El Colegio de la Frontera Sur

Respuesta comportamental y electrofisiológica de ninfas de *Triatoma dimidiata* Latreille (Hemiptera: Reduviidae) a volátiles de heces de conespecíficos

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Dedicatoria

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Introducción

La enfermedad de Chagas es una padecimiento potencialmente mortal causado por el parásito *Trypanosoma cruzi* Chagas (Protozoa, Mastigophora). Esta enfermedad está distribuida principalmente en América Latina, donde se transmite a los humanos principalmente por las heces de insectos triatominos. A nivel mundial, existen cerca de diez millones de personas infectadas y más de 25 millones están en riesgo de adquirir la enfermedad (OMS, 2012).

Triatoma dimidiata Latreille (Hemiptera: Reduviidae) es considerado uno de los vectores mas importantes en la propagación de la enfermedad de Chagas y se distribuye desde el norte de América del Sur (Colombia, Venezuela, Ecuador y Perú), en todos los países de América Central y en las zonas costeras de México (Pacífico y Golfo) (Ibarra-Cerdeña *et al*., 2009). Es la única especie que abarca naturalmente, Norte, Centro y el sur de America, reconociéndose tres genotipos del complejo *dimidiata* en México (Lehmann *et al.,* 2005; Bargues *et al.,* 2008). Esta especie ha sido encontrada en hábitats domésticos en 14 estados de la Republica (Salazar- Schettino *et al.,* 2005), y se sabe que entre 19 y 34% de los individuos *de T. dimidiata* que infestan las viviendas se encuentran infectados con *T. cruzi* (Dumonteil *et al*., 2004; Monroy *et al*., 2003; Nakagawa *et al*., 2005)

En lo que respecta a su comportamiento, los triatominos son insectos que pasan largo tiempo en sus refugios donde se agregan en grupos de individuos de diversos estadios. Esta agregación se debe inicialmente por su tendencia a evitar la luz, una vez dentro de un sitio oscuro, los triatominos buscan mantener un intenso contacto con el sustrato y

con conespecíficos, eligiendo preferentemente lugares estrechos (Lorenzo, 2012). Durante las horas del día, los triatominos se encuentran generalmente agregados en las grietas de paredes y otros lugares protegidos, que dejan después del anochecer en busca de alimento (Lorenzo & Lazzari, 1998). Durante este proceso, los triatominos pueden utilizar diferentes señales físicas y químicas para orientarse hacia los refugios utilizados por conespecificos (Lorenzo & Lazzari, 1998). Se ha documentado que muchas especies de triatominos son atraídos y se agregan alrededor de las heces de conespecíficos y heteroespecíficos (Schofield & Patterson, 1977; Cruz-López *et al*, 1993; Lorenzo Figueiras *et al*., 1994; Lorenzo & Lazzari, 1996; Lorenzo Figueiras & Lazzari, 2002; Vitta *et al*., 2002;.. Vitta *et al*, 2007; Pires *et al*, 2002). La mayoría de los estudios han analizado el efecto de las heces secas en la atracción y agregación de ninfas y adultos (Cruz-López *et al.*, 1993; Lorenzo Figueiras *et al*, 1994) Por el contrario, las heces frescas parecen tener un efecto repelente en triatominos (Lorenzo Figueiras *et al.,* 1994). Las heces mostraron un cambio en la composición volátil durante el tiempo (Lorenzo Figueiras & Lazzari, 2000; Mota *et al.*, 2014.) Y éstas se vuelven atractivas 3 horas después de haber sido depositadas hasta un máximo de 10 días o más si son rehidratadas (Lorenzo Figueiras & Lazzari, 2000).

Hasta el momento pocos trabajos han intentado descifrar la composición química de la señal de agregación de las heces (Cruz-López & Morgan, 1995; Taneja & Guerin, 1997; Mota *et al*., 2014). Los primeros autores encontraron una serie de compuestos hidrosolubles en las heces de ninfas *Triatoma infestans y Triatoma mazzoti,* pero las pruebas comportamentales realizadas no mostraron respuestas de agregación significativas. Los principales compuestos encontrados fueron la o-aminocetofenona, 4

metilquinazolina y 2,4 dimetilquinazolina. Alzogaray *et al. (*2005) demostraron que en ninfas de quinto estadio y en hembras adultas de *T. infestans* hubo un aumento significativo del número de visitas al área de presentación del estímulo a dosis de 50 µg de una mezcla de 4-metilquinazolina y 2,4-dimetilquinazolina. Taneja & Guerin (1997) detectaron emisión de amoniaco a partir de heces humedecidas de ninfas de quinto estadio de *T. infestans* y describieron que este compuesto promovía respuestas electrofisiológicas en sénsulos antenales. Mota *et al. (*2014) a través de técnicas de Microextracción en fase sólida (SPME) y cromatografía de gases lograron identificar cinco compuestos presentes en heces de ninfas de tercer y cuarto estadio de tres especies*: T. infestans, Panstrongylus megistus y Triatoma brasiliensis.* Estas sustancias fueron analizadas para probar atracción y habilidad para reclutar a los insectos a sus refugios. Los compuestos identificados fueron: acetamida, 2,3-butanodiol, ácido acético, ácido 3-metil-butírico y ácido hexanoico. Ninfas de quinto estadio fueron atraídas significativamente a refugios cebados con una mezcla de 160 ng o 1.6 µg de cada sustancia.

Se ha determinado a través de algunos estudios con triatominos que el comportamiento de agregación de estos insectos involucra dos eventos: El primero relacionado con la atracción a las heces, mediada por una señal volátil presente en las deyecciones de las chinches y el segundo ocurre una vez que las chinches hacen contacto con las heces, donde intervienen compuestos poco volátiles que fomentan la permanencia de los insectos dentro de sus refugios y en la proximidad de sus conespecíficos, esto a través de la inhibición de la locomoción (Cruz-López *et al*.,1993; Rojas & Cruz- López, 1994; Lorenzo, 2012).

Como se mencionó anteriormente, poco se sabe de la composición química de las substancias volátiles presentes en las heces a pesar de la clara evidencia comportamental y electrofisiológica de la atracción de varias especies de triatominos. Por lo que surge la necesidad de ampliar el conocimiento de la naturaleza química de las heces en nuevas especies. Cabe mencionar que no hay estudios previos sobre la atracción y agregación mediada por heces, para *T. dimidiata.*

Por lo anterior, en este estudio, se ha analizado la respuesta comportamental y antenal de los dos primeros estadios ninfales (estadios menos evaluados en triatominos) de *T. dimidiata* a volátiles de heces secas y frescas de sus conespecíficos (heces de ninfas de quinto estadio). En primer lugar, se evaluó la respuesta comportamental de *T. dimidata* a papel filtro impregnado con heces y a extractos de heces frescas y secas; En segundo lugar, se evaluó la actividad electrofisiológica de los extractos a través de electroantenografia (EAG); en tercer lugar, se analizaron mediante cromatografía de gases-espectrometría de masas los extractos de heces; y, por último, se evaluó la actividad biológica de los compuestos identificados.

La información sobre el comportamiento y fisiología de *T.dimidiata* puede ofrecer herramientas importantes para su control en las condiciones epidemiológicas y operacionales actuales.

Respuesta comportamental y electrofisiológica de ninfas de *Triatoma dimidiata* a volátiles de heces de conespecíficos

Sometida al Parasites & Vectors

Behavioural and electrophysiological responses of *Triatoma dimidiata* nymphs to conspecific faecal volatiles

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1 **Abstract.**

2 **Background**

3 *Triatoma dimidiata* Latreille (Hemiptera: Reduviidae) is one of the main vectors of
4 Chagas disease; it is distributed from North to Central and South America. Many
5 triatomine species are attracted and aggregate around their conspecific as well as
6 heterospecific faeces in laboratory experiments, highlighting the effect of dry faeces on
7 the attraction and aggregation of nymphs and adults.

8 **Methods**

9 The behavioural and electrophysiological (using EAG) responses of the first two instars
10 of *T. dimidiata* to fresh and dry faecal, extracts and synthetic compounds are analyzed.
11 Gas chromatography-mass spectrometry analyses of faecal extracts demonstrated the
12 presence of 12 compounds in dry faecal.

13 **Results**

14 Recently emerged nymphs (3-5 d-old) aggregated around filter papers impregnated with
15 dry faeces and fresh, and dry faecal extract impregnated filter papers. Older 1st instars
16 (10-15 d-old) and 2nd instars aggregated to fresh and dry faecal impregnated filter
17 paper, and their respective extracts. Dry faecal extracts elicited the strongest antennal
18 responses, followed by fresh feacal extracts. Analyses of faecal extracts demonstrated
19 the presence of 12 compounds in dry faecal, identified as 2-ethyl-1-hexanol, 1,2,4-
20 trimethyl benzene, octadecane, nonadecane, eicosane, heneicosane, tricosane,
21 pentaeicosane, hexaeicosane, octaeicosane, nonanal, and 4-methyl quinazoline. In
22 fresh faeces, only nonanal was clearly detected, although there were other trace
23 compounds, including several unidentified sesquiterpenes. When 12 of these

compounds, were tested (except for 4-methyl quinazoline), only four of the compounds (2-ethyl-1-hexanol, octadecane, nonadecane and tricosane) elicited aggregation behaviour when tested individually at concentrations of 100 ng/µl and 1 µg/µl. A blend containing these four components also mediated the aggregation of nymphs.

Conclusions: In this study, we were able to identify four compounds from faeces that mediate the aggregation of the first two instars of *T. dimidiata* nymphs. Eventually, this information may be valuable to develop monitoring methods and design sensitive strategies to detect and measure *T. dimidiata* infestation.

Keywords

Chagas disease, Triatominae, Faeces volatiles, Aggregation

Background

Chagas disease is caused by the protozoan *Trypanosoma cruzi* Chagas (Protozoa, Mastigophora), mainly distributed in Latin America, and transmitted principally by insects of the subfamily Triatominae. There are about ten million infected persons worldwide, and more than 25 million are at risk of acquiring this Disease [1].

Triatoma dimidiata Latreille is one of the main vectors of Chagas disease; it is distributed from North to Central and South America [2]. In Mexico, three haplogroups of the *T. dimidiata* complex are present [3,4].

Triatomine bugs are haematophagous insects, feeding mostly from the blood of birds and mammals. During daylight hours, triatomine bugs are usually found in burrows, rock

outcroppings or caves, or in wall crevices of houses and other protected places, which they leave after dusk to seek food [5]. Triatomines use different physical and chemical cues to orient towards host refuges used by conspecifics [5]. Many triatomine species are attracted and aggregate around their conspecific as well as heterospecific faeces in laboratory experiments, highlighting the effect of dry faeces on the attraction and aggregation of nymphs and adults [6, 7, 8, 9, 10, 11, 12,13]. Fresh faeces are reported to have a repellent effect on triatomine bugs [8] and there is an apparent shift in volatile composition over time [14,15], with attraction 3 h after being deposited, and for 10 days, or longer if they are maintained in solution [14]. Despite the biological evidence of the attraction and aggregation due to chemical compounds of several triatomine species to their faeces, few studies have analyzed the chemical composition of triatomine faeces [15,16,17]. In the present study, we have analyzed the behavioural and antennal responses ofthe first two instars of *T. dimidiata* nymphs to conspecific faecal volatiles. The behavioural response of *T. dimidata* to faeces impregnated in filter papers and to Porapak Q extracts of fresh and dry faeces were evaluated. Subsequently, the electrophysiological activity of the extracts was evaluated using an electroantennogram (EAG), faecal extracts were analyzed using gas chromatography-mass spectrometry, and the biological activity of identified compounds analyzed.

1 **Methods**

2 **Biological material**

3 *Triatoma dimidiata* bugs were obtained from a colony maintained at theCentro Regional
4 de Investigación en Salud Publica of the National Institute for Public Health (CRISP-
5 INSP) in Tapachula, Chiapas. Insects were originally collected in Manacal, Tapachula
6 county, Chiapas (92°09´38´´N,14°54´59´´ W). The insects used in this study belonged
7 to the fourth generation in insectarium of *T. dimidiata* haplogroup 3 [4]. *Triatoma*
8 *dimidiata* colonies were reared in acrylic flasks containing accordion folded paper to
9 increase climbing surface for the bugs. Insects were maintained with a temperature of
10 25-28 °C and 60-80% of humidity, and an 8:16 hour light/dark illumination cycle. Insects
11 were fed every third day using a live rabbit. First and second instar nymphs of *T.*
12 *dimidiata* were used in these experiments. First instar nymphs were either recently
13 hatched (3-5 day old, group 1A) or older (10-15 day old, group 1B), the latter having
14 greater mobility than the former. Second instar nymphs (2A) were used 7-10 days after
15 primary ecdysis. All nymphs were used unfed for 2-3 days. This starvation level was
16 selected considering that recently fed nymphs will not go for the food source [14]
17 however these nymphs can not spend more than a week without food. (Ramsey
18 personal communication),

19 **Faecal collection**

20 Bug faeces were collected as described elsewhere [8] from 5th instar nymphs, because
21 excreted the largest volume of faeces [18]. Briefly, the faeces were absorbed onto filter
22 paper afterpassing through a plasticized net which separated the bugs during feeding
23 (avoiding contact with other chemical cues). Fresh (between 3-7 hours after deposition)

1 and dry faeces (10 days after being collected) were used for bioassays; volatiles from
2 fresh faeces were collected between 0 and 24 hours. Filter papers impregnated with dry
3 faeces were maintained until use in 100 ml sealed glass vials at 30°C and 60 ± 5%
4 relative humidity.

5

6 **Headspace collection of faecal volatiles**

7 Dry and fresh *T. dimidiata* faecal volatiles were collected by placing 5 to 10 faecal
8 impregnated filter papers (10 cm diameter, Whatman International, Maidstone, England)
9 in a 250 ml glass flask. Headspace volatiles were collected in a glass column with
10 Porapak Q for 24 h at a flow rate of 1 L/min. Volatiles were eluted using 200 µl of
11 dichloromethane (HPLC grade) to a final volume of 2 ml. These samples were slowly
12 evaporated under a gentle stream of nitrogen to a final concentration of 500 µl and
13 extracts were stored at a -20ºC until analyzed and bioassays run.

14

15 **Bioassays**

16 Bioassays were performed using a circular arena (14 cm diameter x 2 cm height)
17 containing two pieces of filter paper (3 x 3 cm) (Whatman no. 1; Whatman International,
18 Maidstone, England) on opposite sides of the arena, one experimental (impregnated
19 with faecal, extract or synthetic compound), and the other with solvent (control). The two
20 filter papers were positioned 2 cm from the edge of the arena, and after each test they
21 were changed to avoid experimental bias. In each bioassay, five insects were placed in
22 the centre of the arena in an upside down 100 ml flask for 5 min before being released;
23 bug placement in the arena was recorded after 15 min. In the first series of experiments,

1 filter paper was impregnated with fresh or dry faeces (20 replicates/compound type)
2 versus control. In the second series, filter paper impregnated with 1 μl of faecal volatile
3 extract (fresh or dry) versus solvent control (30 replicates/compound type). In the third
4 series, the biological activity of 11 synthetic compounds from dry faeces was evaluated
5 (Table 1) (excluding 4-methyl quinazoline). The compounds were evaluated individually
6 at concentrations of 1 ng/μl, 10 ng/μl, 100 ng/μl, and 1μg/μl. After the compounds were
7 tested individually, active compounds were blended to test their combined effect on
8 biological activity. Two blends were tested, the first with 11 compounds (2-Ethyl-1-
9 hexanol, 1, 2, 4-trimethyl benzene, octadecane, nonadecane, eicosane, heneicosane,
10 tricosane, pentaeicosane, hexaeicosane, octaeicosane, nonanal) and the second with 4
11 compounds (2-ethyl hexanol, octadecane, nonadecane and tricosane). Both blends
12 were tested at concentrations of 100 ng/μl and 1 μg/μl versus solvent (30 replicates per
13 treatment). All bioassays were conducted between 08:00 to 16:00 h under dark
14 conditions at 26 ± 2 ºC and 60 ± 5% relative humidity, Insects were tested only once in
15 all series of experiments.
16
17 **Volatile analyses**
18 Headspace samples were analyzed using a gas chromatographer (Varian 3800) linked
19 to a mass spectrometer (Varian Saturn 2200), with a non polar Factor Four capillary
20 column VF-5MS (Varian, 30 m length x 0.25 mm internal diameter). All samples were
21 analyzed in splitless mode with helium at constant flow rate as carrier gas (1 ml/min),
22 and fragmentation by electronic impact at 70eV and trap temperature 200 ºC. The oven
23 temperature was programmed at 50 ºC for 2 min, then 15 ºC /min at 280 ºC and held for

10 min. Compounds were identified by comparison of retention times and mass spectral profile using NIST 98. Mass spectral identifications were confirmed by comparison of retention times and mass spectrum using synthetic standards. Synthetic compounds (95% pure) were obtained from Sigma-Aldrich and Fluka (Toluca, Mexico). The Kovats index was calculated for all compounds not identified by the previous method [19].

Electroantennography

Nymph antennal responses to faecal extracts were measured using electroantennography (EAG). The insect`s head was cut off using dissecting scissors; the reference glass capillary electrode filled with Ringer´s solution was inserted into its base and the tip of the recording glass capillary electrode was inserted into the distal end of the antenna [20]. Antennal signals were displayed on a monitor using Syntech EAG signal software [21]. Antennae were stimulated by air, dichloromethane, fresh and dry faecal extracts. Extracts or solvents (1 µl) were placed on filter paper (0.5 x 3.0 cm Whatman no. 1; Whatman International, Maidstone, England), exposed to air for 20 s, then inserted into a glass Pasteur pipette or sample cartridge, and left 40 s before applying. Stimuli were applied at 2 min intervals, starting with air, followed by dichloromethane, and finally both *T. dimidiata* faecal extracts (20 antenna were tested for 1B and 2A nymphs, and 13 antenna for 1A nymphs). The duration of stimulus was 1 s.

Statistical analyses

Data obtained from the circular arena tests were analysed in a Generalized Linear Model (GLM) using logistic regression with multinomial response (control, sample and without response). Since there was no significant difference between treatments (dry or fresh faeces) or among replicates (5), the data was then analysed using log-likelihood ratio (G-test) for goodness-of-fit with Williams' correction [22]. The null hypothesis was that the distribution of insects in the arena would have a 1:1:1 ratio, assuming that insects had the same opportunity to choose between treatment (1/3), control (1/3) or not to choose (1/3).

EAG data were transformed using the Box-Cox transformation [Y^(-0.2)] for analysis of variance (ANOVA), although untransformed means are presented in the figures for clarity. When significant treatment effects were found, mean comparisons were performed using the Tukey test. Analyses were performed using the software R Development CoreTeam [23].

Results

Behavioural response of nymphs to faecal impregnated filter paper

Youngest nymphs (1A) aggregated around filter paper containing dry faeces (57% vs 33% expected, G=23.47; df= 2; P< 0.001), but not to fresh faecal impregnated filters (29 % vs 33% expected, G=0.87; df=2; P=0.64) (Fig.1A). In contrast, 1B nymphs aggregated around fresh faecal filters (51% vs 33% expected, G=17.87; df=2; P< 0.001) as well as with dry faeces (52% vs 33% expected, G=18.85; df=2; P< 0.001) (Fig.1B). Similarly, 2A nymphs aggregated around fresh faecal impregnated filters (58% vs 33%

expected, G=40.2; df = 2; P< 0.001) and with dry faeces (62% vs 33% expected, G=45.3; df=2; P< 0.001 (Fig.1C).

Behavioural response of nymphs to dry (DFE) and fresh (FFE) faecal extracts

All nymphs aggregated significantly to dry or fresh faecal extracts (Fig. 2). DFE were attractive to 1A nymphs (45.3% vs 33% expected, G=10.05; gl=2; P=0.006), 1B nymphs (47.33% vs 33% expected, G=12.56; df=2; P= 0.001) and 2A nymphs (51.33% vs 33% expected, G=25.54; df=2; P< 0.001), and FFE also attracted 1A nymphs (45.3% vs 33% expected, G=9.46; gl=2; P=0.008), 1B nymphs (48% vs 33% expected, G=17.94; df=2; P< 0.001) and 2A nymphs (52% vs 33% expected, G=23.76; df=2; P< 0.001).

Electroantennography

Antennal responses were distinct for different types of treatments, with the greatest antennal response elicited by DFE, followed by FFE, in 1A nymphs (F= 46.036; df=3,36; P<0.05), 1B nymphs (F= 61.9059; df=3, 57; P<0.05), and 2A nymphs (F= 129.3918; df=3, 57; P<0.05) (Fig. 3).

Chemical identification of faecal volatiles

Dry faeces contained 12 compounds identified as2-ethyl hexanol, 1,2,4 trimethyl benzene, octadecane, nonadecane, eicosane, heneicosane, tricosane, pentaeicosane, hexaeicosane, octaeicosane, nonanal, and 4-methyl quinazoline(Table 1). Only nonanal was clearly detected in fresh faeces, although there were traces of other compounds, as well as several unidentified sesquiterpenes.

1 **Behavioural response of nymphs to synthetic compounds**

2 Eleven of the compounds identified from dry faeces were evaluated individually.
3 Nymphs aggregate to 2-ethyl hexanol, octadecane, nonadecane, and tricosane at 100
4 ng/µl and 1 µg/µl (Table 2) but did not have a preference for 1,2,4 trimethyl benzene,
5 eicosene, heneicosane, pentaeicosane, hexaicosane, octaeicosane, or nonanal at any
6 concentration (data not shown).
7 The1A nymphs did not aggregate to the11-component blend at any concentration,
8 although 1Bnymphsaggregated at 100ng/µl(48.6 % vs 33% expected, G=16.05; df=2;
9 P< 0.001), and 2A nymphs aggregated at 1 µg/µl(50.6 % vs 33% expected, G=19.94;
10 gl=2; P< 0.001) (Fig 4). The 4-component blend provoked a significant aggregation of
11 nymphs of all instars. Aggregation of 1A nymphs ocurred at 100 ng/µl (56.66% vs 33%
12 expected, G=34.96; df=2; P< 0.001)and 1 µg/µl (55.23% vs 33% expected, G=32.28;
13 df=2; P< 0.001), as did 1B nymphs at100 ng/µl (53.3% vs 33% expected, G=27.3;
14 df=2; P< 0.001) and 1 µg/µl (57.3% vs 33% expected, G=36.15; gl=2; P< 0.001).
15 Aggregation of 2A nymphs occurred at 100 ng/µl (56.6 % vs 33% expected, G=34.96;
16 df=2; P< 0.001) and 1µg/µl of this blend (55.33 % vs 33% expected, G=32.28; gl=2; P<
17 0.001) (Fig 5)
18

19 **Discussion**

20 The first two instars of *T. dimidiata* respond electrophysiologically and behaviorally by
21 aggregation to volatilespresent in conspecific faeces. This confirms that *T. dimidiata* has
22 a similar strategy to that of other triatomines for aggregation behaviour mediated, at
23 least in part, by chemical cues present in faeces [6, 7, 8, 9, 10, 11, 12, 13]. While few

studies have identified faecal compounds that promote triatomine aggregation, the present study does identify compounds that mediate, the aggregation behaviour of *T. dimidiata* nymphs. Cruz-López and Morgan [16] identified faecal extracts from *Triatoma infestans* (Klug) and *Triatoma mazzottii* (Usinger) using polar solvents and thermal desorption, compounds such as quinazolines, aldehydes, carboxylic acids and amides. However, these compounds were not attractive when evaluated experimentally, although Alzogaray *et al.* [24] demonstrated that *T. infestans* females and fifth instar nymphs were attracted to blends of two of the compounds previously identified by Cruz-López and Morgan [16] : 4-metilquinazoline and 2,4-dimetilquinazoline. Taneja and Guerin [17] found that *T. infestans*nymphs were attracted to ammonia, a compound released from wet faecal impregnated filter paper and recently Mota *et al.*[15] identified acetic acid, 3-methylbutyric acid, hexanoic acid, acetamide, and 2,3-butanediol in the faeces of *T. infestans*, *Panstrongylus megistus* and *Triatoma brasiliensis*, three species that promote inter-specific aggregation.

Among the compounds identified in *T. dimidiata* faeces, 1,2,4-trimethyl benzene had been not previously reported. Cruz-López and Morgan [16] previously found4-methylquinazoline in the faeces of *T. infestans* and *T. mazzottii,* and2-ethyl-1-hexanolwas reported in faeces of both *P. megistus* and *T.infestans* [25]. Although some of the hydrocarbons found in the faeces, particularly *n*-C23, *n* -C25, *n* -C26 and *n* -C28, have also been identified in the cuticle of *T. dimidiata* females and males [26] , the fact that these compounds have not been found previously in fresh faeces, supports their presence due to blood digestion or metabolic gut by-products, and not cuticle contamination. Since symbionts are present in the triatomine gut, faecal aggregation

compounds could be a metabolic by product of blood digestion or of these and other microorganisms, such as fungi and endosymbionts. Some of the compounds in fact increased from trace to measurable quantities from fresh to dry conditions, providing evidence that they may be of gut bacterial or fungal origin. Endosymbionts of the genus *Arsenophonus* were previously isolated from salivary glands and ovaries of *Triatoma pallidipennis, Triatoma mazzottii* and *Triatoma longipennis*, taxonomically close to the *dimidiata* complex [27] (Ramsey personal communication), and their association with both *Triatoma* and *Panstrongylus* genera midgut and faeces, was recently demonstrated [28]. Future studies will be required to ascertain the tissue/organism origin of *T. dimidiata* faecal volatiles.

Our results show clearly that youngest first instar nymphs (1A) responded only to dry faecal impregnated filter paper, while older first nymphs (1B) and second instars (2A) both responded to fresh, as well as dry faeces. The lack of response of 1A nymphs to fresh faeces, despite the fact that their faeces contain trace concentrations of aggregation compounds, could be the result of several factors. They have less mobility than 1B and 2A nymphs, making it less likely that they find faeces that release compounds that mediate aggregation behaviour. It is also possible that, as in the case of *T. mazzottii* nymphs, aggregation behaviour is affected by faecal quantity [29], supported by the fact that 1A nymphs were attracted to fresh faecal extracts and synthetic compounds. There is also the possibility that antennal receptors of 1A nymphs do not perceive volatiles produced by fresh faeces at those levels of concentration. However, our EAG results demonstrate that fresh faecal extracts provoke the same level stimulation in 1A nymph antennae as 1B or 2A nymphs. Inconsistent results have been

obtained when testing the association of nutritional status with bug responses [6, 7, 8]. Feeding status did not influence the response to faeces of *T*. *mazzotti* nymphs [7] even though *T*. *infestans* had an aggregation response as of eight hours after feeding [14].

Conclusions

In this study, we were able to identify four compounds from faeces that mediate the aggregation of the first two instars of *T*. *dimidiata* nymphs. Future studies will need to clarify the source of these compounds, and their potential specificity and sensitivity for all stages, in order to develop bug monitoring devices. Eventually, this information may be valuable to develop monitoring methods and design sensitive strategies to detect and measure *T. dimidiata* infestation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived the study: LCL, EMR, JCR, ZGM. Collected data: ZGM. Chemical identification: LCR and ZGM; Wrote the manuscript: ZGM, LCL, EMR, JMR and JCR. All authors read and approved the final version of the manuscript.

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1 Table 1. Compounds in dry faeces of *T. dimidiata* nymphs

2

Peak	Retention Time (min)	Compound
1	5.924	1, 2,4-Trimethyl benzene *
2	6.336	2-Ethyl-1-hexanol*
3	7.256	Nonanal*
4	9.916	4-Methyl quinazoline**
5	13.303	Octadecane*
6	14.043	Nonadecane
7	14.640	Eicosane*
8	15.238	Heneicosane*
9	16.462	Tricosane*
10	17.574	Pentaeicosane*
11	18.211	Hexaeicosane*
12	19.780	Octaeicosane*

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13 *Identified by retention time and mass spectrum of synthetic standards.

14 ** Tentatively identified with the mass spectra library NIST

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2 Table 2. Values of G and Probability of response of 1A, 1B, and 2A *T. dimidiata* nymphs to conspecific dry faeces compounds tested individually. Values of non

3 responders were not included. ns= not significant

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		Nymphs								
		1A			1B			2A		
Compound	Concentration	Sample	Control	Values of G and Probability	Sample	Control	Values of G and Probability	Sample	Control	Values of G and Probability
2-Ethyl-1-hexanol	**1 ng/µl**	63	44	ns	66	47	ns	62	49	ns
	10 ng/µl	69	49	ns	62	43	ns	73	44	**G=16.57, gl=2, P<0.001**
	100 ng/µl	65	46	ns	72	37	**G=13.45, gl=2, P<0.001**	79	47	**G=31.22, gl=2, P<0.001**
	1 µg/µl	82	35	**G=28.73, gl=2, P<0.001**	76	32	**G=20.43, gl=2, P<0.001**	82	38	**G=29.62, gl=2, P<0.001**
Octadecane	**1 ng/µl**	65	48	ns	62	46	ns	63	45	ns
	10 ng/µl	69	49	ns	69	41	**G=10.32,gl=2, P=0.005**	61	46	ns
	100 ng/µl	78	42	**G=24.07, gl=2 ,P<0.001**	68	49	ns	68	50	ns
	1 µg/µl	82	31	**G=29.25, gl=2 ,P<0.001**	72	35	**G=14.57, gl=2, P<0.001**	69	41	**G=10.32, gl=2, P=0.005**
Nonadecane	**1 ng/µl**	62	47	ns	65	48	ns	69	53	ns
	10 ng/µl	65	48	ns	75	41	**G=18.32, gl=2 ,P<0.001**	61	42	ns
	100 ng/µl	68	51	ns	73	36	**G=15.32, gl=2 ,P<0.001**	63	48	ns
	1 µg/µl	77	43	**G=22.87, gl=2, P<0.001**	68	48	ns	72	45	**G=15.60, gl=2, P<.001**
Tricosane	**1 ng/µl**	68	51	ns	68	53	ns	67	53	ns
	10 ng/µl	62	46	ns	67	49	Ns	63	49	ns
	100 ng/µl	71	43	**G=13.17, gl=2 ,P<0.001**	75	42	**G=18.75, gl=2 ,P<0.001**	72	43	**G=14.57, gl=2, P<0.001**
	1 µg/µl	68	54	ns	69	50	**G=14.8, gl=2, P=0.005**	74	39	**G=16.36, gl=2, P<0.001**

Figure Legends

Figure 1. Behavioural responses of 1A nymphs (A), 1B nymphs (B), and 2A nymphs (C) of *T. dimidiata* to fresh and dry faecal impregnated filter papers. *G Test, $P < 0.05$.

Figure 2. Behavioural responses of 1A nymphs (A), 1B nymphs (B), and 2A nymphs (C) of *T. dimidiata* to dry (DFE) and fresh (FFE) faecal volatile extract impregnated filter papers. * G Test; $P < 0.05$.

Figure 3. Antennal responses of 1A nymphs (A), 1B nymphs (B), and 2A nymphs (C) of *T. dimidiata* to air, dichloromethane (Dcm), dry faecal extract (DFE), and fresh faecal extract (FFE). Histograms followed by different letters are significantly different using Tukey's test, $P < 0.05$

Figure 4. Behavioural responses of 1A nymphs (A), 1B nymphs (B), and 2A nymphs (C) of *T. dimidiata* to the 11 compounds blend: 2-Ethyl-1-hexanol, 1,2,4-trimethyl benzene, octadecane, nonadecane, eicosane, heneicosane, tricosane, pentaeicosane, hexaeicosane, octaeicosane and nonanal. *G test $P<0.05$

Figure 5. Behavioural responses of 1A (A), 1B (B), and 2A (C) *T. dimidiata* nymphs to a 4 compounds blend: 2-Ethyl-1-hexanol, octadecane, nonadecane and tricosane. * G Test. $P < 0.05$.

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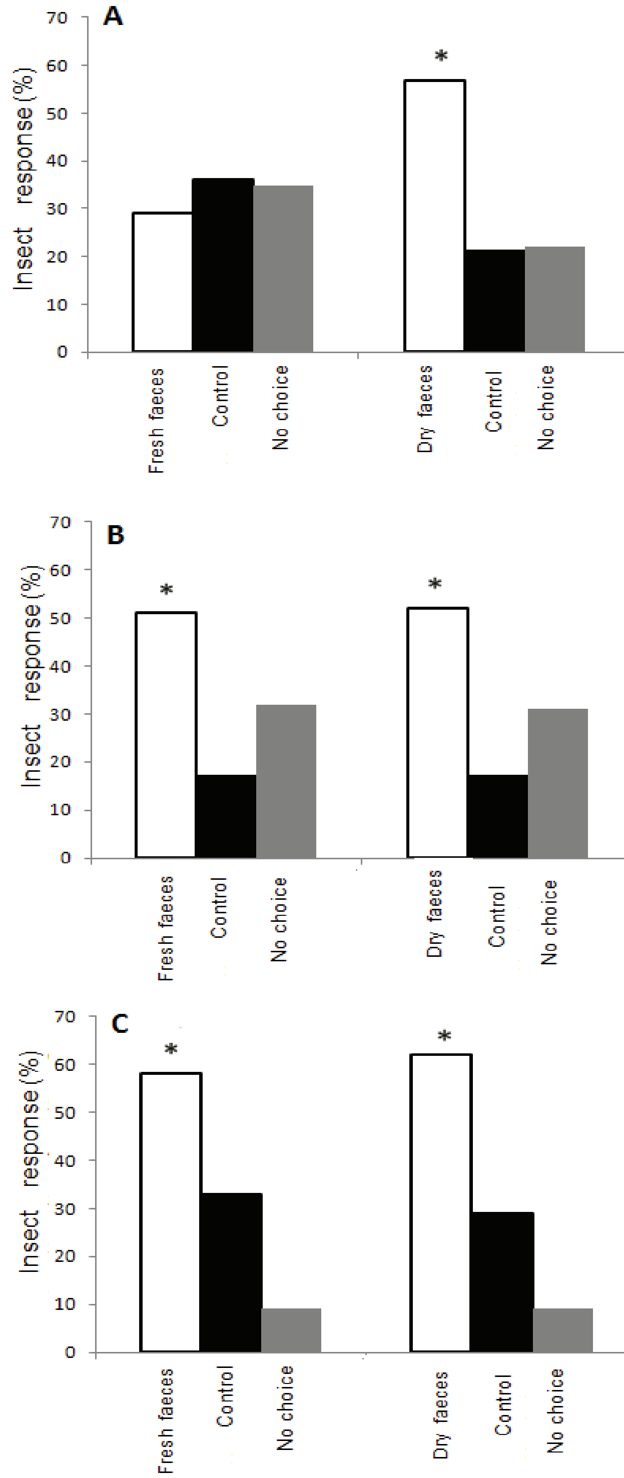

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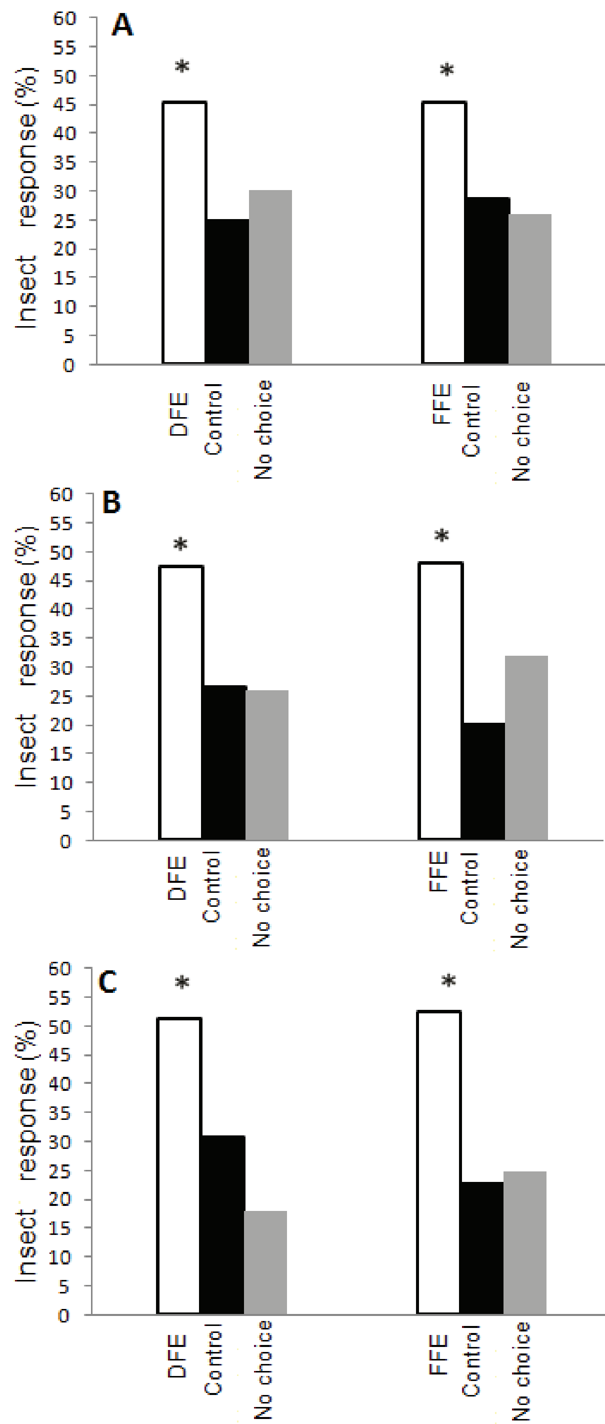

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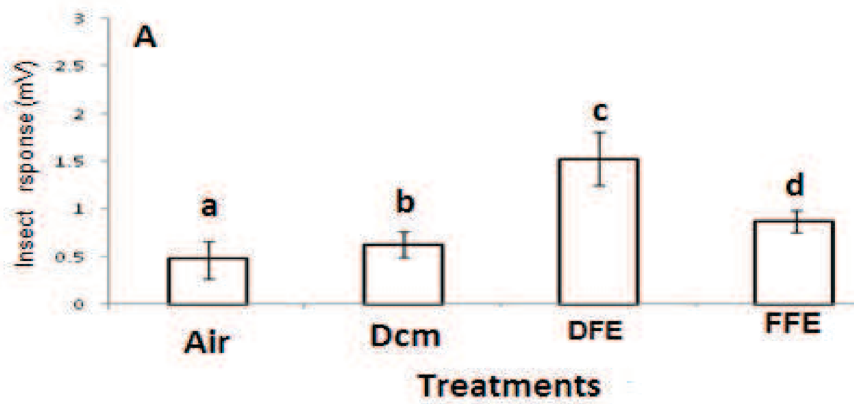

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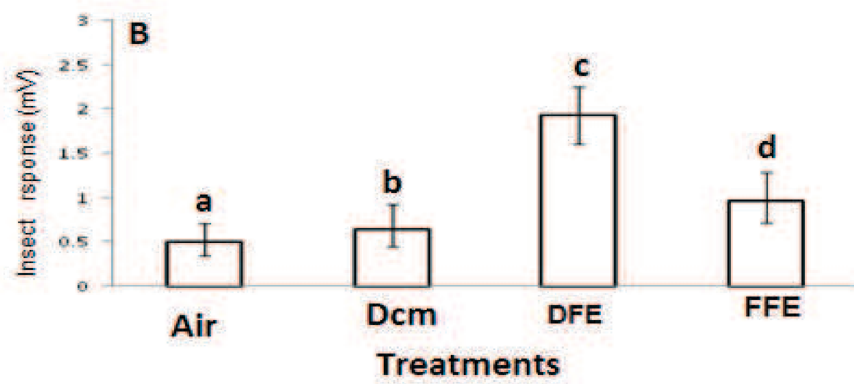

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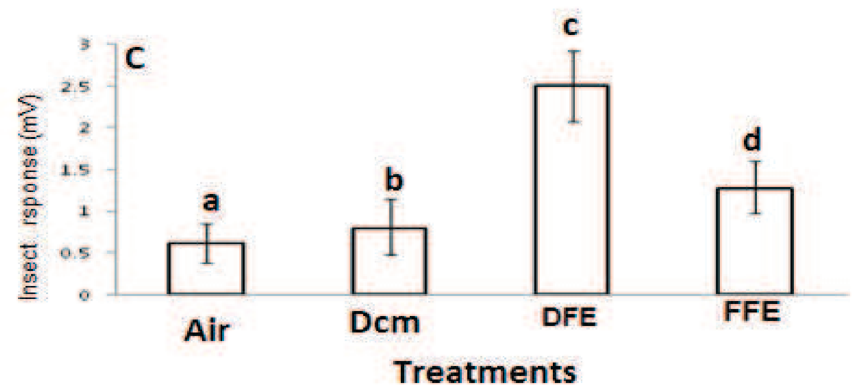

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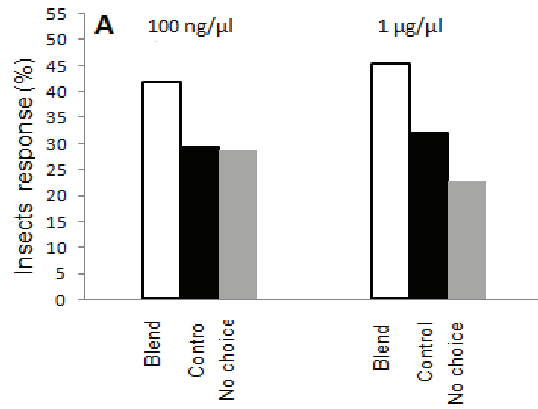

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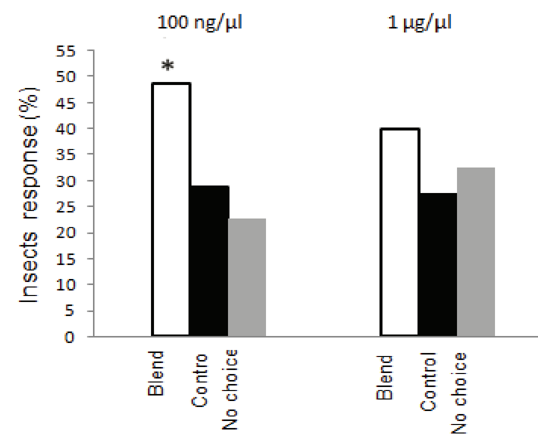

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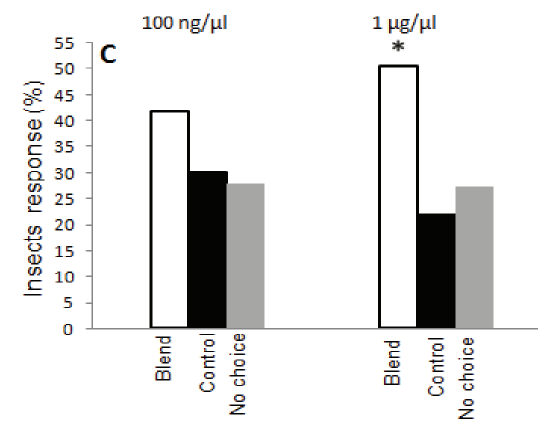

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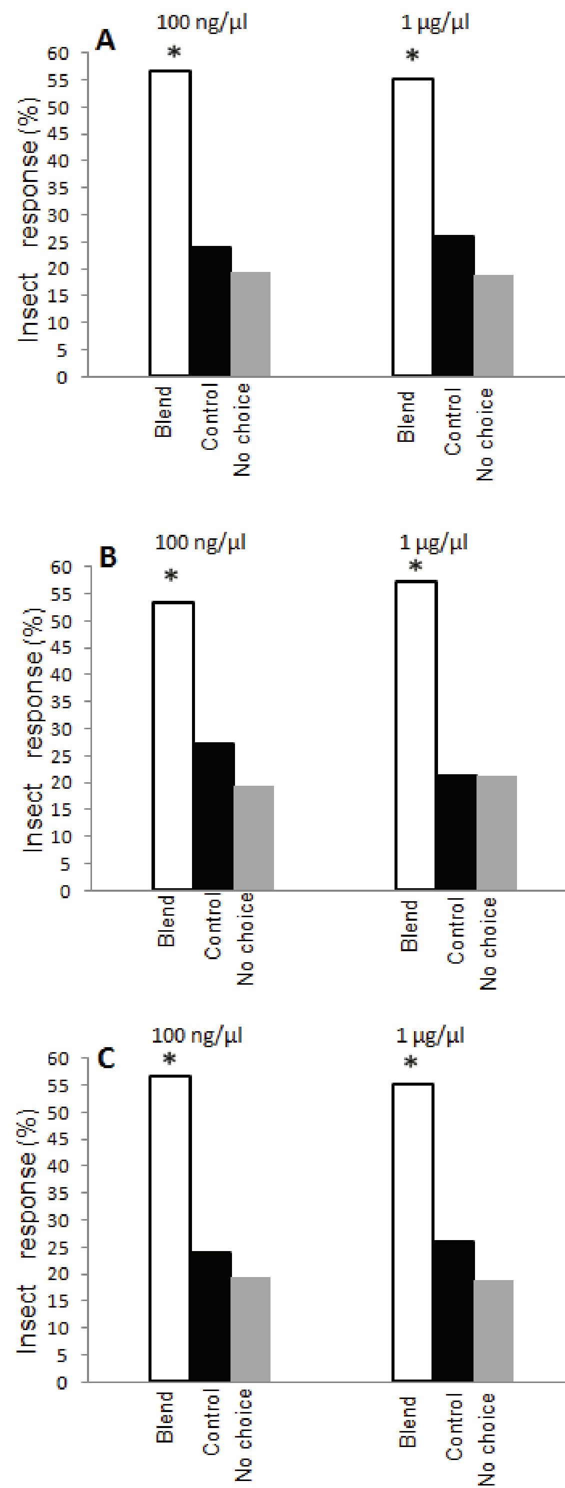

Conclusiones

1. Ninfas de primer y segundo estadio de *T. dimidiata* se ven atraídas a la señal química proveniente de sus heces (frescas y secas), estos insectos mostraron una tendencia significativa a agregarse a papeles impregnados con heces.

2. Los extractos de heces frescas y secas son capaces de atraer a las ninfas de primer y segundo estadio en ensayos comportamentales, al probar ambos extractos por electroantenografía provocaron respuestas significativamente altas a los volátiles de heces secas y en menor grado a los volátiles de heces frescas.

3. Se identificaron 12 compuestos en extractos de heces secas y frescas (el 2-etil-1- hexanol, 1,2,4-trimetil benceno, 4-metilquinazolina, octadecano, nonadecano, eicosano, undeicosano, tricosano, pentaeicosano, hexaeicosano, octaeicosano y nonanal). En heces frescas los compuestos anteriores se observaron en trazas a excepción del nonal que se observó claramente, además en este tipo de heces se logró determinar un grupo de varios sesquiterpenos desconocidos.

4. Se evaluaron los compuestos identificados de manera individual y en mezclas a diferentes dosis, reportamos una mezcla de cuatro compuestos sintéticos (2-etil-1-hexanol, octadecano, nonadecano y tricosano) capaz de atraer a ninfas de primer y segundo estadio de *T. dimidiata*.

Se necesitarán más estudios para aclarar el origen de los compuestos identificados y su posible especificidad y sensibilidad para todas las etapas de desarrollo, a fin de desarrollar dispositivos de monitoreo. La información obtenida en este estudio puede ser valiosa para el desarrollo de métodos de monitoreo y diseño de estrategias sensibles para detectar y medir la infestación de *T. dimidiata*.

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