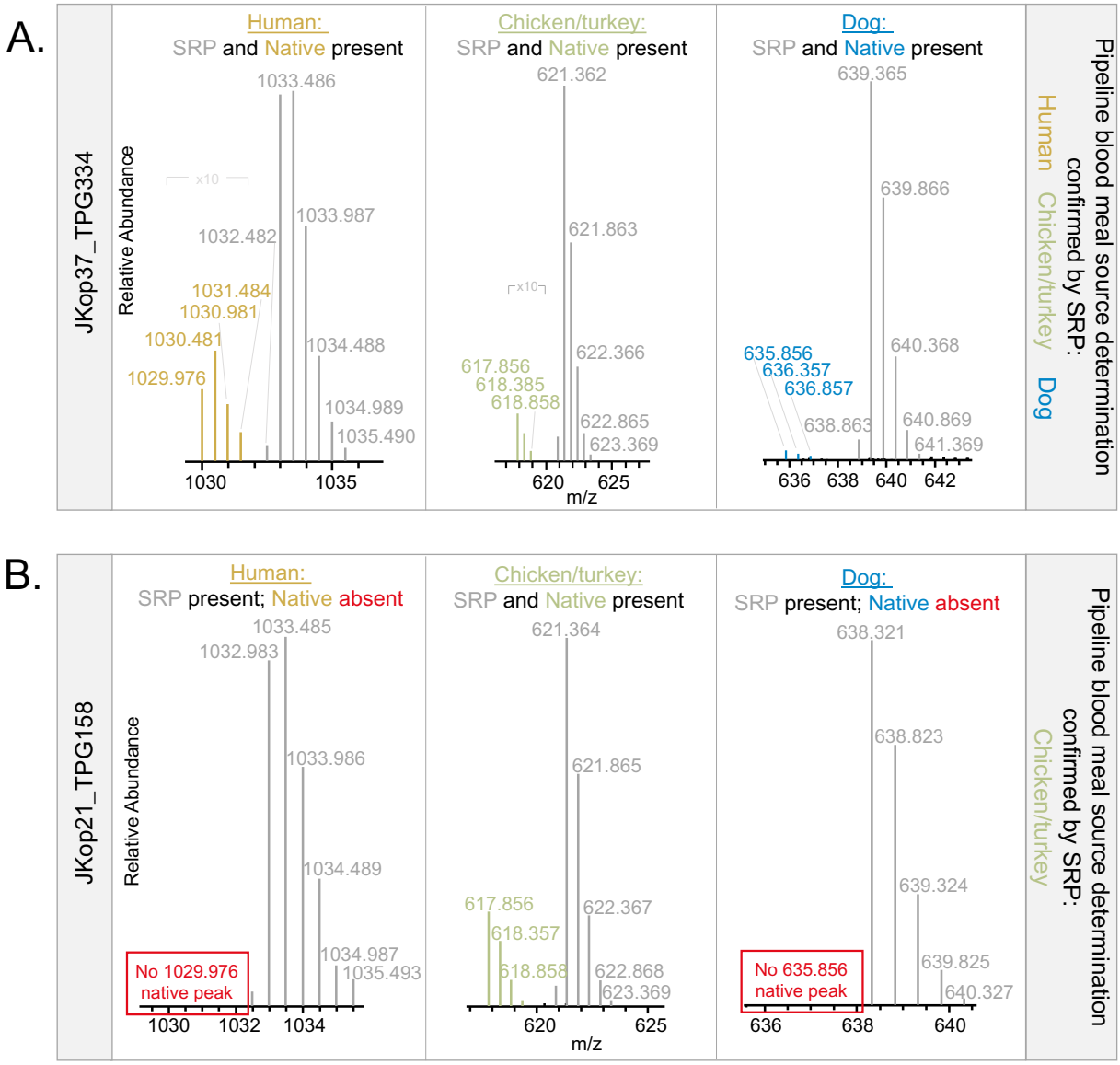

Fig. 3. SRP and native peptide presence/absence validates bloodmeal pipeline. Two representative samples are shown, a multiple and single bloodmeal, where (A) shows the presence of dog, human, and chicken/turkey MS1 isotopic native peptide peaks, which agrees with the bloodmeals identified by the pipeline. (B) shows a single bloodmeal of chicken/turkey, with lacking dog and human native peptide peaks.

3.4. Lowered stringency identifies bloodmeal species for samples with few hemoglobin peptides

Although for most samples, especially those with multiple bloodmeals, our highly conservative stringency filtering accurately identifies blood source species. For samples where few hemoglobin peptides were detected, slightly lower stringency filtering allowed for species-level bloodmeal identification (Supplementary Table 6). For samples JKop35_TPG288 and JKop52_TPG814 vertebrate hemoglobin was detected, yet we were not able to identify the species; notably the spectral count (number of hemoglobin peptides identified) was extremely low. Evaluation with slightly lower stringency enabled species-level identification and manual evaluation of spectra confirmed the assignment. To confirm the necessity of less conservative stringency only when the higher level did not resolve to species, analysis of five additional samples with lower stringency indicated more orders for major bloodmeals, some of which were not supported by the more stringent filtering (Supplementary Table 6).

3.5. Household survey data correlates potential and LC-MS/MS observed bloodmeals

For 45 of the 50 vectors, we had household-level pre-intervention survey data on bio-socio-ecological factors. The 45 vectors came from 32 households because for five we examined more than one vector (Fig. 6). These households owned 0–12 dogs ($\bar{x} = 5.8$), 0–25 birds ($\bar{x} = 2.5$), 0–2 pigs ($\bar{x} = 0.07$), 0–1 livestock (e.g., cows/beasts ($\bar{x} = 0.16$)), and humans ($\bar{x} = 5.8$) occupied the houses (Table 1, Fig. 6).

Although all houses were inhabited, only 20 of 32 households had evidence of human bloodmeals by LC-MS/MS. Evidence for mice/rats/rodents was found in 29 houses (91%) but only 7 (22%) had evidence of rodent bloodmeals and only six of these had evidence of rodents in the home (Fig. 6). All houses except two owned birds (94%), but these two without birds were included in the 16 households that had bird identified as a bloodmeal source. Of the 26 houses with dogs (81%), 12 had evidence of dog bloodmeals compared to two of the six without dogs. Of the five houses that owned pigs or livestock, one had evidence of a pig bloodmeal and one vector from a household without livestock had

Table 3
MS1 monoisotopic peaks of SRPs confirm enhanced pipeline-identified bloodmeals.

SRP		Sample ID										
Name	Species	JKop53_TPG717	JKop51_TPG807	JKop37_TPG334	JKop34_TPG263	JKop30_TPG045	JKop27_TPG630	JKop23_TPG186†	JKop22_TPG168	JKop21_TPG158	JKop19_TPG104†	JKop09_TPG075†
M14–28011	"Dog"	.	.	+	.	.	.	.	.	.	+	+
M14–28012	"Chicken/turkey"	+	.	+	.	.	+	.	+	+	.	.
M14–28013	"Birds"	+	.	+	.	+	+	+	+	+	.	.
M14–28016	"Human"	.	.	+	+	.	.	.	.	.	.	.
Enhanced LC-MS/MS pipeline identified blood meal		Chicken/turkey	Pig, rodentia	Dog, chicken/turkey, human	Human, opossum	Duck	Chicken/turkey	Turkey/chicken	Chicken/turkey	Chicken/turkey	Dog, rodentia, human	Dog, human, rodentia

+ present; • absent; † a native peptide was not present- see text for further details.

evidence of a cow bloodmeal.

3.6. Lower resolution mass spectrometer can identify multiple bloodmeals

Resources in Chagas endemic countries are often minimal, and although proteomics equipment is available (Padrón and Domont, 2014), lower mass accuracy instrumentation could possibly influence the adaptability of our LC-MS/MS pipeline. To investigate this, we compared our enhanced pipeline with stringent filtering on the high mass accuracy orbitrap with that of a lower mass accuracy LTQ instrument. Three samples were examined representing: (1) mouse blood (mouse – 1996); (2) single bloodmeal (JKop016_TPG097) and (3) multiple bloodmeals (JKop17_TPG100). With our enhanced LC-MS/MS pipeline, the orbitrap identified bloodmeal sources to species level, while the LTQ identified blood sources to at least the taxonomic order with high confidence, and some to family and species level (Supplementary Table 5).

3.7. Bloodmeal quantification correlates to spectral count

Analysis with synthetic SRPs demonstrated quantification of bloodmeals could help identify blood sources not currently in the underlying database, and the experiment presented here is the first step in quantifying general hemoglobin peptides. We examined the correlation between the estimated fmol of general hemoglobin peptides (calculated from MS1 peak heights of native vs. SRP) relative to the total number of hemoglobin peptides identified in a sample (Fig. 7). We established a standard curve using our SRPs in their linear range on the instrument, showing that hemoglobin amount correlated to spectral count of hemoglobin peptides identified in a sample.

4. Discussion

Due to the limited availability of treatments for Chagas disease, coupled with the prevalence of Chagas disease in rural communities of Latin America, achievable, alternative disease management methods are needed. Since *T. dimidiata* cannot be controlled using only insecticide applications (Yoshioka et al., 2015), deeper comprehension of the ecology of the species is important to develop new control methods. Implementation science including Ecohealth can decrease disease burdens at local scales. *T. dimidiata* has shown a great diversity of behaviors among its geographical distribution (Lima-Cordón et al., 2018), and a more reliable detection method could help in understanding the role of blood sources in the epidemiology of the disease. Since *T. nitida* is in the process of domestication (Monroy et al., 2003), blood sources prevalence could help in developing new strategies for the control of the species. Knowing the vector blood source is crucial to understanding local transmission cycles, identifying clinically relevant host reservoirs, and understanding the ecology and behavior of the insect, all leading to inform vector control decisions.

Herein we describe an efficient and straight-forward method for identifying multiple vector bloodmeals using LC-MS/MS targeting hemoglobin molecules, which outperformed DNA-based methods in over half of all samples.

In this study we detected bloodmeals in 100% of samples using LC-MS/MS. This is in stark comparison to PCR-based methods which only detected bloodmeals in roughly half. By DNA, bloodmeal species were detected in 28% of specimens from one location in Guatemala (Lima-Cordón et al., 2018) (Fig. 4), a percentage similar to other studies where vectors may not be feeding often, e.g. (Valença-Barbosa et al., 2015). We have previously shown that LC-MS/MS identified bloodmeals at least 4 weeks post-feeding and up to 12 weeks post-molting, whereas DNA only detected bloodmeals up to 1 week post-feeding and not at all post-molting (Keller et al., 2018). Our LC-MS/MS-identified bloodmeals enhanced the ability to make informed vector management decisions. Lima-Cordón et al., 2018 reported dog, bird, humans, and

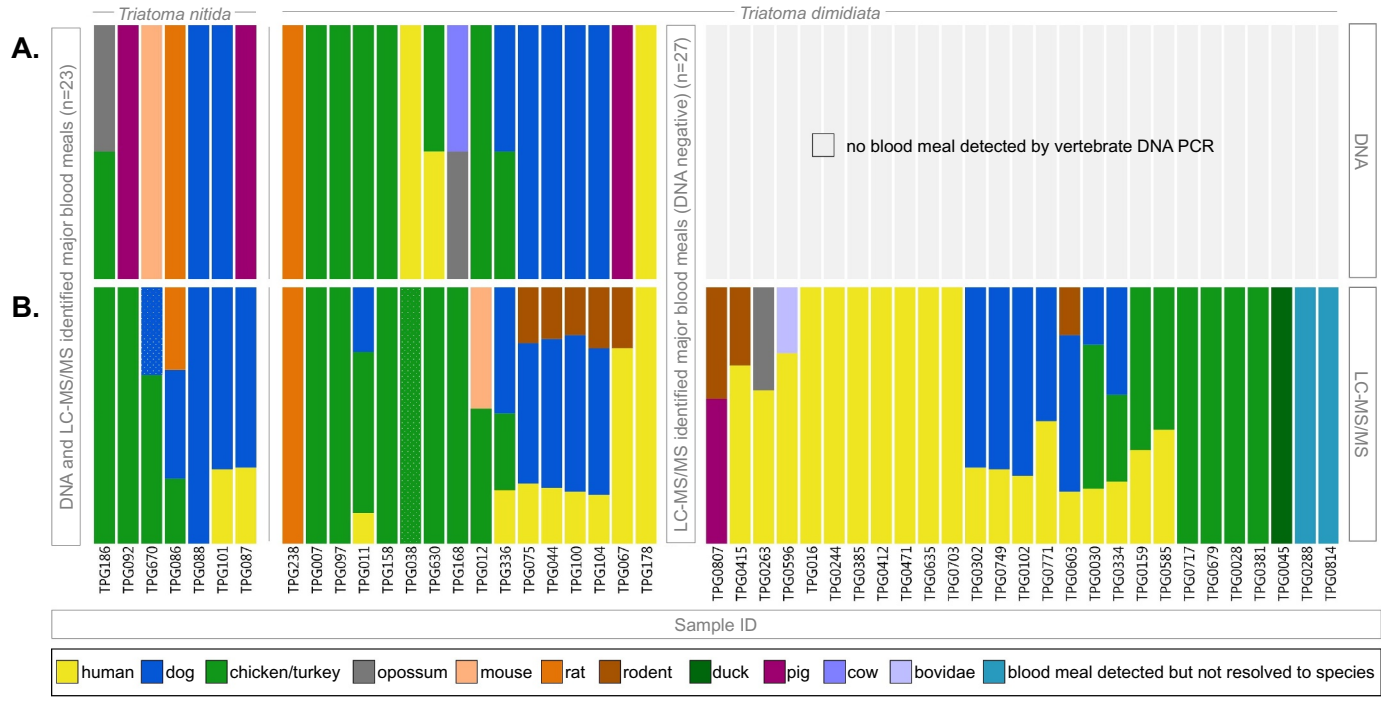

Fig. 4. DNA and LC-MS/MS-identified major bloodmeals in 50 Triatomine vectors from Guatemala. For LC-MS/MS-identified bloodmeals, the proportion of each bloodmeal are shown using the number of peptides supporting each blood source. (A) DNA-identified bloodmeals were only identified to class level for birds and are indicated by green color in comparison to (B) LC-MS/MS that identified to species level (chicken/turkey). JKop06_TPG038 was identified as chicken/turkey/francolin (dotted green). JKop28-TPG670 was identified to 6 Canidae species (dotted blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rodents were the most common bloodmeals among 170 vectors in Guatemala, where an additional 398 vectors had no evidence of a recent bloodmeal. Of the subset of 50 specimens reanalyzed here, we identified the same major bloodmeal sources, but at higher frequency. Notably, LC-MS/MS identified significantly more human bloodmeals (LC-MS/MS $\bar{x} = 1.6$, DNA $\bar{x} = 0.5$, $t = 7.66$, $P < .0001$). Therefore, avoiding vector-human contact should be an important aspect of disease control.

Does handling of vectors by humans cause contamination and inflate DNA-based estimate of human blood meals? Our study is uniquely posed to address this question because contamination is almost impossible when using LC-MS/MS targeting hemoglobin molecules since samples would need to be exposed to blood to become contaminated. The identification of significantly more human blood meals with LC-MS/MS strongly refutes this possibility.

While the most important blood source species in this location (human, chicken, dog) agreed in identification by LC-MS/MS and DNA, there were a few discrepancies. For example, in three samples DNA identified pig as the blood meal source, while LC-MS/MS detected chicken, dog and human, and human and rodent. Pig blood meals were of low abundance in context of the entire study (1.4%, $n = 8$ of 568) (Lima-Cordón et al., 2018), thus this inconsistency does not influence the Ecohealth recommendations from blood meal analysis.

Bio-socio-ecological factors can influence domiciliary infestation of *Triatoma* (Bustamante-Zamora et al., 2015; Lima-Cordón et al., 2018), so it comes as no surprise that the presence of biological factors such as humans, dogs, chickens, rodents, pigs, and livestock being kept in and around houses influences bloodmeal composition of these vectors (Rabinovich et al., 2011). All possible domestic blood sources were present in the majority of houses (with the exception of larger animals like pigs and livestock), and LC-MS/MS identified these blood sources in the vector (Fig. 5). Multiple-blood-source-feeding indicates the ability of the insect to adapt to different conditions and the versatility in adaptation to multiple blood sources. Interestingly, although most (19)

Table 4
Comparison of DNA and LC-MS/MS identified bloodmeals. Detection of bird and dog blood sources significantly agreed across detection methods, while human and rodent blood source identification did not. Human and rodent blood source comparison indicates that LC-MS/MS identified more of these blood sources than did DNA. Only samples where DNA and LC-MS/MS bloodmeal data were available are included. Specimens where bloodmeals were determined using 12 S sequencing were not included.

Blood source	Agreement statistic					
	Degree of Agreement				Bowker's Test	
					Symmetry of Disagreement	
	Kappa Coefficient	Level of Agreement	Prob > z	Prob > |Z|	ChiSquare	Prob > ChiSquare
Human	0	poor/none	0.5	1	6.4	0.0114*
Bird	0.7	moderate/good	0.0005*	0.0010*		
Dog	0.61165	moderate/good	0.0015*	0.0030*		
Rodent	0.146341	poor/none	0.1045	0.2089	7	0.0082*

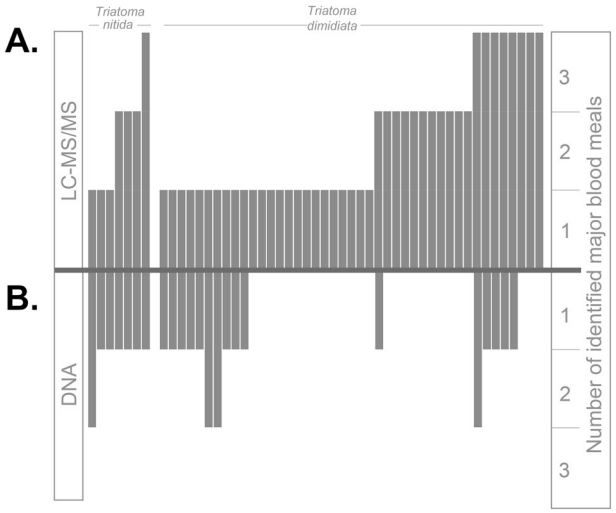

Fig. 5. Number of major bloodmeals detected within an individual specimen with (A) LC-MS/MS and (B) DNA. Two samples where a bloodmeal was detected with LC-MS/MS, but not resolved to species, are represented as a single bloodmeal source.

households had multiple bloodmeal sources, in some households we detected only a single bloodmeal source (human, 4; bird, 7; and dog, 1) (Fig. 6). Determining why vectors in some households are more likely to feed exclusively on humans would enhance Ecohealth efforts, as house improvement within Ecohealth could help avoid human-vector contact (Pellecer et al., 2013).

Movement of vectors or bloodmeal sources among houses can be inferred when LC-MS/MS detected a blood source not present in the household (Fig. 6). For example, dog (three cases) and bird (one case) were detected in households that were not recorded to have these species (Table 1, Fig. 6). However, due to the close proximity of houses, the fact that dogs and some birds are free-ranging, and studies indicating vectors often move among houses (Dumonteil et al., 2018; Gurtler et al., 1997; Stevens et al., 2014), it is not surprising a vector would contain bloodmeals from various sources nearby. While one vector fed on pig in a household that had pigs, another vector also fed on cow in a household that did not own cows, showing the mobility of the vector between intradomicile and peridomicile environments.

Similarly, in one case LC-MS/MS detected a rodent while there was no visible presence of rodents in a home at the time of survey (Table 1, Fig. 6). This could have simply been attributed to visually missing a trace of a rodent during the survey or vector movement among houses. Occasionally, LC-MS/MS detected a rodent bloodmeal, but we were not

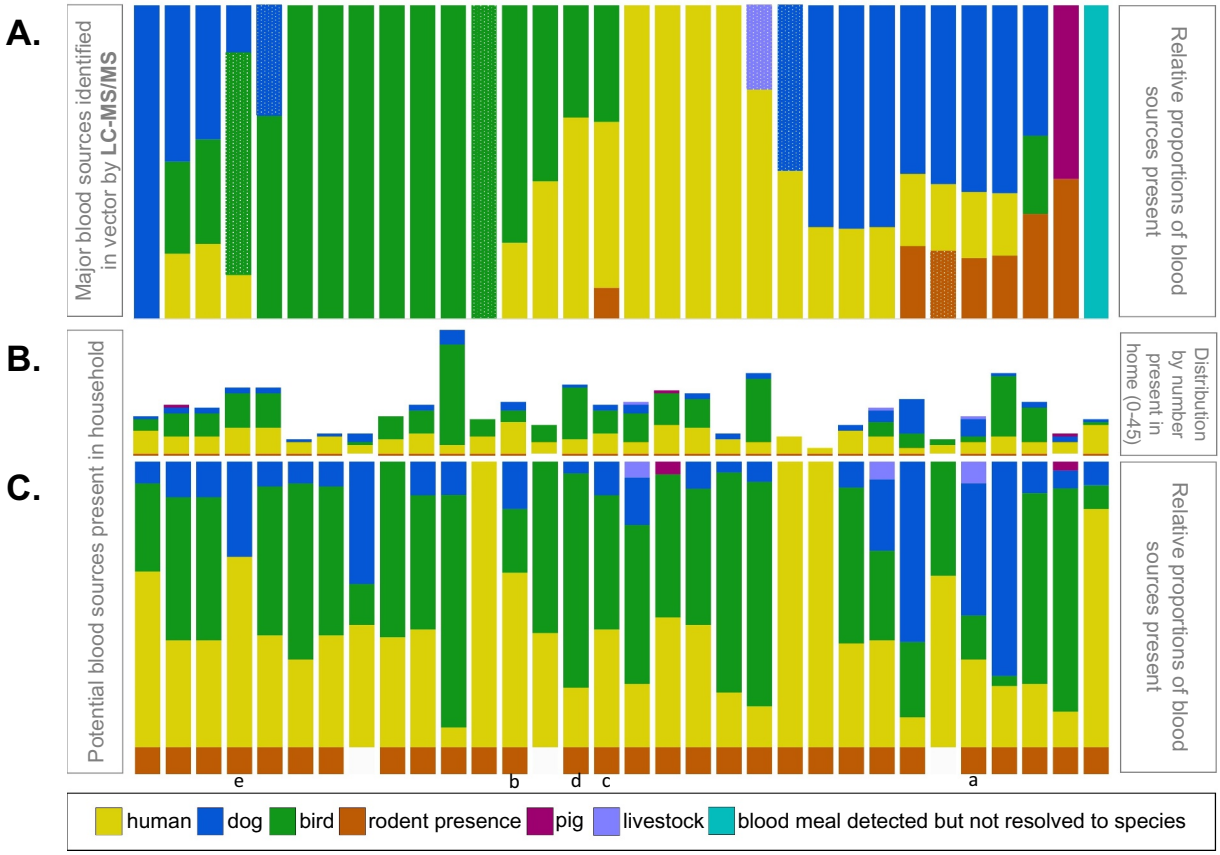

Fig. 6. Comparison of potential blood sources per household based on pre-intervention survey data and LC-MS/MS-identified bloodmeals. (A) The major bloodmeal identified by LC-MS/MS in specimens found in a single household, shown as a relative proportion in terms of number of peptides supporting the identified major bloodmeal source. (B) The distribution of potential bloodmeals per household based on pre-intervention survey data, including the number of inhabitants per house (human blood source). (C) The relative proportion of potential bloodmeals per household. (a-e) indicated households where more than one specimen was collected. Rodent survey data is represented by evidence of presence/absence of rodents in home only. Dotted bloodmeals represent LC-MS/MS-identified blood sources not present in the indicated household. Only households and specimens with complete pre-intervention survey data are shown.

able to identify the species. This could be because it was a small bloodmeal or the vector fed on rodent quite some time ago, possibly even an earlier instar. Rodent presence has been known to be associated with persistent intra-domiciliary *T. dimidiata* infestation in Guatemala, and our results reinforce that rodent control would complement vector control efforts (Bustamante et al., 2014; De Urioste-Stone et al., 2015).

We detected two additional sylvatic blood sources, both ground squirrels in the family Sciuridae. The two species, *Ictidomys tridecemlineatus* and *Otospermophilus beecheyi,* are not found in Guatemala, suggesting a local species not represented in GenBank. Sylvatic bloodmeals were twice as common (8/50, 16%) as previously reported (7%, (Lima-Cordón et al., 2018)). This again shows the mobility of the vector species driven by food source availability, in this case moving from sylvatic to domestic environments.

Not only did hemoglobin-based LC-MS/MS detect bloodmeals in all specimens compared to about 50% with DNA, but also more bloodmeals were detected per specimen (Fig. 4, Fig. 5, Table 4). The multiple blood sources detected by our enhanced pipeline were confirmed by presence/absence of native peptides compared to SRPs (Fig. 3). For two samples, we did not initially identify a bloodmeal to species, yet with relaxing our high stringency filter, we identified the species for both (Supplementary Table 6). Thus, in future implementations of this approach, peptide filters can be made less stringent with the caveat that species-specific identification needs to be examined carefully for specimens where no bloodmeal species are identified. Even with our stringent filtering, we detected 11 different bloodmeal sources, a number comparable to similar studies (Dumonteil et al., 2018; Lima-Cordón et al., 2018; Stevens et al., 2012; Waleckx et al., 2014).

In addition to detecting more blood sources, mass spectrometry for bloodmeal analysis is affordable. We previously documented costs as low as $4.75–$10 per sample (self-run vs. facility charge) (Keller et al., 2017). Cost for DNA analysis by PCR include DNA extraction kits and PCR reagents and which are generally more expensive than preparing samples for LC-MS/MS. In addition, a major advantage of LC-MS/MS is the ability to determine multiple blood sources with a single run, rather than a separate PCR reaction for each species. Cloning materials (approximately $500 for 20 samples) are costly and DNA sequencing is comparable to outsourcing LC-MS/MS samples. Although genomics has the potential to identify multiple blood sources in a single specimen, costs can be over $50 per sample, require a large amount of high-quality DNA, and can require additional reactions (Dumonteil et al., 2018; Kieran et al., 2017). Even when top-of-the-line mass spectrometers are not available, our enhanced pipeline is easily tailored to lower resolution mass spectrometers, such as the LTQ instrument tested here. Further, as with DNA samples, shipping protein samples to a facility is very feasible.

Quantification of hemoglobin peptides can lead to identifying bloodmeal species not currently in the underlying database by comparing the quantity of general hemoglobin peptides to species-specific peptides (Keller et al., 2017). This concept, however, will need further experimentation as peptides decay at varying rates as the insect vector digests the bloodmeal. Careful consideration of SRP sequences should also include attention to modifications on specific amino acids, e.g., oxidation on methionine. Details of the relative decay and amino acid modification over time are needed as this can influence the amount of peptide identified. Therefore, we present here the first step in this endeavor by quantification with our general SRPs, a proxy for total amount of hemoglobin identified within an insect (Fig. 7). Using spectral count as an indirect quantification method for a particular protein in a sample has been previously shown (Lundgren et al., 2010), and as expected, the number of hemoglobin peptides identified in a sample increased with the amount of hemoglobin. Knowing the amount of hemoglobin in a vector can aid in deciding if peptide filtering needs to be of lower stringency to identify a bloodmeal for some specimens.

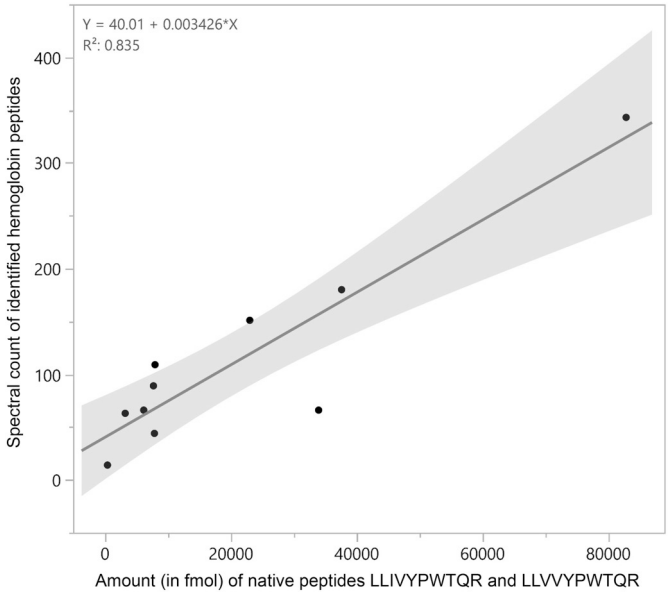

Fig. 7. The fmol amount of native hemoglobin in a vector increased with spectral count, the number of hemoglobin peptides in a sample. Only the linear range prior to saturation was used to establish the linear regression line (R^2 = 0.835).

4.1. Conclusion

Mass spectrometry-based studies are increasing and have wide application, including multi-faceted ecological studies like the one presented here. Hemoglobin peptide-based LC-MS/MS allowed us to identify single and multiple bloodmeals in Triatomine insect vectors with high confidence using our enhanced blood source pipeline. Synthetic reference peptides allowed us to verify our pipeline and quantify hemoglobin in a vector, a first step for further bloodmeal composition studies. While our approach has been very successful, the approach could be modified using multiple reaction monitoring (MRM) analysis depending on equipment available (e.g., triple quadrupole instruments) (Kettenbach et al., 2011). Capturing additional peptide variants using de novo peptide sequencing with PEAKS software or using an additional search tool such as Mascot or Byonic in error-tolerant mode could be used to enhance our underlying hemoglobin database. However, multiple blood meals and variation in decay rates among peptides would limit the utility of this approach. In summary, accurate blood source identification to the species level as shown here, can lead to sound vector management decisions and enhanced Eco-health information for large-scale projects in Latin America.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103998.

Author contributions

JIK, LS, BB, CM conceived and designed the study.
JIK performed experimental lab work and all data analysis.
FZ, AH contributed analytical tools.
AS ran samples on the mass spectrometer.
JIK wrote the manuscript.
JIK, LS, BB, CM, RL, AS edited manuscript drafts.
All authors have read and approve the manuscript.

Declaration of Competing Interest

None.

Acknowledgements

This work was supported by: NIH grant 8P20GM103449 (BAB) from the INBRE program of the NIGMS, NIH grant R03AI26268/1-2 (LS) from NIAID, NSF grant BCS-1216193 (LS, CM) as part of the joint NSF-NIH-USDA EEID program; IDRC grant 106531 (CM) for insect capture; the University of Vermont CAS Seed grant (LS), University of Vermont CAS Suitor Award (JIK), University of Vermont Graduate College MiniGrant (JIK). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the funders. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We thank all collaborators at San Carlos University/LENAP for insect sample collection and initial processing, and Kristiaan Finstad and the UVM Stein Lab for providing a mouse blood control sample. Thank you to Jeffrey Knott and Jason Reynolds at Cell Signaling Technology for synthesizing the stable isotope-containing peptides.

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