JEFFESON JUSCELINO DA SILVA SOBRAL

CONTRIBUIÇÕES PARA A BIOLOGIA E ECOLOGIA DE *Pseudohypocera kerteszi* (DIPTERA: PHORIDAE)

SERRA TALHADA,

2020

UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

UNIDADE ACADÊMICA DE SERRA TALHADA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE E CONSERVAÇÃO

CONTRIBUIÇÕES PARA A BIOLOGIA E ECOLOGIA DE *Pseudohypocera kerteszi* (DIPTERA: PHORIDAE)

Jeffeson Juscelino da Silva Sobral

Dissertação apresentada ao Programa de Pós-Graduação em Biodiversidade e Conservação da Universidade Federal Rural de Pernambuco como exigência para obtenção do título de Mestre.

Linha de pesquisa: Ecologia da polinização

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SERRA TALHADA,

2020

Dados Internacioais de Catalogação na Publicação
Universidade Federal Rural de Pernambuco
Sistema Intergrado de Bibliotecas
Gerada automaticamente, metiante os dados forcenidos pelo(a) autor(a)

S677c Sobral, Jeffeson Juscelino da Silva
CONTRIBUIÇÕES PARA A BIOLOGIA E ECOLOGIA DE Pseudohypocera kerteszi (DIPTERA: PHORIDAE) /
Jeffeson Juscelino da Silva Sobral. - 2020.
78 f.

Orientador: Airton Torres Carvalho.
Coorientador: Paulo Milet Pinheiro. Inclui referências e apêndice(s).

Dissertação (Mestrado) - Universidade Federal Rural de Pernambuco, Programa de Pós-Graduação em Biodiversidade e Conservação, Serra Talhada, 2020.

1. Pseudohypocera kerteszi. 2. contribuição biológica. 3. caracterização química. 4. meliponicultura. 5. Meliponini. I. Carvalho, Airton Torres, orient. II. Pinheiro, Paulo Milet, coorient. III. Título

CDD 338.95

UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO

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TÍTULO

Jeffeson Juscelino da Silva Sobral

Dissertação julgada adequada para obtenção do título de mestre em Biodiversidade e Conservação. Defendida e aprovada em 17/02/2020 pela seguinte Banca Examinadora.

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Dedicatória

Dedico esse trabalho árduo e delicado aos ex-presidentes: **Luiz Inácio Lula da Silva**, pela interiorização das universidades públicas e federais no Brasil. Assim como eu, há milhares de jovens no país de origem humilde, mas interessados e dedicados e que precisam apenas de oportunidades para alcançar sonhos maiores; **Dilma Vana Rousseff**, pela exteriorização e ampliação do conhecimento através do programa Ciências sem Fronteiras. Deste usufrui e colho seus frutos até hoje, pois com a segunda língua adquirida e aperfeiçoada os artigos resultantes desta dissertação foram redigidos apenas em inglês (língua requerida em revistas de qualidade). Além disso, minhas perspectivas de mercado são largamente maiores. Sou imensamente agradecido, pois agora tenho um mundo inteiro pela frente.

Agradecimentos

Agradeço a minha mãe, Francisca, meu pai, Juscelino, minha irmã, Simone e meus sobrinhos tão amados, Isabela e Júlio Sobral. A existência deles é fundamental para minha vida.

Ao meu ilustríssimo orientador, Airton Carvalho, por ter me concedido esse projeto e ter demonstrado ser não só um bom orientador, mas também um grande amigo e companheiro que levarei para o resto da vida.

Ao meu caríssimo coorientador, Paulo Millet Pinheiro, por ser uma excelente pessoa e ter me ajudado grandemente para a construção de todo esse trabalho. Seu papel foi fundamental nessa dissertação.

Agradeço a Artur Maia, por ter colaborado bastante nos experimentos, identificação, construção e correção dos artigos.

A Éricles Charles, Rubem Cláudio e José Augusto por suas contribuições diretas e indiretas para esse trabalho. Além de todo pessoal do grupo de estudos NEEP: Marcos Figueroa, Mariana Feitosa, Vitória Lira, Lucas Reimberg, Fernanda Gomes, Olímpio Neto, Mirella Silva, Maciel Nascimento, Mayalle Carvalho e Marileide Sá.

Aos meus amigos pessoais que fiz durante a graduação e pós-graduação, Érika Millena, Ana Maria Tavares, Paulo Henrique, Victória Souza, João Fernandes, Matheus Emanuel, Cléia Medeiros, Joécio Souza, Joyce Almeida, Evandro Antônio, Jayne Crisleny, Emerson Dias, Aline Rios, Cinara Wanderleia. Especialmente a minha turma do mestrado 2018.1 pelos momentos difíceis e alegres compartilhados durante essa jornada, Thamires, Patrícia, Geanne e Keyla.

A FACEPE pela importante bolsa concedida durante o período de pesquisa, processo IGBG- 0484-5.04/18.

Ao CNPQ pelo financiamento do projeto através do processo 400597/2018-7.

A CAPES pelo auxílio direto ou indireto na graduação e pós-graduação.

A Universidade Federal Rural de Pernambuco por ter sido essa instituição-mãe contribuinte por todo meu desenvolvimento pessoal e intelectual. Especialmente a Unidade Acadêmica de Serra Talhada.

Aos todos os meus professores da graduação e da pós por suas contribuições fundamentais e memoráveis para minha formação. Meu imenso obrigado.

Por fim, a todas as pessoas que contribuíram direta ou indiretamente para que eu chegasse até aqui.

Resumo

A meliponicultura como uma atividade econômica sustentável e em grande crescimento necessita de conhecimento científico sólido para seu pleno desenvolvimento. Há muito a se descobrir, estudar, e técnicas a serem desenvolvidas de manejo e conservação desses polinizadores. Um dos pontos importantes é o estudo de doenças e parasitas. Nesse trabalho, estudamos a relação do cleptoparasita *Pseudoypocera kerteszi* (Diptera, Phoridae) e suas hospedeiras, as abelhas da Tribo Meliponini. Os forídeos, como popularmente conhecidos, são considerados a peste mais importante que aflige a meliponicultura. Essas moscas chegam frequentemente a matar colônias por inteiro. Muito pouco ainda se conhece da biologia e da ecologia de *P. kerteszi.* No primeiro artigo nós investigamos vários aspectos da biologia de *P. kerteszi* como: tempo de desenvolvimento de machos e fêmeas, papel da acidez do meio na oposição, partenogêneses, papel da umidade na emergência de pupas e desenvolvimento de larvas em meio diferente do natural (pólen). Para isso diferentes metodologias foram aplicadas. No segundo artigo nós testamos a hipótese que diferentes ninhos de Meliponini não diferenciam significantemente em relação a compostos voláteis emanados de suas estruturas, já que o forídeo geralmente ataca todas as espécies de abelhas sem ferrão indistintamente. Para isso nós identificamos os compostos voláteis emitidos por diferentes partes dos ninhos através da cromatografia gasosa acoplada a espectrometria de massas. Além disso, realizamos diferentes biotestes em gaiola com *P. kerteszi.* Como resultados nós observamos que em geral fêmeas de *P. kerteszi* vivem por mais tempo do que machos. Larvas se desenvolveram quando alimentadas com um meio não natural. A acidez do meio é um fator de muita importância na ovoposição das moscas. As fêmeas não se reproduzem partenogeticamente. Pupas velhas não emergidas não emergem quando expostas a uma umidade maior. Os compostos voláteis emanados pelos ninhos das diferentes espécies de abelhas Meliponini, usadas nesse estudo, são espécie-específicos, refutando dessa forma, nossa hipótese. Alguns dos principais compostos emitidos pelos ninhos foram: ácido acético, acetato de etila, beta-ocimeno e estireno. As armadilhas contendo pólen como isca foram as mais atrativas às moscas, exceto quando oferecidas ao mesmo tempo de ácido acético glacial. O ácido acético parece ter um papel fundamental na atração de *P. kerteszi* a partir de curtas distâncias. Esses dois artigos trazem importantes informações em relação ao clepto-parasita em questão, as quais podem ser muito relevantes no desenvolvimento e melhora de métodos de controle na meliponicultura.

Palavras-chave: *Pseudohypocera kerteszi*, contribuição biológica, caracterização química, Meliponini, meliponicultura.

Abstract

Meliponiculture as a sustainable and highly developed activity still needs to be extensively explored in terms of scientific knowledge. There is a lot to understand and improve, especially when it comes to diseases and parasites. The klepto-parasite *Pseudoypocera kerteszi* is the most important Meliponini bee pest. These flies often kill entire colonies. Very little is known regarding the biology and ecology of *P. kerteszi*. In the first article we investigated various aspects of *P. kerteszi*'s biology such as: time of development of males and females, parthenogenesis, role of medium acidity in opposition, role of humidity in pupal emergence and development of larvae in a non-pollen source. For this, different methodologies were applied. In the second article, we tested the hypothesis that different Meliponini nests do not differ significantly from volatile compounds emitted from their structures, as the phorid usually attacks all stingless bee species. For this, we identify the volatile compounds emitted by different parts of the nests through mass spectrometry. In addition, we performed different biotests in cage with *P. kerteszi*. As a result, we observed that in general females of *P. kerteszi* live longer than males. Larvae developed successfully when fed on an unnatural medium. The acidity of the environment is a very important factor in the oviposition of flies. Females do not reproduce parthenogenetically. Non-emerged old pupae do not emerge when exposed to higher humidity. The nests of the different species of Meliponini bees used in this study are species-specific in relation to the emanated compounds, thus refuting our hypothesis. Some of the main compounds emitted by the nests were: acetic acid, ethyl acetate, beta-cymene and styrene. Pollen bait traps were the most attractive to flies, except when offered against glacial acetic acid. Acetic acid seems to play a key role in attracting *P. kerteszi* from short distances. These two articles provide important information regarding the klepto-parasite in question, which may be very relevant in the development and improvement of control methods in meliponiculture.

Keywords: *Pseudohypocera kerteszi*, biologic contribution, chemical caracterization, Meliponini, meliponiculture.

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Sumário

1. Apresentação

Essa dissertação consta com uma introdução geral ao tema em língua portuguesa e dois artigos científicos a serem submetidos em revistas científicas internacionais e já escritos em língua inglesa. Essa proposta visa dar celeridade ao processo de publicação dos trabalhos impactando internacionalmente as produções do programa e dentro das perspectivas de crescimento e internacionalização dos programas de pós-graduação geridos pela CAPES. Após cada manuscrito, são anexadas as normas das revistas científicas escolhidas para submissão e seguem o formato sugerido pela revista escolhida. Ao final há uma conclusão geral, também redigida em língua portuguesa.

2. Introdução geral

A criação de abelhas sem ferrão da tribo Meliponini, conhecida como meliponicultura (NOGUEIRA-NETO, 1997) é uma atividade em expansão com grande potencial econômico e socioambiental (CORTOPASSI-LAURINO *et. al.*, 2006; MAGALHÃES E VENTURUERI 2010; CONTRERA *et. al.*, 2011). É uma atividade antiga e tradicional considerada sustentável, de fácil manuseio e baixo custo, além de gerar renda (NOGUEIRA-NETO, 1997). Além disso, possui alta relevância na manutenção da biodiversidade por meio de serviços de polinização prestados a espécies nativas e cultivadas de plantas (HEARD, 1999; CORTOPASSI-LAURINO *et. al*., 2006; MAGALHÃES E VENTURUERI, 2010).

Com cerca de 500 espécies descritas em todo o mundo, a tribo Meliponini possui hábitos, habitats, morfologia e comportamento extremamente diversos. No Brasil, são descritas 237 espécies (MICHENER, 2007; CAMARGO E PEDRO, 2007) e pelo menos uma centena delas tem potencial para produtos meliponícolas, como mel, própolis, pólen, cera, resinas e serviços de polinização (VENTURIERI *et. al.*, 2012). Entretanto, diferentemente da apicultura (criação de *Apis mellifera* L.), que foi exaustivamente estudada por mais de 150 anos (IMPERATRIZ-FONSECA *et. al*., 2012), a meliponicultura ainda está em seu início científico, tendo um aumento substancial de conhecimento apenas nos últimos 40 anos. Estudos visando seleção artificial para aumentar a produtividade, técnicas de padronização da gestão e, principalmente, o conhecimento de doenças e parasitas são ainda mais recentes e escassos (VENTURIERI *et. al.*, 2012; MAIA-SILVA *et. al*., 2013).

A mosca *Pseudohypocera kerteszi* Enderlein (Diptera: Phoridae) entra nesse contexto representando a praga que mais prejudica a meliponicultura (NOGUEIRA-NETO, 1997). São denominadas cleptoparasitas e não têm preferência por espécies ou ninhos de abelhas sem ferrão. Elas parasitam praticamente todas as espécies nos neotrópicos, causando um enorme dano às colônias e perdas econômicas aos meliponicultores (ROUBIK, 1989; OLIVEIRA *et. al*., 2013). As fêmeas entram nos ninhos e ovopositam majoritariamente dentro dos potes de pólen, onde as larvas se desenvolvem. No entanto, as larvas desses forídeos não se alimentam exclusivamente do pólen, pois em certas circunstâncias, como é o caso de uma alta densidade larval, elas chegam a se alimentar das pré-pupas e pupas das abelhas (ROUBIK, 1989). As infestações frequentemente levam ao colapso das colônias (ROBROEK *et. al*., 2003; PORTUGAL-ARAÚJO, 1977).

As contribuições para aspectos biológicos de *P. kerteszi* são muito escassas e normalmente incompletas. Algumas das poucas informações disponíveis relativas à biologia de *P. kerteszi* são relacionadas à fecundidade, acasalamento e características morfológicas (DISNEY, 1988). No entanto, vários outros aspectos como ciclo de vida, partenogênese e diapausa ainda não foram investigados. Dessa forma, em nosso primeiro artigo visamos contribuir para o entendimento atual da história natural de *P. kersteszi*: criamos moscas em laboratório e realizamos alguns experimentos para abordar as seguintes questões: 1) Qual é o tempo de desenvolvimento de ovos, larvas, pupas e adultos?; 2) Existe diferença no tempo de desenvolvimento entre machos e fêmeas?; 3) As larvas são capazes de se desenvolver em fontes não polínicas? 4)Os adultos se alimentam e ovipositam em meio artificial? 5) As fêmeas se reproduzem partenogeneticamente?

Por outro lado, pouco também se sabe a respeito da ecologia de *P. kerteszi*, principalmente da interação química com abelhas Meliponini. Ainda não é conhecido como as moscas encontram os ninhos de abelhas sem ferrão, mas especula-se que a interação seja química, uma vez que as infestações são controladas utilizando-se vinagre comercial (OLIVEIRA *et. al.,* 2013; RAMOS *et al.*,). Para controlar as infestações, armadilhas que consistem em potes de plástico com furos de cerca de 3 mm nas tampas, são dispostos dentro dos ninhos. Isso atrai, especialmente as fêmeas, que acabam morrendo afogadas no vinagre (OLIVEIRA *et. al.,* 2013; NOGUEIRA-NETO, 1997). Especula-se que o ácido acético, componente principal do vinagre comercial e produzido pela fermentação acética dos grãos de pólen dentro dos potes de armazenamento dos ninhos, (NOGUEIRA-NETO, 1997) seja o componente atrativo. No entanto, os resultados dessas pesquisas ainda são anedóticos, e não há evidências fortes que somente esse composto seja utilizado pelas moscas para o encontro de seus hospedeiros, especialmente à longas distâncias. Hipotetizamos que outros compostos também façam parte do processo de comunicação parasito-hopedeiro nesse caso, porém não é conhecido quais são os principais compostos liberados pelos ninhos e se existe diferença em relação aos compostos emitidos por ninhos de diferentes espécies de Meliponini. Como *P. kerteszi* não parasita apenas uma espécie de abelha sem ferrão, nossa hipótese é que ninhos de diferentes abelhas sem ferrão não diferem significativamente em relação aos voláteis emitidos. Em nosso segundo artigo, nós testamos essa hipótese. Para isso, nós coletamos os voláteis de diferentes partes de ninhos de Meliponini (pólen, cerume e geoprópolis) de três espécies diferentes: *Melipona scutellaris*, *Melipona subnitida* e *Scaptotrigona* sp. (grupo *tubiba*). Além disso, testamos a atratividade de

diferentes estruturas dos ninhos e outros compostos químicos em relação ao *P. kerteszi* em bioensaios.

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4. Capítulo 1. – Artigo a ser submetido ao Journal of Economic Entomology, Oxford academic press

New contributions to the biology of *Pseudohypocera kerteszi* (Diptera: Phoridae), a major pest of stingless bees (Meliponini: Apidae)

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Abstract The phorid fly *Pseudohypocera kerteszi* is the main pest and prioritary concern of stingless beekeeping, leading to decreases in honey yield and even death of the colonies. Our current understanding of even the most basic aspects of the natural history of *P. kerteszi* remains, nonetheless, insipient. In this work, we comprehensively investigated the biology of *P. kerteszi*, focusing on post-embryonic development times of female and male flies and whether the larvae can adequately grow on a non-pollen diet. Furthermore, we carried out parthenogenesis tests and determined the role of medium acidity levels in oviposition. In general, female *P. kerteszi* live longer than males, both in the larval and adult life stages (10/2.5 days, respectively). Most of the larvae (74%) that were fed at a non-pollen protein source underwent full development. A more acidic medium positively influenced oviposition in terms of the number of eggs laid per female. In general, not all individuals reached adult phase. Females reproduced parthenogenetically. Our results are extremely important to the conservation of stingless bees because they bring novel and necessary information for improving the controlling methods in bee cultures regarding their main pest.

Key-words: Bees; Meliponiculture; Meliponini, Parasite, Phorid flies; productivity

INTRODUCTION

Stingless bees (Meliponini: Apidae) are a diverse group of tropical bees, with about 500 species (Michener, 2007; Camargo and Pedro, 2007). They are effective pollinators of many economically important fruit crops (e.g. macadamia, mango, strawberries, watermelon, avocado, citrus plants, lychee), thus their activities generate a considerable impact in contemporary agriculture (Jaffé et al., 2015; Malagodi-Braga and Kleinert 2002). Stingless beekeeping, also known as meliponiculture, is an old tradition in the New World, being practiced by the precolombiam native populations in South and Central America (Camargo and Posey, 1990; Villanueva-G et al., 2005; González-Acereto et al., 2006, Poots el al 2016). Meliponiculture is growing fast and provides important income to local farmers. It supplies beekeepers with high quality honey and other direct products, such as pollen, propolis and beeswax (Heard, 1999; Venturieri et al., 2012, Poots et al., 2016). Some of its problems is the lack of management techniques that optimize production. However, parasites such as ants, termites, kleptoarasitic bees and forids, are the main problem concerning this activity and have brought great damage to nest os Meliponini bees and highlighted the need to collect data on the general biology of these parasites. (Nogueira-Neto, 1997; Roubik, 2006; Pasteels et al., 1983).

However, some natural parasites such as Phorid flies (Diptera: Phoridae), black soldier flies (Diptera: Stratiomyidae) and even some mites (Acari: Pyemotidae) can cause noticeable damages to nest of Meliponini bees (Nogueira-Neto, 1997; Hashim et al., 2017; Menezes et al., 2009). Therefore, understanding the natural history of the natural enemies of this activity is essential for its full and sustainable development.

Pseudohypocera kerteszi Enderlein (Diptera: Phoridae) is considered the most important pests in meliponiculture (Nogueira-Neto, 1997). These small flies parasitize nearly all species of Neotropical stingless bees, causing damage to colonies and considerable economic losses to beekeepers (Roubik, 1989; Oliveira et al., 2013). Female *P. kerteszi* flies enter the nests and

oviposit mainly in the pollen pots, the major protein source for the developing larvae (Nogueira-Neto 1997; Michener 2007). However, *P. kerteszi* larvae do not feed exclusively on pollen and in certain circumstances, such as overcrowding, they might also prey on pupae and pre-pupae of the parasitized nests. This leads to larger infestations and, finally, to the colapse of entire colonies (Roubik, 1989, Robroek et al., 2003, Portugal-Araújo, 1977).

Several other aspects on the biology of *P. kerteszi*, such as life cycle, parthenogenesis and oviposition on alternative substrates remain unknown. In the literature, contributions to the natural history of *P. kerteszi* are very scarce and mostly incomplete. Little available biological data is related to fecundity, courtship and morphological traits (Disney, 1988). What we know is that fecundity ranges from 31 to 102 eggs per female (66.4 on average) (Chaud-Netto, 1980). Males are slightly smaller than females and present a black abdomen, whereas females display pale abdomens (Robinson, 1981). Mating is usually airborne and close to stingless bees' colonies, which the females access when fertilized (Portugal Araújo, 1977). The infestation starts as female flies enter the nest, pass the guarding bees, and oviposit on pollen pots and waste dumps, resulting on the first set of emerged offspring ca. 14 days later (Robroek at al., 2003).

One of the main issues for stingless beekeepers is how poorly cleptoparisitic phorid flies are understood. The main knowledge gaps concern the strategies for the combat and control (Maia et al., 2015, Jaffé et al., 2015). Traps containing vinegar or solutions of acetic acid have been used in the capture of the *Pseudohypocera kerteszi* which have already been studied and proved to be effective in minor infestations (Nogueira-neto 1997, Ramos et al., 2003, Wolff and Nava, 2007, Oliveira et al., 2013, review in Contrera and Venturieri 2008). However, these traps do not prevent from massive infestations in the nests, which occur very often. Thus, new and more effective control methods need to be studied and created.

With the aim of contributing to the current knowledge on the natural history of *P. kersteszi*, we reared flies in the lab and performed several controlled experiments to address the following questions: 1) What is the development time of eggs, larvae, pupae and adults; 2) Is there difference on the time of development of males and females?; 3) Are the larvae able to develop on non-pollen sources? 4) Will adults feed and oviposit on an artificial medium? 5) Do females reproduce parthenogenetically? With this work, we expect to generate important basic knowledge for the reduction of this pest in meliponaries, leading to a positive impact on the meliponiculture.

MATERIAL AND METHODS

Study site and system

Sampling of phorid flies and nest materials (pollen, honey, geopropolis and cerumen) were conducted at the meliponary ROCC, in a residential area of Aldeia, municipality of Camaragibe, Pernambuco state (GPS DATA), NE-Brazil from September 1st, 2018 to November 30th, 2019. *Melipona scutellaris*, *Melipona subtnitida*, *Scaptotrigona* sp. are the major species found in the Meliponary ROCC, from which the nest material used in the experiments were extracted. *Melipona scutellaris* is the most numerous species from the Melipnary, this species is popularly known as uruçu and is naturally distributed along the east coast of the country. Currently, it occurs only between the states of Rio Grande do Norte and Bahia, corresponding to less than half of its original distribution. The species was originally domesticated by the indigenous peoples of the northeast, such as the Kariri and Xucuru, who passed on their management techniques to the colonists, giving continuity to the rational breeding. With the intense suppression of humid forests in the northeastern coast, the natural occurrence of this species is increasingly scarce, which enhences the importance of improving management techniques,

which have been the only alternative alternative for the survival of this species. (Mariano-Filho, 1911; Kerr et al., 1996; Kerr, 2002).

The vegetation of the area is characterized by the presence of representatives of the native Atlantic Rainforest flora, intermingled with exotic cultivated plant species and tropical fruit trees like *Annona muricata* (Annonaceae), *Malpighia emarginata* (Malpighiaceae) and *Eugenia uniflora* (Myrtaceae).

Controlled experiments were carried out in the laboratory of chemical ecology at the Federal University of Pernambuco (UFPE), Recife, Pernambuco and in the Laboratory of arthropods ecology at the Rural Federal University of Pernambuco (UFRPE), Serra Talhada, Pernambuco, NE-Brazil.

2.3 Trap Sampling of for flies

In order to attract flies for getting eggs, larvae and adults for the experiments, we used small empty stingless bee nests, which were baited with a mixture consisting of different nest materials. The trapnests consisted of a square wooden box (15 cm^2) with a removable lid of the same material and an entrance of 7 mm in diameter (Fig. 1a). To prepare the bait, we mixed 10 grams of stingless beebread, 10 grams of macerated commercial honeybee pollen, 5 grams of honey and ca. 3 grams of other nest materials (namely geopropolis and cerumen, to give the bait a scent note similar to a real bee hive. The baiting material was placed inside the nest, whose entrance was swabbed fresh pollen. The nest was then closed and purposely placed about 50 cm next to stingless bee nests and left there for 72 hours. After 3 days, we sealed the entrance and the complete nest and moved it to the lab. These procedures was performed everytime we were running out of flies in the laboratory, on average about twice a month from September 2018 to November 2019 to get enough flies for the experiments. Eventually we collected flies from naturally infested nest of *Melipona scultellaris* at the same meliponary.

Sampling of flies

In order to attract flies for getting eggs, larvae and adults for the experiments, we used small empty stingless bee nests, which were baited with a mixture consisting of different nest materials. The trapnests consisted of a square wooden box (15 cm^2) with a removable lid of the same material and an entrance of 7 mm in diameter (Fig. 1a). To prepare the bait, we mixed 10 g stingless beebread, 10 g macerated commercial honeybee pollen, 5 g honey and ca. 3 g other nest materials (geopropolis and cerumen) in order to give the bait a scent note similar to a real beehive. The baiting material was placed inside the nest, whose entrance was swabbed fresh pollen. The nest was then closed and purposely placed at about 50 cm next to stingless bee nests and left there for 72 hours. After 3 days, we sealed the entrance and the complete nest and moved it into the lab. These procedures were performed about twice a month from September 2018 to November 2019 to get enough flies for the experiments. Eventually we collected flies from naturally infested nest of *Melipona scutellaris* at the same meliponary.

Controlled breeding and rearing of *P. kerteszi*

We developed a protocol for the breeding and rearing of *P. kerteszi* under controlled laboratory conditions. In order to rear flies for the experiments in the lab, we first developed an artificial "phorid fly food", a cheaper breed, since the stingless beebread available was not enough for the rearing. In natural conditions, female flies oviposit in open pollen pots and the larvae first eat this mass of pollen grains. The pollen grains inside the cerumen pots undergo a natural fermentation process that transforms them into a moist and sticky mass. This mass has a strong acetic acid odor, as result of the activity of acetogenic bacterias (Nogueira-Neto 1997). After some attempts, the best combination for the "phorid fly food" was a mixture of honey (15 g) and dehydrated commercial honeybee pollen granules (7.5 grams) (*Apis mellifera* commercial pollen) and stingless beebread (saburá, 2 grams) plus 1.5 ml of acetic acid, in addition to these we use whey protein (whey) in order to assess the nutritional importance of protein food in the maturation of larvae and pupation. In a petri dish, we macerated the pollen

mass using a mortar and pestle, added and mixed the honey in order to keep a moist consistency (similar to the *saburá*). Finally, we added the beebread and acetic acid and mixed again.

This food was made available to the flies inside flight cages (1 m^3) in a petri dish, where the flies could eat and oviposit. Into the fly cages we observed several copulas and all the females used were considered able to oviposit. This experiment was performed at room temperature (25º to 28º C) and relative humidity of 50 to 60 %.

Life cycle of *P. kerteszi*

To describe the development of *P. kerteszi* from eggs to larvae stage, we first observed the traps when there were only eggs (see section 2.3) and observed every 3 hrs in order to record when larvae first appeared. To describe the development from egg to adult stage, 140 freshly hatched larvae were individually transferred to labeled Eppendorf tubes (2 ml) containing 0.5 g stingless beebread (see rearing of flies). We observed the larvae every 12 hrs after initial transfer and recorded the duration of each developmental stage (larva, pupa and imago). Additionally, we describe each stage in detail, regarding sizes, shapes, color and other morphological traits. Sex was determined after death of adults. In order to determine the sex, we observed observed the flies individualy under an estereomicroscope (Leica MZ6), following the descriptions for male and female presented in Roubik, 2003.

Larval development in artificial substrate

To describe the larval development on non-pollen sources (Phorid fly food), a hundred freshly hatched larvae were transferred from an infested colony individually labeled 2 mL Eppendorf tubes containing 0.5 g of the substrate. The larvae were observed every 12 hours after initial transfer to check whether they were alive and feeding on the medium. To determine a complete development, we considered only larvae that reached the adult stage. All the experiment was performed at room temperature (25º to 28º C) and relative humidity of 50 to 60 %.

The role of protein content and acidity in oviposition

The whey protein substrate or "artificial protein larval food" was made to understand the role of protein content in the females ovoposition and larval development. This non-pollen medium consisted of 70 g of filtered honeybee honey and 35 g commercial protein supplement (Six protein Body Builders®, containing 50% peptides and amino acids, 35% carbohydrates and 5% fats). Honeybee pollen pellets have similar general nutrional content (Brasil, 2001).

In order to understand the role of protein content and acidity in oviposition and development, we performed an experiment in flight cages, consisting of 4 treatments and one control. In the treatment cages, we placed a square wooden box (10 cm^2) with different substrates one at a time: A) 10 grams of dry and macerated commercial pollen of *Apis mellifera*; B) 10 g of whey protein substrate (see above) mixed with 1 mL of acetic acid; C) 10 g of whey protein substrate with 1 mL pure lime juice; D) 10 g whey protein substrate without any acid. As control, we placed 10 grams of our phorid fly food within the cage. Acetic acid and lime juice were replaced after the initial 24 hrs. To each experimental cage we added 200 adult *P. kerteszi* (random sex). Experiments were conducted for 48 hrs and repeated four times for each treatment and for the control. Cages were kept at 25º - 28º C and 50 to 60% relative humidity. At the end of the experiment, we counted the eggs deposited at the medium under stereomicroscope.

Parthenogenetic reproduction

Aiming to determine whether *P. kerteszi* reproduce parthenogenetically, we performed an experiment in which females were kept either isolated (treatment; n = 5) or together with males (control; n = 5) inside arenas made of closed clear-plastic petri dishes. In order to obtain virgin females for the experiment, we placed pupae individually in 2 mL Eppendorf tubes and waited until they hatch. After hatching, but still inside the Eppendorf, insects were visually sexed. We inserted three females (one at a time) in each of the 10 experimental arenas through

a 7 mm ⌀ apperture drilled on the lid (Fig. 1C). Females did not have any previous external contact with other individuals, only this way we could asssure that all the females used were virgin. To five of the experimental arenas we additionally inserted one male. Each experimental arena contained 2 g *P. kerteszi* food with lime juice (see topic 2.2) as substrate for oviposition. This experiment was carried out for 48 hrs at 25º - 28º C and of 50 to 60 % relative humidity. After 24 hours we added 1 mL of pure lemon juice on the medium in order to keep its acidity. After 48 hours, the eggs laid on the substrate available in each petri dish were counted under a stereomicroscope.

Statistics

Normality of data (Anderson-Darling) was tested using the software Minitab-18 (Inc., State College, PA). We used the Mann-Whitney test (α 5%) to assess differences between times of development of male and female individuals (life cycle and development experiment); fully and non-fully larvae development at non-pollen source vs. control. We also used Mann-Whitney test (α 5%) to check for differences between 1) oviposition in medium with acid x medium without acid; 2) oviposition in petri dishes with and without males (parthenogenesis experiment); pupae emerged x non-emerged pupae. For sex ratio, we used Chi-square test for association to check whether differences were significant.

RESULTS

Life cycle of *P. kerteszi*

From the 140 larvae used, 111 reached adult stages (79.3%), whereas 29 (20.7%) fail to develop (Mann-Whitney, $p < 0.001$). Fourteen individuals died as larvae (9.3%) and 15 reached the pupa stage but were no able to emerge (10.70%). From the 111 individuals which had a full development, 84 (75.7%) were male, while 27 (24.3%) were female, a sex ratio of ~3/1 (Chi Square =1, $p < 0.005$) (Fig. 3).

In general, from freshly hatched larva onwards, female development took longer than that of males (median 10 d ± 1.65 ♀ vs 6 d ± 1.81 ♂; Mann-Whitney, $p < 0.001$). This duration is biased by the larval phase, as no significant difference was observed between sexes on the pupal stage (ca. 3 d for both sexes; Mann-Whitney, $p = 0.57$) (Fig. 4). Adult female flies had longer lifespans than males (10 d ± 7.05 vs 2.5 d ± 2.35 Mann-Whitney, $p < 0.001$; Fig 4).

The eggs of *P. kertezi* are pale, banana-shaped and measure a median of 0.81 mm in lenght and 0.25mm in diameter. Larvae undergo at least three different stages that can be easily recognized according to their size and color. In the first stage, which ranges from 0 to 24 hrs, larvae have a median of 1.16 mm long, 0.26 mm diameter. In the second stage, ranging from 1 to 3 days depending on the sex, larvae have a median of 3.88 mm long and 0,45mm diameter. On the third stage, the larvae become more yellowish with 5 mm in lenght and 1.31 mm in diameter. This stage demonstrates to be the most voracious, when the larvae eat more and walk around the colony. Here is where the chitinization process begins (Fig 2). Larvae can remain at this stage for 3 to 10 days depending on the sex. At the end of this stage, the larvae stop eating and moving, and start covering up by chitin (pre-pupa). The pupa is 4mm and can be either copperish (fresh pupa) or old wood color (mature pupa). All these stages can be visualized in Figure 2.

Oviposition and larval development using a non-pollen medium as food

Larvae did not survive when fed on substrates of low protein content. Larvae only survived and developed to pupae when fed on the phorid food, pollen bread and whey protein. We observed that the whey protein medium is a valuable source of food not only for larvae but also for adults, since we oberserved adult flies eating on the whey protein substratei. 74% of the larvae fed on the whey protein medium had full development, while 26% either died at a larval stage (18%) or did not emerge from the pupal stage (8%) (Fig 5).

Oviposition regarding the protein content and acidity of the substrate

Female flies oviposited 83 eggs on the artificial feeding substrate containing acetic acid and 16 eggs on the artificial feeding substrate containing lime juice (Mann-Whitney, $p < 0.001$). Another interesting observation is that before the flies lay the eggs on the substrate, they touch it, walk upon it and taste it, conducting the legs to the mouth. On the other hand, we did not observe ovipositions on the non-acidic substrates (Mann-Whitney, $p<0.001$).

Parthenogenetic reproduction

Female adult *P. kerteszi* inside petri dish arenas containing a male fly oviposited on the feeding substrate. The number of eggs varied from 3 – 9 with a median of 5 per arena (Mann-Whitney, $p = 0.025$). No eggs were recovered from the female-only arenas.

DISCUSSION

This study provides novel information on the biology of P. kerteszi, which might contribute to the combat and control of the main pest of the meliponiculture. Females of *P. kerteszi* did not oviposit on substrates with low protein content and acidity. In other words, they only grow in substrates with high protein content and with a certain level of acidity, as revealed by our experiments testing commercial pollen from *Apis mellifera*, stingless bee pollen and whey protein sustrate. On the other hand, it does not exclude the possibility that these flies will develop on other substrates with high protein content, but only if a high degree of acidity is respected, as revealed by our experiments. This clearly shows that acidity plays a major role in oviposition behavior by *P. kerteszi* flies.

Our observations show that females taste the substrate and feed on it prior to oviposition. This behavior is probably related to the chemical clues used by the kleptoparasites to find their food, a behavior that might avoid waste of eggs in a low-quality substrate. This type of behavior is well known among other species of flies such as Caliphoridae and Muscidae (Mitchell and Soucie, 1993; Larsen et al., 1966; Dethier 1961). In experiments using liver homogenate as substrate for gravid *Sarcophaga bullata* (Calliphoridae), Mitchell and Soucie (1993) showed

248 that tasting and larviposition were closely related. Larsen et al. (1966) observed that house flies
249 (*Musca domestica*, Muscidae) had to feel (by touch) the substrate in order to lay the eggs,
250 suggesting an inspection by the flies before the actual oviposition. A similar observation was
251 made by Dethier (1961), who reported that the black blow flies (*Phormia regina*, Calliphoridae)
252 were able to distinguish between protein and carbohydrate through contact of chemoreceptors
253 present on their legs and mouthparts. Furthermore, he also stated that olfactory cues are not the
254 determinant factors in food choice by flies, which instead preferably use contact
255 chemoreceptors (Dethier, 1961).

256 Usually kleptoparasites use chemical cues to identify their hosts (Eisner et al., 1991;
257 Heiduk et al., 2016); in the specific case depicted in our study, nonetheless, once locating their
258 hosts the female flies must also find ideal sites for oviposition inside the nests of stingless bees.
259 Thus, as observed in other studies (Roubik 1989; Robroek et al., 2003), *P. kerteszi* oviposit
260 preferably in the pots of pollen or in the waste dumps inside the nests, where there is a high
261 acidity and high amount of protein. Once the oviposition has taken place and the eggs have
262 hatched, population can substantially increase and the larvae can eventually feed on pupae and
263 pre-pupae of the stingless bees (Roubik, 1989, Robroek et al., 2003, Portugal-Araújo, 1977).

264 The results of this work imply important observations for combating and controlling this
265 pest in meliponiculture. Our data indicate the importance of the acidity of the medium for the
266 oviposition of the females, showing that the importance of the acetic fermentation as the main
267 acidity occurring inside the pots of pollen. As already observed in other works, the infestation
268 starts mainly with females that enter the nests of bees with copulation occurring outside the
269 nests (Oliveira et al., 2013, Portugal-Araújo 1977).

270 **Development time**

271 Our observations demonstrate that the development of the larvae, especially in favorable
272 conditions, can be so rapid that as soon as the first adult offspring arise and become mature,

273 they start ovipositing producing more than on generation per infestation. Therefore, the
274 beekeeper must take emergency measures to control the phorids as soon as possible. We
275 indicate the prompt manual removal of larvae using a spatula and placing traps containing
276 vinegar within the nests to capture the females that have not yet oviposited, as well as new
277 females that will emerge from pupae inside the nest, as also suggested by Contrera and
278 Venturieri (2008). Another valuable alternative suggestion is the use of sticker traps containg
279 a little amount stored pollen. However, this sticker traps must be previously teste, once stingless
280 bees might also be trapped on them.

281 In this paper we categorized three different stages of the development of *P. kerteszi*
282 larvae, where the third is the longest stage during up to 10 days. These results are in accordance
283 with observations by Disney (1988), that states three free-living larval instars for the phorid
284 species *Spiniphora bergenstammi* (Mik, 1864), *Megaselia rufipes* (Meigen, 1804),
285 *Phalacrotophora berolinensis* (Schmitz, 1920), and *Dohrniphora cornuta* (Bigot, 1857). He
286 asserts that the third instar also tends to last longer than the other two as observed for *D. cornuta*.
287 Our observations show that this third stage was the most voracious, when the larvae have more
288 eating and walking activity. It is important that beekeepers remove most of these large larvae
289 from their nests, preventing them from entering the pupal stage and a new generation reinfesting
290 the nest.

291 Female larvae can live up to 7 d longer than male larvae, while adult female flies usually
292 live over 7 d more than males. Moreover, males emerge earlier than females (3-4 days). This is
293 very common in parasites and Benner and Ostermeyer (1980) also reported this finding in
294 population of *Megaselia scalaris* (Phoridae). Males of this phorid emerged around 4 days before
295 the females.

296 To our best knowledge, there are so far no studies describing the life cycle of *P. kerteszi*.
297 In 2003, Roubik and collaborators investigated the behavior of *P. kerteszi* in infested beehives.

298 They mentioned the appearance of fly stages (eggs, larvae, pupae and the first offspring adults)
299 since the beginning of the infestation, without providing more information on how long each
300 stage last or on morphological characteristic of immatures. Even so, we could roughly infer by
301 their work the length period of larval and pupal stage by the first appearance of the previous
302 stage and the first appearance of the nest stage. In their work, larval stage lasted approximately
303 4 days and pupal stage 5 days. Our results were very similar and shows that the dynamics of
304 the infestation are very comparable to amazon Region to Atlantic Rain forest. It would be also
305 interesting to verify whether the life cycle of this pest is similar in regions where precipitation
306 levels are lower, such as in the Caatinga and Cerrado, Brazilian tropical dry forests. Studies
307 showing the seasonality of *P. kerteszi* are only available for the Amazon region, where the
308 highest rates of infestation occur during the rainy season (Peruquetti et 2012).

309 **Larvae development using a non-pollen substrate as food**

310 The fact that the flies were able to develop in the commercial protein supplement (Six
311 protein Body Builders®) is very important for future studies because it broadens the possibility
312 for rearing this pest under laboratory conditions. Honeybee pollen are expensive and stingless
313 bee polen is more than expensive or is not easily available. Our findings are, therefore,
314 important to reduce the cost with rearing, facilitating future experimental studies. However,
315 important questions to be tested is whether the quality of food (protein content) play a role in
316 an offspring's sex ratio or body size variation. We have not performed this experiment until this
317 study.

318 The extent to which the larvae of *P. kerteszy* depend on pollen for development was
319 speculative until this work. Chaud-Netto (1980) has mentioned a type of food for fish as
320 substrate culture for these flies. However, he did not detail what type of food it was or how to
321 make it.

322 **Sex ratio**

We are not able to affirm that our results of 3 males for each female ascertain a natural sex ratio in the wild. Previous different experiments resulted in different sex ratios. When reared in the laboratory in similar conditions but different substrate, Chaud-Netto (1980) found a sext ration of ~1:1. Robroek at al. (2003) observed that only females enter in the hives of meliponine bees. On the other side, Costa and Hime (1981) found that 97% of the *P. kerteszi* individuals encountered pollinating *Aristolochia gigantea* were male. It is suggested that male *P. kerteszi* are rather attracted by sexual pheromones than brood-site cues (Martin et al., 2016). Nevertheless, we wonder if a cluster of female flies in a hive would not be enough to attract any male inside the nests and where they encounter the males to copulate, since our findings suggest a non-parthenogenetic condition for these flies.

Oviposition regarding the acidity of the substrate

Our results clearly show that the acidity of the substrate plays a crucial role on the oviposition behavior of female *P. kerteszi* flies. We observed that even though the adult flies might feed on the non-acid artificial food (Fig. 1b) the decision to oviposit or not depends especially on the acidity of the substrate. We could observe that before laying eggs, flies tasted the food by touching their legs upon it and taking them to the mouth (tasting) as well as eating the substrate. Thus, it seems that oviposition in *P. kerteszi* is not a response to the smell of the substrate, but a touch response to the quality of the food, of which female flies test before laying eggs. The fact that *P. kerteszi* adult did feed on our artificial food is result of great importance because the natural food, bee-stored pollen, is a very expensive product for the bees and has low availability in the market.

Parthenogenec reproduction

Our experiments provide strong evidence that *P. kerteszi* flies do not reproduce partenogenetically. As support to our findings, parthenogenesis has never been described

among phorids, although the males of some species among the termitophilous subfamily Termitoxeniinae have never been described (Rohdendorf, 1974; Ferrar, 1987; Disney 1992).

Humidity

P. kerteszi larvae are strongly dependent on a high humidity environment in order to have a complete development. Robinson (1981) states a strong positive correlation between relative air humidity and the presence of *P. kerteszi*. He asserts that even under the same weather conditions, nest located in sites that are more humid are more likely to get greater infestations (Robinson, 1981). Portugal-Araújo (1977) asserts that the phoridae flies will not attack even weak stingless bees' colonies (low population and limited food) if their internal humidity in the nest is low. Indeed, we also observed this pattern through our periodic collection in the field. Even though it was not possible to test the role of seasonality, we perceived that in the hotter and drier months of November and December (dry season in the Brazilian Northeast), we captured very few adult *P. kerteszi* using our trapping methodology (see traps for flies in the field). Even when keeping the traps exposed for longer periods (8 days or more), we did not capture any *P. kerteszi* in experiments carried out by the end of November. On the other hand, in months from March to July, not only we captured a signicant amount of *P. kerteszi* but also some nests were naturally infested in this period. Thus, both *P. kerteszi* adults and larvae are severely impacted by a relatively low humidity. Therefore, it is important to think about alternative ways to control humidity in meliponaries, especially in rainy period.

Conclusions and future perspectives

This work is extremelly important for the conservation of stingless bees. Not only because it brings novel information regarding the biology of their main pest, the *P. kerteszi*, but also because its results open a vast possibility for further research concerning these flies and their ecological and chemical interaction with Meliponini bees. Yet, there is a lot of work to be done. Understanding the chemical communication in this system will be the key to discorver how *P.*

kerteszi flies indeed find their hosts. Thus, new studies in this field of research is urgent and crucial.

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Figures captions

Figure 1. **A)** Square wooden box of approximately 15 cm^2. Arrow A indicates the top removable part of the wooden box used as nest; the arrow B indicates the pollen swabbed around the hole entrance; arrow C shows the shaped-hole entrance of 7 mm diameter. **B**) Wooden box used to place the fly food. Arrows indicates some of the feces of *P. kerteszi* fly as feeding result on the artificial food. **C)** Petri dishes with 2 grams of *P. kerteszi* food. On the first petri, arrow indicates the 7 mm diameter hole on the lid used to transfer the flies. On the second arrows indicate the Eppendorf tubes of 2 ml used transfer the flies individually and a female fly already inside the dish. On the third petri, arrow indicates the adhesive used to tape the hole used to transfer the flies.

Figure 2. Development stages of *P. kerteszi*. **A**: Eggs. **B**: First stage of larva **C**: Second stage of larva (1 to 3 days). **D**: Third stage of (3 to 10 days). **E**: Pre-pupa (larva stops moving and

begins covering up by chitin). **F**: fresh pupa. **G:** Mature pupa. Figure bellow represents a larvas of *P. kerteszi* in the third stage (3 to 10 days). Eyespot of the larva used to see through different gradients of light. Seta of the larva, used as mechanoreceptor and controller of larva's movement through surfaces

Figure 3. Larvae development in natural stingless beebread. ~79.3% of the larvae reached the adult stage (fully developed), whereas ~20.7% did not have full development ($p < 0.001$). 14 died as larvae (9.29%) and 16 reached the pupal stage but did no emerge (~11.43%). From the individuals which had a full development, ~75.68% were male, whereas ~24.32% were female, a sex ratio of ~3:1 (Chi Square: =1.00, $p < 0.005$)

Figure 4. A) Boxplot displaying the life span difference between male and female adult flies. * stands for significant difference between males and females (Chi Square: $p < 0.001$). B) Life span difference between larvae leading to male and female flies. * stands for significant difference between males and females ($p < 0.001$).

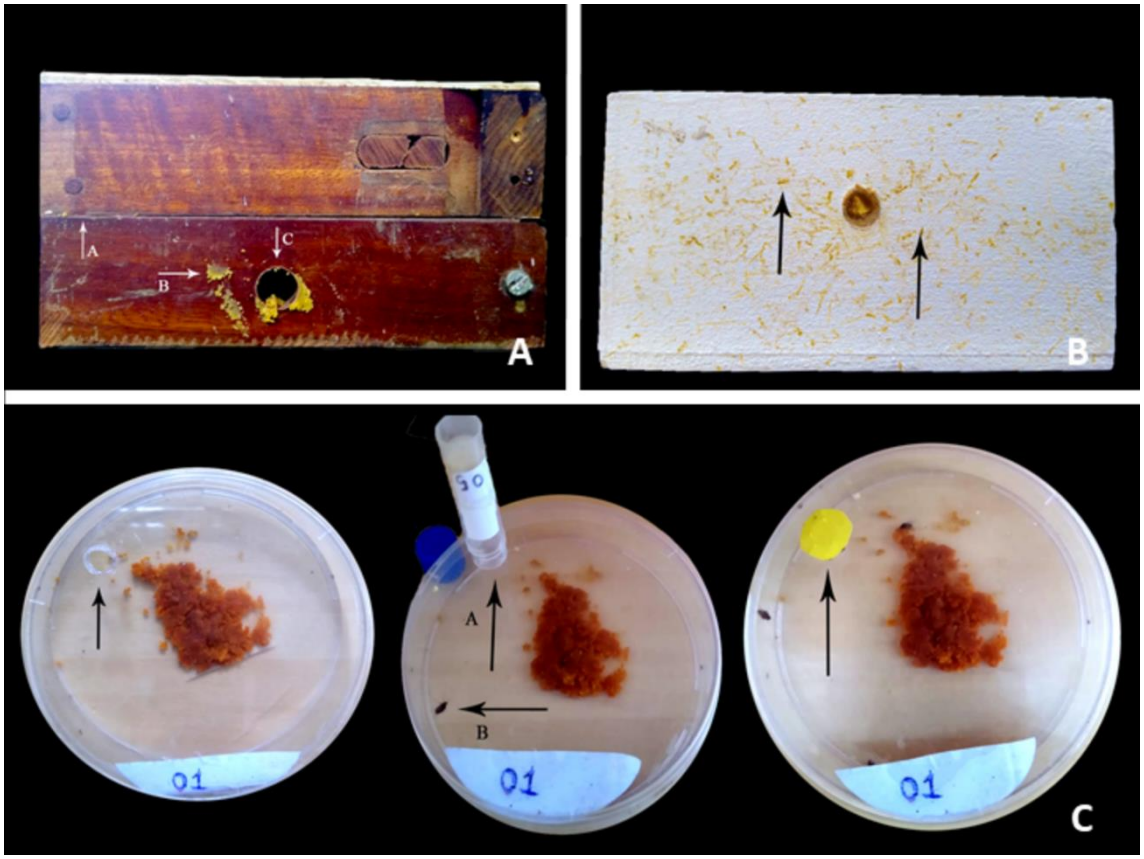
Figure 5: Larval development at a non-pollen source (whey protein).

Tables captions

Table I: Oviposition of females and development of larvae of *Pseudohypocera kerteszi* in different substrates under controlled conditions. Experiment 1: four replicates of 20 copulated females on one-liter plastic pots at B.O.D conditions; Experiment 2: four replicates of 200 individuals at fly cage conditions.

Table II: Larvae newly emerged from Pseudohypocera kerteszi eggs at different temperatures and relative humidity. Test of 5 repetitions per matrix.

Figures



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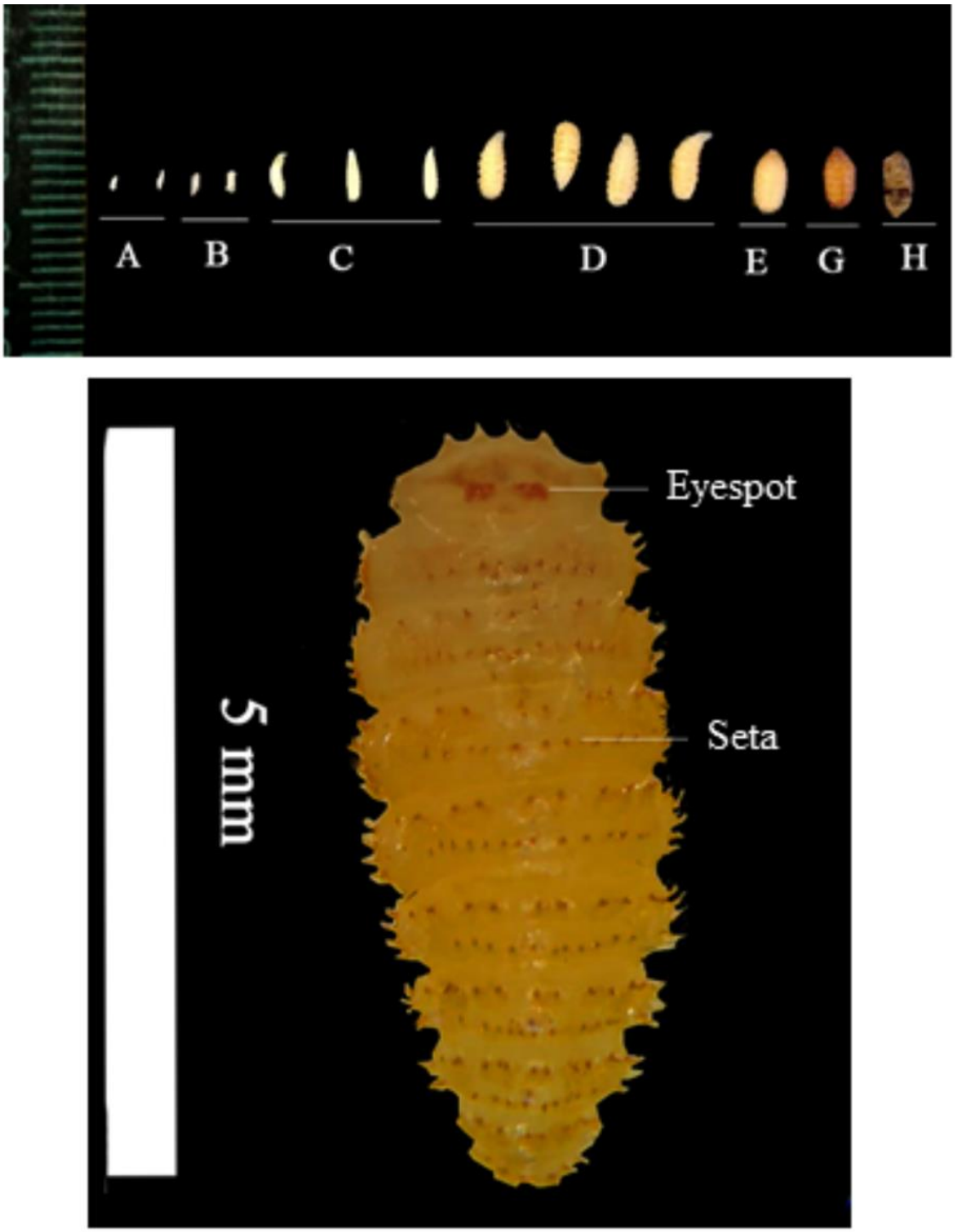

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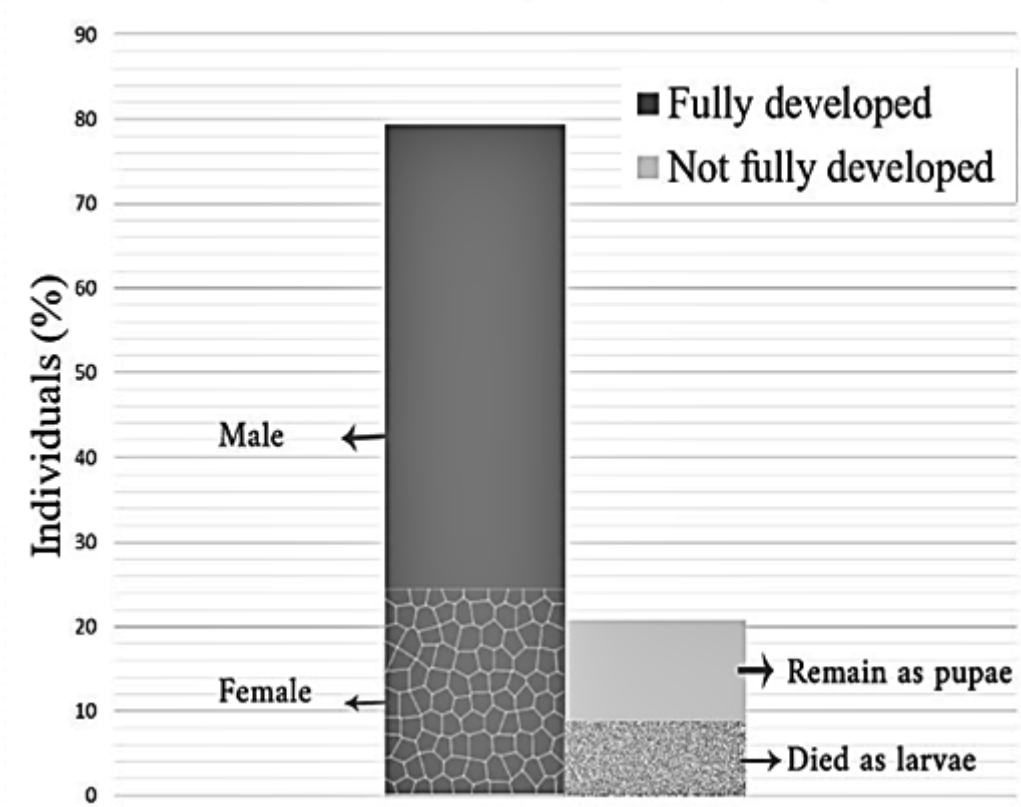

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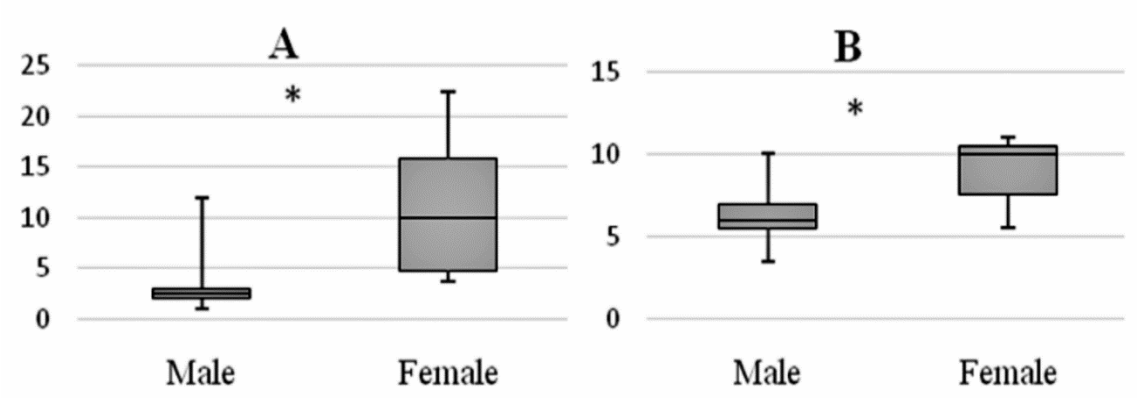

Figure 4. A) Boxplot displaying the life span difference between male and female adult flies. Y axis indicates the time of development (days). * indicates significant difference between males and females ($p < 0.001$). **B)** Life span difference between larvae leading to male and female flies. * stands for significant difference between males and females ($p < 0.001$).

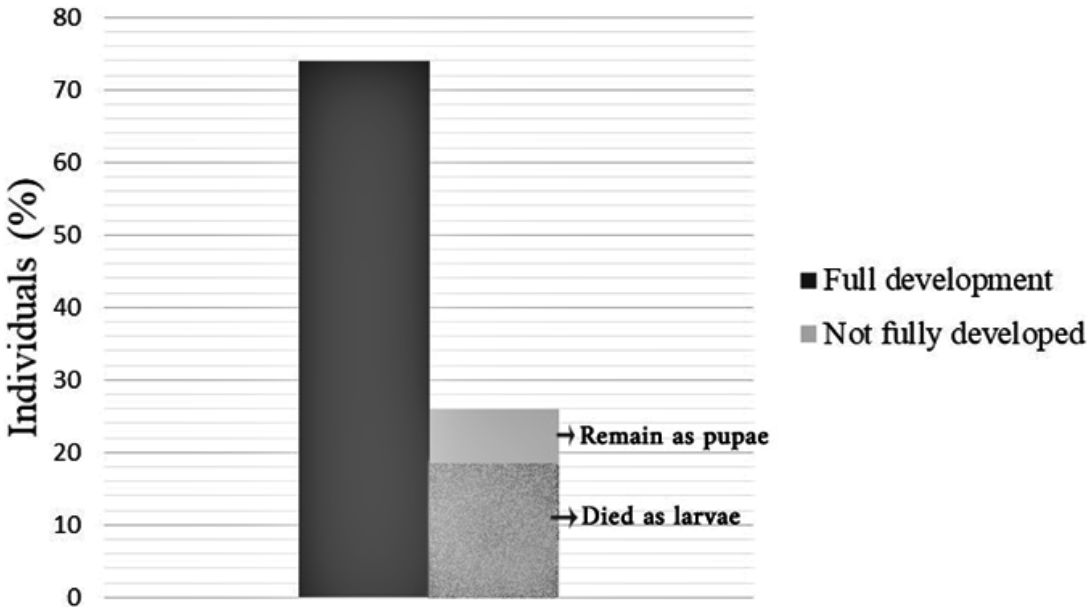

Figure 5: Larval development at a non-pollen source (whey protein substrate).

5. Capítulo 2 - Artigo a ser submetido ao Journal of Chemical Ecology, Spriger press

THE IMPORTANCE OF POLLEN AND ACETIC ACID IN THE ATTRACTION OF *Pseudohypocera Kerteszi* ENDERLEIN (DIPTERA: PHORIDAE), THE MAIN PARASITE IN BRAZILIAN MELIPONICULTURE

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Abstract

Pseudohypocera kerteszi is the most important parasite of stingless bees, which causes a vast damage to the productivity and may end up killing entire colonies. These flies enter in the colonies and oviposit mainly in the pots pollen and in the waste dumps. However, we do not know what rules the other structures of the nests play in this interaction. Here we aimed at investigating the role of different nest structures involved in the recognition of meliponini nests by the kleptoparasitic *P. kersteszi* and what are the major compounds emitted from them. As *P. kerteszi* do not parasite only one species of stingless bees, our hypotheses was that there is no significant difference among Meliponini species regarding the scent emitted by their nests. To test this, we identified the volatile compounds emitted by different structures of the nests (pollen, cerumen and geopropolis) of meliponine bees and test the attractiveness in bioassays. We used gas chromatography coupled to mass spectrometry to obtain the chemical characterization of the nests and identified them using CGMS solution Software (Shimadzu®). We also verified in behavioral tests the attractiveness of the substances to understand the functioning of the olfactory signals used by the parasite to encounter its hosts. Pollen was the structure that most attracted the flies as well as acetic acid. The major compounds emitted by the nest were: acetic acid, ethyl acetate, trans-ocimene, 4-ethylphenol and butanediol. In general, the traps containing pollen were the most attractive to flies of P. kerteszi except when it was offered againstglacial acetic acid. Pollen and acid acetic seem to play a very important role towards the *P. kersteszi* attraction. In addition, the microbiota associated with the production of acetic acid from pollen must have a significant importance in this klepoparasitic behavior. However, much of work needs to be done for a better understanding of this interaction.

Key words

Pseudohypocera kerteszi; Meliponini; Stingless bees; Phoridae; Meliponiculture; kleptoparasite.

INTRODUCTION

The tribe Meliponini has about 500 species described worldwide (Michener 2007) with habits, habitats, morphology, and behavior extremely diverse. In Brazil, 237 species are described (Camargo and Pedro 2007) and at least a hundred of them have the potential for bee products, such as honey, propolis, pollen, wax, resins and pollination services (Contrera et al. 2011, Venturieri et al 2012). However, unlike beekeeping (*Apis mellifera* L.), which has been exhaustively studied over more than 150 years (Imperatriz-Fonseca et al. 2012), meliponiculture is still in its scientific beginning, having a substantial increase in knowledge only in the last 40 years. Studies aiming artificial selection to increase productivity, management standardization techniques and especially knowledge of diseases and parasites are even more recent and scarce (Contrera et al. 2011; Venturieri et al. 2012; Maia-Silva et al 2013; Jaffé et al 2015; Nunes-Silva et al 2016; Potts et al 2016).

Pseudohypocera kerteszi Enderlein 1912 (Diptera: Phoridae) is considered the major kleptoparasite of meliponiculture (Sommeijer and De Bruijn 1994; Nogueira-Neto 1997). These small rapid flies do not have preferences among species of Meliponini, invading most of the nests in the neotropics (Roubik 1989; Nogueira-Neto 1997), causing enormous damage to the productivity (Contrera and Venturieri 2008; Oliveira et al 2013). Although the natural history of this pest is little known, the adults of these flies enter the nests and oviposit inside or next to the pots of pollen in the colony. The larvae, which have very rapid development, devour the pollen supply and then begin to eat the larval supply of the bee larvae and even the pupae, causing large infestations that can lead colony to collapse (Roubik 1989; Robroek et al. 2003).

Traditionally, in order to control this pest, the beekeepers used traps containing vinegar that capture the adults inside the colony and manual removal of infested larvae and food pots (Nogueira-Neto 1997; Ramos et al 2003; Wolff and Nava, 2007; Oliveira et al. to 2013). The trap consists of a pot (of varied volume) containing commercial vinegar inside and a lid with small holes (1.5 to 2mm in diameter). This trap attracts especially the females, who enter the pot and end up drowning inside it. The idealization of the trap was conceived from the sensory observation that the pollen pots exude strong acetic odor during the fermentation process (Imperatriz-Fonseca personal communication). However, several questions about the interaction between *P. kerteszi* and meliponine bees remain unresponsive, especially those

questions about the chemical tracks used by the flies to find the nests (Nogueira-Neto 1997, Oliveira et al. 2013).

The infestation of the bee nests occurs initially by females of *P.kerteszi* (Oliveria et al. 2013; Portugual Araújo 1977; Robroek et al. 2003). They find the nests and invade the colony by entering a gap in the nest structure or passing through the entrance of the colony that is usually protected by workers or soldiers (Nogueira Neto 1997; Grüter et al. 2012). After this initial barrier, the females oviposit in the pots of stored pollen, in the waste dumps of the colonies or close to the young brood discs (Roubik 1989; Nogueira Neto 1997). Infestations in nests under natural conditions are relatively rare, however in managed colonies, infestations become common, especially in weak or recently divided nests, constructed with low quality wood, with poorly closed lids or more commonly during the honey collection or other meliponic products (management) made by the breeders (Nogeueira Neto 1997).

Considering the extreme damage that phorid flies can cause to stingless beekeeping, few contributions about *P. kerteszi'*s ecology were published. Some works focusing on control techniques has been published (Oliveira et al. 2013; Moretto 2000; Ramos et al. 2003; Freire et al. 2006; Wolff and Nava 2007), but no work has been carried out regarding the basic question about this interaction: How can these flies find de nests? To start answering this big question, in this work we aim to verifying the attraction of *P. kerteszi* to different structures of the stingless bees' nests. Here we described what are the major compounds released from the nests, and whether there is difference among nests of different species of Meliponini. It is important to mention that no work has been published regarding the volatiles emitted by the nests of stingless bees. As *P. kerteszi* do not parasite only one species of stingless bees, our hypothesis is that nest of different Meliponini bees do not differ significantly regarding volatiles emitted. In this work, we collected and indentify the volatiles from different structures of nests of Meliponini (pollen, cerumen and geopropolis) from three different species: *Melipona (Michmelia) scutellaris* Latreille, 1811, , *Melipona (Melipona) subnitida* Ducke, 1910 and *Scaptotrigona* sp. (*tubiba* group). In addition, we tested the attractiveness of different structures of the nests and other chemical compounds toward the *P. kerteszi* in bioassays.

MATERIAL AND METHODS

Trap for Flies

To obtain enough adults of *P. kerteszi* to perform biotests in the laboratory, we developed a technique to obtain eggs and larvae in the field and then rear them in lab. The technique consisted of placing small empty stingless bee nests containing a mixture of different nest materials as baits. The nests are square wooden boxes (15 cm^2) with a removable lid of the same material and an entrance of 7 mm in diameter (Fig. 1). As bait, we mixed 10 grams of beebread (fresh pollen), 10 grams of macerated honey commercial bee pollen, 5 grams of honey and ca. 3 grams of other nest materials, namely geopropolis and cerumen, to give the bait a scent note similar to a real bee hive. The baiting material was placed inside the nest, whose entrance was swabbed fresh pollen. The nest was then closed and purposely placed about 50 cm next to stingless bee nests and left there for 72 hours. After 3 days, we sealed the entrance and the complete nest and moved it to the lab. These procedures were performed every time we were running out of flies in the laboratory, on average twice a month from September 2018 to November 2019 at Meliponary RCCO Camaragibe, Pernambuco, Northeast Brazil. The Meliponary is located in a semi natural area of Atlantic Rain Forest with native end exotic cultivated species of plants including trees and scrubs surrounded by secondary Atlantic Rainforest and tropical fruit crops like *Annona muricata* (Annonaceae), *Malpighia emarginata* (Malpighiaceae) and *Eugenia uniflora* (Myrtaceae). Eventually we collected flies from naturally infested nest of *Melipona scultellaris* at the same meliponary.

Most of the flies we collected were females, since it is observed that only females attack the nests and are found inside of the nests in the beginning of infestation (Oliveira et al 2013, Portugal Araújo 1977 e Roubik 1989). The sex ration was proceeded only for those flies captured within the traps, the remaining flies inside the cage were not taken into consideration.

Rearing of Flies

In order to make the food for the adults and larvae of the flies, we weighed 15 grams of honey and 7.5 grams of *Apis mellifera* commercial pollen, 3 grams of stingless bee bread (fresh pollen) plus 1.5 ml of acetic acid. A Petri dish and an analytical balance were used for this purpose. We macerate the pollen granules using a mortar and pestle, added and mixed the honey in order to keep a moist consistency (similar to fresh beebread). At last, we added the beebread and acid acetic and mixed again. Larvae and adults were kept inside fly cages (~1 m^3).

Biotests

Biotests were conducted using adult flies obtained from the cage where rearing was taking place. Biotests consisted of a two choice bioessay occurring in a fly cage (~1 m^3) under temperature ranging from 25ºC to 27ºC. In biotests, flies had the choice to enter a trap containing the product to be tested (treatment) or to enter the control trap. The traps consisted of plastic stool collector with a 2mL clear vial bonded inside, each one containing 1 gram of the different nest structures or 1 ml of the liquid substance (Acetic acid, vinegar or ethyl acetate). Holes were evenly made on the lid in order to the flies get in (Fig. 2). The products tested consisted of different nest structures, i.e. pollen, cerumen, geopropolis and also a mix of them. Besides, we also tested glacial acetic acid and commercial vinegar in the biotests (1 ml) against negative controls. A solution of water and odorless detergent (70/0,3 ml) was used as the negative control for all the experiments. Secondly, the nest structures and the substances were tested against each other. The traps were placed in different cages containing 200 or 500 flies, depending on the availability of flies. Two traps were placed inside each cage (nest material x control) 40 cm away from each other. The cages were covered with fabric to prevent light influence. Fourteen (14) different combinations were applied (Table I). The duration of the biotests has also varied according to the availability of flies and cages so we could perform more tests before the adults die (n=200, 24h.; n=500 12h. See table I.). After half time of each experiment, we switched the places of the traps.

Sampling of Volatiles

We collected the volatiles of different nest structures using standard dynamic headspace method (adapted from Dötterl et al., 2005). For this study, we selected three species Meliponini: *Melipona scutellaris, Melipona subnitida* and *Scaptotrigona* sp. (*tubiba* group). For each species, we repeated 5 times (5 different nests), total of 15 nests. In each nest we collected 3 nest structures: 1) pollen; 2) cerumen (encasement of the pollen), and 3) geopropolis. In total, we collected 45 samples. We removed the samples of each nest structure using sterilized and scentless tweezers and placed them within polyester bags (ToppitsBratschlauch ®, 10 cm x 10 cm). The air inside the bags, enriched with volatiles of the individual nest structures, were drawn through an adsorbent tube for 5 minutes using a vacuum pump (G12 / 01EB, Thomas, Puchheim, Germany) at a constant flow of 200 ml/m. The adsorbent filter consisted of a quartz vial cylinder (3cm long, 0.25 cm i.d) filled with 3mg of a 1:1 mixture of Tenax-TA (mesh 60-80, Supelco) and Carbotrap (mesh 20-40, Supelco).

Negative control samples (empty bags; n = 6,) were collected to control for environmental contaminants. All the samples were stored in 2mL screw cap clear vials at -20 ° C until further analysis.

Chemical analysis

In order to identify the compounds, the headspace samples were analyzed by gas chromatography coupled to mass spectrometry (GC/MS). We used an injector for thermal desorption of the chromatoprobes, using the Direct Injection Method on an Agilent quadrupole system 5975C Series GC/MSD (Agilent Technologies, Palo Alto, USA), equipped with a DB-5 apolar column (Agilent J & W; mx 0.25 mm di, 0.25 μm film thickness). The injector was heated at 200ºC and held there for 4.2 min and a split of 5:1 was used throughout the analysis. Electronic flow control was applied to keep a constant helium carrier gas flow of 1.5 ml min. The oven temperature program was at 40°C for 2 min and then increased by 6ºC per min to 240º C and held there for 7 min. The GC–MS data were processed by using the CGMS solution Software (Shimadzu®) The identification of the compounds was carried out by using the NIST and SATURN data bases and confirmed by a comparison of retention times and retention index with published data (Adams, 2007). The compounds with a relative amount percentage lower than 0.05 % on average were not taken into account.

To quantify the absolute amount of each compound emitted in a sample, known amounts of monoterpenes, fatty acid derivatives, and aromatics were injected into the GC/MS system, and their mean peak areas were used to determine the total amount of each compound (for more details see Dötterl et al. 2005b).

To quantify the absolute amount of each compound emitted in a sample, the relative amount for each compound was calculated regarding the total sum of all compounds released in that sample. We used their mean peak areas for this purpose (for more details see Dötterl et al. 2005). To calculate the average of relative amount of the compound released by each structure, we used the total sum each structure and divided by the number of repetition (n=5).

Statistical Analysis

For each two-choice biossays, we used the binomial test of goodness-of-fit to test whether the difference between the traps were significant. We tested the null hypothesis that all trap samples were equally attractive to the *P. kerteszi* for all the combinations. Data analysis was performed in Minitab statistical software (Minitab Inc., State College, PA).

Possible differences in scent chemistry among nest structures (i.e. pollen, geopropolis and cerumen) and bee species *Mscutellaris, M. subnitida, Scaptotrigona* sp were assessed by comparing (1) the relative ratio of compounds (semi-quantitative comparisons) or (2) the presence/absence of compounds (qualitative comparisons). For this, we first generated semi-quantitative and qualitative similarity matrices based on Bray-Curtis and Sørensen similarity indices, respectively. The relative ratios of compounds were transformed to their square root for the semi-quantitative analysis. Based on the obtained similarity matrices, we performed a two-factorial PERMANOVA analyses (factors: species and nest structures) and used nonmetric multidimensional scaling (NMDS) to depict variation in scent chemistry among samples. The similarity matrices, the PERMANOVA analyses and the NMDS plot were run in the software PRIMER 6 (version 6.1.15; PRIMER-E Ltd. 2012) in combination with the add-on PERMANOVA + (version 1.0.5; PRIMER-E Ltd. 2012).

RESULTS

Biotests

In general, the traps containing pollen were the most attractive to flies of *P. kerteszi* except when it was offered against glacial acetic acid. While 25.62% of Phoridae flies were captured in the pollen trap, 63.4% flies were caught in the trap containing glacial acetic acid ($p < 0.001$). In the assay testing pollen vs. control, the trap with pollen attracted 47% of individuals, while in the control 14% flies were counted ($p < 0.001$). When the pollen was set against the mixture of the 3 main structures of the net, we also observed a higher attraction by P. kerteszi (45.8% vs. 21% ($p < 0.001$). Pollen traps also captured more flies than that filled with honey (66.4% vs. 3.2%; $p < 0.001$). The pollen was also more attractive than the geopropolis and commercial vinegar. We observed only 1% of flies in the traps of geopropolis and 3.4% for the vinegar, while in the pollen traps 47% and 28.5% were captured respectively ($p < 0.001$). Acetic acid has also shown a higher attraction when tested against a negative control, 67.5% versus 5% in the first repetition, and 63.5 vs 2.5% in the second repetition (p-<0.001). In the test regarding ethyl acetate versus control, the control trap captured a higher number of flies, 61% against only 3.5% in the trap containing ethyl acetate ($P<0.001$). For the geopropolis versus control experiment, there was no significant difference, in the sample with geopropolis 26.5% of Phoridae flies were found, while in the negative control sample 23% of flies were trapped ($p = 0.482$). We also tested geopropolis against cerumen and while the trap with geopropolis captured 26%, the trap with cerumen caught 38.5% (P= =0,028). In the

experiment containing only cerumen as bait 24.5% of flies were captured, while in the negative control there were only 4.5% ($p < 0.001$). For the honey versus control experiment, there was no significant difference; while in the sample with honey 19% of flies were found, the control sample trapped 19.5% ($p = 0.909$). When the mixture was tested against the control it showed higher attraction towards the kleptoparasitc flies, in the mix 75% of flies were trapped, while in the negative control 17% of individuals (Ps <0.001). Most of the flies caught in our traps were female (~85%) and the sex ratio was 4/1 for most of the structures, expect for some control traps and other specific ones (see table I).

Chemical Characterization of Nest Volatiles

Styrene (16.17%), acetic acid (10.75%), beta-ocimene (10,27%), ethyl acetate (7.35%), 2,3-butanediol (7.17%) and ethyl lactate (3%) were the most abundant compounds in our analysis. We detected more than 200 compounds. Some compounds were exclusive to some structures of the nests. Other compounds were frequently present in more than one structure in all the species analyzed.

The statistical analyses revealed a significant semiquantitative (PERMANOVA: Pseudo-$F_{2,42} = 2.31$, $p < 0.01$) and qualitative difference (PERMANOVA: Pseudo-$F_{2,42} = 1.8482$, $p<0.05$) among bee species (Fig. 3). Similar chemical profiles of nest structures also differed significantly semiquantitavely (PERMANOVA: Pseudo-$F_{2,42} = 10.26$, $p<0.001$) and qualitatively (PERMANOVA: Pseudo-$F_{2,42} = 9.19$, $p<0.001$). The statistical analyses also revealed that a significant difference on both semiquantitative and qualitative chemical profile for the interaction between the factors bee species x nest structure (Fig. 4). A posteriori pairwise tests for pairs of levels of factor species revealed (1) that the scent profile of pollen of the pair *Scaptotrigona* sp./*M.subnitida* was similar, whereas the scent profile of *M.scutellaris* was significantly different from both *Scatotrigona* sp. ($T=1.52$, $p<0.05$) and *M. subnitida* ($T=1.7$, $p<0.05$); (2) that the scent profile of the cerumen of the pair *M. scutellaris* and *Scaptotrigona* sp. but not of the other pairs, differ significantly ($T=1.5$, $p<0.02$) and (3) that the scent profile of batumem of the pairs *M. scutellaris/M.subnitida* ($T=1.64$, $p<0.05$) and *Scaptotrigona* sp/*M.subnitida* differ significantly ($T=1.56$, $p<0.02$).

Pollen

In the samples of pollen, we found 142 compounds, of which 97 were identified and 45 were not. In spite of this great diversity of constituents, 96 compounds account together for about 75% of the total scent bouquet, whereas acetic acid was the most abundant compound

in samples of pollen (25.28% on average). Acetic acid was present in 12 out of 15 samples. Ethyl acetate was the second most abundant compound (14.90% on average) and it was present in 13 out of 15 samples. Other significantly abundant scents were: Trans-Ocimene (11.05%), 2,3-butanediol (10.80%), ethyl lactate (6.5%), trans-cimene, propyl butanoate (2.67%), benzyl alcohol (2.00%), benzyl acetate (1.28%) and beta-Caryophyllene (1.24%) (Table II). All the other pollen-originated compounds were emitted in relative amounts lower than 1% (refer to appendix to see the complete list of compounds).

Cerumen

The cerumen produced 132, of which 100 were identified and 33 remained unknown. trans-Ocimene was the most abundant compound (29.31%), followed 2,3-Butanediol (15.16%), Ethyl Acetate (8.66%), alpha-Pinene (5.98%), acetic acid (4.0%), styrene (5.24%), 1,8-Cineole (2.94%), methyl p-anisate (2.05%) toluene (1.53%), pentacosane (1.42%), heptanone (1.05%), ethyl propanoate (1.03%), p-anisyl acetate (1.9%) (Table II).

Geopropolis

In the geopropolis, we reported 133 compounds, of which 98 compounds were identified. The most abundant compound in the samples of geopropolis was Styrene with relative mean of 42.66%. Other major compounds were, 4-ethylphenol, (7.04%), alpha-Copaene (5.36%), Tetracosanol (3.35%), Gurjunene (2.40%), Ethyl lactate (2.10%), Acetic acid (2.06%) Benzene, 1-ethyl-4-methoxy (1.73%), (Table II). The remaining compounds were emitted in mean relative amounts lower than 1% (refer to appendix to see the complete list of compounds).

DISCUSSION

In our biotests, the vast majority of the flies were attracted to the traps containing pollen or acetic acid. Indicating that *P. kerteszi* is preferably conducted by products with a very volatile scent, especially acid. However, this is only true for females, since only females were found to be attracted and invade the hives of stingless bees (Robroek at al., 2003; Oliveira et al 2013; Protugual Araújo, 1977 e Roubik 1989). Consequently, most of the flies captured in our traps were females, a sex ratio of 4/1 in general. As we did not identify the sexes of the remaining flies that did not fall into the traps it is reasonable to think that among those outside, most flies must be male. It is very important to refer that these observations should only be valid for this specific interaction between the flies and nest of Meliponini, since male

flies play important keys in other interactions and environments such as the pollination of *Aristolochia gigantea* (Costa and Hime, 1981).

It is widespread among the stingless beekeepers the common observation that *P. kerteszi* are also attracted to some decaying fruit. Costa and Hime (1981), describe the odor of an individual of *Aristolochia gigantea* as strong and sweet similar to decaying fruits. Hoehne et al (1927), also reports the *A. gigantea*'s smell and states that the scent may vary according to the altitude. Interestingly, there is an intriguing relationship between *P. kerteszi* and *A. gigantea*, which might be helpful for the understanding of this study. The most frequent pollinator insects to the *A. gigantea* are Diptera belonging to the Phoridae family (Costa and Hime, 1981; Hipólito et al 2012). In his work, Hipólito et al (2012), states that about 81% of the visitors found in *A. gigantea* are *Megaelia* sp. and *Pseudohypocera* sp. (Phoridae Family). Costa and Hime (1981), also found that *P.kerteszi* is the specie that most often visits *A. gigantea* at any time of the year. In both studies *P.kerteszi* was also reported carrying the pollen of this flower (Costa and Hime 1981; Hipólito et al 2012). However, 96% of the flies observed by Costa and Hime (1981) were male, an observation that suggests sexual rather brood-site deception that usually attract female flies of *P. kerteszi* (Martin et al 2018). On the other hand, in the study carried out by Hipólito et al (2012) 82% of the *Pseudohypocera* sp. were females, even though they were not identified to the species level. The results between Hipólito et al (2012) and Costa and Hime (1981) regarding the main sex that pollinates of *P. kerteszi* that pollinates *A. gigantea* are significantly contrasting. Thus, it is possible that there might have been some confusion in the identification of flies' sexes. Few studies have been published regarding *A. gigantea* floral scent and much work still necessary to understand the relationship between this flower and phoridae flies.

Unlikely what we thought, each species of Meliponini has its own scent profile, as well as each nest structure. Thus, the ecological interaction regarding the chemical cues used by *P. kerteszi* to find the nests of Meliponini bees must be related to specific compounds released in common by all the nests. Meliponini bees are quite generalists in the collection of floral resources, because of this, we should have found a generic scent as a whole. Nonetheless, our results show a significant discrepancy between the odors present in the nests, even though, most of the odors on the list are also floral volatiles (Knudsen et al. 2006.). The generalist habit is as important as the storage of food and must be linked to the social habit of each Meliponini species (Ramalho et al., 2007).

On the other hand, some compounds are common to more than one structure of the nests. The most abundant ones are acetic acid, styrene, trans-ocimene, ethyl acetate, 2, 3-Butanediol and ethyl lactate. It is plausible to think that these compounds, or maybe a combination of them, might be the answer why Phoridae flies are able to find the nests, without distinguishing the different species of stingless bees. However, is much more reasonable to consider that the pollen, as the structure that most releases acetic acid odor regardless the species (~25% of relative amount), is the most important structure in the attraction of *P. kertezi*, especially when we consider the behavioral aspects observed in our biotestes. In this work, it was evidenced that *P. kerteszi* had greater attraction for the traps of pollen and glacial acetic acid and this might be associated with acetic acid odors produced by the acetic acid bacteria. Not to mention that the biological aspects are crucial in this context, since pollen is the substrate upon which the flies oviposit the most when they first invade the nests. In particular, it is interesting to note that cerumen was more attractive than the negative control, but in this case, a contamination with the acetic acid originated from pollen must have taken place, since cerumen is hard to collect without touching it.

Furthermore, acetic acid alone has shown to play a key role in the attraction of *P. kerteszi* towards the nests of Meliponini bees. Even when it was tested against the fresh pollen bread, which is the main source of food and oviposition for the *P. kerteszi*, acetic acid demonstrated to attract a larger number of flies. Not by chance, traps of vinegar have been used within the nests by the stingless beekeepers in order to control these pests (Nogueira-neto, 1997, Ramos et al 2003, Wolff and Nava, 2007, Oliveira et al. to 2013). Nevertheless, it is worth mentioning that the amount of acetic acid used in our bioassays must be much higher than what is naturally emitted by the amount of pollen offered simultaneously.

In nature, acetic acid is produced by acetic acid bacteria (AAB) from the family Acetobacteraceae, within the order Rhodospirillales (Matsushita et al 2016). They are microorganism commonly found in the environment, and easily found in association with plants, fruits and flowers (Kersters et al 2006). Moreover, AAB has been frequently described as symbionts of different insect species that depend on sugar-based diets. That is why AAB play an important role in microbiota of bees in general, since they are also found in the digestive system of these insects (Crotti et al 2010). However, pure acetic acid or even commercial vinegar are not attractive to *P. kertiszi* outside or around the nests (Ramos et al., 2003, Oliveira et al. 2013; personal observation). We do not know in this way how the kleptoparasite finds the nests of the bees and what chemical components are connected to the

encounter of the host. It is not reasonable to think that, over a long distance, the visual component should play a minor role in nesting by these flies, since the entrances of the Meliponini nests are usually inconspicuous and small (Camargo, 1970).

Another acid, which might be very relevant in the chemical attraction of *P. kerteszi*, is the lactic acid. Not by chance, ethyl lactate, a derivative of lactic acid, was found in most of our pollen samples, representing 6.5% of relative amount. Pollen storage pots of Meliponini bees are usually enclosed, and although it has some tiny orifices allowing gas exchange, the environment inside might be propitious for the lactic acid bacteria functioning. Microbial action, especially lactic acid fermentation produced by bacteria and yeast, is the major contributors for the conversion of pollen grains to bee bread (stored pollen) (Gilliam 1989). Thus, beebread has a higher acidity than the pollen grains, possessing a different chemical composition (Haydak 1942). Vázsquez and Olofsson (2009) reported that lactic acid bacteria (LAB) belonging to the genera *Lactobacillus*, *Bifidobacterium* and the Pasteurelaceae family were found to be in bee's digestive fluids as well as stored pollen (beebread) from *Apis mellifera*. In the production of scents, they play a key role since a variety of lactic acid bacteria are known to synthesize ethyl esters and thioesters (Gupta et al 2015). *Lactococcus lactis*, for instance, has an esterase enzyme responsible for the aroma of ester compounds (Nardi et al 2002). Moreover, L-lactic acid has been reported to be an important factor in the attraction of other Diptera such as *Aedes aegypt* (Diptera: Culicidae) and *Anopheles funestus* (Diptera: Culicidae) (Murphy et al 2001; Steib et al 2001).

However, in contrast with AAB, LAB should only play minor role in the attraction of *P. kerteszi* since, in this case, their optimal conditions are inside the closed pots of pollen. Besides, the usual infestations of the flies occurs when there is disturbance of this natural arrangement, such as an predator attack or even an usual handling by the stingless bee keeper (Roubik 1989, Nogueira Neto 1997, Contrera and Venturieri 2008). This leaves the pollen storage pots open and in contact with air (favorable condition for the acetic acid bacteria functioning, since they are obligates aerobes and oxidize sugar, producing acetic as the main final product (Raspor and Goranovic 2008).

Overall, our results, indicate that the microbiota present the fermentation of pollen grains inside the food pots are the key to understanding the chemical interaction between *P. kerteszi* and Meliponini bees. Undoubtedly, pollen is the most important among all the other nest structures in the attraction of *P. kerteszi*, as well as its major compound released, acetic

acid. Nevertheless, a thorough understanding of the microbiota inside the pots of pollen and in the nests as a whole is extremely necessary for future studies. Much work needs to be done regarding *P. kerteszi's* ecology in order to distinguish what other compounds are linked to the attraction of these flies toward the nests of Meliponini bees and what are the major roles of the microbiota in this interaction.

ACKNOWLEDGMENT

We deeply appreciate all the support from FACEPE for the important scholarship granted during the research period, process IGBG-0484-5.04 / 18. To CNPQ for financing the project through process 400597 / 2018-7. CAPES for direct and indirect assistance to carry out this research. To Daniela Navarro e Júlio César R. de O. F. de Aguiar for the support in the Chemical Ecology Laboratory.

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FIGURE LEGENDS

Figure. 1. Nest of *Melipona scutellaris* infested by thousands of *P. kerteszi* larvae.

Figure. 2 Drawing of a plastic stool collector used as trap, with holes on the lid and a 2 ml clear vial bounded inside (a). Drawing of a cage used in the biotests with two traps inside (b).

Figure 3. NMDS representing the Meliponini species regarding their compounds emitted.

Figure 4**.** NMDS representing the different structures of the nests of different Meliponini species (P = pollen; I= cerumen; B; geopropolis).

TABLES LEGENDS

Table I. List of the results of all bioassays performed, as well as, sex ratio (F-female/M-male), time and number of flies (N).

Table II. List of the major compounds found in the structure of the nests of tree species Meliponini bees. The order of the compounds is according to their relative amount (%) (decreasing). The retention index (RI), retention time (RT) are also included.

Figure. 1. Nest of *Melipona scutellaris* infested by thousands of *P. kerteszi* larvae.

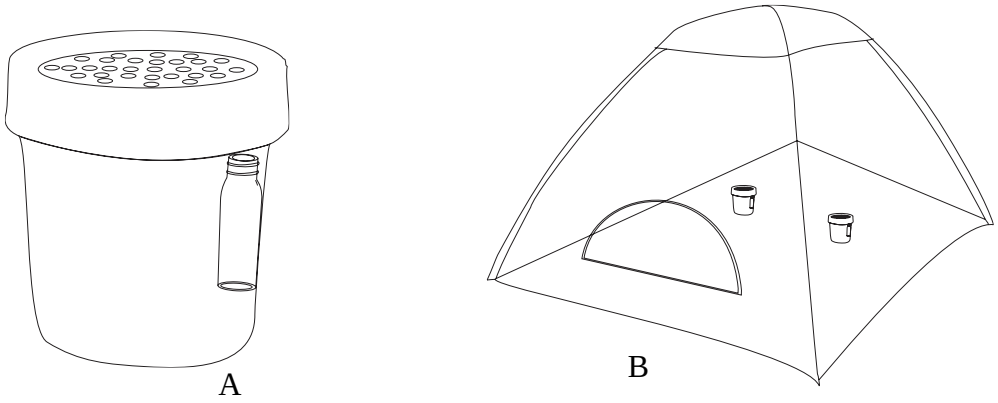
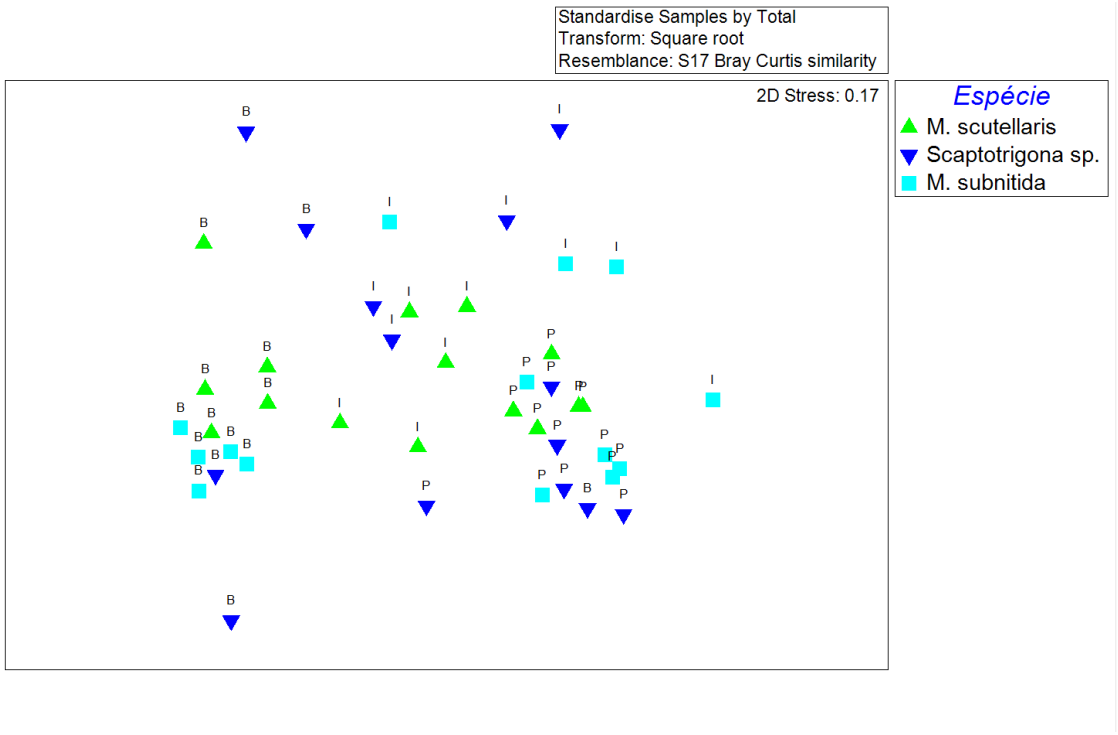


Figure. 2 Drawing of a plastic stool collector used as trap, with holes on the lid and a 2 ml clear vial bounded inside (a). Drawing of a cage used in the biotests with two traps inside (b).



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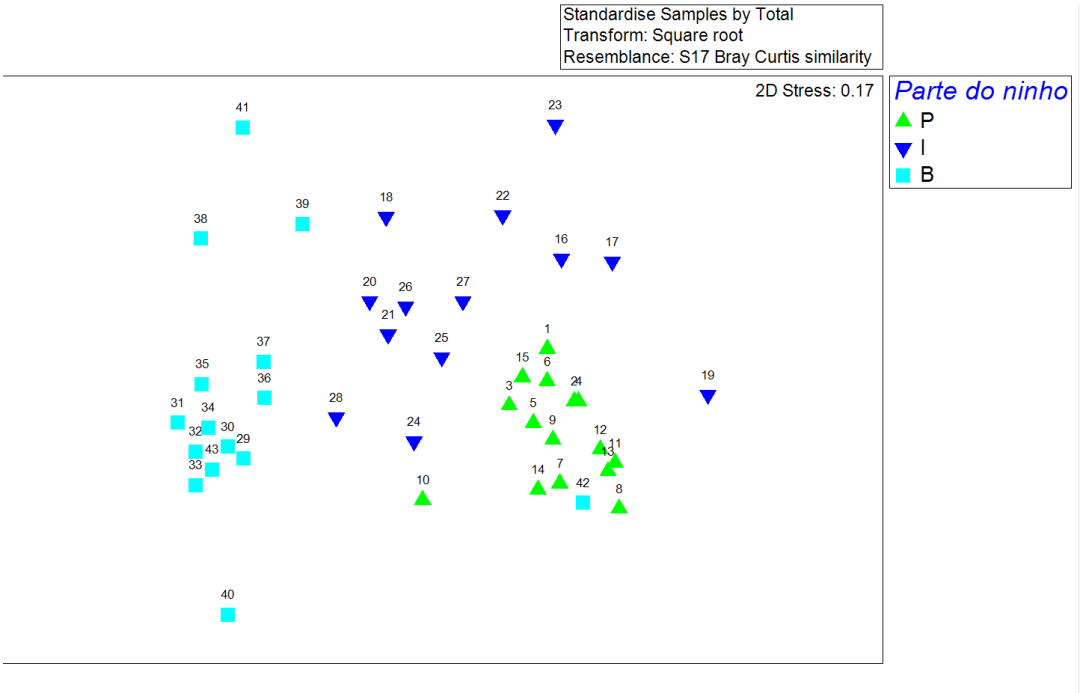

Figure 4. NMDS representing the different structures of the nests of different Meliponini species (P = pollen; I= cerumen; B; geopropolis).

Table I. List of the results of all bioassays performed, as well as, sex ratio (F-female/M-male), time and number of flies (N).

Combination (Trap-A x Trap-B)		**Trap -A (N)**	**Sex ratio**	**Trap -B (N)**	**Sex ratio**	**N**	**Time**	**P**
Trap-A	**Trap-B**							
Pollen	Control*	94	87F/7M	28	16F/12M	200	48h.	<0.001
Pollen	Acetic acid	128	105F/23M	317	289F/28M	500	24h.	<0.001
Pollen	Mix**	229	195F/34M	105	81F/24M	500	24h.	<0.001
Pollen	Honey	332	228F/44F	16	7F/9M	500	24h.	<0.001
Pollen	Geopropolis	94	88F/8M	2	2M	200	12h.	<0.001
Pollen	Vinegar	57	51F/6M	7	6F/1M	200	12h.	<0.001
Acetic acid	Control	135	131F/4M	10	4F/6M	200	12h.	<0.001
Acetic acid	Control	127	119F/26F	5	1F/4M	200	12h.	<0.001
Ethyl acetate	Control	7	2F/5M	122	97F/25M	200	12h.	<0.001
Geopropolis	Control	53	22F/31M	46	8F/38M	200	12h.	=0.482
Geopropopolis	Cerumen	52	40F/12M	77	34F/33M	200	12h.	=0,028
Honey	Control	38	33F/5F	39	35F/4M	200	12h.	=0.909
Mix*	Control	150	131F/19F	34	24F/10M	200	12h.	<0.001
Vinegar	Control	72	57F/15F	36	29F/7M	200	12h.	=0.001
Cerumen	Control	49	20F/29M	9	3F/6M	200	12h.	<0.001
0.5g(P)+ 0.5(AA) *** ***	0.5g(P)+ 0.5(AE) ***	116	111F/5M	0	-	200	12h	<0.001

*Negative control (water and detergent – 70/0,03ml)

**Mix of the material of the nests (pollen, cerumen, geopropolis and honey)

** Mix of 0.5 grams of polen + 0.5 grams of acetic acid X 0.5 grams of polen + 0.5 grams of Ethyl acetate

Table II. List of the major compounds found in the structure of the nests of tree species Meliponini bees. The order of the compounds is according to their relative amount (%) (decreasing). The retention index (RI), retention time (RT) are also included.

Structure	Compound	Functional group	R.I.	R.T.	%
Pollen	Acetic acid	Carboxylic acid	665	2.134	25.28
	Ethyl Acetate	Ester	612	1.742	14.90
	Trans-Ocimene	Terpene	1048	9.517	11.05
	2,3-Butanediol	Alcohol	785	3.690	10.80
	Ethyl Lactate	Carboxylic acid/Alcohol	815	4.122	6.50
	Unknown (43!55,88,101,117...)		935	6.728	3.93
	Propyl butanoate	Ester	899	5.860	2.67
	Benzyl alcohol	Alcohol	1035	9.192	2.0
	Benzyl acetate	Ester	1165	12.381	1.29
	Beta-Caryophyllene	Sesquiterpene	1423	18.194	1.24
Cerumen	Trans-Ocimene	Terpene	1048	9.517	29.31
	2,3-Butanediol	Alcohol	785	3.690	15.16
	Ethyl Acetate	Ester	612	1.742	8.66
	Unknown (43,57,83,97!,208)		2468	35.333	6.71
	Alpha-Pinene	Terpene	931	6.631	5.98
	Acetic acid	Carboxylic acid	665	2.134	4.0
	Styrene	Aromatic hydrocarbon	894	5.641	5.24
	1,8-Cineole	Ether	1031	9.091	2.94
	Methyl p-anisate	Ester	1375	17.183	2.05
	p-Anisyl acetate	Ester	1419	18.113	1.9
Geopropolis	Styrene	Aromatic hydrocarbon	894	5.641	42.66
	4-Ethyl Phenol	Benzene derivative	1167	12.450	7.04
	Unknown(38,43!,45,68,73,74...)		888	5.632	5.69
	Alpha-copaene	Sesquiterpene	1378	17.250	5.37
	Tetraconasol	Alcohol	2477	35.452	3.34
	Unknown (43,55,69,97!,139...)		2487	35.612	2.93
	Gurjunene	Sesquiterpene	1413	18.011	2.41
	Ethyl Lactate	Ester	815	4.122	2.10
	Acetic acid	Carboxylic acid	665	2.134	2.06
	Benzene, 1-ethyl-4-methoxy	Methoxy	1114	11.146	1.73

6. Conclusão

Os resultados obtidos nessa pesquisa são de extrema importância para a conservação das espécies de abelhas sem ferrão (meliponini). Através desses dois artigos conseguimos informação totalmente novas e relevantes para o combate e controle da principal praga da meliponicultura *P. kerteszi*. É essencial, entretanto que estudos futuros continuem sendo feitos para melhor entender a biologia e ecologia de *P. kerteszi*, e principalmente suas interações e comportamento cleptoparasitas em relação a abelhas sem ferrão.

Supplementary material (XLSX format from submition)

Table III- Complete list of the compounds emitter by each structure (decreasing order).

Pollen			
Compound	RI	RT	%
Acetic acid	665	2134	25,28124
Ethyl Acetate	612	1742	14,90061
Ocimene <(E)-, beta->	1048	9517	11,05614
2,3-Butanediol	785	3690	10,80144
Lactate <ethyl->	815	4122	6,501647
Unknown	935	6728	3,936036
Propyl butanoate	899	5860	2,674524
Benzyl alcohol	1035	9192	2,002782
Benzyl acetate	1165	12381	1,287384
ß-Caryophyllene	1423	18194	1,240664
f. 2,3-Butanediyl diacetate	1063	9883	1,110157
Unknown	940	6850	0,833084
Unknown	2429	34829	0,813423
cis-Linalool oxide pyranoid	1170	12505	0,593122
Methyl benzoate	1095	10688	0,580518
cis-Ocimene	1039	9281	0,518195
Linalool	1100	10813	0,488336
Unknown	888	5632	0,481388
1,2-Dimethoxybenzene	1147	11951	0,448113
Hexanoic acid, ethyl ester	1001	8340	0,443121
Unknown	758	3218	0,440833
Toluene	761	3262	0,440413
Isoamyl acetate_S	877	5414	0,415791
Butyl butyrate	996	8202	0,399765
Hexyl acetate	1015	8679	0,389554
2-Undecanon	1294	15386	0,370447
Unknown	1117	11239	0,368535
alpha-Copaene	1378	17250	0,3601
Rosa-5,15-diene	1935	27623	0,351648
Styrene	894	5641	0,350534
Unknown	1010	8560	0,346552
Unknown-	902	5934	0,338077
Ar-Curcumene	1484	19462	0,316723
(Z)-Linalool oxide (furanoid)	1074	10143	0,298287
1-Hexanol, 2-ethyl-	1030	9052	0,287684
2-Phenylethanol	1114	11139	0,287063
Carene <delta-3->	1010	8567	0,284558
|allo|-Ocimene	1131	11560	0,273434

Unknown	1359	16778	0,251682
Pinene<alpha->	931	6631	0,25117
Unknown	1871	26579	0,243127
2-Tridecanone	1495	19683	0,233699
Unknown	1579	21336	0,212106
Unknown	914	6207	0,202937
beta.-Phenethyl acetate	1258	14558	0,20002
2-Methylbutanoic acid	870	5223	0,194894
Octanoic acid, ethyl ester	1198	13169	0,193619
1,2-Propanediol, diacetate	1030	9067	0,192853
Butenoic acid, 3-methyl-2-	881	5498	0,183503
2-Methylbutanoic acid	859	5040	0,182675
unkown	1009	8535	0,181765
Benzaldehyde	960	7342	0,180059
Benzeneacetonitrile	1140	11762	0,179314
Isovaleric acid	847	4894	0,176534
Curcumene<beta-	1513	20044	0,171789
Unknown	1759	24652	0,150074
n-propyl acetate	714	2585	0,137349
Germacrene D	1485	19478	0,136195
Amyl acetate	915	6263	0,127825
Bergamotene <alpha-, trans->	1437	18499	0,127704
Farnesene <(E,E)-, alpha->	1509	19951	0,123983
Uknown	745	3034	0,123431
unkown	880	5591	0,123046
trans-Linalool oxide pyranoid	1175	12618	0,10328
Hexanol	869	5244	0,103194
Unknown	2134	30713	0,100176
Methyl eugenol	1405	17820	0,096867
Ethyl propanoate	709	2523	0,092015
Humulene<alpha->	1457	18906	0,090166
Muurolene <gamma->	1480	19386	0,089457
Unknown	1073	10129	0,083717
Unknown	1499	19766	0,080533
Unknown	1124	11394	0,079872
jasmone (CIS OR TRANS?)	1400	17717	0,079422
Octadecanoic acid	2158	31054	0,077888
Unknown	854	4986	0,073977
Unknown	1704	23686	0,073419
Unknown	929	6588	0,07221
Propanoic acid, butyl ester	907	6058	0,071441
Ethyl hexadecanoate	1991	28517	0,070153
Unknown	1059	9769	0,069407
Unknown	2165	31170	0,065453

Cubebene<alpha->	1353	16693	0,06471
Unknown	1072	10122	0,061484
Ionone<(E)-beta->	1488	19531	0,060923
Benzene acetic acid, ethyl ester	1246	14282	0,060416
Unknown	1118	11249	0,057905
Hexadecanoate <methyl->	1924	27441	0,057543
Unknown	1275	14956	0,056917
Octanol acetate	1212	13497	0,055587
Unknown	1094	10661	0,054777
Unknown	1897	26996	0,054569
(e)-Linalool oxide (furanoid)	1090	10542	0,053213
Unknown	912	6172	0,053161
BETA. ELEMENE	1394	17600	0,052721
Methyl p-anisate	1375	17183	0,046454
Kaurene	2045	29354	0,043068
Unknown	1172	12538	0,039478
Unknown	1089	10535	0,036502
Unknown	1165	12386	0,036263
Pentyl furan <2->	992	8113	0,034204
Prenyl acetate	925	6485	0,033091
Unknown	2135	30724	0,030042
Unknown	1300	15530	0,029566
Unknown	1270	14840	0,028341
p-Anisyl alcohol	1283	15146	0,026614
Linalool oxide acetate<trans->(pyranoid)	1288	15263	0,025541
Geranyl acetate	1384	17361	0,022917
Geranylacetone_S	1453	18836	0,022652
Unknown	1462	18997	0,022237
Unknown	1158	12209	0,022178
Unknown	1584	21430	0,020669
Benzene, 1-ethyl-4-methoxy	1114	11146	0,020227
Hexenyl butanoate	1190	12975	0,019698
Dodecane	1200	1310	0,018071
Heptanone	892	5713	0,018007
Unknown	1544	20660	0,017776
Coumaran	1220	13699	0,016863
n-Hexadecanoic acid	1958	27985	0,016649
Unknown	937	6781	0,016257
Unknown	2128	30615	0,015372
Diethyl succinate	1182	12797	0,015305
Unknown	1014	8524	0,014555
Unknown	854	4942	0,014399
Unknown	1350	16621	0,012888
2-Heptanol	901	5943	0,011416

Unknown	1296	15448	0,011366
Copaene <beta->	1433	18410	0,010806
Unknown	990	8079	0,009932
Unknown	1694	23498	0,009354
Unknown	1440	18550	0,008303
Unknown	1314	15850	0,007752
Unknown	1079	10199	0,007706
Unknown	2369	34025	0,0073
alpha.-Guaiene	1442	18599	0,006875
Unknown	1159	12223	0,006848
Zonarene	1527	20311	0,006663
Octalactone	1286	15205	0,006444
p-Anisyl acetate	1419	18113	0,005937
Unknown	2069	29726	0,005872
Cerumen			
Compound	RI	RT	%
Ocimene <(E)-, beta->	1048	9517	19,88043
2,3-Butanediol	785	3690	11,15862
Ethyl Acetate	612	1742	7,053144
Unknown	2468	35333	6,345903
Pinene<alpha->	931	6631	4,103319
Acetic acid	665	2134	3,996196
Styrene	894	5641	3,843119
1,8-Cineole_S	1031	9091	2,28152
Methyl p-anisate	1375	17183	1,810204
p-Anisyl acetate	1419	18113	1,61436
Unknown-	902	5934	1,568983
Unknown	937	6781	1,545159
Toluene	761	3262	1,535668
Pentacosane	2494	35682	1,429711
Unknown	1072	10122	1,426724
Unknown	955	8524	1,211176
Heptanone	892	5713	1,054155
Ethyl propanoate	709	2523	1,033409
Unknown	2134	30713	1,021877
Prenyl acetate	925	6485	1,018046
n-propyl acetate	714	2585	1,011265
Unknown	2074	29805	1,007388
Benzyl alcohol	1035	9192	0,942927
Benzene, 1-ethyl-4-methoxy	1114	11146	0,942324
l-Limonene	1029	9024	0,933084
Unknown	1436	18468	0,814411
Unknown	952	7279	0,732694
Unknown	2292	32979	0,678415

Unknown	1350	16621	0,657717
Ar-Curcumene	1484	19462	0,6089
Phenol, 4-ethyl-	1167	12450	0,606591
Bergamotene <alpha-, trans->	1437	18499	0,603291
Cadinene<gamma->	1518	20152	0,594099
Methyl eugenol	1405	17820	0,53649
Unknown	1499	19766	0,522091
Acetoin	706	2437	0,453418
Unknown	1159	12223	0,448155
|allo|-Ocimene	1131	11560	0,4187
(e)-Linalool oxide (furanoid)	1090	10542	0,409372
Geranylacetone_S	1453	18836	0,39352
Unknown	1462	18997	0,374845
Methyl benzoate	1095	10688	0,372428
Cubebene <beta->	1393	17564	0,358592
Hexanoic acid, ethyl ester	1001	8340	0,343828
Unknown	2490	35628	0,334811
Benzyl acetate	1165	12381	0,316917
Unknown	1270	14840	0,298631
Unknown	1390	17532	0,295196
2-Methylbutanoic acid	859	5040	0,291875
Unknown	1314	15850	0,279806
Pentyl furan <2->	992	8113	0,245964
Octalactone	1286	15205	0,238221
alpha-Copaene	1378	17250	0,234928
Maaliene <gamma->	1432	18387	0,217867
Naphthalene	1184	12821	0,212738
Unknown	1418	18099	0,210106
Unknown	2165	31170	0,200965
2-Phenylethanol	1114	11139	0,198191
2-Tridecanone	1495	19683	0,18667
ß-Caryophyllene	1423	18194	0,180018
Linalool	1100	10813	0,176891
unkonwn	924	6478	0,174597
Cubebene<alpha->	1353	16693	0,17323
Unknown	811	4039	0,16806
Unknown	947	7033	0,167786
Unknown	1073	10129	0,165614
Propanoic acid, butyl ester	907	6058	0,161363
cis-Linalool oxide pyranoid	1170	12505	0,154687
Gurjunene<gamma->	1475	19279	0,153942
Unknown	940	6850	0,153425
Unknown	2348	33742	0,152308
Unknown	2365	33982	0,152048

Unknown	1165	12386	0,14798
Unknown	813	4106	0,145147
Unknown	1079	10199	0,142717
Unknown	1059	9769	0,141691
Unknown	1511	20007	0,139891
1,2-Propanediol, diacetate	1030	9067	0,139467
Unknown	935	6728	0,131939
Unknown	2446	35051	0,123651
Unknown	1118	11249	0,120528
Unknown	1144	11877	0,11878
Terpinene <gamma->	1059	9788	0,115117
Unknown	1158	12209	0,113429
Elemene<delta->	1340	16404	0,104185
Unknown	1361	16868	0,098897
Unknown	1694	23498	0,096297
alpha.-Guaiene	1442	18599	0,095628
Unknown	1089	10535	0,093642
Unknown	993	8134	0,088006
Butyl butyrate	996	8202	0,084914
2-Undecanon	1294	15386	0,08197
ß-Pinene	976	7729	0,081509
Unknown	1117	11239	0,080438
Octanoic acid	1174	12599	0,078508
Selinene	1490	19611	0,077027
Unknown	1136	11677	0,076905
cis-Ocimene	1039	9281	0,075205
Unknown	1025	8935	0,075179
Unknown	1010	8560	0,066196
Benzeneacetonitrile	1140	11762	0,064256
N-Methyl-2-furohydroxamic acid	1082	10346	0,062838
aristolochene	1473	19227	0,06145
Isoamyl acetate_S	877	5414	0,059989
Unknown	2305	33166	0,058777
Hexyl acetate	1015	8679	0,056759
Selinene <beta->	1498	19770	0,054815
1,2-Dimethoxybenzene	1147	11951	0,054517
Benzene acetic acid, ethyl ester	1246	14282	0,047612
Farnesene <(E,E)-, alpha->	1509	19951	0,047173
Selinene <alpha->	1500	19802	0,046076
beta.-Phenethyl acetate	1258	14558	0,044643
Coumaran	1220	13699	0,042717
n-Hexadecanoic acid	1958	27985	0,042268
Muurolene <alpha->	1503	19861	0,042174
Kaurene	2045	29354	0,041683

2-Nonanone	1093	10635	0,041588
Zonarene	1527	20311	0,039052
Gurjunene	1413	18011	0,039042
Tricosane	2295	33023	0,038756
Butenoic acid, 3-methyl-2-	881	5498	0,037711
Unknown	1094	10661	0,037357
Acetophenone	1066	9956	0,036036
Unknown	2234	35708	0,035828
Benzaldehyde	960	7342	0,035623
Unknown	1275	14956	0,034758
2-Heptanol	901	5943	0,032656
Hexadecanoate <methyl->	1924	27441	0,032099
(Z)-Linalool oxide (furanoid)	1074	10143	0,028794
Methyl dihydrojasmonate<cis->	1656	22789	0,024388
Unknown	1296	15448	0,020966
Unknown	1481		0,019906
Geopropolis			
Compound	RI	RT	%
Styrene	894	5641	42,66482
Phenol, 4-ethyl-	1167	12450	7,047564
Unknown	888	5632	5,659102
alpha-Copaene	1378	17250	5,366488
TETRACOSANOL	2477	35452	3,344988
Unknown	2487	35612	2,933864
Gurjunene	1413	18011	2,407079
Lactate <ethyl->	815	4122	2,095697
Acetic acid	665	2134	2,069486
Benzene, 1-ethyl-4-methoxy	1114	11146	1,732163
ß-Caryophyllene	1423	18194	1,661392
Unknown	2069	29726	1,319808
Cyclosativene	1370	17061	1,225972
Ocimene <(E)-, beta->	1048	9517	1,165767
Caryophyllene <9-epi-(E)->	1466	19081	1,034268
unkonwn	894	5760	0,922007
Cubebene<alpha->	1353	16693	0,827717
Zonarene	1527	20311	0,702138
Methyl p-anisate	1375	17183	0,654463
Ethyl hexadecanoate	1991	28517	0,589752
Humulene<alpha->	1457	18906	0,542857
Farnesene <(E,E)-, alpha->	1509	19951	0,533631
Pinene<alpha->	931	6631	0,476589
Germacrene D	1485	19478	0,458256
Unknown	2454	35157	0,442806
Unknown	2074	29805	0,429498

Cubebene <beta->	1393	17564	0,416998
Selinene	1490	19611	0,41383
Muurolene <gamma->	1480	19386	0,389973
Carene <delta-3->	1010	8567	0,385813
Isoamyl acetate_S	877	5414	0,360575
Benzaldehyde	960	7342	0,352922
Methyl eugenol	1405	17820	0,342532
Unknown	1418	18099	0,336766
Benzyl alcohol	1035	9192	0,336392
Unknown	2135	30724	0,318007
Copaene <beta->	1433	18410	0,312673
aristolochene	1473	19227	0,311952
Bergamotene <alpha-, trans->	1437	18499	0,30517
Selinene <alpha->	1500	19802	0,299531
Unknown	1073	10129	0,282945
Unknown	2134	30713	0,267053
alpha.-Guaiene	1442	18599	0,25831
p-Anisyl alcohol	1283	15146	0,253449
diethyl acetal	727	2754	0,25245
Unknown	2446	35051	0,243715
Unknown	1372	17111	0,219982
Ethyl propanoate	709	2523	0,175961
Bulnesene <alpha->	1510	19991	0,1704
BETA. ELEMENE	1394	17600	0,170062
Butyl butyrate	996	8202	0,165992
n-Hexadecanoic acid	1958	27985	0,161212
n-propyl acetate	714	2585	0,160021
Butenoic acid, 3-methyl-2-	881	5498	0,151401
Unknown	1436	18468	0,143386
Geranylacetone_S	1453	18836	0,143323
2-Methyl-3-dimethylamino-2-isopropylthio(2H)azirine	1386	17414	0,14222
Unknown	1462	18997	0,1402
Selinene <beta->	1498	19770	0,135269
Muurolene <alpha->	1503	19861	0,127234
Unknown	2234	35708	0,127072
Ionone<(E)-beta->	1488	19531	0,124479
Cadinene<gamma->	1518	20152	0,124434
Unknown	2269	32658	0,120028
Barbatene<beta->	1447	18692	0,117462
Bourbonene<beta->	1389	17479	0,114551
Unknown	980	7788	0,109608
Methyl benzoate	1095	10688	0,107348
Octadecanoic acid	2158	31054	0,097568
Unknown	1445	18650	0,096042

Unknown	1443	18616	0,093788
ß-Pinene	976	7729	0,091623
Unknown	2468	35333	0,087362
Unknown	927	6549	0,086518
2,3-Butanediol	785	3690	0,078088
Unknown	1089	10535	0,073376
Acetophenone	1066	9956	0,070821
unkonwn	887	5621	0,070351
Octanoic acid, ethyl ester	1198	13169	0,062516
Elemene<delta->	1340	16404	0,062006
Unknown	937	6781	0,061646
Unknown	1511	20007	0,0597
Ethyl Acetate	612	1742	0,056763
Gurjunene<gamma->	1475	19279	0,054574
Ar-Curcumene	1484	19462	0,054027
Isopropyl tetradecanoate	1825	25798	0,053883
Unknown	2277	32771	0,053638
Terpinene <gamma->	1059	9788	0,048591
(Z)-Linalool oxide (furanoid)	1074	10143	0,046954
Coumaran	1220	13699	0,044989
Rosa-5,15-diene	1935	27623	0,04153
2-Heptanol	901	5943	0,040566
Unknown	2292	32979	0,031743
Unknown	1897	26996	0,030832
Linalool	1100	10813	0,03079
Unknown	1390	17532	0,030025
1,2-Propanediol, diacetate	1030	9067	0,027655
trans-Linalool oxide pyranoid	1175	12618	0,027226
Hexyl acetate	1015	8679	0,025792
Methyl dihydrojasmonate<cis->	1656	22789	0,025298
Unknown	1440	18550	0,023783
Cyperene	1404	17806	0,022661
f. 2,3-Butanediyl diacetate	1063	9883	0,016397
Unknown	1294	15386	0,014059
beta.-Phenethyl acetate	1258	14558	0,013994
Hexadecanoate <methyl->	1924	27441	0,013874
N-Methyl-2-furohydroxamic acid	1082	10346	0,013519
Coniferyl alcohol<Z->	1737	24271	0,013325
Naphthalene	1184	12821	0,012466
Unknown	1694	23498	0,010892
Salicylate <2-ethylhexyl->	1805	25464	0,010769
Unknown	2371	34025	0,010602
Unknown	2128	30615	0,010435
Benzoate <(3Z)-hexenyl->	1572	21195	0,010421

Terpinen-4-ol $$ 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	1179	12715	0,010367
Unknown	1474	19261	0,01026
Unknown	952	7279	0,009695
Unknown	1610	21927	0,009497
Unknown	1177	12650	0,009317
Propanoic acid, butyl ester	907	6058	0,008847
Unknown	1079	10199	0,008205
1,2-Dimethoxybenzene	1147	11951	0,007832
l-Limonene	1029	9024	0,007411
Prenyl acetate	925	6485	0,003992
Octanoic acid	1174	12599	0,000219