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Electroantennogram responses of the *Triatoma dimidiata* complex to volatiles produced by its exocrine glands

I.J. May-Concha[a,b,*], P.G. Guerenstein[b], E.A. Malo[c], S. Catalá[d], J.C. Rojas[c]

[a] Laboratorio de Investigación en Triatominos (LIT), Centro de Referencia de Vectores (CeReVe), Ministerio de Salud de la Nación, Santa María de Punilla, Córdoba, Argentina
[b] Laboratorio de Estudio de la Biología de Insectos, Centro de Investigaciones Científicas y Transferencia de Tecnológica a la Producción (CICyTTP), Diamante, Entre Ríos, Argentina
[c] Grupo de Ecología y Manejo de Artrópodos, El Colegio de la Frontera Sur (ECOSUR), Tapachula, Chiapas, Mexico
[d] Centro Regional de Investigaciones Científicas y Técnicas de La Rioja (CRILAR), Mendoza y Entre Ríos, 5301, Anillaco, La Rioja, Argentina

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ABSTRACT

Members of the *Triatoma dimidiata* complex are vectors of the protozoan parasite *Trypanosoma cruzi,* the etiologic agent of Chagas disease. Morphological and genetic studies indicate that *T. dimidiata* complex has three principal haplogroups in Mexico. However, whether there are differences in the olfactory physiology among the haplogroups of this complex and a possible correlation with their antennal phenotype are not yet known. Antennal responses to 13 compounds released from the metasternal and Brindley´s glands, which are involved in the alarm and mating-related behaviours of *T. dimidiata* were investigated using electroantennography (EAG). Overall, of the 13 compounds tested, seven triggered EAG responses in both sexes of three Mexican haplogroups. The sensitivity of the EAG responses show some relationship with the total number of chemo-sensilla present on the antennae. Antennal sensitivity was different between sexes and haplogroups of the *T. dimidiata* complex. Discriminant analysis of EAG sensitivity was significant, separating the three haplogroups. Our finding is consistent with morphological and genetic evidence for haplogroups distinction within the complex.

1. Introduction

The olfactory system plays an important role in many aspects of the life of insects, including the searching for food, refuges and mates, as well as the avoidance of natural enemies. For instance, the olfactory system of many insect species has evolved a number of specializations that allow them to detect volatile compounds from conspecifics and hosts (Barrozo et al., 2016). Thus, a number of studies have shown that the olfactory receptors (housed in olfactory sensilla) from antennae are involved in the reception of semiochemicals used in the intra and interspecific communication of triatomine bugs (Pontes et al., 2014; Guidobaldi et al., 2014). However, those studies have only been performed in *Triatoma infestans* (Bernard, 1974; Taneja and Guerin, 1997; Guerenstein, 1999; Guerenstein and Guerin, 2001; Diehl et al., 2003), *Triatoma brasilensis* (Vitta et al., 2009), and *Rhodnius prolixus* (Lorenzo et al., 1999; Rojas et al., 2002). Therefore, studies on other important triatomine disease vector species are needed.

As other triatomine species, *Triatoma dimidiata* (Latreille) is an important vector of Chagas disease in Latin America. This species is now considered a complex of species because different populations vary in colour, size, morphological characters, exocrine compounds, and characters at the molecular level (Dorn et al., 2007; Bargues et al., 2008; Ibarra-Cerdeña et al., 2009; May-Concha et al., 2013; Monteiro et al., 2013; Ibarra-Cerdeña et al., 2014; Galvão and Justi, 2015; Gómez-Palacios et al., 2015; May-Concha et al., 2015, 2016). This species complex is distributed in Mexico, Guatemala, Belize, El Salvador, Honduras, Nicaragua, Costa Rica, Panama, Colombia, Venezuela, Guinée Française, Ecuador, and Peru (Dorn et al., 2007; Gourbière et al., 2012). This species complex represents a set of morphologically and morphometrically different populations throughout its distribution and is highly tolerant to habitat modification (e.g., high tolerance for secondary vegetation and human presence) in tropical evergreen and seasonal dry landscapes (Ibarra-Cerdeña et al., 2009; Grisales et al., 2010). In Mexico, there are three haplogroups of *T. dimidiata*: the haplogroup 1 (hg1) distributed in the Yucatán Peninsula (Lehmann et al., 2005; Calderón-Fernández et al., 2011), haplogroup 2 (hg2), distributed along the Gulf of Mexico and all the populations of the North Pacific of the Isthmus of Tehuantepec, and haplogroup 3 (hg3) distributed in the coast of the Pacific Ocean of Chiapas (Bargues et al., 2008; Ibarra-Cerdeña et al., 2009; Calderón-Fernández et al.,

* Corresponding autor at: Laboratorio de Investigación en Triatominos, (CeReVe), Hospital Rawson s/n, Santa María de Punilla (X5164), Córdoba, Argentina.
E-mail address: irving_jmc@hotmail.com (I.J. May-Concha).

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Table 1
Collection sites of *Triatoma dimidiata* used in this study. hg1: haplogroup Yucatan-Peninsula; hg2: haplogroup Gulf of Mexico; hg3: haplogroup Pacific-Coast.

Haplogroups	Community	County	State	Longitude (deg/min/)	Latitude (deg/min/)
	Eknakan	Cuzamá	Yucatán	89°22'15"	20°45'31"
hg1	San Pedro Chacabal	Motul de Carrillo Puerto	Yucatán	89°13'00"	21°07'06"
	Kantunil	Kantunil	Yucatán	89°02'04"	20°47'45"
hg2	Rio Blanco	Berriozabal	Chiapas	93°10'10"	16°25'26"
	Montecristo	Berriozabal	Chiapas	93°09'56"	16°25´´27"
hg3	Los Mangos Manacal, Lomas de Chiapas	Tuzantán	Chiapas	92°14'38"	14°54'59"

2011; Gómez-Palacios and Triana, 2014). Several aspects of the biology and ecology of the *T. dimidiata* complex have been studied in detail (Zeledón et al., 2008; Ibarra-Cerdeña et al., 2009; Abad-Franch et al., 2010) but no studies on the physiology of its olfactory system have been performed.

In this study, we investigated the electroantenogram responses of both sexes of the three haplogroups of the *T. dimidiata* complex, to odours emitted by adults of this complex, to determine whether there are differences in the olfactory physiology between haplogroups and to study a possible correlation with their antennal phenotype. The information obtained from this work may be useful for designing better strategies for behavioural manipulation of this triatomine species complex.

2. Materials and methods

2.1. Insects

Insects used in this study belonged to the *T. dimidiata* complex and were collected in domestic sites from the Yucatan and Chiapas states (Mexico), where the three haplogroups have been consistently isolated (Table 1). The haplogroup of the individuals used in this study was identified using molecular markers: one nuclear, (ITS2) and the other mitochondrial, (ND4) (Monteiro et al., 2013; Richards et al., 2013). The nomenclature used herein corresponds to that first used to designate Mexican haplogroups (Marcilla et al., 2001; Lehmann et al., 2005; May-Concha et al., 2015). Insects were reared and maintained separately for four generations at 27 ± 1 °C, 70 ± 5% RH, a photoperiod of 12: 12 (Light: Dark) h, and were allowed to feed every week on rabbit blood (New Zealand White).

2.2. Chemicals

The compounds used were selected considering previous studies and included volatiles from the metasternal and Brindley´s exocrine glands, which were involved in the alarm and mating behaviours of this species (May-Concha et al., 2013, 2015). The compounds used were 1-octen-3-ol, 3-methoxy-2-5-dimethylpirazine, octanal, 1-octen-3-one, 2-methyl-3-buten-2-ol, decanal, α-pinene, 6-methyl-5-hepten-2-one, nonanal, 3-methyl-2-pentanone, 3-methyl-2-hexanone, 3-5-dimethyl-2-hexanone and 3-5-dimethyl-2-hexanol. Most of the synthetic compounds were obtained from Sigma-Aldrich (Toluca, Mexico). The compounds were 97–99% pure according to the manufacturer. The compounds 3-methyl-2-pentanone and 3-methyl-2-hexanone were donated by Dr. William F. Wood (Chemistry Department, Humboldt University, Arcata, CA, USA). The compounds 1-octen-3-one, 3, 5-dimethyl-2-hexanone and 3,5-dimethyl-2-hexanol were prepared as described elsewhere (May-Concha et al., 2013). All compounds were dissolved in HPLC-grade dichloromethane (Sigma-Aldrich, Toluca, Mexico).

2.3. Electroantennograms

The antennal response of both sexes of the three haplogroups to the stimuli selected were determined by electroantennogram (EAG). Insects were placed in a freezer at −20 °C for 3 min to anesthetize them. The insect´s head was carefully removed and the reference electrode was inserted in a small hole at the base of the head using a glass capillary electrode filled with physiological saline solution (Malo et al., 2004). The recording glass capillary electrode contacted the tip of the antenna. The electrical signal generated by the antenna was sent to a high-impedance amplifier (NL 1200 with a IDAC2; Syntech GmbH), and displayed on a monitor using the Syntech software to process EAG signals.

A current of humidified filtered air (0.7 L/min) was directed continuously onto the antenna through a glass tube (diameter 10 mm). A stimulus controller (CS-05; Syntech GmbH) was used to generate 1-s puffs at 1-min intervals. Serial dilutions of the compounds were prepared in dichloromethane to make 0.01 (dose 3), 0.1 (dose 2) and 1 μg/μl (dose 1) solutions. A standard aliquot of each test dilution was applied to a piece of filter paper of 0.5 x 3.0 cm (Whatman no. 1, Whatman, Maidstone, England). Filter paper strips were inserted into glass Pasteur pipettes (odour cartridges), after the solvent was allowed to evaporate. New cartridges were prepared for each antennal preparation. To present a stimulus, the pipette tip containing the test compound was inserted through a side hole located at the midpoint of the glass tube. The continuous flow of clean air through the glass tube and over the preparation ensured that odours were removed immediately from the antenna. In each experiment, the antenna was stimulated first with dichloromethane as control. In preliminary tests, we found that dichloromethane elicited responses similar to clean air. Afterwards, the stimuli were applied in random order. The statistical analysis was performed using the amplitude of the EAG peak (mV) in response to each stimulus. All EAG recordings were performed from 18:00 to 22:00 h in a room at 27 ± 1 °C, and 55% HR. At least 10 insects, one antenna per insect, of each sex were used for each stimulus, that is a total of 20 per haplogroup.

Two series of experiments were performed. In the first experimental series, female and male antennae of hg3 were stimulated with 1 μg of 13 compounds. This experimental series was used to help choose compounds for further analysis in the second series. In the second series, we stimulated the antennae of both sexes of the three haplogroups with different doses of the seven responsive compounds selected from the first series. In this series, the mass of the test compounds spanned from 0.01 to 1 μg. Dichloromethane was used as a control stimulus.

2.4. Morphological analysis of the antenna

The antennal phenotype of the three haplogroups of the *T dimidiata* complex was studied using the procedure reported previously (May-Concha et al., 2016). In this study, we analysed the chemo-sensilla in the dorsal and ventral sides of the pedicel (P), flagellum 1 (F1) and flagellum 2 (F2). The chemo-sensilla were identified and counted using a stereo microscope DME Leica at 400 x and a cell counter CT-270 (Cientifica Schönfeld, Argentina). The following chemo-sensilla were studied: thin walled trichoid (TH), thick walled trichoid (TK) and basiconic (BA) (Catalá and Schofield, 1994). We analyzed 48 antennae of adults from the three haplogroups of the *T. dimidiata* complex.

2.5. Data analysis

All statistical analyses were performed using statistical software R version 3.3.3 (R Core Team, 2017). When necessary, EAG data were transformed using Johnson´s method (Lagos and Vargas, 2003). The EAG responses (mV) from both sexes of the first experimental series,

were analysed using a one-way analysis of variance (ANOVA). The EAG values of the second experimental series (dose-response experiment) were analysed using multivariate analysis of variance (MANOVA) to determine the effect of haplogroup and dose on the antennal responses. Differences in the interaction were analysed by multiple comparison by means of orthogonal contrasts ($\alpha = 0.05$). Data from antennal phenotype from the three haplogroups were analysed using the Kruskall-Wallis non-parametric test. A complementary analysis of EAG sensitivity (EAG_s) was performed. For this analysis EAGs was calculated as follows: $EAGs = EAG_{1\mu g} - EAG_{0.01\mu g}$. For the EAGs, the control was not included because this rank represents the difference between the EAG response to the highest dose and the lowest dose of each compound; thus, representing the sensitivity of the antennal response to changes in dose of the compound (it is related to the slope of the dose-response curve). Data from antennal phenotype and antennal sensitivity were analysed using a discriminant analysis to estimate functions that identify possible study subgroups (Dujardin, 2004). The significance of analysis was calculated by Wilks and Mahalanobis distance values using permutation tests (1000 permutations). This analysis uses crosschecked classification tests to validate the re-classification of discriminant analysis. The Bonferroni correction was used for multivariate comparisons. The discriminant analysis was performed using seven variables of EAG sensitivity (1-octen-3-ol, octanal, decanal, 6-methyl-5-hepten-2-one, nonanal, 3-5-dimethyl-2-hexanone, 3-5-dimethyl-2-hexanol) and nine variables of antennal phenotype (P-TH, P-TK, P-BA, F1-TH, F1-TK, F1-BA, F2-TH, F2-TK and F2-BA) in order to define the differences between haplogroups and between sexes.

3. Results

3.1. First experimental series

The one-way ANOVA of EAG amplitude showed that the compound tested to 1 μg influenced the antennal responses of females ($F = 8.20$; $df = 13, 126$; $P < 0.001$). The compounds 3,5-dimethyl-2-hexanol, nonanal, decanal, octanal, and 1-octen-3-ol evoked higher EAG responses than the control. There were no differences between the antennal responses evoked by the other eight compounds evaluated and that elicited by the control (Fig. 1a).

In the case of males, the statistical analysis revealed that the compound tested to 1 μg influenced their antennal responses ($F = 20.76$; $df = 13, 135$; $P < 0.001$). The compounds, 3,5-dimethyl-2-hexanol, nonanal, decanal, octanal, 6-methyl-5-hepten-2-one, and 3,5-dimethyl-2-hexanone elicited significantly higher antennal responses compared to control. 3,5-dimethyl-2-hexanol, nonanal decanal, and octanal evoked higher EAG responses than those elicited by 6-methyl-5-hepten-2-one, and 3,5-dimethyl-2-hexanone. The other seven compounds tested did not show differences in antennal responses comparing to the control (Fig. 1b).

3.2. Second experimental series

Female EAG responses were affected by the haplogroup ($F = 3.42$; $df = 14, 152$; $P < 0.0001$), dose ($F = 6.76$; $df = 14, 152$; $P < 0.0001$) and the interaction haplogroup*dose ($F = 3.19$; $df = 28, 312$; $P < 0.0001$). When the dose factor was fixed, we found that at the dose of 1 μg, there were significant differences between haplogroup 1vs haplogroup 2, haplogroup 1 vs haplogroup 3 and haplogroup 2 vs haplogroup 3; octanal, 6-methyl-5-hepten-2-one, 3-5-dimethyl-2-hexanone and 3-5-dimethyl-2-hexanol the compounds than evoked higher EAG responses in haplogroup 2 compared to haplogroup 1. While nonanal showed the highest EAG responses in haplogroup 2 compared to haplogroup 1 and haplogroup 3. At the dose of 0.1 μg, there was a significant difference between the haplogroup 1 vs haplogroup 3; 3-5-dimethyl-2-hexanone the compounds than evoked higher EAG responses in haplogroup 1 compared to haplogroup 3 (Table 2a). When the haplogroup factor was fixed, there were significant differences, except in the comparison 0.1 μg vs 0.01 μg in the three haplogroups ($P > 0.05$, Table 2b).

Males EAG responses were affected by haplogroup ($F = 12.99$; $df = 14, 152$; $P = 0.0001$), doses ($F = 8, 57$; $df = 14, 152$; $P = 0.0001$) and for the interaction haplogroup*doses ($F = 3.46$; $df = 28, 312$; $P = 0.0001$). When the dose factor was fixed, we observed that there were significant differences in all comparison, except in the comparison between haplogroup 1 vs haplogroup 2 and haplogroup 2 vs haplogroup 3 at the dose of 0.01 μg (Table 3a). The compound 1-octen-3-ol evoked higher EAG responses at the dose of 1 μg and 0.1 μg in haplogroup 2 compared to haplogroup 1 and haplogroup 3. Whereas, when the haplogroup factor was fixed, we found that in most of the comparisons there were significant differences, except between the comparison 1 μg vs 0.1 μg, and 0.1 μg vs 0.01 μg in the haplogroup 1, in the comparison between 0. μg vs 0.01 μg in the haplogroup 2 and in the haplogroup 3 (Table 3b).

3.3. Effect of haplogroup on EAG sensitivity

The sensitivity of the EAG response of females was affected by the haplogroup ($F = 3.72$; $df = 14, 44$; $P < 0.0022$). Six-methyl-5-hepten-2-one, 3-5-dimethyl-2-hexanone and 3-5-dimethyl-2-hexanol showed significant differences between haplogroups (Table 4a). The higher antennal sensitivity to changes was shown by haplogroup 3 to the doses tested. The sensitivity of the EAG of males was significant affected by the haplogroup ($F = 4.06$; $df = 14, 44$; $P < 0.0002$). Octanal, decanal, 6-methyl-5-hepten-2-one, nonanal and 3-5-dimethyl-2-hexanol, showed significant differences between haplogroups (Table 4b), and the higher antennal sensitivity to changes was found in haplogroup 2 to the doses tested.

3.4. Multivariate analysis

The number and location of the three chemo-sensilla on the antennae of both sexes were significantly discriminated between haplogroups using nine antennal variables (see Materials & Methods section). For the females, the first function explains 78% of the total variation, while the second function explained 22%. The Mahalanobis distance (D2) was significantly different between haplogroups ($P < 0.0001$), with the discriminant plot indicating that haplogroup 2 is the most distant from both haplogroup 1 and haplogroup 3 (Fig. 2a). The Mahalanobis distance (D2) for the female group centroid indicated that haplogroup 2 is more distant from haplogroup 3 (D2 = 7.58) than from haplogroup 1 (D = 5.34), while haplogroup 1 was least distant from haplogroup 3 (D = 4.35) (Fig. 2a). The first discriminant function for males explained 86% of total variation, whereas the second explained 14%, and the Mahalanobis distances for males indicated significant differences between haplogroups ($P < 0.01$). The discriminant plot indicated that haplogroup 1 is most distant from haplogroup 2 and haplogroup 3 (Fig. 2b). The Mahalanobis distance from the male group centroid, indicated that haplogroup 1 is more distant from haplogroup 2 (D = 4.45) than from haplogroup 3 (D = 3.05), whereas haplogroup 2 was least distant to haplogroup 3 (D = 2.35) (Fig. 2b).

The discriminant multivariate analysis considering EAG sensitivity to seven compounds (see Materials & Methods section) showed that the Mahalanobis distance for females and males were significantly different between haplogroups ($P < 0.01$) (Fig. 3). Females of haplogroup 1 were the most distant from haplogroup 2 and haplogroup 3 (Fig. 3a). The Mahalanobis distance for the female group centroids, indicated that haplogroup 1 was more distant from haplogroup 2 (D = 3.69) than from haplogroup 3 (D2 = 3.01), while haplogroup 2 was closest to haplogroup 3 (D = 1.51) (Fig.3a). A similar pattern of distance was observed in males (Fig. 3b). The Mahalanobis distance for the male group centroids indicated that haplogroup 1 is more distant from haplogroup 2 (D = 5.42) than from haplogroup 3 (D = 4.18), while

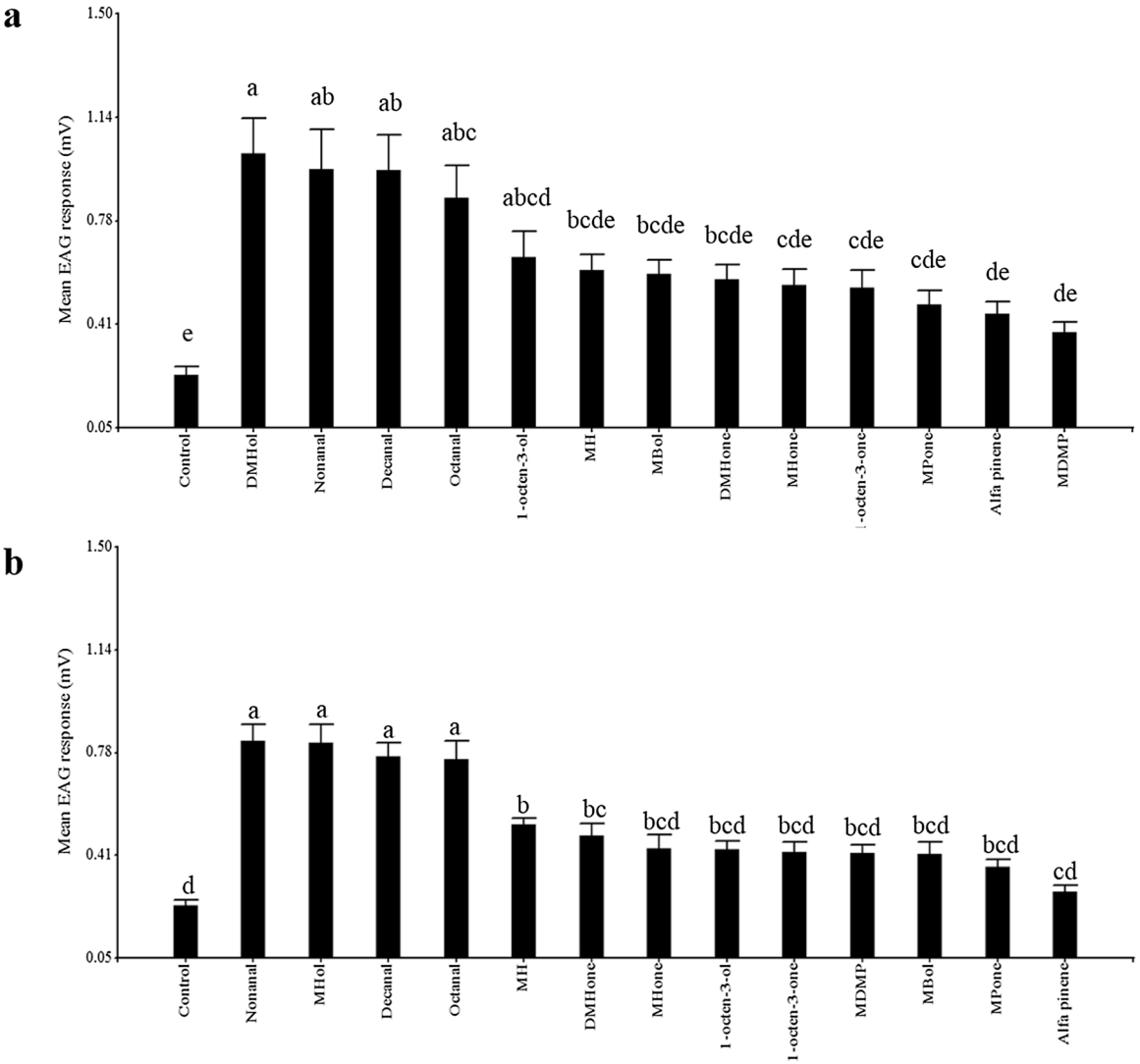

Fig. 1. Electroantennogram (EAG) responses of *Triatoma dimidiata* (hg3) to 1 μg of semiochemicals. (A) females and (B) males. Significant differences are indicated by diferent letters (Tukey test, $P < 0.05$). DMHol: 3-5-dimethyl-2-hexanol, MH: 6-methyl-5-hepten-2-one, MBol: 2-methyl-3-buten-2-ol, DMHone: 3-5-dimethyl-2-hexanone and, Mhone: 3-methyl-2-hexanone, MDMP: 3-methoxy-2-5-dimethylpirazyne.

haplogroup 2 was closest to haplogroup 3 (D = 1.91) (Fig. 3b).

3.5. Comparison of the total number of chemo-sensilla of the T. dimidiata haplogroups

Three types of chemo-sensilla (thin walled trichoid, thick walled trichoid and basiconic) were found in both the ventral and dorsal sides of the pedicel and both flagella of all adult insect (Table 5). The total number of chemo-sensilla and type, varied according to the sex of each haplogroup. The Kruskal-Wallis univariate analysis of the nine morphological variables showed significant differences between haplogroups in females for P-TH, P-BA, F1-TH, and all F2 sensilla ($P < 0.01$) (Table 5). The highest number of chemo-sensilla was found in haplogroup 3. In contrast, there were differences in the P-TK and F1-TK ($P < 0.01$) of males between haplogroups (Table 5). In this case, it was observed that haplogroup 2 showed the highest number of chemo-sensilla on whole antenna.

4. Discussion

In this study, we determined the antennal responses of both sexes of the three Mexican haplogroups of *T.* dimidiata complex to selected volatiles produced by exocrine glands in order to investigate possible olfactory physiological differences between haplogroups and a possible correlation with the antennal phenotype. In general, the antennal responses of *T. dimidiata* to the tested compounds were different between the haplogroups. In a number of insect species, it has been documented that the detection of odours produced in exocrine glands results in species-specific responses (Sudd and Franks, 1987; Domínguez-Sánchez et al., 2008). In this study, these differences may be due to variability in the number of chemo-sensilla between the haplogroups.

We found that females show higher EAG responses than males. This result is possibly related to the numerical difference of chemo-sensilla in the antennae of both sexes, which may contribute to the sexual dimorphism in the sensory responses. The compounds that evoke higher antennal responses at the dose of 1 μg in both sexes (1-octen-3-ol, octanal, nonanal and decanal), have been found to be key and indicative of the presence of a host in other triatomines (Guerenstein and Guerin, 2001; Fontan et al., 2002; Barrozo and Lazzari, 2004a; González-Audino et al., 2006). This is the first study to find an electrophysiological response to 1-octen-3-ol in triatomines, demonstrating that this compound evokes an EAG response in both sexes of the three haplogroups. Some authors have shown the potential of 1-octen-3-ol as attractant for triatomine bugs (Barrozo and Lazzari, 2004b; Milne et al.,

Table 2
Multiple comparisons of dose and haplogroup effects by orthogonal contrasts, A) fixed factor dose and B) fixed factor haplogroup on electroantennogram (EAG) responses of *Triatoma dimidiata* complex females. hg1: haplogroup Yucatan-Peninsula; hg2: haplogroup Gulf of Mexico; hg3: haplogroup Pacific-Coast.; d1: 1 µg, d2: 0.1 µg and d3: 0.01 µg.

A) Dose fixed

Comparison	*F*	*df*	*P*-level
hg1-hg2: d1	13.06	7, 75	**0.00001**
hg1-hg3: d1	5.80	7, 75	**0.0001**
hg2-hg3: d1	4.07	7, 75	**0.004**
hg1-hg2: d2	1.83	7, 75	0.37
hg1-hg3: d2	4.26	7, 75	**0.003**
hg2-hg3: d2	2.85	7, 75	0.053
hg1-hg2: d3	0.70	7, 75	0.88
hg1-hg3: d3	1.75	7, 75	0.37
hg2-hg3: d3	0.99	7, 75	0.88

B) Haplogroup fixed

Comparison	*F*	*df*	*P*-level
d1-d2:hg1	5.34	7, 75	**0.0002**
d1-d3:hg1	4.49	7, 75	**0.001**
d2-d3:hg1	1.24	7, 75	0.29
d1-d2:hg2	18.34	7, 75	**0.00001**
d1-d3:hg2	10.68	7, 75	**0.00001**
d2-d3:hg2	1.81	7, 75	0.19
d1-d2:hg3	7.33	7, 75	**0.00001**
d1-d3:hg3	10.22	7, 75	**0.00001**
d2-d3:hg3	2.13	7, 75	0.14

Table 3
Multiple comparisons of doses and haplogropus effects by means of orthogonal contrasts, A) fixed factor dose and B) fixed factor haplogroup on electro-antennogram (EAG) responses of *Triatoma dimidiata* complex males. hg1: haplogroup Yucatan-Peninsula; hg2: haplogroup Gulf of Mexico; hg3: haplogroup Pacific-Coast.; d1: 1 µg, d2: 0.1 µg and d3:0.01 µg.

A) Dose fixed

Comparison	*F*	*df*	*P*-level
hg1-hg2: d1	27.64	7, 75	**0.00001**
hg1-hg3: d1	7.37	7, 75	**0.00001**
hg2-hg3: d1	12.59	7, 75	**0.00001**
hg1-hg2: d2	2.89	7, 75	**0.02**
hg1-hg3: d2	4.43	7, 75	**0.001**
hg2-hg3: d2	5.57	7, 75	**0.0002**
hg1-hg2: d3	2.23	7, 75	0.08
hg1-hg3: d3	4.73	7, 75	**0.0009**
hg2-hg3: d3	1.59	7, 75	0.1

B) Haplogroup fixed

Comparison	*F*	*df*	*P*-level
d1-d2:hg1	2.54	7, 75	0.08
d1-d3:hg1	3.42	7, 75	**0.01**
d2-d3:hg1	2.18	7, 75	0.11
d1-d2:hg2	20.20	7, 75	**0.00001**
d1-d3:hg2	23.78	7, 75	**0.00001**
d2-d3:hg2	2.25	7, 75	0.11
d1-d2:hg3	7.32	7, 75	**0.00001**
d1-d3:hg3	7.49	7, 75	**0.00001**
d2-d3:hg3	0.88	7, 75	0.52

2009). In addition, our study showed a high antennal response to aldehydes in both sexes of *T. dimidiata*. The TH sensilla of *T. infestans* respond to aldehydes, including heptanal, octanal, and nonanal (Guerenstein and Guerin, 2001), which suggests that in *T. dimidiata* similar olfactory sensory neurons exist and may be even housed in similar TH sensilla. However, further studies using single sensillium

Table 4
Effect of haplogroup on the *EAG* sensitivity of *Triatoma dimidiata* complex to selected exocrine gland volatiles. A) females and B) males. Asterisks represent significant differences between the haplogroups respect to the semiochemical evaluated ($P < 0.05$). MH: 6-methyl-5-hepten-2-one, DMHone: 3-5-dimethyl-2-hexanone and DMHol: 3-5-dimethyl-2-hexanol.

A

Compound	*F*	*df*	*P*
1-octen-3-ol	0.089	2	0.422
Octanal	2.74	2	0.082
Decanal	0.92	2	0.410
MH	6.07	2	**0.006***
Nonanal	0.99	2	0.383
DMHone	8.62	2	**0.001***
DMHol	17.00	2	**0.001***

B

Compound	F	*df*	P
1-octen-3-ol	3.10	2	0.061
Octanal	15.53	2	**0.001***
Decanal	23.63	2	**0.001***
MH	5.80	2	**0.008***
Nonanal	27.09	2	**0.001***
DMHona	2.53	2	0.098
DMHol	20.17	2	**0.001***

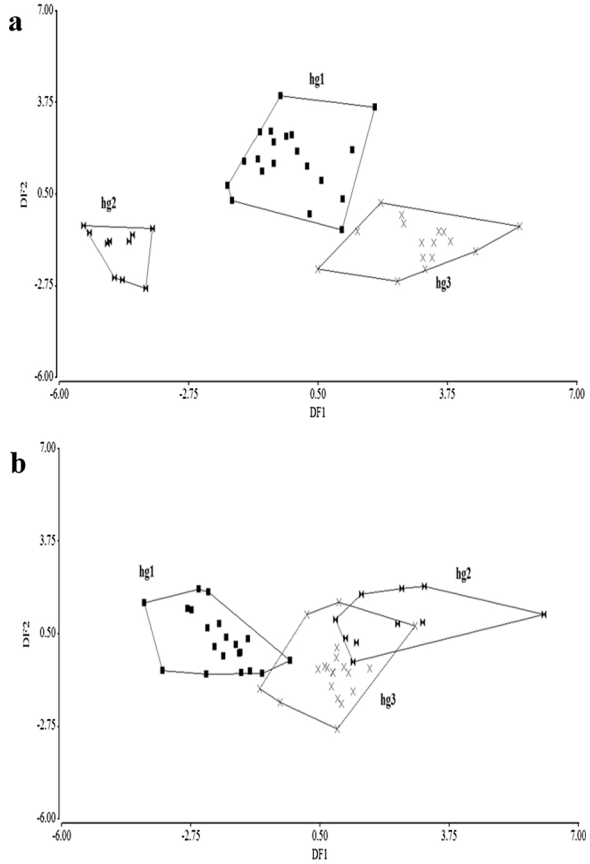

Fig. 2. Discriminant analysis of the number and location of the chemo-sensilla on the antenna of adults, A) females and B) males using nine antennal chemosensilla variables. Each point represents one specimen on the canonical axis. Polygons enclose specimens of each haplogroup of the *T. dimidiata* Complex: hg1: haplogroup Yucatan-Peninsula; hg2: haplogroup Gulf of Mexico; hg3: haplogroup Pacific-Coast. DF1 and DF2 represent the discriminant function 1 and 2, respectively.

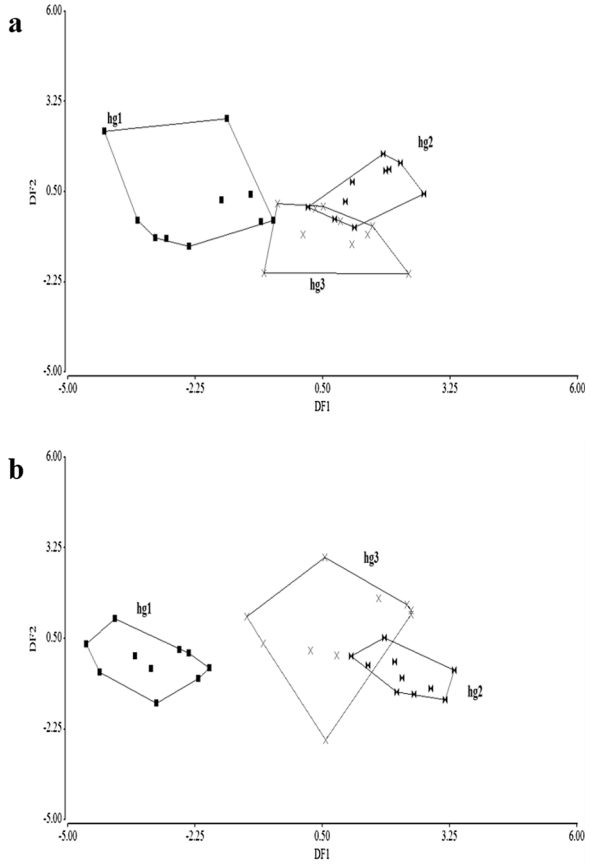

Fig. 3. Discriminant analysis of EAG sensitivity of adults, A) females and B) males using seven EAG sensitivity variables. Each point represents one specimen on the canonical axis. Polygons enclose specimens of each haplogroup of the *T. Dimidiata* Complex: hg1: haplogroup Yucatan-Peninsula; hg2: haplogroup Gulf of Mexico; hg3: haplogroup Pacific-Coast. DF1 and DF2 represent the discriminant function 1 and 2, respectively.

recording techniques are necessary to confirm if TH sensilla are involved in the reception of aldehydes in *T. dimidiata*.

Our results also showed that the olfactory sensitivity of the *T. dimidiata* complex was different between haplogroups. Similar results have been observed in behavioral bioassays in *T. dimidiata*, demonstrating differences in the behavioral response to exocrine compounds between sexes and the three Mexican haplogroups of *T. dimidiata* (May-Concha et al., 2015). On the other side, the analysis of the antennal phenotype of the whole antennae (ventral + dorsal) showed that the haplogroup 3 females and haplogroup 2 males have the higher number of chemo-sensilla compared to the other two sex/haplogroups. The ecological niche of haplogroup 2 and haplogroup 3 is conserved (Ibarra-Cerdeña et al., 2014), and evidence now exists for the sympatry of haplogroup 1 and haplogroup 2 in the Yucatan Peninsula, Chiapas and Tabasco (Herrera-Aguilar et al., 2009; Garcia et al., 2013; López-Cancino et al., 2015), and the three haplogroups are sympatric in northern Chiapas (Pech-May, personal communication), suggesting that macroecological tolerances may not have affected these differences. This however, does not exclude microecological influences, such as landscape modification, its frequency or degree. To our knowledge, no study has analyzed multiple populations from within a haplogroup to analyze association of antennal phenotype with habitat modification. In addition, our observations support the hypothesis of a sexual dimorphism at a sensory level as suggested by different studies in other triatomine bugs (Carbajal de la Fuente and Catalá, 2002; Catalá et al., 2005; Moreno et al., 2006; Rodríguez-Rodríguez et al., 2009; May-Concha et al., 2016).

Morphometric analyses of cuticular hydrocarbons and volatile compounds emitted by exocrine glands, cytogenetic analysis, antennal phenotype and molecular data have provided unequivocal evidence of cryptic taxa and suggest that *T. dimidiata* is a complex of species (Dorn et al., 2007, 2009; Bargues et al., 2008; Ibarra-Cerdeña et al., 2009; Monteiro et al., 2013; Ibarra-Cerdeña et al., 2014; Gómez-Palacio et al., 2015; May-Concha et al., 2015, 2016). The results here presented support this idea. The multivariate analysis of the antennal phenotype in females separates haplogroup 2 from the other two haplogroups. One of the characteristics of haplogroup 2 females is lower number of TH sensilla on the pedicel. This could be linked to a low expansion area and habitat range of this haplogroup, as it has been suggested for other triatomines (Catalá, 1997). On the other hand, a shorter Mahalanobis distance was evident between the antennal phenotype and sensitivity in the EAG response from haplogroup 2 and haplogroup 3 males which may suggest different olfactory sensitivity respect to haplogroup 1 males. These same results were observed in studies of antennal phenotype made with the same complex (May-Concha et al., 2016).

Olfactory sensitivity analysis in both sexes shows shorter Mahalanobis distance between haplogroup 2 and haplogroup 3 with respect to haplogroup1. Therefore, our results suggest that haplogroup 2 and haplogroup 3 at the physiological level are close haplogroups compared to haplogroup 1, and this is in agreement with results of genetic studies obtained with molecular markers (Bargues et al., 2008; Dorn et al., 2009; Monteiro et al., 2013; Gómez-Palacios et al., 2015). This suggests that at a sensory level these haplogroups can be considered a complex of species, as suggested by several authors, and supports the idea that in Mexico there are three haplogroups of the species *T. dimidiata* (Marcilla et al., 2001; Jurberg et al., 2005; Lehmann et al., 2005; Ibarra-Cerdeña et al., 2014; Galvão and Justi, 2015; May-Concha et al., 2015, 2016). Likewise, our results of antennal phenotype at sensory level and olfactory sensitivity analysis, separate the sexes from the three haplogroups.

In summary, our study evidence that the EAG response of the haplogroups to the glandular compounds evaluated, is a useful variable to

Table 5
Number of chemo-sensilla on the antenna of adults of the *Triatoma dimidiata* complex. Data shown are means and standard deviations for thin walled trichoid (TH); thick walled trichoid (TK) and Basiconica (BA). The number of specimens analyzed is shown between parentheses and the numbers between clasps represent the standard deviation of the data. hg1: haplogroup Yucatan-Peninsula; hg2: haplogroup Gulf of Mexico; hg3: haplogroup Pacific-Coast. F = female, M = male. Different letters indicate significant differences between haplogroups for each sex (Kruskal-Wallis tests; $P < 0.01$).

			Pedicel			Flagellum 1			Flagellum 2		
Sex	N	Haplogroup	TH	TK	BA	TH	TK	BA	TH	TK	BA
	10	**hg1**	136.95^{a} (11.09)	77.30 (7.51)	16.90^{b} (1.36)	67.55^{a} (6.07)	76.45 (6.61)	31.70 (2.59)	29.10^{a} (2.75)	82.55^{b} (6.75)	27.00^{b} (1.97)
Females	5	**hg2**	121.30^{a} (12.11)	86.90 (5.91)	7.60^{a} (0.91)	71.50^{ab} (9.48)	99.60 (6.11)	23.40 (2.10)	51.10^{b} (5.07)	53.00^{a} (4.83)	17.80^{a} (1.33)
	8	**hg3**	198.5^{b} (5.92)	73.63 (4.50)	22.06^{b} (1.35)	101.00^{b} (5.03)	91.88 (6.80)	26.06 (1.75)	36.50^{ab} (3.35)	88.56^{b} (7.24)	28.00^{b} (1.69)
	10	**hg1**	259.35 (19.8)	65.05^{a} (4.41)	20.55 (1.37)	124.70 (10.71)	65.90^{a} (4.38)	48.65 (4.83)	52.80 (4.49)	103.95 (6.88)	35.60 (3.07)
Males	5	**hg2**	284.40 (27.6)	99.30^{b} (9.33)	19.60 (1.01)	107.20 (7.25)	116.80^{b} (9.22)	29.80 (3.84)	44.50 (3.79)	122.00 (10.49)	28.60 (2.02)
	10	**hg3**	266.85 (17.4)	67.60^{a} (3.22)	16.65 (1.02)	97.50 (6.81)	98.30^{b} (3.05)	30.80 (1.81)	41.85 (2.37)	98.95 (6.39)	30.00 (2.55)

analyze intraspecific variability within the complex. The study of the olfactory sensilla number and location, and sensory responses could be an important tool to analyse differences in the olfactory system of populations with genetic diversity, as well as environmental influences on populations. It is important to mention that the responses described in this study are limited to a small number of compounds emitted by their conspecifics. Therefore, it could be interesting to evaluate a greater number of compounds in order to find components of attractive blends. This information could contribute to the list of volatiles compounds that modulate the behaviour of this species complex, with the aim to develop odour-baited traps for the control of triatomines.

5. Conclusions

Discriminant analysis of antennal phenotype at peripheral level and olfactory sensitivity of the three Mexican haplogroups of *T. dimidiata* indicate significant separation among the three haplogroups. Olfactory sensitivity analysis showed that there are similarities between haplogroup 2 and haplogroup 3, in contrast to the ancestral haplogroup 1. Antennal responses have provided additional evidence to discriminate the *dimidiata* complex. Antennal responses are possibly related to the numerical difference of chemo-sensilla in the antennae of both sexes, which may contribute to the sexual dimorphism in the sensory responses. Similar analyses of other North American species complexes, will provide a complete understanding of the physiology of the olfactory system of these insects, knowledge that may useful for developing novel methods to manipulate their behavior.

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