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ORIGINAL PAPER

An unusual recruitment strategy in a mass-recruiting stingless bee, *Partamona orizabaensis*

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Abstract Foragers of several stingless bee species deposit attractive scent marks on solid substrates to precisely recruit nestmates to food. Interestingly, *Partamona* workers quickly recruit large numbers of nest mates to resources, likely even without the deposition of attractive scent marks. However, systematic studies of the recruitment system of these bees are lacking. We now studied the recruitment behavior of *P. orizabaensis*. Our findings show that foragers of this species can recruit large numbers of nestmates to food sources at a particular location. The precise nestmate recruitment does not rely on attractive scent marks deposited on substrates. We never observed any scent marking behavior and feeders baited with labial or mandibular gland extracts were not attractive for the bees. Chemical analyses showed that the foragers' labial gland secretions exclusively contain long chain hydrocarbons, which render their role in recruitment communication unlikely. Whether mandibular gland secretions, which contain esters and alcohols that are known as attractive pheromones in other bee species, are used to guide recruits toward food during flight, remains elusive. We conclude that *Partamona*'s quick recruitment system that does not rely on conspicuous scent marks has evolved as a strategy against competition with sympatrically occurring and more aggressive bee species.

Keywords Mass recruitment · Guiding flights · Cephalic gland chemistry · Labial gland hydrocarbons · Mandibular gland esters

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Introduction

Sophisticated communication systems are key to the success of social insects because they permit an effective allocation of workers to the different tasks that have to be met at colony level (Wilson 1971). The recruitment of nestmate foragers to resources to quickly exploit them can be imperative to nourish the entire colony, which includes larvae and workers that do not leave the nest to feed (Kerr 1969). Within the taxonomic group of stingless bees, which comprises several hundred species (Michener 2000), a variety of foraging strategies and recruitment communication mechanisms have evolved (Biesmeijer and Slaa 2004; Nieh 2004; Barth et al. 2008). These recruitment mechanisms include the mobilization of unemployed foragers inside the nest to stimulate them to leave and to search for food at random, as well as the communication of precise food locations by scent marks deposited on substrates at, or near resources that attract recruited bees toward the marked sites (Lindauer and Kerr 1958, 1960; Kerr et al. 1963; Hrncir et al. 2000, 2006a, b; Hrncir 2009; Schmidt et al. 2003; Jarau et al. 2003, 2004; Nieh et al. 2003, 2004; Sánchez et al. 2004; Aguilar et al. 2005; Schorkopf et al. 2007, 2011). The use of scent marks, to communicate the exact food location from the colony at a particular direction, distance, and height above ground, has been observed for several species within the genera *Trigona*, *Scaptotrigona*,

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Geotrigona, *Cephalotrigona,* and *Oxytrigona* (reviewed in Jarau 2009). This highly effective recruitment mechanism has attracted the attention of researchers because of the great numbers of workers that can be quickly and precisely recruited to a newly discovered food source by experienced foragers; indeed, stingless bees using this communication mechanism are able to compete with the recruitment efficiency of honey bees (Lindauer and Kerr 1958, 1960; Jarau et al. 2003). The deposited attractive scent marks, which include species-specific pheromones as well as nest-specific signature mixtures (Jarau et al. 2010, 2011; John et al. 2012; Reichle et al. 2013; for the distinction between pheromones and signature mixtures see also Wyatt 2010), are secreted from the foragers' labial glands (Jarau et al. 2004, 2006; Schorkopf et al. 2007; Lichtenberg et al. 2011). The behavior exhibited by a scent marking forager is very conspicuous and involves sequences of alternating short flights and quick landings, during which labial gland secretions are released from the base of the bee's extended glossa that is simultaneously rubbed against a solid surface (photographs illustrating this peculiar behavior have been published in: Jarau et al. 2004; Barth et al. 2008; Jarau 2009). Compounds produced by the mandibular glands, by contrast, do not attract recruited foragers to food sources but rather have a deterrent effect on both bees that approach a food source or already feed on it, indicating that they are important for alarm communication and trigger defensive behavior (Lindauer and Kerr 1958, 1960; Jarau et al. 2004, 2006, 2010, 2011; Stangler et al. 2009; Schorkopf et al. 2009).

Stingless bees of the genus *Partamona* are quite special regarding their foraging strategy and recruitment communication. Keppner and Jarau (2016) found that the flight activity of *P. orizabaensis* peaks when its competing stingless bee species are less active, i.e., in the early morning hours and before sunset, as well as during rainfalls, even rather heavy ones. In addition, *P. orizabaensis* foragers quickly recruit nestmates in large numbers to food sources, which gives them a good chance to outcompete more aggressive competitors, such as *Trigona fuscipennis* (Keppner and Jarau 2016). Interestingly, unlike other mass-recruiting stingless bee species *Partamona* appears to recruit large numbers of workers without the deposition of attractive scent marks at food sources or in its close surroundings. This assumption, however, is only supported by a single observation reported by Kerr (1969). This author hypothesizes that the foragers of the species *Partamona helleri* release attractive pheromones while flying from the nest to a food source, thereby creating an "aerial odor tunnel" that is followed by recruited nestmates and leads them to a specific feeding site. However, a scientific approach testing this hypothesis by means of several repetitions of standardized recruitment experiments has never been provided (Jarau 2009). Therefore, we studied the recruitment behavior of the species *Partamona orizabaensis* to answer the question whether experienced foragers indeed can recruit large numbers of nestmates to a specific feeding site without the deposition of attractive scent marks on, or near it. In addition, we recorded the bee's temporal recruitment pattern and tested whether recruits are attracted to chemical compounds extracted from the foragers' labial- or mandibular glands in order to reveal the origin of potential recruitment pheromones. Finally, we analyzed the chemical composition of labial and mandibular gland secretions of foragers from different *P. orizabaensis* colonies and looked for nest-specific qualitative and quantitative differences.

Methods

Bee colonies and study sites

All investigations were carried out in Costa Rica between January and July 2011 with four colonies of *Partamona orizabaensis* (nest A–D) that were left at their nesting sites and observed in their natural habitats. Nests A and B, which were about 30 m apart, were located in the Tropical Field Station of the University of Vienna in La Gamba near Golfito, Puntarenas (8°42′61″N, 83°12′97″W, 70 m above sea level), whereas nests C and D were from Barrio Jesús near Atenas, Alajuela (9°58′20″N, 84°25′05″W, 698 m above sea level) with a distance of approximately 2 km between them. The two study sites were separated by approximately 196 km.

Recruitment experiments

Training phase

The general experimental setup and procedure for training foragers followed the method described in Jarau et al. (2000). No recruitment experiment was carried out with nest D because the foragers of this colony never visited our feeders for unknown reasons. With the remaining nests, we conducted between 6 and 15 repetitions within the recruitment experiments (henceforth named 'trials'). During the training phase, the feeders were filled with a 0.5 mol l^{-1} (nests A and B) or 1.5 mol l^{-1} (nest C) refined sugar solution, respectively. The difference in sugar solution concentration was due to the differences in the bee's motivation to collect it. Importantly, however, these sugar concentrations were sufficient to repeatedly attract the trained bees but without triggering any recruitment process. At least 15 foragers (marked with water-based color on their thoraces) were trained to a final feeding site at a distance of 15, 30 or 50 m from the nest by moving a

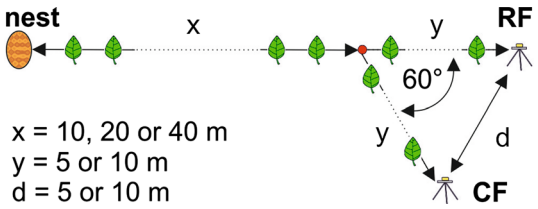

Fig. 1 Setup for the recruitment experiments. Trained and color-marked foragers were allowed to collect sugar solution at a recruitment feeder (RF) that was located at a particular distance ($x + y$) from their colony and to recruit additional foragers inside the nest. To test whether newcomers are recruited to a specific feeding site, the RF, an additional control feeder (CF) was installed at the same distance (y) from the branching point (*red circle*) but shifted in an angle of 60° from the flight direction between nest and RF. Thus, the distance (d) between RF and CF was 5 or 10 m, depending on the experiment. The leaves mounted on sticks were provided between the nest and the feeders in intervals of 2 m to observe whether the foragers show any scent marking behavior

feeder in intervals of 5 m while bees were sitting on it. At the final position, the trained bees were allowed to collect sugar solution for 30 min before the training feeder was replaced by two identical clean feeders with equal quantities of 3 mol l^{-1} refined sugar solution. A recruitment feeder (RF) replaced the training feeder and a control feeder was installed at a distance of 5 m (in the case of the 15 m distance trials) or 10 m (30 and 50 m distance trials). To provide potential substrates for the deposition of scent marks, short sticks with *Clusia valerioi* (La Gamba) or *Syzygium malaccense* (Barrio Jesús) leaves mounted on them were installed in intervals of 2 m between nest and recruitment feeder, as well as along the last 5 m (15 m distance trials) or 10 m (30 and 50 m distance trials) toward the control feeder to provide similar structural conditions between the nest and the two feeders (Fig. 1). The arrangement of the feeders and leaves was adopted from studies investigating species that use attractive scent marks in their recruitment communication (Jarau et al. 2006, 2010).

Test phase

The test phase of each trial started as soon as the recruitment feeder filled with 3 mol l^{-1} sugar solution was offered to the trained bees, which were allowed to freely move between feeder and nest and to recruit new bees from their colony. These experienced bees were continuously monitored to observe any distinctive behavior that resembles the easily detectable scent marking behavior described for scent trail laying stingless bee species (Lindauer and Kerr 1958, 1960; Kerr et al. 1963; Schmidt et al. 2003; Nieh et al. 2003, 2004; Jarau et al. 2004; Sánchez et al. 2004; Aguilar et al. 2005).

During the subsequent 60 min, all newly arriving bees that landed on either the recruitment feeder or the control feeder were counted and captured with a suction tube. The captured bees were marked with color dots on their thoraces prior to their release at the end of a trial. For the analyses of data collected during the single trials of the recruitment experiments only unmarked newcomers to our feeders, i.e., bees that had never visited a feeder during the training phase or a previous trial, were taken into account. To illustrate the temporal recruitment pattern of *P. orizabaensis,* we defined the total number of unmarked bees that arrived at the recruitment feeder during a trial as 100 %. We then calculated the percentage of bees that had reached the feeder for each time interval of five minutes to get a cumulative representation of the newly recruited bees.

Preparation of gland extracts

Neat extracts of labial and mandibular gland secretions were prepared by dissecting the glands from the heads of foraging workers collected at sugar solution feeders. All tissues other than the respective glands and their reservoirs were carefully removed prior to extraction. The dissected pair of glands of a single individual was extracted in 100 µl hexane for 24 h at room temperature. All extracts were subsequently stored in a freezer (−8 °C) until they were used for the bioassays or chemical analyses (see below).

Gland compounds bioassays

We conducted feeder choice experiments with bees from nest A to test whether compounds from the labial or mandibular glands attract recruited bees to food sources. For these bioassays, we prepared pooled gland extracts from foragers (8 labial glands in 400 µl hexane; 8 mandibular glands in 400 µl hexane). One hundred microliter of gland extract equaled the gland content of one foraging bee.

Foragers were trained to collect sugar solution (0.5 mol l^{-1}) at the training feeder at a distance of 50 m from their colony for 15 min. After that time the training feeder was replaced by two unvisited and clean test feeders filled with 3 mol l^{-1} sugar solution. Both feeders were placed 1 m away from the position of the training feeder and in an angle of 120° between their directions from that position (Fig. 2). Once the test feeders were in place, one of them was baited with either labial or mandibular gland extract and the other with the pure solvent hexane. Ten microliter test extract, corresponding to 0.1 bee equivalent of labial or mandibular gland content, or hexane were applied to filter papers (1 cm^2 in size) that were positioned on top of the feeders. After 15 min, the test compounds were renewed (application of another 10 µl) and each trial ended after 30 min. The gland extracts were only applied on

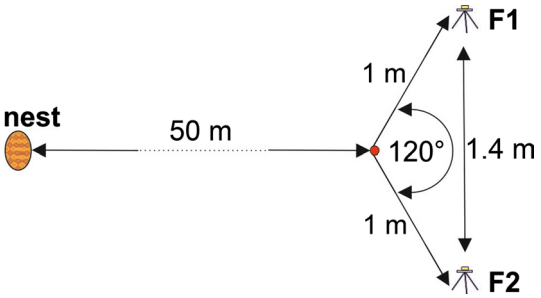

Fig. 2 Setup for the gland extract bioassays. Trained bees were allowed to take up sugar solution at a training feeder at the branching point (*red circle*) 50 m away from the nest. This feeder was then replaced by two clean feeders (F1, F2) that were established 1 m away from the branching point and with an angle of 120° between the directions toward them. F1 and F2 were baited with 10 µl of gland extract or with the pure solvent hexane and the arriving bees had to choose between them

the feeders because scent marking of substrates along the flight route by *P. orizabaensis* foragers was never observed during any of the trials in the recruitment experiments (see results) but gland secretions could have been potentially released while feeding at food sources. We conducted nine trials testing the attractiveness of labial gland extract and ten trials testing the attractiveness of mandibular gland extract. All bees that landed on a feeder and took up sugar solution were immediately captured with a suction tube to prevent any recruitment behavior and their feeder choice was registered. The captured bees were marked with water-based color on their thoraces to prevent double counting during subsequent tests and released not until the end of a trial. The bees marked from prior experiments were only captured, but not counted to assure that only the preference of foragers that were not tested in previous bioassays was analyzed. After each test, all items of the setup were cleaned with ethanol (99 %). The positions of the feeders baited with extract or hexane were alternated between trials to avoid an influence caused by potential side biases of the bees.

Chemical analyses

For the chemical analysis, we collected foragers from all four colonies (nest A–D) and extracted their labial or mandibular glands as described above. We analyzed the labial gland extracts from 9 to 12 foragers from different nests and the mandibular gland extracts from 4 or 5 foragers per nest. For the quantitative analyses, 1 µl of each extract was injected into a gas chromatograph (Agilent Technologies 7820A GC Sytems) equipped with a DB-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness, J & W Scientific, Folson, CA, USA) and a flame ionization detector (FID). Hydrogen was used as carrier gas (constant linear flow rate 2 ml/min). The GC was operated splitless at 50 °C for 1 min, followed by a programed increase to 310 °C at a rate of 10 °C/min and held at the final temperature for another 18 min.

Structure elucidation of extracted compounds was performed by GC–MS analyses on a HP 6890 GC (Hewlett Packard Series, Palo Alto, CA) connected to a mass selective detector (MS, Agilent Quadrupol 5973). All labial and mandibular gland extracts, respectively, made from the bees of a particular nest were pooled. Prior to the chemical analyses, the volume of the pooled extracts was reduced to 40 µl under a mild stream of nitrogen. The GC parameters were the same as described before, but helium was used as a carrier gas (constant flow 1.5 ml/min). Mass spectra were taken in EI mode with 70 eV. For the identification of the compounds, the GC retention times and mass spectra were compared with those of synthetic reference compounds from our stock collection, as well as with literature data (NIST library). To identify the position of methyl branches in hydrocarbons, we used retention indices and diagnostic ions. The position of double bonds in unsaturated compounds was determined by dimethyl disulfide derivatizations of crude extracts and analyses of the resulting mass spectra (Buser et al. 1983).

Statistical analyses

For the statistical analyses of the behavioral experiments, we used the software R version 3.3.0 (R Development Core Team 2016). We calculated two-sided binomial probabilities based upon the null hypothesis that foragers will arrive equally at both feeders if they use no information to discriminate between them ($P = 0.5$) to test for differences in the absolute numbers of recruits arriving at the recruitment feeder or the control feeder, as well as to compare the numbers of bees choosing between feeders baited with labial gland extract, mandibular gland extract or hexane.

For the comparisons of the chemical composition of labial gland extracts and mandibular gland extracts from bees of the different colonies, we used the program PAST 3.10 (Hammer et al. 2001). The relative proportions (in percent) of single compounds identified from extracts were calculated from the peak areas of GC chromatograms with the Software Agilent Chemstation 9.03. We conducted nonparametric multivariate analyses based on 35 compounds (labial gland secretions) and 9 compounds (mandibular gland secretions) identified from the corresponding extracts. A nonmetric multidimensional scaling (NMDS) analysis was carried out based on the calculation of Bray–Curtis distances to visualize differences between the gland secretions' composition from foragers of the different nests. Bray–Curtis distances were also used to perform one-way analyses of similarities (ANOSIM) to test for significance

Table 1 Directed recruitment to food sources in *Partamona orizabaensis*

Experiment	*N*	Number of recruits at RF	Number of recruits at CF	Probabilty to arrive at RF	Binomial probability (*P*)
Nest A, 30 m	15	5666	907	0.86	<0.00001
Nest A, 50 m	14	3228	1741	0.65	<0.00001
Nest B, 50 m	12	675	94	0.88	<0.00001
Nest C, 15 m	6	151	0	1.00	<0.00001

In all experiments done with different nests and different feeder distances, the majority of newcomers arrived at the recruitment feeder (RF) rather than the control feeder (CF)

N = number of trials, *P* values calculated with two-sided binomial tests

in dissimilarities between the bee's gland compositions depending on nest origins (sequential Bonferroni adjusted *P* values). Compounds that predominantly contributed to Bray–Curtis dissimilarities between the foragers from different nests were identified by calculating similarity percentages (SIMPER).

Results

Recruitment precision and temporal pattern

In all recruitment experiments, a significantly higher number of bees as would be expected by chance landed on the recruitment feeder visited by the trained bees as compared to the control feeder (two-sided binominal probability, $P < 0.00001$; see Table 1 for details). In experiments with nest A, 5666 (86 %) of the recruited bees arrived at the recruitment feeder when it was located at a distance of 30 m from the nest, whereas only 907 newcomers came to the control feeder. When the recruitment feeder was located 50 m away from nest A, 3228 (65 %) of the recruits arrived there, while 1741 bees were recorded at the control feeder. Likewise, a significantly larger number of the recruits from nest B arrived at the recruitment feeder (675 bees, 88 %) compared to the control feeder (94 bees), both placed at distances of 50 m. All recruits from nest C (151 bees, 100 %) were guided to the recruitment feeder situated 15 m away from the nest entrance and no bee was seen landing and feeding on the control feeder.

During the 47 trials, the typical scent marking behavior described for foragers of other species, by which bees land on solid substrates and rub their glossa against it to deposit labial gland secretions (Jarau 2009) was neither observed at the feeders nor along the bees' flight path toward the nest. Experienced foragers always flew immediately back to the entrance of their nest once they had stopped feeding. However, we frequently observed piloting behavior in experienced foragers, which led newcomers in small groups of up to 15 bees from the nest entrance to the food source. Close to the feeders the recruits showed searching flight behavior and hovered in front of them before landing.

The temporal recruitment pattern of *P. orizabaensis* was marked by a quick increase of the number of newly recruited bees at the feeders within the first 5 min after the experienced bees had started to take up the 3 mol l^{-1} sugar solution. Subsequently, the number of newcomers at the recruitment feeders increased continuously. Thus, a constant recruitment of newcomers was maintained throughout the trials (two examples for this recruitment pattern are shown in Fig. 3).

Gland extract bioassays

During the choice experiments testing labial gland extracts, the bees showed no preference for either feeder; 168 (51 %) of the inexperienced bees had chosen the feeders baited with labial gland extract and 161 (49 %) the hexane baited feeders (two-sided binominal probability, $P = 0.741$; Table 2). The mandibular gland extract, however, had a significant repellent effect on the bees (two-sided binominal probability, $P = 0.00004$; Table 2), with the majority of them favoring the hexane over the mandibular gland extract baited feeders (hexane: 1111 bees, 55 %; mandibular gland extract: 926 bees, 45 %).

Chemical analyses

Labial gland secretions

The hexane extracts prepared from labial glands of *P. orizabaensis* foragers collected in La Gamba (nests A, B) contained 34 compounds and that from bees from Barrio Jesùs (nests C, D) 35 compounds (Table 3). The extracts exclusively contained straight chain and branched hydrocarbons (alkanes, alkenes, alkadienes, methyl-branched alkanes). The only qualitative difference in the extracts from foragers of the two different locations was the alkene 7-tricosene, which was only present in the labial glands of bees from Barrio Jesús. However, the labial gland compositions of foragers from La Gamba and Barrio Jesús showed

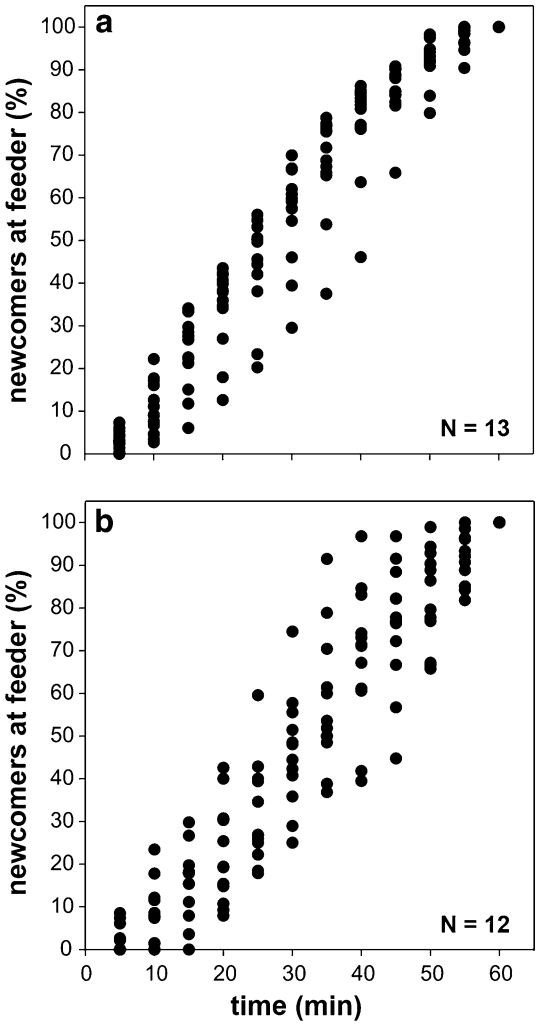

Fig. 3 Temporal recruitment pattern to sugar solution feeders in *Partamona orizabaensis*. Recruitment of workers from **a** nest A to a distance of 30 m and from **b** nest B to a distance of 50 m. The number of recruited bees arriving at the feeders is given as percentage of the total number of recruits at the end of a trial for each time interval of 5 min. *N* = number of trials

marked quantitative differences for most of the compounds (Table 3). This difference is well visualized by a NMDS plot based on the relative proportions of single compounds within the glands' entire bouquets (Fig. 4). In addition, ANOSIM results showed significant dissimilarities between the chemical profiles of the labial gland secretions from the foragers of different nests (all nests: global $R = 0.707$, $P < 0.0001$ nest A vs. nest B: $R = 0.4138$, $P < 0.0006$ nest A vs. nest C: $R = 0.9974$, $P < 0.0006$ nest A vs. nest D: $R = 0.8703$, $P < 0.0006$ nest B vs. nest C: $R = 0.9794$, $P < 0.0006$ nest B vs. nest D: $R = 0.8073$, $P < 0.0012$). The only exception was the rather small and non-significant dissimilarity detected between the composition of labial gland secretions from foragers from nests C and D ($R = 0.1175$, $P = 0.249$). SIMPER analyses revealed that the differences between the compositions of labial gland secretions from foragers of all nests compared in a single analysis, as well as in all pairwise nest comparisons, were mainly caused by a single compound, 9-hentriacontene, which contributed more than 10 % to the according dissimilarities. One additional compound that remains unidentified so far (Table 3, unidentified#3) substantially (> 10 %) contributed to the difference in the labial gland bouquets of foragers from nest A and B. The only comparison in which 9-hentriacontene did not primarily contribute to the difference in the foragers' labial gland bouquets was between nests C and D; here, 9-nonacosene was the sole compound contributing more than 10 % to the dissimilarity between the secretions' chemical composition.

Mandibular gland secretions

The mandibular gland extracts prepared from *P. orizabaensis* foragers of all three nests contained nine compounds and showed some quantitative differences in the proportions of the single compounds between bees from La Gamba and Barrio Jesús, respectively (Table 4). Interestingly, the mandibular gland extracts contained esters and alcohols in addition to hydrocarbons (Table 4). The calculated NMDS plot based on the relative proportions of single compounds indicated no significant dissimilarities between the mandibular gland secretions of foragers from the different nests (Fig. 5). Likewise, ANOSIM showed no convincing dissimilarities between the chemical profiles of the foragers from the three nests (global $R = 0.1234$, $P = 0.1676$ nest A vs. nest B: $R = 0.0375$, $P = 0.9102$ nest A vs. nest C: $R = 0.1$, $P = 0.5802$ nest B vs. nest C: $R = 0.1667$, $P = 0.6249$). Results of the SIMPER analyses showed that the compounds that predominately

Table 2 Two feeder choice bioassays performed with *Partamona orizabaensis* bees from nest A

Extract tested	*N*	Number of recruits at TF	Number of recruits at CF	Probabilty to arrive at TF	Binomial probability (*P*)
LGE	9	168	161	0.51	0.741
MGE	10	926	1111	0.45	0.00004

Absolute number of bees attracted to a test feeder (TF) baited with either labial gland extract (LGE) or mandibular gland extract (MGE), respectively, or to a control feeder (CF) bearing the pure solvent hexane

N = number of trials, *P* values calculated with two-sided binomial tests

Table 3 Relative abundance (%) of compounds extracted from the labial glands of *Partamona orizabaensis* foragers from four different nests

Compound name	Nest A	Nest B	Nest C	Nest D
Alkanes				
Tricosane	0.28 ± 0.17	0.85 ± 0.40	0.94 ± 0.45	0.81 ± 0.32
Pentacosane	0.71 ± 0.43	1.95 ± 0.70	1.76 ± 0.82	1.48 ± 0.60
Heptacosane	2.44 ± 0.69	3.59 ± 0.70	2.77 ± 1.12	2.97 ± 1.58
Nonacosane	1.68 ± 0.30	2.34 ± 0.66	2.00 ± 1.11	2.23 ± 1.38
Hentriacontane	0.52 ± 0.26	1.09 ± 0.57	0.91 ± 0.69	0.88 ± 0.66
Dotriacontane	0.15 ± 0.09	0.33 ± 0.36	0.13 ± 0.14	0.17 ± 0.12
Tritriacontane	1.24 ± 0.21	1.09 ± 0.19	0.74 ± 0.28	1.18 ± 0.39
Alkenes				
9-Tricosene	0.05 ± 0.03	0.08 ± 0.12	0.44 ± 0.35	0.30 ± 0.22
7-Tricosene	–	–	0.25 ± 0.23	0.10 ± 0.08
9-Pentacosene	0.14 ± 0.07	0.26 ± 0.26	2.92 ± 2.60	1.58 ± 1.37
7-Pentacosene	0.12 ± 0.07	0.23 ± 0.22	1.00 ± 0.39	0.74 ± 0.27
9-Heptacosene	2.65 ± 0.93	3.88 ± 1.44	3.72 ± 0.58	3.00 ± 0.84
7-Heptacosene	1.41 ± 0.43	1.75 ± 0.52	4.07 ± 0.70	3.39 ± 1.01
9-Octacosene	0.76 ± 0.24	0.80 ± 0.11	0.70 ± 0.16	0.64 ± 0.09
9-Nonacosene	**22.41 ± 2.10**	**20.30 ± 2.83**	**18.96 ± 3.68**	**18.11 ± 3.73**
7-Nonacosene	5.59 ± 0.53	4.81 ± 0.75	**11.06 ± 2.31**	**10.60 ± 2.75**
9-Triacontene	0.97 ± 0.08	0.84 ± 0.10	0.53 ± 0.09	0.61 ± 0.10
9-Hentriacontene	**27.94 ± 3.41**	**24.23 ± 3.50**	**12.12 ± 1.65**	**14.45 ± 3.41**
Alkadienes				
9,21-Nonacosadiene	0.73 ± 0.30	0.94 ± 0.56	6.38 ± 1.11	5.44 ± 1.38
9,19-Nonacosadiene	1.35 ± 0.39	1.74 ± 0.69	3.74 ± 0.77	3.64 ± 1.29
9,21-Hentriacontadiene	1.32 ± 0.89	1.83 ± 1.07	4.68 ± 0.88	4.40 ± 1.24
9,19-Hentriacontadiene	4.11 ± 1.13	3.11 ± 1.51	5.44 ± 0.64	5.07 ± 1.24
9,17-Hentriacontadiene	2.81 ± 0.37	2.37 ± 0.60	3.28 ± 0.56	4.52 ± 1.11
9,21-Tritriacontadiene	5.39 ± 0.55	4.42 ± 1.17	3.97 ± 0.88	4.50 ± 1.55
9,19-Tritriacontadiene	2.21 ± 0.37	1.65 ± 0.27	2.56 ± 0.75	2.83 ± 0.96
9,17-Tritriacontadiene	1.02 ± 0.12	0.81 ± 0.17	0.95 ± 0.42	1.42 ± 0.52
9,21-Pentatriacontadiene	4.09 ± 1.49	2.90 ± 1.03	1.44 ± 0.85	1.77 ± 0.94
9,19-Pentatriacontadiene	1.05 ± 1.12	0.22 ± 0.28	0.47 ± 0.40	0.67 ± 0.46
Methyl-branched alkanes				
13-; 11-Methyl-heptacosane	0.61 ± 0.33	1.16 ± 0.61	0.39 ± 0.43	0.36 ± 0.18
15-; 13-; 11-Methyl-nonacosane	0.82 ± 0.39	1.15 ± 0.56	0.41 ± 0.22	0.56 ± 0.30
15-; 13-Methyl-hentriacontane	0.82 ± 0.36	1.03 ± 0.51	0.38 ± 0.18	0.57 ± 0.31
Unidentified#1	0.87 ± 0.54	1.50 ± 0.94	0.21 ± 0.13	0.32 ± 0.23
Unidentified#2	0.13 ± 0.15	0.46 ± 0.30	0.20 ± 0.13	0.19 ± 0.10
Unidentified#3	3.08 ± 1.36	5.94 ± 4.66	0.30 ± 0.29	0.29 ± 0.21
Unidentified#4	0.54 ± 0.15	0.34 ± 0.23	0.19 ± 0.16	0.22 ± 0.14

Nests A and B were located in La Gamba and nests C and D in Barrio Jesús. The relative amount of a compound was calculated as its percentage in the glands' entire bouquet

Compounds shown in bold are major components contributing >10 % to the composition of the labial gland secretions in at least one colony

Given are mean values (±standard deviation), sample sizes are $n_A = 12$, $n_B = 9$, $n_C = 10$, $n_D = 11$

(>10 % each) accounted for the still remaining dissimilarities between the compositions of mandibular gland secretions from all nest compared in a single analysis, as well as in all pairwise nest comparisons, were 9-octadecen-1-ol, 9-heneicosene, and 9-octadecen-1-ylbutyrate. The additional compound heptadecene also contributed substantially (>10 %) to the difference in the mandibular gland bouquets of foragers from nest A and B.

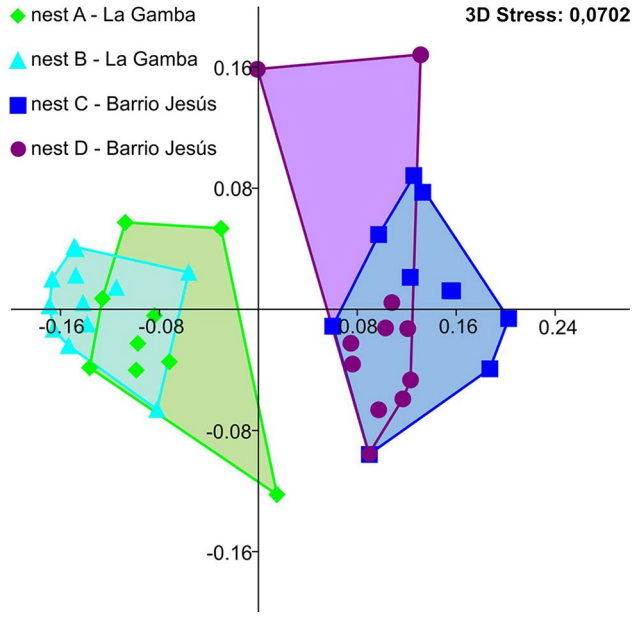

Fig. 4 Nonmetric multidimensional scaling (NMDS) visualization of differences in the chemical composition of labial gland secretions of *Partamona orizabaensis* foragers collected from four different nests

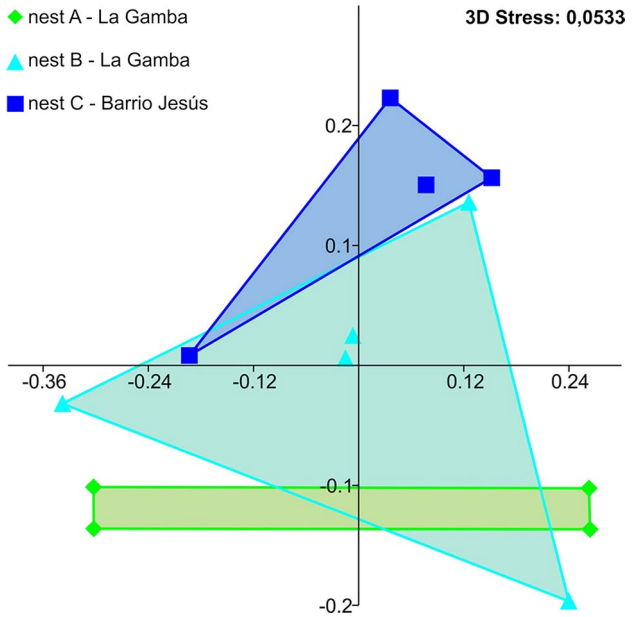

Fig. 5 Nonmetric multidimensional scaling (NMDS) visualization of differences in the chemical composition of mandibular gland secretions of *Partamona orizabaensis* foragers collected from three different nests

Table 4 Relative abundance (%) of compounds extracted from the mandibular glands of *Partamona orizabaensis* foragers from three different nests

Compound name	Nest A	Nest B	Nest C
Alkanes			
Heptadecane	5.94 ± 6.67	1.07 ± 1.24	3.42 ± 0.87
Nonadecane	1.98 ± 1.36	1.73 ± 2.00	1.47 ± 0.98
Alkenes			
Heptadecene	9.30 ± 2.29	8.66 ± 1.62	**10.35 ± 3.67**
Nonadecene	**14.14 ± 2.37**	**14.64 ± 2.09**	**17.41 ± 2.03**
9-Heneicosene	**28.43 ± 8.18**	**31.34 ± 19.31**	**35.61 ± 10.22**
Alcohols			
Hexadecan-1-ol	2.89 ± 1.79	1.23 ± 1.43	0.54 ± 0.67
9-Octadecen-1-ol	9.95 ± 6.25	7.85 ± 9.16	**10.96 ± 0.94**
Esters			
2-Pentadecylester	7.03 ± 2,47	8.11 ± 1.31	5.39 ± 0.86
9-Octadecen-1-ylbutyrate	**20.34 ± 5.13**	**25.38 ± 6.44**	**14.86 ± 6.66**

Nests A and B were located in La Gamba and nest C in Barrio Jesús. The relative amount of a compound was calculated as its percentage in the glands' entire bouquet

Compounds shown in bold are major components contributing >10 % to the composition of the labial gland secretions in at least one colony

Given are mean values (±standard deviation), sample sizes are $n_A = 5$, $n_B = 4$, $n_C = 4$

Discussion

Recruitment precision, pattern, and mechanism

Although almost 50 years have passed since Kerr (1969) postulated that bees within the genus *Partamona* create "aerial odor tunnels" with mandibular gland secretions to transfer information about the location of food sources to their nestmates, the recruitment behavior of *Partamona* was never scientifically studied. Our experiments now show that experienced foragers of *P. orizabaensis* are able to recruit other workers from their nest in large numbers to food sources at a specific spatial location. We also confirmed that the quick recruitment of nestmates did not require any scent marking of substrates between the food source and the nest. Marking behavior in stingless bee species that use scent "trails" to recruit nestmates to food is very conspicuous (Lindauer and Kerr 1958, 1960; Kerr et al. 1963; Schmidt et al. 2003; Nieh et al. 2003, 2004; Jarau et al. 2004; Sánchez et al. 2004; Aguilar et al. 2005). Moreover, the majority of these marks are deposited at the food source itself or in its close surroundings. Therefore, it is highly unlikely that we would have overlooked scent marking behavior in *Partamona* if the bees deposited any gland

secretions at or near our experimental feeders. In addition, neither labial gland secretions nor mandibular gland secretions attracted *P. orizabaensis* workers to feeders baited with these substances. Mandibular gland extracts even had a repellent effect on the approaching bees, which is in accord with earlier findings showing that compounds from these glands act as pheromones eliciting alarm behavior in stingless bees (Blum et al. 1970; Luby et al. 1973; Weaver et al. 1975; Johnson and Wiemer 1982; Keeping et al. 1982; Smith and Roubik 1983; Johnson et al. 1985; de Korte et al. 1988; Jarau et al. 2004; Schorkopf et al. 2009). Therefore, communication by means of attractive scent marks secreted from labial- or mandibular glands and deposited on solid substrates for target-oriented recruitment can be excluded as the underlying recruitment mechanisms in *P. orizabaensis*. This is in strong contrast to other mass-recruiting stingless bee species within the genera *Trigona*, *Scaptotrigona*, and *Geotrigona* which all rely on recruitment pheromones that are produced in the bees' labial glands and deposited by experienced foragers mainly at food sources and their close surroundings (Jarau et al. 2004, 2006, 2010, 2011; Schorkopf et al. 2007; Barth et al. 2008; Jarau 2009; Stangler et al. 2009; Lichtenberg et al. 2011; Reichle et al. 2013). Interestingly, however, workers of *Scaptotrigona* aff. *depilis* and *Trigona spinipes* are also able to effectively recruit nestmates to a particular feeding site even if the bees are restrained from using extended scent trails for recruitment communication by forcing them to cross a large lake when flying back and forth between sugar solution feeders and their nest (Schorkopf et al. 2011).

The question how *Partamona* foragers manage to recruit large numbers of their nestmates to a particular feeding site in a short time without using substrate-attached scent marks even at the food source itself remains to be answered. Kerr (1969) postulated that experienced bees guide workers that they had previously recruited within the nest toward a food source by releasing attractive pheromones from their mandibular glands during flight. Alternatively, recruits could follow a piloting forager in flight simply by visually keeping track with her. However, considering the habitat of *P. orizabaensis*, which often is the understory of dense tropical rainforests with dim light conditions and rather complex background vegetation, this possibility seems unlikely (at least for *P. orizabaensis*).

Gland chemistry and possible functions

Our analyses has shown that the mandibular glands of *P. orizabaensis* foragers contain high amounts of alkyl esters (9-octadecen-1-yl butyrate and a 2-pentadecyl ester), as well as alcohols with medium chain lengths (hexadecane-1-ol and 9-octadecen-1-ol). The presence of esters in mandibular gland secretions is interesting because the attractive recruitment pheromones of several stingless bee species comprise carboxylic acid alkyl and terpenyl esters; however, in these cases the compounds are always secreted from the foragers' labial glands and deposited on solid substrates (Jarau et al. 2004, 2006, 2010, 2011; Schorkopf et al. 2007; Barth et al. 2008; Jarau 2009; Stangler et al. 2009; Lichtenberg et al. 2011; Reichle et al. 2013). Whether esters released from the mandibular glands of *P. orizabaensis* foragers during flight attract recruited nestmates, thereby leading them toward food sources, remains to be tested. Mandibular gland secretions, which had a repellent effect at food sources in our bioassays, may serve a different function in another context, i.e., the guidance of recruits during flight. Further compounds from the mandibular glands that are well suited to attract flying bees are hexadecane-1-ol and 9-octadecen-1-ol. These two alcohols, as well as others with similar chemical structures, have been identified from labial gland secretions of males from a variety of bumble bee species (reviewed in Ayasse and Jarau 2014; see their Supplemental Material). It is possible (although unproven) that male bumble bees deposit their labial gland secretions at particular spots in the vegetation to attract virgin queens from some distance for the purpose of mating (Ayasse and Jarau 2014).

The labial glands of *P. orizabaensis* foragers likely do not play any role in the extranidal recruitment communication of this species because they exclusively contain saturated and unsaturated straight chain, as well as methyl-branched hydrocarbons. These compounds are not well suited to attract bees in flight from a distance, although they may be important for intranidal communication involving direct antennal contacts between workers.

Interestingly, we found colony-specific differences in the composition of secretions from labial glands, which are based on differences in the relative amounts of the secretions' single constituents, but not from mandibular glands. The colony-specific mixtures of different straight chain- and methyl-branched hydrocarbons from labial glands may be well involved in nestmate recognition in *P. orizabaensis* because similar compounds have been found to serve this function in a variety of different social insect species (Howard and Blomquist 2005; van Zweden and d'Ettorre 2010). The lack of colony specificity in mandibular gland secretions is interesting because they contained esters and alcohols, along with alkanes and alkenes. Species- specific mixtures as well as colony-specific mixtures of esters comprise the attractive recruitment scent marks of *Trigona*, *Scaptotrigona*, and *Geotrigona* foragers, which secrete them from their labial glands, though (Jarau 2009; Stangler et al. 2009; Jarau et al. 2010, 2011; John et al. 2012; Reichle et al. 2013). The chemical specificity of substrate-attached recruitment scent marks is important because it enables stingless bee recruits to recognize and visit food

sources marked with the gland secretions of their nestmates, thereby minimizing competition with bees from foreign colonies at resources (Jarau 2009; Jarau et al. 2010, 2011; Reichle et al. 2012, 2013). If esters from mandibular gland secretions were involved in the attraction of *Partamona* recruits that follow a nestmate forager in flight, as suggested by Kerr (1969), rather than being deposited at resources, nest-specific compositions would not be required to keep recruits from different nests apart. However, in our bioassays compounds extracted from the bee's mandibular glands had a repellent effect on foragers' behavior when they encountered them at food sources. This repellent effect may argue against a role of mandibular gland secretions in the recruitment communication of *P. orizabaensis*. Nevertheless, specific compounds (or a different subset of compounds secreted from a particular gland) may evoke different behavioral responses in receivers according to the context in which they are perceived (Wyatt 2014). This may well apply to bees that either keep track with a nestmate forager in flight or approach a potential food source. Furthermore, a particular compound may serve different functions in communication depending on its concentration, as has been demonstrated for 4-methyl-3-heptanone, a compound that attracts *Atta texana* leafcutter ants at low concentrations but releases aggressive behavior in the same ants when present at high concentrations (Hölldobler and Wilson 1990). Future experiments testing the effect of different concentrations of *P. orizabaensis* mandibular gland extracts (and of synthetic mixtures of identified compounds) on foragers at the nest entrance, as well as at food sources could help to clarify this issue.

Conclusions

An interesting question is why *Partamona* workers have evolved a recruitment system that is highly efficient and even faster than that of species that mark food sources with attractive pheromones. We assume that competition with other, often more aggressive stingless bee species that inhabit the same habitats may be the reason. Conspicuous signals that endure for some time, such as scent trail marks, are more likely to be exploited by unintended receivers, i.e., by eavesdroppers (Peake 2005). Thus, eavesdropping harms signalers and opts against noticeable signals (Brandley et al. 2013). However, if signal conspicuousness discourages eavesdroppers by suggesting costs that may occur when receivers that take advantage of food recruitment signals have to fight to gain access to the food source, signaler and eavesdropper indeed may benefit (Lichtenberg et al. 2011). Regarding the fierce competition for food sources, various species that mark resources, for instance *Trigona* species, are known to defend their resources intensely against competitors (Lichtenberg et al. 2010). This results in the decision of eavesdropping individuals to recruit only to weakly marked resources to avoid costly takeover attempts (Lichtenberg et al. 2014). *Partamona orizabaensis* workers possibly try to avoid competition caused by eavesdroppers by not marking food sources at all, thereby impeding takeover attempts by other stingless bee species. This remarkable recruitment strategy, thus, may have evolved to avoid eavesdropper imposed costs in foraging.

Although *P. orizabaensis* showed no chemical marking on the feeder and the surrounding environment, our study shows that this species has a very effective way of recruitment communication. Only 5 min after an experiment had started the first newly recruited bees reached the recruitment feeder. Indeed, Keppner and Jarau (2016) demonstrated that *P. orizabaensis* workers can outcompete foragers of the more aggressive species *Trigona fuscipennis* by quickly increasing the number of foragers at a food source, thereby reaching a threshold size of its forager force that cannot be defeated by the otherwise dominant bees. In addition, foragers of *P. orizabaensis* are particularly active during times of the day that are less favorable for bees, i.e., during early morning hours, right before sunset, and even during heavy rainfall (Keppner and Jarau 2016). Thus, the quick and "inconspicuous" guidance of recruits by means of pheromones released during flight (if true) rather than deposited at solid substrates and the increase of forager activity during times of the day with dim light and during unfavorable weather conditions may all be the adaptations that have evolved in *P. orizabaensis* to cope with the aggressive species that are strong competitors for the resources in their habitats.

Our study clearly shows that foragers of *P. orizabaensis* can communicate the location of food sources to their nestmates. However, the exact mechanism by which they do so remains to be elucidated. Future experiments testing whether dummy foragers that emit labial or mandibular gland extracts (or synthetic copies of identified compounds) while being moved from the nest toward a food source attract workers could help to solve the question about the potential involvement of "odor tunnels" (Kerr 1969) in the recruitment process of *Partamona* bees.

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