Chemical Emission of Two *Eccritotarsus* Species (Hemiptera: Miridae) Released as Biological Control Agents of Water Hyacinth[1]

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Abstract In the family Miridae (Hemiptera), females and males attract each other by means of sex pheromones. Among insects, these pheromones are characterized by a variety of chemical structures, including saturated and unsaturated, long- and short-chain esters, as well as unsaturated ketoaldehydes. The aim of this study was to assess the chemical emissions in *Eccritotarsus catarinensis* (Carvalho) and *Eccritotarsus eichhorniae* Henry to determine their similarity and their possible role in reproductive isolation mechanisms that led to speciation. Chemicals emitted by adults inserted in air-entrainment chambers were collected in absorbent tubes and were analyzed using gas chromatography–mass spectrometry (GC-MS). Results from the GC-MS library indicate that *E. catarinensis* females and *E. eichhorniae* males have chemical emissions that their conspecific and the same sex of the other species lack. Also, *E. catarinensis* males lack benzenebutanoic that the other sexes have, while *E. eichhorniae* males have 1,2,3,4-tetrahydro-6-(phenyl methyl) that other sexes lack. Further analysis using statistical approaches (e.g., cluster analysis, multidimensional scaling plot, and principal component ordination) indicated that cross-breeding pairs have similar chemical emissions in that *E. eichhorniae* females had similar chemical emissions to those of *E. catarinensis* males, while *E. catarinensis* females had similar chemical emissions to those of *E. eichhorniae* males. These unique differences in chemical emissions could be caused by the recently identified differences in the metathoracic scent glands and the antennae of the two *Eccritotarsus* species, and they may serve as a basis in explaining the interbreeding and mating incompatibilities reported in these two *Eccritotarsus* species.

Key Words *Eccritotarsus*, chemical emissions, metathoracic scent gland, antennae

The metathoracic scent glands (MSG) of mirids (Hemiptera: Miridae) produce defensive chemical compounds emitted when insects are disturbed or provoked (Zhang and Aldrich 2003b). In addition to defensive compounds, the MSG also release sexual, aggregation, alarm, and dispersal pheromones that function intraspecifically (e.g., Aldrich et al. 1991, Aldrich 1994, 1996, McBrien and Millar

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1999, Millar et al. 1997, Millar and Rice 1998, Smith et al. 1991, Wardle et al. 2003, Zhang and Aldrich 2003b); but they also can be used by various other insects interspecifically as kairomones (e.g., Aldrich and Barros 1995, Eisner et al. 1991, Zhang and Aldrich 2003b). Additionally, Millar et al. (1997) found that chemical compounds also can be emitted from the thorax. Pheromones in Miridae include simple esters and monoterpenes; linear, monocyclic and multicyclic sesquiterpenoids; and novel acetogenins (Millar 2005), while typical defensive compounds include short-chain alcohols, aldehydes, esters, alkenals, alkanes, monoterpenes, and aromatic alcohols and aldehydes (Millar 2005), although these can be interchangeable.

This study investigated chemical emissions of two sympatric species: *Eccritotarsus catarinensis* (Carvalho) and *Eccritotarsus eichhorniae* Henry (Hemiptera: Miridae). These herbivorous bugs were once thought to be morphologically identical. Both measure 2–3 mm and are primarily black with darkly marked, but mostly transparent, wings (Stanley and Julien 1999). Both are leaf-sap–sucking biological control agents of a problematic invasive weed known as water hyacinth, *Eichhornia crassipes* (Martius) Solms-Laubach (Pontederiaceae) (Hill et al. 1999). The nymphs and adults of these species are mobile and feed gregariously underneath water hyacinth leaves, causing chlorosis that leads to an eventual leaf death because of excessive acquisition of chlorophyll from the palisade parenchyma (Hill et al. 1999). Feeding stunts the growth rate of the weed by reducing photosynthesis, and that eventually leads to a reduction in overall biomass of the weed (Coetzee et al. 2007). Their development is about 23 d, with 15 of those in 4 to 5 nymphal instars, after which the adult lifespan is approximately 50 d (Hill et al. 1999). These insects can live for several weeks in suitable laboratory conditions and can persist even when the leaves are subjected to chlorosis (Mnguni 2019a).

Eccritotarsus catarinensis was imported from Florianopolis (Santa Catarina), Brazil in 1994. This collection was subjected to host-specificity testing after which the insect was released throughout South Africa in 1996 (Taylor et al. 2011). The Brazilian collection was subjected to a severe genetic bottleneck event after importation into quarantine, and it was later shown to be unable to cope with cold winter temperatures, so resampling was subsequently conducted in South America (Paterson et al. 2016). The recently described *E. eichhorniae* was then imported from Yarapa River near Iquitos, Peru, in 1999, and was introduced in South Africa in 2007, after having gone through host-specificity testing that deemed it suitable for release (Paterson et al. 2016). Initially, it was thought that these were two introductions of the same species. A few years later, an interbreeding of the two populations of the same species occurred. The redescription of *E. eichhorniae* was justified by findings reported in the genetic analysis conducted by Taylor et al. (2011) as well as the subsequent behavioral experiments reported in Paterson et al. (2016), which are briefly discussed below. Therefore, Henry (2017) rightfully redescribed the two species into separate *E. catarinensis* (Carvalho) and *E. eichhorniae* Henry.

Taylor et al. (2011) found a significant 5.2% haplotype sequence divergence, infertile hybrids, and 29 fixed differences. Paterson et al. (2016) found an interbreeding incompatibility since *E. eichhorniae* females × *E. catarinensis* males did not produce any offspring while *E. catarinensis* females × *E. eichhorniae* males produced nonviable offspring. Ismail and Brooks (2016) found differences in fitness

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traits caused by differences in thermal limits, while Henry (2017) found slight morphological differences in the genitalia, MSG, and antennae that are known to store, emit, and receive chemicals. Ismail and Brooks (2018) found morphological differences and assessed male mating preferences while Mnguni (2019b) found a mating incompatibility since *E. eichhorniae* females and *E. catarinensis* males preferred to mate with their respective conspecifics only. Nevertheless, the most relevant finding for this chemical emission assessment work is that *E. eichhorniae* has a shorter antennal segment II than *E. catarinensis* (Henry 2017).

However, at the time, the intention of the second collection of the same agent from a different population was to increase genetic diversity and variability in the hope that this would increase the efficacy of the biocontrol agent in the country (Paterson et al. 2016). It also was thought that interbreeding and, therefore, hybridization of these two populations, would increase the chances of establishment in colder regions in South Africa. The underlying assumption was that the resulting population from these two interbreeding populations would yield a more resistant, resilient, efficient, and effective biological control agent that would easily overcome unforeseen catastrophic events (Mnguni 2019b, Paterson et al. 2016, Taylor et al. 2011).

The differences in the MSG and the antennae of these two mirids could be a key factor concerning their chemical emissions. They may enable or disable one species from detecting a chemical emission from a bouquet of pheromones released in an ecosystem. They may also change the functional groups of some of the chemical emissions, causing these two species to have one or more different chemical emissions. They may also cause a unique emission that is shared by the two species to be emitted in different ratios, proportions, or relative abundances. Therefore, the aim of the study was to assess the chemical emissions in the two species to investigate whether or not they are identical, and if they can explain the behavioral patterns reported in the mating studies that have been briefly discussed above. We tested the hypothesis that the chemical emissions of the two *Eccritotarsus* species are different. Also, knowing the chemical emissions of the two species is the first step towards identifying major emissions, which could possibly be sex pheromones. Furthermore, this first step will help in identifying possible sexual, aggregation, kairomones, alarm, and dispersal pheromones.

Materials and Methods

Insect culture. The two species of *Eccritotarsus* were kept in the Department of Zoology and Entomology at Rhodes University in Grahamstown. *Eccritotarsus catarinensis* was maintained at the Biological Control Quarantine Facility, while *E. eichhorniae* was housed at the Waainek Mass Rearing Facility, approximately 1 km away from the quarantine facility. Both species were maintained at 25 ± 1°C and 65 ± 5% relative humidity (RH) for many generations over 10 yr, under 12:12-h light:dark conditions. The cultures were kept separate from each other to avoid interbreeding between the two species. The invasive alien plant, water hyacinth, was provided as food and was constantly changed to consistently provide optimum nutrition for the mirids.

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Insect collection and maintenance. Mirids were collected as third- to fifth-instar nymphs from the respective *E. catarinensis* and *E. eichhorniae* cultures using an aspirator. Forty petri dishes (90 mm, Munktell, Lasec, Ahlstrom, Fisher Scientific, Waltham, PA) lined with filter paper, with one nymph in each, were prepared for both species. Water hyacinth leaves were placed into the petri dishes. Humidity in the petri dishes was maintained by adding distilled water using a 3-ml Pasteur pipette. Petri dishes were then inserted into plastic wrappers to increase humidity and placed under a 12:12-h light:dark fluorescent growing light conditions. The experiment was conducted at 23 ± 5°C and 45 ± 20% RH.

When nymphs had developed into adults, the insects were individually removed from the petri dish using a small paintbrush and viewed either ventrally or laterally to observe their genitalia. The genitalia identification followed Hill et al. (1999). Nymphs were placed individually to ensure that all adults used for the experiment had not mated. Each petri dish was labelled with the species and sex of the individual contained within. When sufficient numbers of adult insects were available, air-entrainment chamber experiments were conducted.

Air-entrainment chambers. The entire air-entrainment system was sterilized by thoroughly washing using distilled water and ethanol. All components were then dried in an oven at 30°C for 12 h. Emissions by adults were collected by placing insects in air-entrainment chambers and using Tenax sorbent tubes (SKC Inc., Eighty Four, PA) to trap the emissions. The test organisms were females and males from both *Eccritotarsus* species. Throughout the experiment, air flowed through Teflon tubes (ClearAir Engineering Inc., Chicago, IL). A pump (Sonic Silent Power 108, Sonic Aquarium Air Pump, Zhenhua Electric, Zhejiang, China) was used to generate air throughout the system. Charcoal-filtered air passed through 500 ml of distilled water. The air then passed through the air-entrainment chamber, which had openings on either side. Air entering and existing the air-entrainment chambers was adjusted at 35 ± 5 L/h using a flowmeter. Insects were then placed inside the air-entrainment chamber. A Tenax sorbent tube was placed in the opening, where air was exiting, to trap all the insect emissions that had been placed inside the chamber.

When emissions were trapped from test organisms into the Tenax tube, the Tenax tube was quickly sealed and stored in a refrigerator at −20°C. Five replicates were conducted concurrently for both female and males of *E. catarinensis* and *E. eichhorniae* species. Each replicate had 10 insects placed in the air-entrainment chamber. All replicates ran for 12 h. All these experiments were conducted from 6 a.m. to 5 p.m. Only 2- to 3-d-old adults were used throughout the experiment. In *Eccritotarsus*, adults need only 2 d to be considered to be sexually mature (Ismail and Brooks 2016).

Chromatographic separation. Tenax tubes used in the volatile chamber experiment were transported to the Central Analytical Facility (CAF) at Stellenbosch University. Analysis of volatile compounds was performed on a Thermo Scientific TRACE^{TM} 1300 gas chromatograph coupled to a TSQ 8000 Mass Spectrometer detector. Empty Tenax tubes were analyzed as controls to distinguish between contaminants and insect emissions. Peaks 9.06, 10.80, 12.19, 13.41, and 17.94 were shown to be siloxanes (breakthrough material from the Tenax), and these were treated as contaminants. As such, these peaks had to be ignored when interpreting chemical emissions of insects. The analysis and the separation of the

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chemicals was performed on a nonpolar ZB-MultiResidue-1 instrument (30 m, 0.25-mm inner diameter, 0.25-μm film thickness; part number 7HG-G016-11). The initial oven temperature was adjusted to 40°C, held for 1 min, and finally adjusted to 250°C at 15°C/min, and held for 5 min. The injector temperature was maintained at 250°C. The injection was split-less, and the carrier gas was helium. The transfer line temperature was held at 300°C. The ionization source temperature was set at 250°C.

Statistical analysis. The widely used gas chromatography–mass spectrometry (GC-MS) data were received from the Stellenbosch University CAF as chromatogram graphs. The graphs were received with slightly different retention times as it was well established that peaks were only being recorded after 5 min (Lucky Mokoena, Stellenbosch Univ., pers. comm.).

All the peaks were given with relative abundances that ranged between 0 and 100. As such, all the measures were equated to percentages for females and males of both species. Names of the peaks, adopted from the GC-MS library, are listed in Table 1. Presence and absence analyses were conducted using the chromatograms (Table 2). Since five replicates were conducted for both females and males of both species, each peak had five different measurements. These measurements were summed to calculate the mean and the standard error (SE) for all the peaks (Table 3).

Data from both *Eccritotarsus* species (mean ± SE) were log transformed using log $(X + 1)$ to fit the assumption of homogeneity. Generalized linear modelling was used to test for significant differences among the treatments using Statistica software (version 13). The log-transformed data also were subjected to cluster analysis (CA), multidimensional scaling (MDS), and principal component ordination (PCO) graphs using PRIMER 6.1.8 and PERMANOVA+.

Results

In both species of *Eccritotarsus,* females and males share chemical compounds: propionic acid; 5-t-butyl-1,2,3-trimethylbenzene; 1-methyl-1,3-propanediyl; n-ethyl-2-methyl; 1,2,3,4-tetrahydro-1-phenyl; and n-ethyl-4-methyl (Fig. 1; Table 3). There is no chemical compound unique to either *E. catarinensis* or *E. eichhorniae* females. Males from *E. catarinensis* lack benzenebutanoic acid that other sexes have, while males from *E. eichhorniae* have 1,2,3,4-tetrahydro-6-(phenyl methyl) that other sexes lack.

When comparing within species, *E. catarinensis* females have chemical compounds benzenebutanoic acid; phenol (4-cyclohexyl); 1-methyl-1-propene-1,3-diyl; and 1,1,3-trimethyl-3-phenyl that *E. catarinensis* males lack. On the other hand, *E. eichhorniae* females have chemical compounds phenol (4-cyclohexyl); 1-methyl-1-propene-1,3-diyl; 1,2,3,4-tetrahydro-6-(phenyl methyl); and 2-pentene-1,5-diyl that *E. eichhorniae* males lack.

When comparing the species, *E. eichhorniae* males have 1,2,3,4-tetrahydro-6-(phenyl methyl) that *E. catarinensis* females lack, while *E .eichhorniae* females have benzenebutanoic acid that *E. catarinensis* males lack. When comparing the sexes between species, *E. catarinensis* males have chemical compounds phenol (4-cyclohexyl); 1-methyl-1-propene-1,3-diyl; and 2-pentene-1,5-diyl that *E. eichhor-*

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Table 1. Chemical emissions of *Eccritotarsus* spp. (Hemiptera: Miridae) as denoted by the retention time (in minutes) of both females and males, for both *E. catarinensis* (Carvalho) and *E. eichhorniae* (Henry) in the gas chromatographs.

Retention Time (min)	Chemical Compound Name
9.06; 10.80; 12.19; 13.41 & 17.94	Siloxanes (breakthrough material from the Tenax); ignored
12.89	2-Phenylpropenal
14.89	Benzenebutanoic acid (methyl ester) (ç-methyl-)
15.65–15.66	3-(5,6,7,8-Tetrahydro-1-naphtyl)-propionic acid
16.58	5-t-Butyl-1,2,3-trimethylbenzene
16.66	3-(5,6,7,8-Tetrahydro-1-naphtyl)-propionic acid
17.00	Phenol (2-cyclohexyl)
17.01–17.01	Phenol (4-cyclohexyl)
17.05–17.06	Benzene: 1,1′-(1-methyl-1,3-propanediyl)-bis-
17.12–17.12	Benzene: 1,1′-(1-methyl-1-propene-1,3-diyl)-bis-
17.15–17.16	Naphthalene: 1,2,3,4-tetrahydro-6-(phenyl methyl)-
17.28–17.28	1H Indene: 2,3-dihydro (1,1,3-trimethyl-3-phenyl)
17.55	Benzene: 1,1′-(2-pentene-1,5-diyl)-bis-
17.89–17.90	Benzenesulfonamide (n-ethyl-2-methyl)
17.96–17.97	Sclareoloxide
18.19–18.20	Naphthalene: 1,2,3,4-tetrahydro-1-phenyl
18.37	Benzenesulfonamide (n-ethyl-4-methyl)

niae females lack, while *E. eichhorniae* males have chemical compounds benzenebutanoic acid; phenol (4-cyclohexyl); 1-methyl-1-propene-1,3-diyl; 1,2,3,4-tetrahydro-6-(phenyl methyl); and 1,1,3-trimethyl-3-phenyl that *E. catarinensis* males lack.

In summary, GC-MS results suggest that *E. catarinensis* females and *E. eichhorniae* males have chemical emissions that their conspecifics and the same sex of the other species lack. These differences could be caused by the differences in the MSG, thorax, and antennae of the insects that were reported by Henry (2017). More importantly, when this pair was crossed in an interbreeding experiment, they produced very few offspring (Paterson et al. 2016). Furthermore, these results may explain why *E. echhorniae* males and *E. catarinensis* females preferred to mate only with their respective conspecifics (Mnguni 2019b).

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Table 2. Gas chromatography–mass spectrometry (GCMS) presence-absence of *Eccritotarsus* spp. (Hemiptera: Miridae) chemical emissions. The left column represents five replicates of each sex for both *Eccritotarsus* species. Each replicate had 10 females and 10 males placed in separate air entrainment collecting chamber. The top column represents the observed peaks based on retention times (mins). The presence of the peak was represented by (I) while the absence was represented by (0). Symbols: EC stands for *E. catarinensis*, EE for *E. eichhorniae*, f for females, and m for males ($n = 5$).

Retention Time (min) of Peaks Denoting Chemical Compounds of Mirids Using GCMS Analysis Excluding Siloxanes																
Sex	**12.89**	**14.89**	**15.66**	**16.58**	**16.66**	**17.00**	**17.01**	**17.06**	**17.12**	**17.16**	**17.28**	**17.55**	**17.89**	**17.97**	**18.20**	**18.37**
ECf1	0	I	I	I	I	0	I	I	I	0	I	I	I	I	I	I
ECf2	0	I	I	I	I	0	I	I	I	0	I	I	I	I	I	I
ECf3	0	I	I	I	I	0	0	I	I	0	I	I	I	I	I	I
ECf4	0	I	I	I	I	0	I	I	I	0	I	I	I	I	I	I
ECf5	0	I	I	I	I	0	I	I	I	0	I	I	I	I	I	I
ECm1	0	0	I	I	I	0	0	I	0	0	0	0	I	0	I	I
ECm2	0	0	I	I	I	0	0	I	0	0	0	I	I	I	I	I
ECm3	0	0	I	I	I	0	0	I	0	0	0	I	I	0	I	I
ECm4	0	0	I	I	I	0	0	I	0	0	0	0	I	I	I	I
ECm5	0	0	I	I	I	0	0	I	0	0	0	I	I	I	I	I
EEf1	0	I	I	I	I	0	0	I	0	0	0	0	I	I	I	I
EEf2	0	I	I	I	I	0	0	I	0	0	I	0	I	I	I	I
EEf3	0	I	I	I	I	0	0	I	0	0	I	0	I	I	I	I
EEf4	0	I	I	I	I	0	0	I	0	0	0	0	I	I	I	I

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Table 2. Continued.

Retention Time (min) of Peaks Denoting Chemical Compounds of Mirids Using GCMS Analysis Excluding Siloxanes																
Sex	**12.89**	**14.89**	**15.66**	**16.58**	**16.66**	**17.00**	**17.01**	**17.06**	**17.12**	**17.16**	**17.28**	**17.55**	**17.89**	**17.97**	**18.20**	**18.37**
EEf5	0	I	I	I	I	0	0	I	0	0	0	0	I	I	I	I
EEm1	0	I	I	I	I	0	I	I	I	I	I	I	I	I	I	I
EEm2	0	I	I	I	I	0	I	I	I	I	I	I	I	I	I	I
EEm3	0	I	I	I	I	0	I	I	I	I	I	I	I	I	I	I
EEm4	0	I	I	I	I	0	I	I	I	I	I	I	I	I	I	I
EEm5	0	I	I	I	I	0	I	I	I	I	I	I	I	I	I	I

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Table 3. Relative abundances (mean ± SE) of the gas chromatography–mass spectrometry spectra found in the metathoracic scent glands of the two *Eccritotarsus* species.

Peaks[a]	*E. catarinensis* Females	*E. catarinensis* Males	*E. eichhorniae* Females	*E. eichhorniae* Males
12.89	0	0	0	0
14.89	2.4 ± 1.8	0	2	2
15.66	30.4 ± 1.24	22.4 ± 0.82	20.4 ± 0.82	17.2 ± 1.43
16.58	12.8 ± 0.66	28.4 ± 0.44	26.8 ± 0.92	20.4 ± 2.23
16.66	50.8 ± 2.40	99.6 ± 0.18	98.8 ± 0.36	100
17.00	0	0	0	0
17.01	3.2 ± 0.22	0	0	6 ± 0.40
17.06	10.4 ± 0.95	7.6 ± 0.33	5.6 ± 0.18	11.2 ± 0.54
17.12	3.2 ± 0.36	0	0	3.6 ± 0.33
17.16	0	0	0	2
17.28	3.6 ± 0.33	0	2	2
17.55	7.6 ± 0.52	6.8 ± 0.22	0	4.4 ± 0.18
17.89	5.6 ± 0.18	4.4 ± 0.18	4.4 ± 0.18	3.6 ± 0.18
17.97	14 ± 1.65	10.4 ± 0.18	8.4 ± 0.33	7.2 ± 0.36
18.20	36 ± 2.61	27.6 ± 0.72	22.4 ± 0.52	10.8 ± 0.96
18.37	29.6 ± 2.25	36.8 ± 1.34	34 ± 1.44	23.6 ± 1.78

[a] 12.89 = 2-phenylpropenal; 14.89 = benzenebutanoic acid (methyl ester) (ç-methyl-), lacked by *E. catarinensis* males; 15.66 = propionic acid; 16.58 = 5-t-butyl-1,2,3-trimethylbenzene; 16.66 = propionic acid; 17.00 = phenol (2-cyclohexyl); 17.01 = phenol (4-cyclohexyl); 17.06 = 1-methyl-1,3-propanediyl; 17.12 = 1-methyl-1-propene-1,3-diyl; 17.16 = 1,2,3,4-tetrahydro-6-(phenyl methyl), exists only in *E. eichhorniae* males; 17.28 = 1,1,3-trimethyl-3-phenyl; 17.55 = (2-pentene-1,5-diyl; 17.90 = n-ethyl-2-methyl; 17.97 = sclareoloxide; 18.20 = 1,2,3,4-tetrahydro-1-phenyl and 18.37 = n-ethyl-4-methyl. ($\chi^2 = 2.20$, df = 3, $P = 0.532$).

The cluster analysis suggests *E. eichhorniae* males and *E. catarinensis* females have similar chemical emissions inasmuch as *E.catarinensis* males and *E.eichhorniae* females also have similar chemical emissions based on relative abundances. The MDS plot and the PCO also suggest that *E. eichhorniae* females and *E. catarinensis* males have similar chemical emissions that have very little variability inasmuch as *E. catarinensis* females and *E. eichhorniae* males have similar chemical emissions that have very high variability. Also, *E. catarinensis* males and *E. eichhorniae* males, as well as *E. catarinensis* females and *E. eichhorniae* females are at opposite ends of the graph from each other and, therefore, have significantly different chemical emission variations (Fig. 2).

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Discussion

Similar to Yasuda and Higuchi (2012), the chemical emissions reported in our study involving the two sympatric species of *Eccritotarsus* helps us better understand the patterns reported in previously discussed genetic, interbreeding, and mating studies. More importantly, the chemical emissions support the observed minimal mating incidences between the two species that have been reported in Mnguni (2019b). They also support the interbreeding incompatibility reported in Paterson et al. (2016). The unique chemical emission differences revealing that *E. catarinensis* females and *E. eichhorniae* males have chemical emissions that their respective conspecifics and the same sex of the other species lack could be important. A few statistical techniques used in this study in a form of a cluster analysis, MDS plot, and PCO have further supported the observation that the cross-breeding pairs, *E. catarinensis* females and *E. eichhorniae* males have similar chemical emissions inasmuch as *E. eichhorniae* females and *E. catarinensis* males also have similar chemical emissions.

The fact that *E. catarinensis* males lack benzenebutanoic acid that other individuals have, while *E. eichhorniae* males have 1,2,3,4-tetrahydro-6-(phenyl methyl) that other individuals lack could also prove to be significant, especially if males attract females in *Eccritotarsus*. The presence or absence of a unique chemical emission could be the barrier that exists between the two species, and they may explain the interbreeding and mating incompatibilities that have somewhat exposed the presence of prezygotic reproductive isolation mechanisms. Nevertheless, the differences in chemical emissions between the two species still need further investigation.

These unique differences could be key in explaining the interbreeding and mating incompatibilities reported in Paterson et al. (2016) and Mnguni (2019b). These differences may have been caused by the subtle morphological differences in the MSG, thorax, and antennae of these two species of *Eccritotarsus* as reported by Henry (2017). The results suggest that, although the chemical emissions of the two species are very similar, there are some consistent differences present between the species, particularly between the sexes, and these could be of evolutionary importance. The quantity and quality of chemical emission assessments as key determinants in reproductive isolation were necessary in *Eccritotarsus* as suggested by Groot et al. (1999), Millar et al. (1997), Millar and Rice (1998), Zhang and Aldrich (2003a), and Yang et al. (2015). Furthermore, it is possible that emissions present in small amounts also could have an important role in the attraction and mate recognition of one sex towards the other. Therefore, an antennal stimulation assessment of these two species is warranted for further study.

In the Miridae, research has shown that it is common for females and males to have very similar or identical chemical emissions within and between species, but the reason for that is not clear yet (Zhang et al. 2015). Some authors have argued that the same emissions that females use to attract males are instead used by males of the same species as defense pheromones, allomones, or by individuals from other species as kairomones (Millar et al. 1997). Although there are slight differences in chemical emissions produced by the two species, the similarities and differences of the functions served by the major compounds remain unknown.

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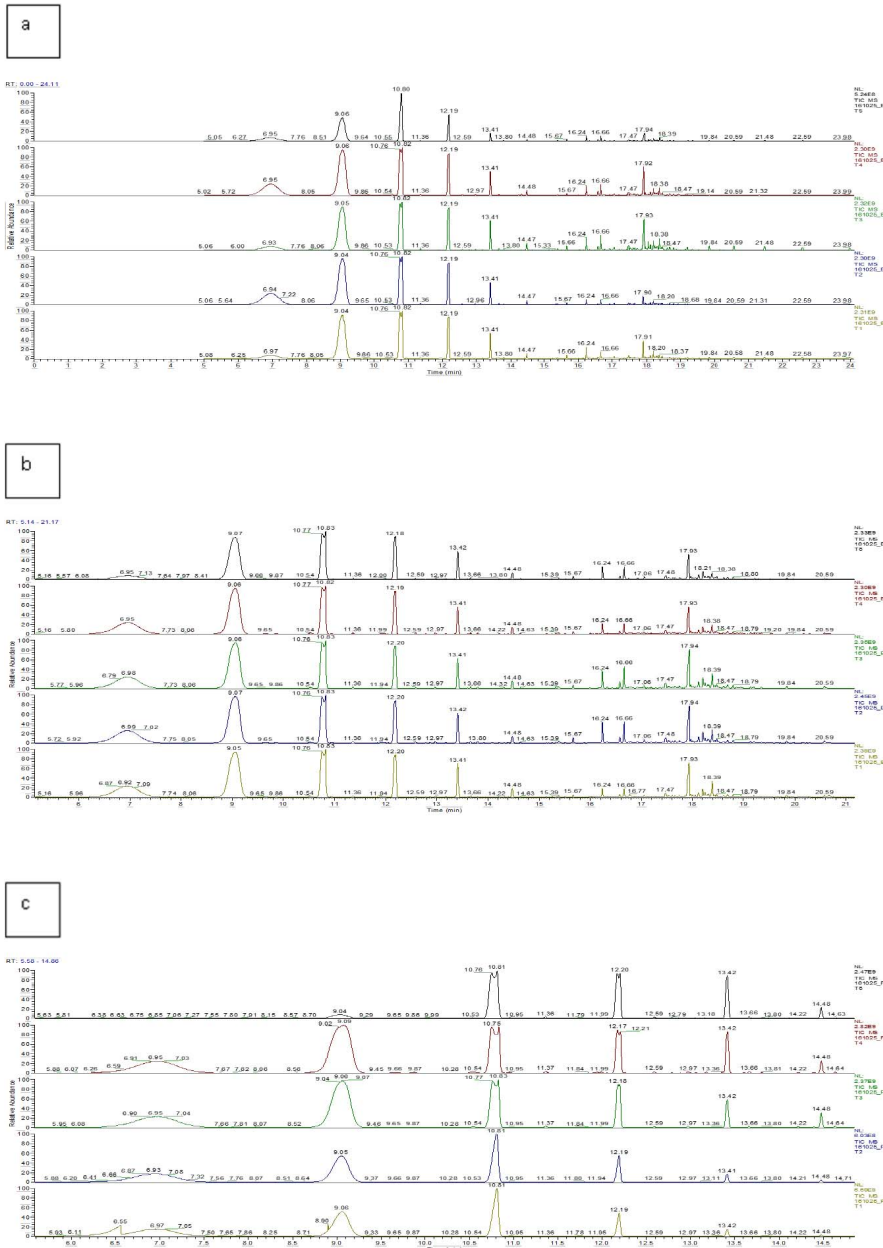

Fig. 1. Gas chromatographs of *Eccritotarsus*: (a) *E. catarinensis* females, (b) *E. catarinensis* males, (c) *E. eichhorniae* females, (d) *E. eichhorniae* males, and (e) single replicates of females and males from both species. The five replicates for both sexes of both species are color-coded as follows: T1 (gold), T2 (navy), T3 (green), T4 (red), and T5 (black); the single replicate is color-coded in this way: *E. catarinensis* females (blue), *E. catarinensis* males (green), *E. eichhorniae* females (red), and *E. eichhorniae* males (black).

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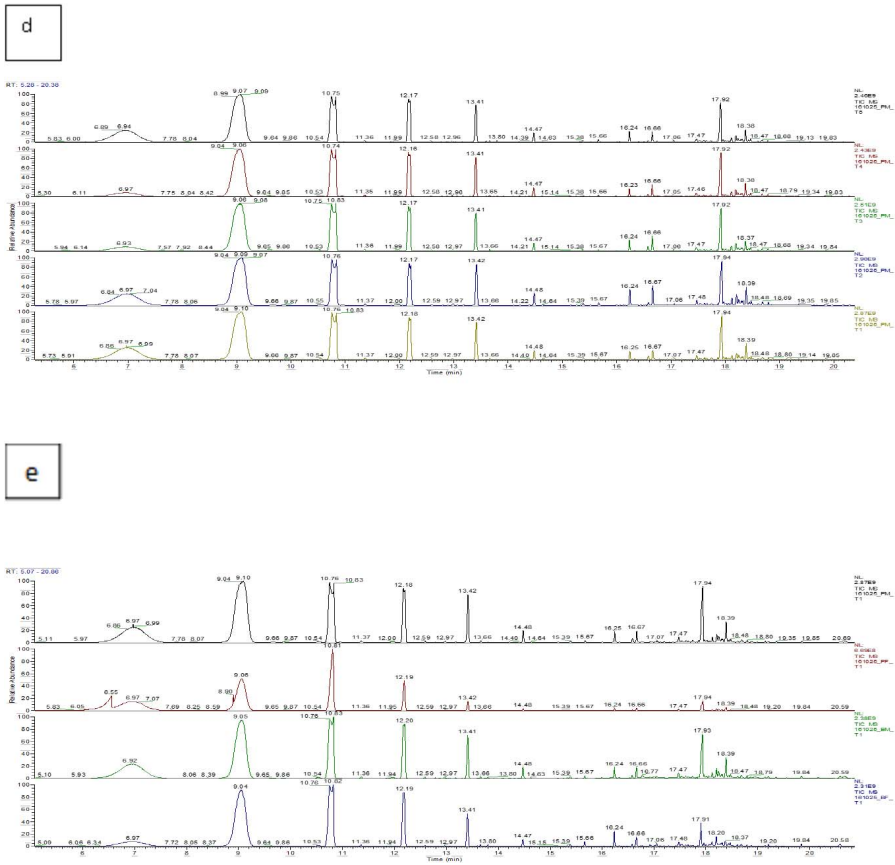

Fig. 1. Continued.

Establishing which sex attracts the other sex in *Eccritotarsus* will be important going forward and is warranted.

Furthermore, establishing the role played by the chemical emissions reported here, individually, binary, and in multiple combinations, would drive towards establishing the major chemical emissions in the two species. From then, it would be ideal to use those major emissions to try to pinpoint specific sex pheromones. Interaction studies will have to be conducted to assess whether one species will affect the fitness of the other species positively, negatively, or neutrally. More pheromone communication investigations are needed to make a better distinction between these two species assessed in this study, and one way could be through an antennal stimulation. Zhang and Aldrich (2008) have further suggested that assessing the specificity of receptor neurons involving the behaviorally and physiologically active chemical compounds using a single-cell recording technique could prove useful.

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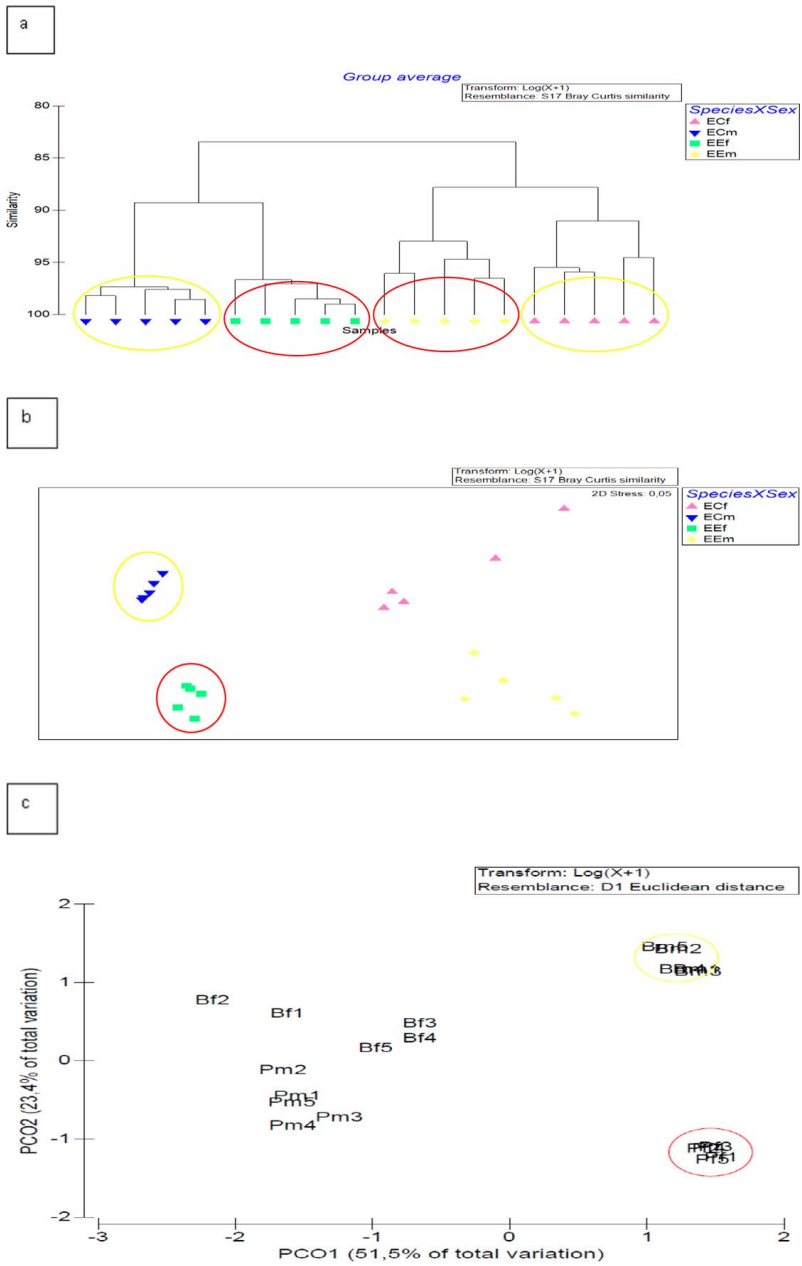

Fig. 2. Graphs showing (a) cluster analysis (CA), (b) multidimensional scaling (MDS) scatterplot (2D stress: 0.06) and (c) principal component ordination (PCO1, 51.5% and PCO2, 23.4% of total variation) using log-transformed (mean ± SE) chemical compound compositions of two *Eccritotarsus* species. EC and B indicate *E. catarinensis*, EE and P

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Other aspects worth considering are the sensilla on the antennae, as well as the neurons and chemoreceptors that pick up pheromone blends, as they have also been shown to play a key role in chemical communication (Teal and Tumlinson 1992). Most importantly, investigating how the MSG regulates the production of pheromones between the *Eccritotarsus* species could also give an indication as to what might have caused the reproductive isolation and eventual speciation caused by sexual selection mechanisms. This could prove to be key since the compartmentalized MSG produces many forms of pheromones that serve different functions and could be intertwined, making it difficult to be able to tease apart sex pheromones from other pheromones produced and released for other purposes (Zhang and Aldrich 2008).

The chemical emission assessment of the two species of *Eccritotarsus* consist of saturated or unsaturated esters, as well as unsaturated ketoaldehyde, known to be consistent throughout the Miridae (Yang et al. 2015). However, it will be worthwhile to tweak the functional group of the chemical emissions reported here because a change in the functional group may cause species to react very differently to a pheromone (Schwarz et al. 1990). Schwarz et al. (1990) highlighted the importance of functional groups, to an extent that they may be a key determinant of whether one sex succeeds in attracting the opposite sex or not. Functional groups could also give an indication of the mechanisms that resulted in speciation.

In conclusion, the chemical emissions of *Eccritotarsus* spp. (Hemiptera: Miridae) has shown a sex difference involving the *E. catarinensis* females and the *E. eichhorniae* males, which could be a key mechanism that led to reproductive isolation and the eventual speciation between the two species. The two species share several chemical emissions, but *E. catarinensis* females and *E. eichhorniae* males have chemical emissions that are unique to them, but their conspecifics and the same sex of the other species lack those same emissions. There is also a difference observed in males since *E. catarinensis* males lack an emission that other sexes have, while *E. eichhorniae* males have an emission that other sexes lack. These results may be explaining the variation in mating preferences reported in Ismail and Brooks (2018) and Mnguni (2019b), and the crossbreeding results reported in Paterson et al. (2016). Chemical emissions confirm the presence of prezygotic reproductive isolation mechanisms that possibly led to speciation in the two sympatric *Eccritotarsus* species.

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indicate *E. eichhorniae*, f indicates female, and m indicates male. The *E. catarinensis* females were represented by pink, *E. eichhorniae* males were represented by green, *E. catarinensis* females were represented by blue, and *E. eichhorniae* males were represented by yellow ($n = 5$).

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