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Effect of Larval Topical Application of Juvenile Hormone on Cuticular Chemical Composition of *Mischocyttarus consimilis* (Vespidae: Polistinae) Females

EF Neves[1,2,3], TS Montagna[1,2], LC Santos-Junior[1,2], KB Michelutti[2,4], CAL Cardoso[4], LHC Andrade[4], SM Lima[4], WF Antonialli-Junior[1,2,4]

1 - Programa de Pós-graduação em Entomologia e Conservação da Biodiversidade, Universidade Federal da Grande Dourados, Dourados-MS, Brazil

2 - Laboratório de Ecologia Comportamental, Universidade Estadual de Mato Grosso do Sul, Dourados-MS, Brazil

3 - Instituto Federal de Educação Ciência e Tecnologia de Mato Grosso do Sul, Naviraí-MS, Brazil

4 - Programa de Pós-graduação em Recursos Naturais, Universidade Estadual de Mato Grosso do Sul, Dourados-MS, Brazil

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Corresponding author
Kamylla Michelutti
Programa de Pós-graduação em Recursos Naturais
Universidade Estadual de Mato Grosso do Sul
CEP: 79804-970, Mato Grosso do Sul, Brasil.
E-Mail: kamylla_michelutti@yahoo.com.br

Abstract

Juvenile Hormone (JH) is considered the main determinant of caste in social insects, though little is known about how this hormone acts in social wasps, especially the independent-founding species. The known relationship between JH titer and caste in the colony and we suggest a relationship among the effects of JH and the cuticular chemical profile. Therefore, this study aimed to test the hypothesis that topical application of JH to larvae of different instars alters the cuticular chemical composition of newly emerged females of *Mischocyttarus consimilis* (Zikán), influencing the dynamics of colony. Two techniques were used to evaluate the variation in cuticular chemical composition: Fourier transform infrared photoacoustic spectroscopy and gas chromatography coupled to mass spectrometry (GC-MS). Indeed, the application of JH did significantly alter the cuticular chemical composition of adult females that received treatment at the larval stage in comparison to control. The effects of JH were instar-dependent in that the results of topical application were significant when performed at third larval instar. Overall, these results add evidence that caste determination may, at least in part may be pre-imaginal in species of independent-founding social wasps.

Introduction

Among other factors, the success of perfect social organization in insects is built on recognition among nestmates. This phenomenon is a determinant for avoidance of predators and parasites, or even loss of resources (Mitra et al., 2014).

By the detection and emission of chemical compounds, insects find mating partners, food, or prey; choose oviposition locations; defend themselves against predators; and organize their colonies, in the case of social insects (Zarbin et al., 2009). Compounds that form the basis of interaction between and among individuals of the same species are known as pheromones, while in different species, these compounds are called allelochemicals (Gullan & Cranston, 2012).

Pheromones are chemical compounds produced by exocrine glands and are released from dermal structures. Other compounds in the cuticle of insects can also act as pheromones, promoting interactions between individuals of the same species. These compounds are mainly represented by hydrocarbons produced in the oenocytes, absorbed by lipophorin and transported by hemolymph (Howard & Blomquist, 2005; Blomquist & Bagnères, 2010; Gullan & Cranston, 2012).

Surface pheromones express patterns that vary according to sex, caste, or age, as described by Antonialli-Junior et al. (2007) for the ant *Ectatomma vizottoi* (Almeida), by Kather et al. (2011) for *Apis mellifera* (L.), and by Neves et al. (2012) for the social wasp *Mischocyttarus consimilis* (Zikán).

These chemical compounds are known as cuticular hydrocarbons (CHCs), and they are regulated by exogenous and genetic mechanisms that act together to express a unique chemical profile that reflects the cuticle composition of each individual. The chemical profile can be acquired within the first few hours after emergence, as reported by Panek et al. (2001) for *Polistes fuscatus* (F.) and by Neves et al. (2012) for *M. consimilis*.

This chemical profile is specific to each individual, signaling, for example, the role of each worker within the colony (Ferreira-Caliman et al., 2010; Kather et al., 2011). Specifically, alpha females responsible for reproduction possess cuticular chemical compounds different from those found in workers, as described in studies of the wasp species *Polistes versicolor* (Olivier) (Torres et al., 2014) and *Polistes ferreri* (Sassure) (Soares et al., 2014).

In addition, some CHCs of social insect queens may induce sterility in workers, as seen in the study of Van Oystaeyen et al. (2014) with the wasp species *Vespula vulgaris*, the buff-tailed bumblebee *Bombus terrestris* and the desert ant *Cataglyphis iberica*.

On the other hand, an important inducer of caste determination in social insects is Juvenile Hormone (JH), which is present in higher titers in the hemolymph of queen wasps, compared to the titers in workers (Giray et al., 2005; Tibbetts & Huang, 2010). In ants, different levels of JH also generate morphological differences between castes and subcastes (Nijhout & Wheeler, 1982). In bees, such as *A. mellifera*, JH titers are regulated by differential feeding at the larval phase, which originates queens and/or workers (Laidlaw, 1992). Similarly, in termites, different JH titers in worker larvae are responsible for the formation of pre-soldiers, soldiers, and nymphs (Park & Raina, 2003).

In swarming wasps, Kelstrup et al. (2014) suggested that the titers of JH prepare the ovaries for further development since the titer of JH of possible future queens increases in the absence of established queens. However, in independent-founding wasps with an almost imperceptible morphological differentiation between queens and workers, caste determination, which is primarily based on behavioral and physiological patterns, is poorly understood (Weaver, 1996). In this case, caste determination is proposed to be post-imaginal (Solís & Strassmann, 1990). In these wasps, JH activity can affect the aggression of queens, prevent ovarian development of workers, and determine the guard and foraging activities in workers (Robinson & Vargo, 1997; Giray et al., 2005). However, Kelstrup et al. (2015) pointed out that the role of JH is not restricted since even founders of closely related wasps that share the same environment (*Polistes dominula* and *Polistes smithii*) have divergent endocrine profiles.

Nevertheless, some studies found evidence that, at least in part, caste determination in independent-founding wasps may be pre-imaginal, with JH playing an important role (Gadagkar et al., 1990; Judd et al., 2010). In *Polistes metricus* (Say, 1831), differential feeding at the larval phase produces larger adults with greater reproductive potential (Rossi & Hunt, 1988; Judd et al., 2010). Only one study has tested the effect of topical JH application in larvae of independent-founding wasps. It found a significant enhancement of the signals for pre-imaginal caste determination among this group of wasps (Montagna et al., 2015).

The relationship between JH titer and caste in the colony suggests a potential relationship also between the JH titer and the cuticular chemical profile in *Mischocyttarus* species, as already evaluated in colonies of the wasp *Polistes dominula* (Christ, 1791) (Sledge et al., 2004; Izzo et al., 2010) and the ant *Myrmicaria eumenoides* (Lengyel et al., 2007).

Nonetheless, a few studies have reported on the relationship between the effects of JH titers in cuticular chemical profiles (Lengyel et al., 2007; Kelstrup et al., 2017; Oliveira et al., 2017), especially in independent-founding paper wasps. Therefore, the objective of this study was to experimentally treat larvae from different instars by topical application of JH and analysis the effect of this application in the chemical perfil of *M. consimilis* newly emerged females. By so doing, we tested the hypothesis that such treatment would alter the chemical cuticular profile of *M. consimilis* newly emerged females and prove an alignment between the endocrine system and chemical expression.

Material and Methods

Treatments and collection of individuals

Experimental manipulation was performed using eight colonies of the eusocial wasp *M. consimilis* nested in rural areas in the municipality of Dourados (Mato Grosso do Sul State, Brazil; 22º13'16"S, 54º48'20"W). These colonies were in the worker-producing phase (Jeanne, 1972). All colonies were transferred to wooden artificial shelters, measuring 1.2 x 1.2 x 2.5 m and covered with tiles for thermoregulation, constructed as described by Montagna et al. (2015). The interior of each shelter contained four movable boards, positioned horizontally 1.90 m off the ground, which were attached to transversal clapboard using a hinge that allowed individual rotation of 180 degrees. A single colony was transferred and attached to each board, as described by Prezoto and Machado (1999).

The rotation of the boards enabled the topical application of JH (Hormone III Code J2000, Sigma-Aldrich, Protons Cientifica LTDA) by gravity, precisely onto the larvae selected for treatment. For maximal accuracy of JH application onto the larvae, an automatic 2 µL capacity micropipette was used, following the methodology of Montagna et al. (2015).

The concentration and volume of JH applied to the larvae followed the protocol used by Montagna et al. (2015) who evaluated if the responses to the application of JH in larvae of *M. consimilis* would vary according to developmental stage, especially from the 3rd instar. The topical applications of JH were performed in the 3rd, 4th and 5th instars. The application protocol of 1 µg of JH in 1 µL of acetone, as described by Shorter and Tibbetts (2009), was adopted here.

A total of 460 larvae received JH application, 202 larvae of 3rd instar, 164 larvae of 4th instar and 94 of 5th instar. As a form of control, the development of 37 larvae that did not receive any type of treatment or manipulation was monitored.

The population size of each colony, together with the very high mortality rate caused by manipulation, as reported by Montagna et al. (2015), made it impossible to apply the pure solvent in individuals from the same colony in order to assess its effect in isolation. Larval instar determination was performed by measuring the cephalic capsule of the larvae used in each treatment, based on an established rule (Dyar, 1890). For instar control, a larva of the same age as treated larvae was removed from the nest for cephalic capsule measurement. Previous studies have recognized five instars during the larval development of this species (Michelutti et al., 2017).

In order to facilitate application to the different larval instars and collect newly emerged females, daily nest mapping was performed on hexagon-printed paper (Giannotti, 1998). As a result, it was possible to perform a daily monitoring of each treated larva/control, allowing us to detect the cell from which the adult emerged. Each newly emerged female, treated, or not, during the larval stage, received a mark with a colored dot on the leg. Then, when the females completed 12 days of life, they were collected, anesthetized and sacrificed by freezing for further analysis of the cuticular chemical profile. All the newly emerged females from the larvae under treatment, together with the same number from the group without any treatment, were collected, separated, sacrificed, and preserved by freezing, hence avoiding the use of any type of chemical fixative or preservative that might react with the chemical components of the cuticle.

Analysis of the effect of JH topical application on cuticular chemistry by Fourier Transform Infrared Photoacoustic Spectroscopy

The variation in cuticular chemical compounds was accessed by the nondestructive Fourier transform infrared photoacoustic spectroscopy technique (FTIR-PAS). This technique has been successfully used to evaluate differences between groups, but without evaluation of compounds that might be responsible for the differences, and it has already been applied in studies with ants and social wasps (Neves et al., 2012; Torres et al., 2014). FTIR-PAS is faster and less expensive than gas chromatography, the method most commonly employed, and requires minimal sample preparation. It enables the analysis of opaque and solid materials, is nondestructive (Michaelian, 2003), and can provide rapid and reliable results.

Our study complements the one performed by Montagna et al. (2015), who evaluated ovarian development on the same samples used here; therefore, we standardized by analyzing the thorax of all females. These samples were preheated in a vacuum oven for 48h to minimize the absorption of moisture, which could interfere with the spectrum. Infrared photoacoustic spectroscopy was used to measure the radiation absorbed by the samples in the mid-infrared region (4000 - 400 cm^{-1}). This region is sensitive to vibrations of molecular chemical groups, enabling the identification of molecular radicals and the types of chemical bonds in the sample (Smith, 1999). During the analysis, the system was purged with dry air to remove particles, water vapor, and CO_2, and the photoacoustic chamber coupled to the microphone was purged with helium gas to increase sensitivity. For each sample, a mean spectrum was obtained from 64 scans with resolution of 8 cm^{-1}.

Possible differences among the cuticular chemical compositions of newly emerged females from the treated larvae were evaluated using discriminant function analysis based on peak intensities of the mean spectra (Quinn & Keough, 2005). Using the same form of analysis, we compared the differences between the groups on a par.

Analysis of the effect of JH topical application on cuticular chemistry by Gas Chromatography coupled to Mass Spectrometry

To complement the analysis and evaluate the qualitative and quantitative differences caused by JH application, the same thorax samples evaluated by FTIR-PAS were also analyzed by gas chromatography coupled to mass spectrometry (GC-MS). Each sample was immersed in 2000 µL of hexane for 2 min. Subsequently, the solute resulting from the extraction was dried in a fume hood and stored frozen for a maximum of 30 days. For the chromatographic analysis, each extract was dissolved in 200 µL of hexane.

The samples were analyzed using a gas chromatograph (GC-2010 Plus, Shimadzu, Kyoto, Japan) equipped with a mass detector (GC-MS Ultra 2010, Shimadzu, Kyoto, Japan). Separation was achieved with a fused silica DB-5 capillary column (5% phenyl dimethylpolysiloxane, 30 m x 0.25 mm x 0.25 µm thickness; J & W, Folsom, California). The analytical conditions were as follows: helium (99.99%) carrier gas at a flow rate of 1.0 mL min-1; 1 µL injection volume, splitless injection mode; initial oven temperature of 150 °C, followed by a ramp to 300 °C at 3 °C min-1, and holding the final temperature for 10 min; injector, detector, and transfer line temperatures of 220, 300, and 200 °C, respectively. MS was operated in scan mode with electron impact ionization voltage of 70 eV, mass band from m/z 45 to 600, and scan interval of 0.3 s.

Identification of the compounds was based on the calculated retention indexes (Van den Dool & Kratz, 1963), employing a series of linear alkanes (C_{14}-C_{38}, Sigma-Aldrich, purity ≥ 90%), comparison with literature indexes (Bonavita-Cougourdan et al., 1991; Howard et al., 2001; Bonckaert et al., 2012; Costanzi et al., 2013; Weiss et al., 2015), and mass spectral data from the equipment libraries (NIST 21 and Wiley 229) for interpretation of the spectra when no standard was available for comparison. We only considered the peaks that occurred in at least 30% of each of the groups of samples evaluated.

The differences among the cuticular chemical compositions of newly emerged females from larvae under different treatments were evaluated by discriminant function analysis, considering the relative abundances of the peaks in the chromatograms, using all the peaks detected (Quinn & Keough, 2005) and Systat 12 software. Using the same form of analysis, we compared the differences between the groups on a par.

Results

Of all 460 larvae that received JH treatment in the larval phase, 202 corresponded to 3rd instar larvae, out of which only 29 larvae reached adulthood; of the 164 larvae of 4th instar, 34 reached adulthood, and of the 94 larvae that received JH application in the 5th instar, only 18 individuals completed their development to reach adulthood.

Analysis of the effect of JH topical application on cuticular chemistry by FTIR-PAS.

Visual inspection of the mean spectra obtained by FTIR-PAS suggested that differences among the studied groups were subtle (Fig 1). However, discriminant analysis showed that the cuticular chemical profiles of the controls and newly emerged females from larvae that received topical applications of JH were significantly different (Wilks' lambda = 0.329; F = 2.847; p = 0.0012). The first canonical root explained 69% of the results, while the first and second roots together explained 84% of the results. Fourteen peaks were analyzed, and five (at 1041, 1373, 1650, 2962, and 3093 cm^{-1}) were significant for separation of the groups (Figs 1 and 2; Table 1). The analysis between groups, pair by pair, shows that only females, the larvae of which were applied with JH in the 3rd instar, and controls had significant difference in cuticle composition (F = 5.310; p = 0.027) and that functional group 8 (1650) N-H and/or C-N (Amide II) was/were the most important compound(s) for the separation of these two groups.

Analysis of the effect of JH topical application on cuticular chemistry by Gas Chromatography

Visual inspection of the chromatograms obtained by GC-MS (Fig 3) showed that the groups receiving JH application at the third, fourth, and fifth larval instars presented

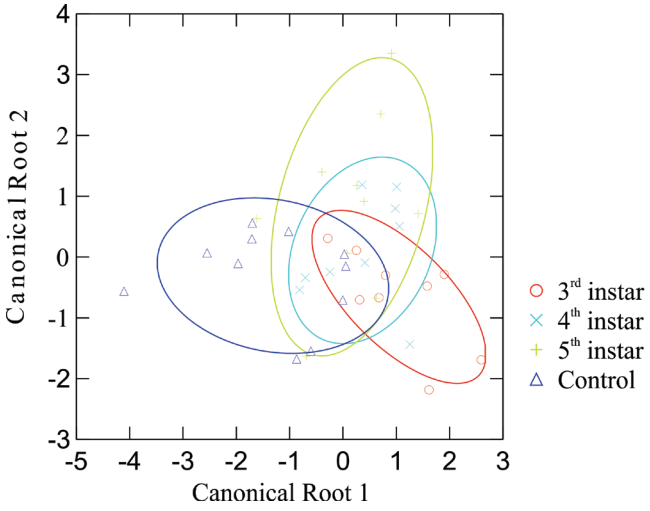

Fig 2. Dispersion diagram of the results of discriminant analysis applied to the FTIR-PAS data, showing the two canonical roots of differentiation between the control group and the groups with application of Juvenile Hormone to different larval instars of *Mischocyttarus consimilis*.

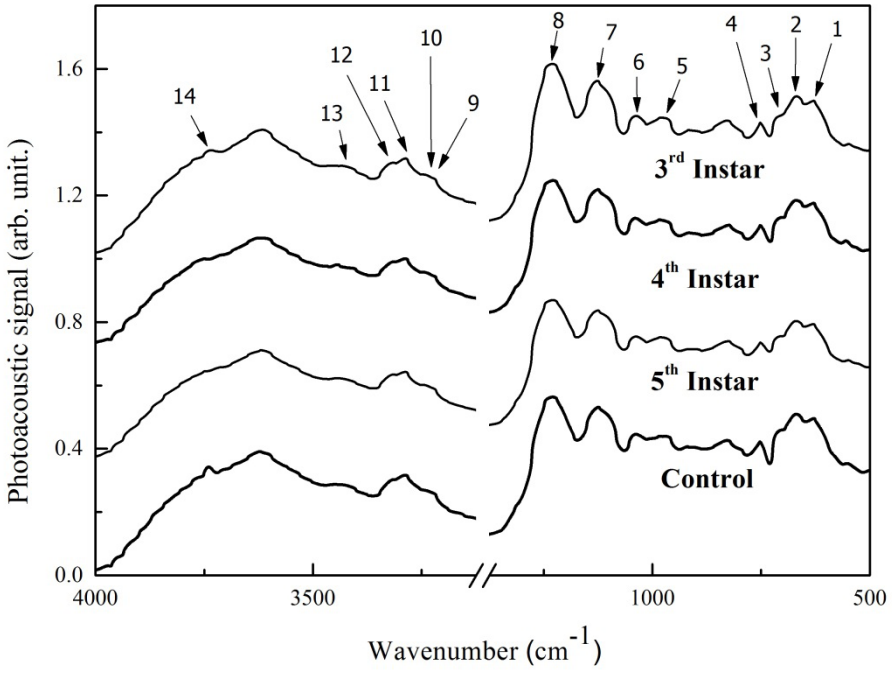

Fig 1. Mean FTIR-PAS spectra of *Mischocyttarus consimilis* thoraxes following JH application to different larval instars. Arrows indicate the peaks used in the statistical analysis.

Table 1. Wavenumber, coefficients of the two canonical roots, functional group, and vibration mode of peaks identified in the mean FTIR-PAS spectra for wasps with JH application to different larval instars.

Peak	Wavenumber (cm^{-1})	Functional Group	Vibration Mode	First Canonical Root	Second Canonical Root
1	1041	C-H	Bending in the plane	2.724	3.529
2	1079	C-H	Bending in the plane	-	-
3	1110	C-H	Bending in the plane	-	-
4	1157	C-H	Bending in the plane	-	-
5	1373	$C\text{-}CH_3$	Symmetrical bending	-1.017	2.290
6	1450	CH_2	Scissors	-	-
7	1542	N-H and/or C-N (Amide II)	Balance in plane	-	-
8	1650	N-H and/or C-N (Amide II)	Plane bending and/or asymmetrical stretching	1.723	2.973
9	2854	CH_2	Symmetrical Stretching	-	-
10	2877	$CH\text{-}CH_3$	Symmetrical Stretching	-	-
11	2931	CH_2	Asymmetrical Stretching	-	--
12	2962	CH_3	Asymmetrical Stretching	-0.890	4.841
13	3093	N-H	Harmonic Bending	2.949	1.965
14	3425	N-H	Stretching	-	-

some compounds with greater intensity compared to the control group. For example, 7-Methylheptacosane was more intense for the group that received JH at the 4th and 5th larval instar, while heptacosane and 3-Methylpentacosane were more intense for all the treatments compared to the control group (Fig 3).

A total of 30 peaks were detected, of which 29 were identified (Table 2). The statistical analysis showed that 16 peaks were significant for separation of the groups and that 10 stood out as being the most statistically significant (Fig 3; Tables 2 and 3). The discriminant analysis demonstrated that the cuticular chemical profiles of the control group and newly emerged females from larvae that received topical application of JH at different instars were significantly different (Wilks' lambda = 0.00; $F = 2.83$; $p < 0.05$). The first canonical root explained 71.6% of the results, and the first and second roots together explained 20% of the results (Fig 4).

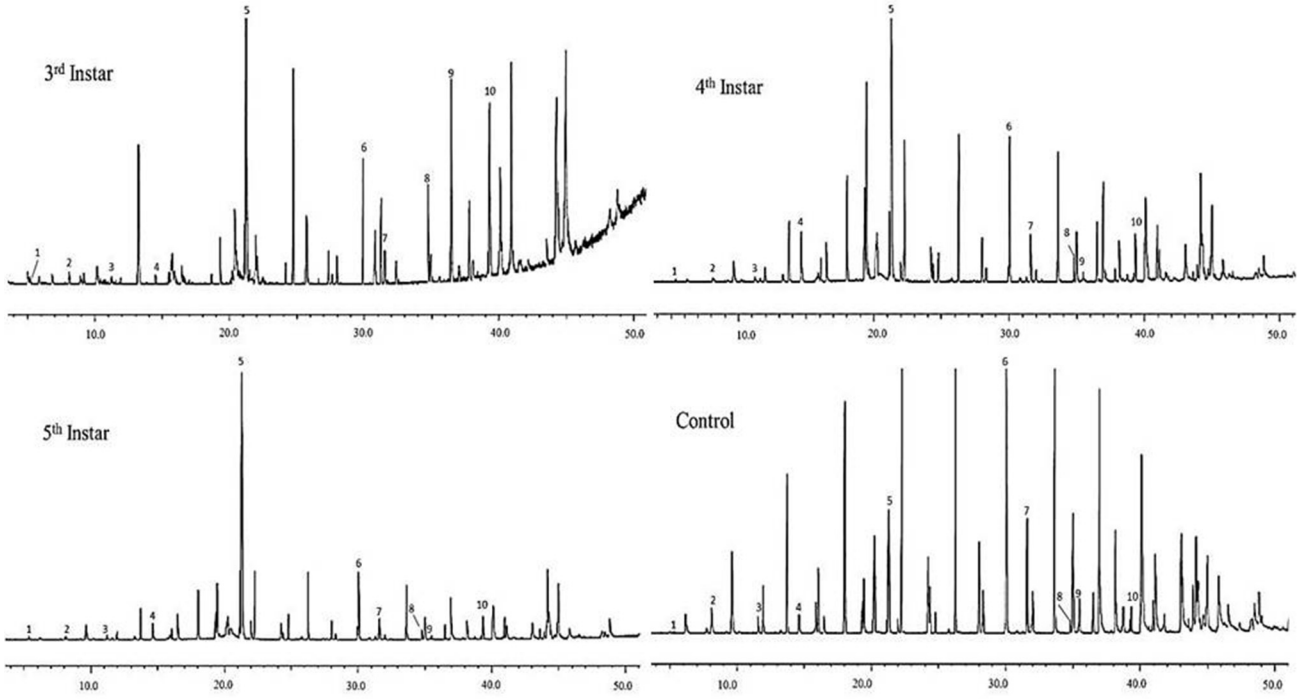

Fig 3. Representative chromatograms for the control group and the groups with the 3rd, 4th, and 5th larval instars subjected to application of Juvenile Hormone, indicating the 10 compounds that were most statistically significant for separation of the groups.
1 = Pentadecane; 2 = Unknown; 3 = Octadecane; 4 = Unknown; 5 = 3-Methylheneicosane; 6 = Pentacosane; 7 = 9,13-Dimethylpentacosane; 8 = Y-Methylheptacosane; 9 = 7-Methylheptacosane; 10 = Nonacosane.

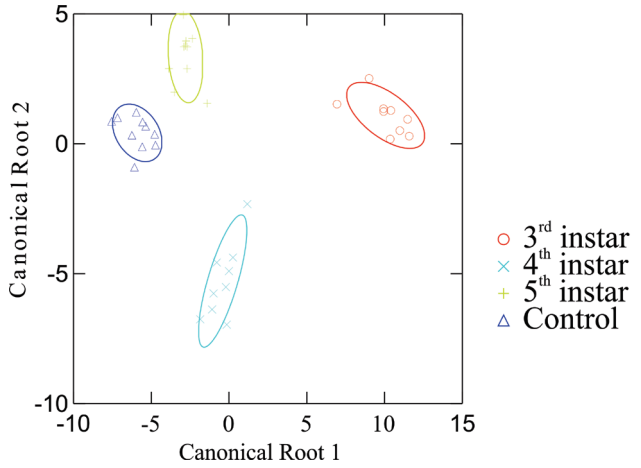

Fig 4. Dispersion diagram of the results of discriminant analysis applied to the GC-MS data, showing the two canonical roots of differentiation between the control group and the groups with application of Juvenile Hormone to different larval instars of *Mischocyttarus consimilis*.

In all samples that received JH treatment and in the control, higher levels of branched alkanes were found, followed by linear alkanes and a lower percentage of alkenes (Fig 5). All identified compounds were shared by all types of samples (Fig 5). The compound with the highest content in all types of samples was tricosadiene (Table 2).

Major compounds found for specific treatment groups were as follows: 7-Methylheptacosane (5th instar); heptacosane (4th, 5th instars and control); heneicosane (3rd instar); 2-Methyloctacosane and 3-Methyltriacontane (4th instar); 9,21-Dimethyltritriacontane (3rd instar); 3-methylnonacosane (3rd and and 5th instars and control); 9-Methylheneicosane (5rd instar and control) and nonacosane and 13-Methylnonacosane (3rd and 4th instars) (Table 2). The analysis between the groups, pair by pair, shows that only the females, the larvae of which was applied with topical JH in the 3rd instar, and controls had significant difference in cuticular composition ($F = 5.310$; $p = 0.027$). In this analysis, heneicosane, 3-methylpentacosane, 13-methylnonacosane, 9,21-dimethyltritriacontane, and 11,15-dimethyltritriacontane were the most important compounds for the separation of these two groups.

Discussion

The results demonstrated that topical application of JH at different larval stages significantly altered the cuticular composition of newly emerged females and that the results were instar-dependent. Our findings complement the results of Montagna et al. (2015) who found that an increase of JH titer at the larval stages altered the phenotype of adult females. In this sense, behavioral and morphological aspects are closely related to cuticular chemical composition (Kelstrup et al., 2017; Oliveira et al., 2017). Indeed, Kelstrup et al. (2014) also found a relationship between cuticular compounds and castes, age and reproductive status of females in the social wasp *Polybia micans*.

Even though the effects of JH application found here were similar to those of studies such as Kelstrup et al. (2014) and Oliveira et al. (2017), some studies, such as Kelstrup et al. (2015), report that the effects of JH may not be the same, even in closely related species.

Although the results of both techniques showed that cuticular chemical composition was significantly altered by the treatments, the techniques provided the significant difference between treatments at the 3rd and the control. Montagna et al. (2015) also reported that 3rd instar was the larval stage for achieving the most significant results following application of JH. When JH application was performed at the 3rd instar, adult

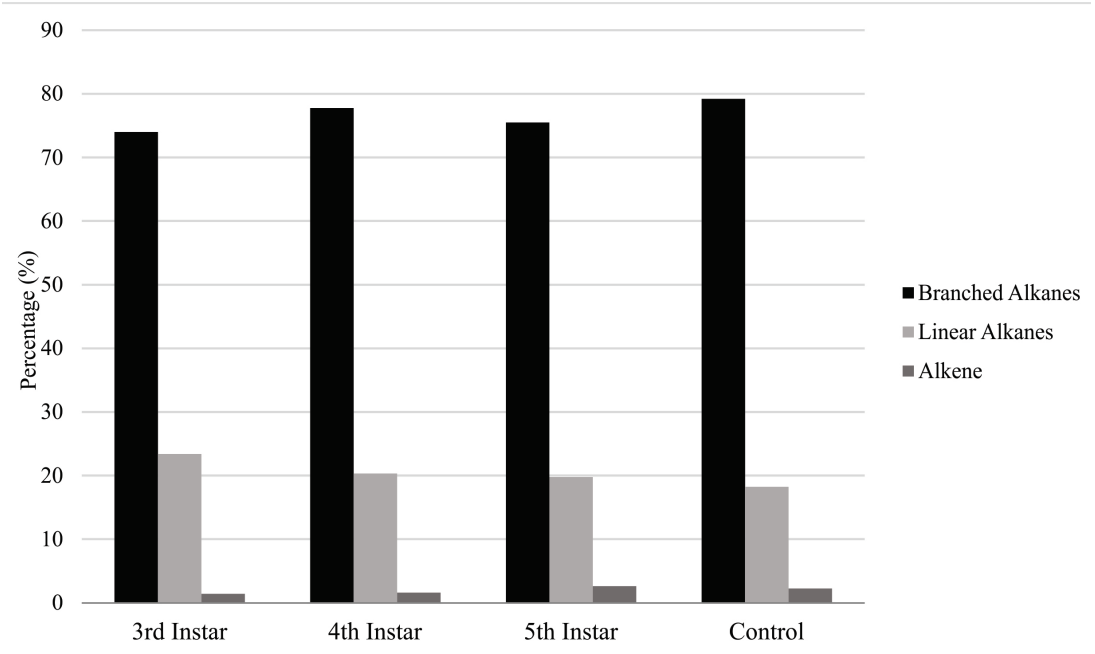

Fig 5. Relative abundances of the compounds identified by GC-MS for the control group and the groups with application of Juvenile Hormone to different larval instars of *Mischocyttarus consimilis*.

Table 2. Mean relative abundances and standard deviations for cuticular compounds identified by GC-MS in the control group and the groups with application of Juvenile Hormone to different larval instars of *Mischocyttarus consimilis*. 1= major compounds.

Compounds	ECL	3rd Instar	4th Instar	5th Instar	Control
		Percentage (% ± standard deviation)			
Octadecane	1800	0.24±0.21	0.11±0.09	0.22±0.23	0.43±0.75
3-Methyloctadecane	1876	0.34±0.14	2.09±1.68	1.35±1.32	1.96±3.70
Unknown	1970	1.21±1.28	0.32±0.35	2.06±5.66	0.31±0.42
X-Methylnonadecane	1975	0.73±0.85	0.63±0.64	0.31±0.24	0.63±1.12
Eicosane	2000	3.16±1.36	2.63±1.77	2.46±1.22	4.19±3.53
Heneicosane	2100	6.45±5.63[1]	1.97±1.81	0.72±0.34	0.71±0.63
9-Methylheneicosane	2146	4.51±2.66	3.24±2.85	6.95±9.98[1]	16.05±19.58[1]
Tricosadiene	2172	34.38±18.13[1]	26.53±16.14[1]	39.67±21.99[1]	37.39±22.22[1]
Docosane	2200	1.44±0.44	1.19±0.58	2.10±1.29	2.09±1.01
Tricosane	2300	2.86±1.45	2.72±1.69	3.78±2.36	2.99±2.63
9-Methyltricosane	2331	0.54±0.49	0.48±0.57	0.45±0.29	0.57±0.43
Pentacosane	2500	1.34±0.59	1.63±1.09	2.39±1.67	1.28±0.96
13-Methylpentacosane	2538	0.47±0.27	0.36±0.50	0.33±0.28	0.31±0.33
5-Methylpentacosane	2555	0.20±0.08	1.06±1.26	1.14±1.22	0.33±0.39
3-Methylpentacosane	2567	2.05±1.71	1.99±2.50	0.56±0.85	0.22±0.45
5,9-Dimethylpentacosane	2584	0.40±0.42	0.72±0.82	0.94±1.44	1.27±2.04
Heptacosane	2700	2.39±1.78	5.06±7.01[1]	4.06±5.55	2.26±2.10
7-Methylheptacosane	2738	1.07±1.00	7.30±11.98	7.23±15.01[1]	2.31±2.95
3-Methylheptacosane	2778	6.17±4.99[1]	4.82±6.19	5.45±4.53[1]	6.05±5.59[1]
14-Methyloctacosane	2834	0.33±0.17	2.26±5.81	0.99±1.48	0.56±0.49
2-Methyloctacosane	2859	0.91±1.28	7.50±10.67[1]	1.58±1.60	0.45±0.66
4,8-Dimethyloctacosane	2894	0.76±0.72	0.25±0.32	0.23±0.22	0.56±0.66
Nonacosane	2900	5.51±4.16[1]	5.03±3.43[1]	4.07±3.27	4.28±2.58
13-Methylnonacosane	2937	7.26±2.83[1]	5.03±6.10[1]	1.49±0.91	1.53±1.27
7-Methylnonacosane	2942	0.95±0.34	0.23±0.39	0.48±0.43	1.52±3.80
3-Methylnonacosane	2975	4.19±3.12	4.36±2.50	3.77±2.45	4.71±2.69
Triacontene	2983	1.43±1.49	1.59±2.24	2.65±3.75	2.24±3.92
14-Methyltriacontane	3035	1.18±2.34	1.83±1.84	0.84±0.99	0.47±0.67
3-Methyltriacontane	3074	2.02±1.84	6.00±5.65[1]	1.59±2.05	1.63±2.72
9,21-Dimethyltritriacontane	3364	5.54±3.04[1]	1.09±1.21	0.15±0.31	0.69±0.58

females grew significantly larger, remained longer in the nest, and became less aggressive, compared to the control group and the other treatments.

Studies with social bees showed that topical application of JH leads to significant effects that are especially dependent on the instar to which the hormone is applied. In studies performed by Hartfelder and Rembold (1991) with the bee *Scaptotrigona postica depilis* (Latreille), it was observed that the period most sensitive to increased JH titer was between the fourth and fifth larval instars. In another work conducted by Antonialli-Junior and Cruz-Landim (2009) with *A. mellifera*, it was found that application of JH at the 3rd larval instar led to results that were more significant, leading to greater preservation of the ovaries. Similar findings have also been reported for other social hymenopterans, such as the ant *Harpegnathos saltator* (Jerdon), as demonstrated by Penick et al. (2012), who concluded that application of JH at the 3rd and 4th larval instars resulted in adult females with greater propensity to become queens.

The compound 3-Methylheptacosane, although not major, presented a percentage 2-fold higher after JH treatment relative to control. Indeed, this compound is important in females with greater hierarchical status in colonies, as reported by Oi et al. (2015). In this study, the author demonstrated that this compound is used by queens of the species *Vespula vulgaris* to mark their eggs and thus signal their presence to the workers.

Table 3. Compounds that presented statistically significant values, highlighting the 10 most significant ones for separation of the different larval instars that received JH treatment and control of the species *Mischocyttarus consimilis*, as assessed by GC-MS.
1= 10 most significant compounds for separation of the groups.

Compounds	Calculated Index	Root 1	Root 2
Octadecane[1]	1800	**3.905**	0.588
3-Methyloctadecane[1]	1876	**3.335**	- 0.460
Unknown	1970	1.858	1.955
x-methylnonadecane	1975	0.888	0.135
Eicosane	2000	0.988	2.264
Heneicosane[1]	2100	3.356	1.591
9-Methylheneicosane[1]	2146	**2.022**	**5.055**
Tricosadiene[1]	2172	**9.763**	**8.481**
Docosane	2200	1.574	-0.691
9-Methyltricosane	2331	-0.896	1.793
Pentacosane	2500	2.924	-1.599
13-Methylpentacosane	2538	1.308	3.162
5-Methylpentacosane	2555	0.057	0.204
3-Methylpentacosane	2567	1.963	1.066
5,9-Dimethylpentacosane	2584	0.433	-2.122
Heptacosane	2700	0.921	1.722
7-Methylheptacosane[1]	2738	**4.974**	1.252
3-Methylheptacosane[1]	2778	2.798	**5.742**
14-Methyloctacosane[1]	2834	2.078	**3.678**
2-Methyloctacosane	2859	0.724	1.480
4,8- Dimethyloctacosane[1]	2894	**3.895**	0.693
Nonacosane	2900	-0.733	0.070
13-Methylnonacosane	2937	-2.008	-0.426
7-Methylnonacosane	2942	1.541	2.229
3-Methylnonacosane[1]	2975	**-4.364**	0.275
Triacontene	2983	1.291	0.291
14-Methyltriacontane	3035	0.607	1.420
3-methyltriacontane	3074	0.033	-0.284
9,21-Dimethyltritriacontane[1]	3364	**5.312**	1.644

The analyses show that the significant differences found between JH treatments with the cuticle composition of the control females were between larvae with application of JH in the 3rd instar and, still, 4 compounds (heneicosane, 3-methylpentacosane, 13-methylnonacosane, 9,21-dimethyltritriacontane or 11,15-dimethyltritriacontane) were important for the separation between these two groups. Of these four compounds, 3-Methylpentacosane was found in higher levels in the cuticle of queens of *Polybia micans* compared to the cuticle contents of workers (Kelstrup et al., 2014), indicating its role in differentiating reproductive status within the wasp colony. On the other hand, 13-Methylnonacosane was described in the study of Soares et al. (2017) as one of the compounds that occurred in the cuticle of all studied wasp species of the genus *Mischocyttarus*, suggesting its importance for colonies of these social wasps. Linear alkane heneicosane is a key component in the recognition among nesting companions of *Vespa crabro* (Ruther et al., 2002*)*. Thus, of the four compounds, evidence suggests that at least 3 appear to mediate important interactions in the colonies of the species where they are present.

Kelstrup et al. (2017) also found a clear association between JH level, ovarian status and the proportions of hydrocarbon classes in *B. longitarsus* females. Similarly, Oliveira et al. (2017) demonstrated that *Vespula vulgaris* workers treated with JH acquired a CHC profile that became similar to that of the queen. All the samples showed a greater abundance of branched and linear alkanes and lower abundance of alkenes (Fig 5) in agreement with the findings of Sledge et al. (2001) in *P. dominula*, Tannure-Nascimento et al. (2007) in *Polistes satan* (Bequaert) and Michelutti et al. (2017) in *M. consimilis*.

Methyl-branched compounds represented the majority of compounds and were also the most responsible for separating the groups. According to Kather and Martin (2015), this category of methyl-branched compounds, together with alkenes, consists of the most important compounds for the separation of groups of Hymenoptera. Gibbs (2002) pointed out that branched alkanes and alkenes are fundamentally important in chemical communication, whereas linear alkanes are compounds mainly responsible for protecting insects against desiccation. Nonetheless, it is possible that linear alkanes might play a role in intraspecific recognition since Lorenzi et al. (2004) showed that *P. dominula* workers less than a day old, when treated with linear alkane extracts, were not accepted by their nestmates, suggesting that these compounds may also be used as signals for intracolonial recognition.

Based on the results of this study, it can be concluded that the topical application of JH in different larval instars alters the chemical profile of newly emerged females in *M. consimilis*. The findings complement the results of the work performed by Montagna et al. (2015) who reported that treatment of the larvae of this species at the 3rd instar led to larger females with greater potential to become gynes. Therefore, the present results add further evidence that caste determination may, at least in part, be pre-imaginal in species of independent-founding social wasps.

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Author Contributions

Erika F. Neves and Thiago S. Montagna collection, data analysis; Claudia A. L. Cardoso, chemical data analysis; Luis H. C. Andrade and Sandro M. Lima, FTIR-PAS analysis; Erika F. Neves, Luiz C. Santos-Junior and Kamylla B. Michelutti manuscript writing and William F. Antonialli-Junior, general supervision, discussion and writing of the data.

The authors declare no conflict of interest.

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