Chemical signatures of egg maternity and *Dufour*'s gland in Vespine wasps

Authors: Rafael Carvalho da Silva[1*], Fabio Santos Nascimento[1], Tom Wenseleers[2] & Cintia Akemi Oi[2,3]

Affiliation: [1] Universidade de São Paulo – USP/ Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Departamento de Biologia, SP, Ribeirão Preto, Brasil

[2]Laboratory of Socioecology and Social Evolution, KU Leuven – University of Leuven, Belgium.

[3]Department of Genetics, Evolution and Environment, University College London, London, United Kingdom

*Corresponding author: Avenida Bandeirantes, 3900 – Vila Monte Alegre, Ribeirão Preto – SP, 14040-900 / E-mail: rcswasp@gmail.com

ORCID: 0000-0003-2674-8976 (RCS); 0000-0002-3682-8219 (CAO); 0000-0002-3731-7111 (FSN); 0000-0002-1434-861X (TW)

Abstract

Cuticular hydrocarbons (CHCs) are often used in the chemical communication among social insects. CHCs can be used in nestmate recognition and as queen pheromones, the latter allows the regulation of the reproductive division of labor. In the common wasp *Vespula vulgaris*, CHCs and egg-marking hydrocarbons are caste-specific, being hydrocarbon queen pheromones and egg maternity signals. Whether these compounds are conserved among other Vespinae wasps remains unknown. Queens, virgin queens, reproductive workers, and workers belonging to four different wasp species, *Dolichovespula media*, *Dolichovespula saxonica*, *Vespa crabro*, and *Vespula germanica* were collected and studied. The cuticular hydrocarbons, egg surface, and *Dufour's* gland composition were characterized and it was found that chemical compounds are caste-specific in the four species. Quantitative and qualitative differences were detected in the cuticle, eggs, and *Dufour's* gland. Some specific hydrocarbons that were shown to be overproduced in the cuticle of queens, were also present in higher quantities in queen-laid eggs and in their *Dufour's* gland. These hydrocarbons can be indicated as putative fertility signals that regulate the division of reproductive labor in these Vespine societies. Our results are in line with literature for *V. vulgaris* and *D. saxonica*, in which hydrocarbons were shown to be conserved queen signals. This work presents correlative evidence that queen chemical compounds are found not only over the body surface of females but also in other sources, such as the *Dufour*'s gland and eggs.

Keywords: cuticular hydrocarbons, egg surface, *Dufour*'s gland, Vespine wasps, fertility cues

Introduction

Social insects live in complex societies, leading them to be referred to as superorganisms (Strassmann and Queller 2010; Boomsma and Gawne 2017). One or a few individuals reproduce (queens), whereas the remaining majority engage in other high-cost tasks (workers). A high level of coordination and task partitioning exists among females in social insects (Wenseleers et al. 2020a). Even in such cooperative circumstances, conflicts emerge among females (queen versus workers, workers versus workers, see Ratnieks et al. 2006), as individuals are not clones and express intermediate or low relatedness levels (Hamilton 1964). In some species, workers can retain the capacity to activate their ovaries and lay unfertilized eggs (male destined) (Foster and Ratnieks 2000; Barron et al. 2001; Foster and Ratnieks 2001; Tsuchida et al. 2003; see Toth et al. 2004). These worker-laid eggs are perceived and often destroyed by

their nestmates, a behavior also referred to as policing (Foster et al. 2002; Foster and Ratnieks 2001; Saigo and Tsuchida 2004; Liebig et al. 2005; Wenseelers et al. 2005; Oi et al. 2015b). Policing behavior results in worker-laid eggs being destroyed, or in the reproductive workers being directly attacked by other workers or the queen (Ratnieks et al. 2006; Wenseelers and Ratnieks 2006; Wenseelers et al. 2020b). Policing has evolved as a mechanism to regulate the reproductive division of labor. Reproductive workers do not go unnoticed in their colonies because they produce certain fertility cues, which distinguish them from sterile nestmates (Smith et al. 2008; Smith et al. 2012; Oi et al. 2021b). Reproductive workers would benefit by not being detected, however, fertility cues are probably produced as by-products of their ovary activation (Oi et al. 2015a; Wenseleers et al. 2020b).

Hydrocarbons are often used as cues for egg policing in social insects (Wenseleers et al 2021b). In honeybees, in which policing behavior was firstly described (Ratnieks and Visscher 1989), esters derived from the *Dufour's* gland - an accessory gland of the reproductive system in Hymenoptera females (Mitra 2013) - were identified as the main cues driving policing behavior. Queen-laid eggs have higher proportions of these esters when compared with worker-laid eggs (Katzav-Gozansky et al. 1997). Empirical evidence, however, suggests that neither esters nor hydrocarbons work as signals allowing policing (e. g. Katzav-Gonzansky et al. 2001; Martin et al. 2002). In ants, queens also release queen characteristic compounds on eggs and hydrocarbons that cover the egg's surface that are the molecules that allow policing behavior (Schultner and Pulliainen 2020). A study integrating chemical data and electroantennography sensibility in workers of *Pachycondyla inversa*, revealed that a dimethyl hydrocarbon (3,11-diMeC27) acts as a fertility signal (D'Ettorre et al. 2004). The proportion of 3,11-diMeC27 was positively correlated with total egg length and the antennae of workers were highly sensitive to this hydrocarbon, and worker-laid eggs coated with 3,11-diMeC27 were generally more accepted than non-treated control eggs (D'Ettorre et al. 2004; van Zweden et al. 2009). In the ant *Camponotus floridanus*, the cuticular hydrocarbons (CHCs) and compounds covering egg surface are caste specific and the transference of queen's chemicals onto worker-laid eggs prevented them from being destroyed by workers (Endler et al. 2004). In the primitively eusocial ant *Dinoponera quadriceps*, the presence of several branched and linear alkanes on the egg's surface possibly helps nurses to discriminate maternity information (Tannure-Nascimento et al. 2009), however bioassays were not conducted in this study. In social wasps *Polistes dominula*, subordinate eggs do not chemically match those laid by queens, and consequently, they are policed (Dapportto et al. 2007). In *Polistes chinensis antennalis*, workers reproduce under queen-right conditions, however, their eggs are often destroyed (Tsuchida et al. 2020). In the common wasp *Vespula vulgaris*, methyl alkanes are present in higher concentrations over the queen-laid eggs, whereas worker-laid eggs have a higher proportion of long-chain alkenes (Bonckaert et al. 2012) and the methyl alkane 3-MeC29 drove egg policing (Oi et al. 2015b).

The *Dufour's* gland is an exocrine gland that evolved more than 200 million years ago. The compounds derived from *Dufour's* gland in social insects are used to fixate eggs, during nest building, as cues for nest recognition, nestmate recognition, and fertility signaling (Ross and Matthews 2018; Mitra 2013; Sarmiento 2020). This gland displays several roles across the Hymenoptera (Mitra 2013), and it is found in all eusocial wasps (Landolt and Akre 1979). Interestingly, morphological differences of *Dufour's* glands are seen between queens and workers in social wasps (Landolt and Akre 1979). Considering that this gland is associated to the sting apparatus in females of several Hymenoptera species (Apocrita) (Mitra 2013), a key

function of this gland is to help egg attachment as the gland is derived from colleterial glands in insects (Mitra 2013). This would facilitate the release of chemical compounds covering the eggs (Abdalla and Cruz-Landim 2001). During oviposition, eggs are likely coated with chemical substances from the *Dufour*'s gland (Mitra 2013), especially in bees and wasps (Sarmiento 2020).

Considering that CHCs and egg-marking compounds are often produced in different amounts by females bearing different fertility status (Dapporto et al. 2007; Oi et al. 2015b; Oliveira et al. 2017; Oi et al. 2021b) and that the *Dufour*'s gland could be a source of egg-marking compounds (shown in Mitra 2013; Ferreira et al. 2022), in the present study, the main goal was to chemically characterize four species of Vespine wasps. To do that, different groups of females were used: queens, virgin queens, workers and reproductive workers. Three different sources of chemical compounds were investigated: CHCs, egg-marking cues and *Dufour*'s gland content. The hypothesis was that female's fertility (= ovary activation) would be correlated with chemical similarity among the four studied species. Thus, the predictions of the study were: (1) queens and reproductive workers would chemically resemble each other when compared with virgin queens and workers; (2) a higher chemical resemblance between *Dufour*'s gland and egg-marking compounds would exist in the four species. This would reinforce the possible role of *Dufour*'s gland in egg-marking. A chemical similarity between egg-marking compounds in the four species would indicate a conserved strategy linking fertility and egg maternity. The results of the present study contribute to the understanding of the chemical communication in highly eusocial wasps, as we identified putative queen and egg marking pheromones to be tested in bioassays in the future.

Material and Methods

Study site and nest sampling

Nests of the four Vespinae species, *Dolichovespula saxonica*, *Dolichovespula media*, *Vespa crabro*, and *Vespula germanica* were collected between July and September 2019 in the vicinity of Leuven - Belgium (50° 52′ 46″ N; 4° 42′ 3″ E, 41 m elevation). Nests were found hanging under the roof, in tree branches, inside birdhouses, or in the ground. Following collection, nests were kept in plastic boxes and transported to the laboratory, where samples were collected. The first step was to anesthetize the nests with CO_2. Once all the wasps were anesthetized, the queen, workers, and virgin queens (if present) of each nest were frozen in -20°C until chemical analyses. After that, we sampled queen-laid eggs from the newest comb. Eggs were removed from their cell wall with the aid of surgical scissors and tweezers. To avoid eggs being destroyed during their removal, a tiny piece of the nest where the eggs were attached was removed together with the egg. To collect worker-laid eggs, about 10 to 20 workers were settled in a wooden box provided with water, sugar syrup, sugar paste (Apifonda), and pieces of mealworms (*Tenebrio mollitor*), together with a comb from their original nests without eggs. The wooden box consisted of two main parts, the first part where the comb was placed over a thin wire (reproductive area) was kept covered with a plastic lid and a piece of thick black plastic. In this case, pieces of wire were used to hang the comb in the experimental box. The pieces of wire allowed the comb to remain far from the box floor and hence allowed females to check cells and lay eggs. The black plastic kept the area under conditions to recreate the dark interior of their nests. The second part consisted of the foraging area, where tubes containing sugar syrup and water were placed.

The foraging area was covered with a transparent plastic lid. A small plastic tube was placed between the first and second boxes, which allowed workers to go from the foraging arena to the comb area. This procedure was adapted from Oi et al. (2015b). The combs were checked for the presence of worker-laid eggs after one week (we kept workers under these conditions for maximum two weeks). A total of 154 samples of CHCs, 169 samples of eggs, and 118 samples of the *Dufour*'s gland were collected and analyzed (Table 1).

Table 1: Number of samples analyzed per species.

Species	**Nests**	**Cuticular hydrocarbons (CHC)**			
		Queens	Virgin queens	Reproductive workers	Workers
Dolichovespula saxonica	6	4	4	5	17
Dolichovespula media	6	5	10	7	29
Vespa crabro	10	8	-	-	35
Vespula germanica	10	4	-	6	20
Species	**Nests**	**Eggs**			
		Queens	Workers		
Dolichovespula saxonica	5	12	15		
Dolichovespula media	6	24	21		
Vespa crabro	9	38	14		
Vespula germanica	6	27	18		
Species	**Nests**	***Dufour*'s gland**			
		Queens	Virgin queens	Reproductive workers	Workers
Dolichovespula saxonica	4	2	2	3	12
Dolichovespula media	5	5	5	7	27
Vespa crabro	10	8	-	-	16
Vespula germanica	10	8	-	6	17

CHCs analysis

For CHC extraction, queens, virgin queens, reproductive workers and workers, and whole body samples were placed in individual glass vials and covered with pentane (HPLC grade, Sigma-Aldrich), 1 ml for workers and 2 ml for queens for one minute, except for *V. crabro* in which 3 ml were used for workers and 10 ml for queens, and the queens of *D. media* were also extracted with 10 ml of pentane. After that, all the extracts were dried under a fume hood. After CHC extraction, wasps were dissected under a Leica MZ125 stereomicroscope to sample their *Dufour's* gland. The *Dufour's* gland was removed and placed in glass inserts filled with 50 µl of pentane (HPLC grade, Sigma-Aldrich). The extracts were dried under a fume hood and the glass vials were kept frozen. The eggs were extracted in 50 µl of pentane for 1 minute. Subsequently, the liquid content was transferred to another vial and evaporated. A total of 1000 µl for CHC of queens, 250 µl for CHC of workers were used to resuspend the CHC samples. For eggs and *Dufour*'s glands 50 µl of hexane were used (HPLC grade, Sigma-Aldrich). All the samples were analyzed in a Gas Chromatography coupled with Mass Spectrometer (GC-MS) (Thermo Fisher Trace 1300 ISQ) system. In

the GC-MS system, 1 µl of each sample was injected by using a splitless mode, and the chemical compounds were separated in a Restek MXT-5 column (30 m, 0.25 mm of internal diameter, and 0.25 µm of film thickness), with helium as a carrier gas (constant flow of 0.9 mL min-1). We used GC-MS parameters adapted from Oi et al. (2021c). The injector temperature was kept at 320 ºC. Initially, the oven temperature was set at 40 ºC for 2 minutes, then increased to 120 ºC with a rate of 20 ºC/minute. Next, the temperature increased to 200 ºC at a rate of 10 ºC/minute, then 7 ºC/minute until 250 ºC. Finally, the temperature increased at a rate of 5 ºC/minute until 350 ºC, which was held for 4 minutes. Electron impact ionization at 70 eV was adopted during mass spectrometry at a temperature of 300 ºC. To help identify the chromatograms and to calculate cubic spline interpolated retention indexes (Messadi et al., 1990), linear alkane ladders (Supelco) from *n*-C7 to *n*-C40 were used. Hydrocarbons were identified based on the expected mass spectrometric fragmentation patterns (Carlson et al., 1998) and retention indexes by the NIST 2014 retention index database (Linstrom and Mallard, 2016; Pherobase El-Sayed, 2016). Chromatograms were integrated using a custom R script on R version 4.0.2 (available from the authors upon request).

Statistical analysis

For the first analysis, in which the aim was to test whether chemical cues would consistently vary according to females' fertility status across the four study species, the peak area of each compound from the three types of samples (CHCs, eggs, and *Dufour's* gland) was transformed into relative abundances (%) and they were compared in PERMANOVA tests (9999 permutations). The aim of the permutation analysis was to check whether there were chemical differences among castes. To conduct this analysis, the package *vegan* (Oksanen et al. 2015) was used. The data corresponding to CHCs, eggs and *Dufour*'s gland were plotted in order to verify the relative percentage of the different groups of compounds. To check which compounds contributed the most to group differentiation, a percentage similarity test (SIMPER) by adopting a Bray-Curtis index was used with the package *vegan* (Oksanen et al. 2015). To visualize whether *Dufour*'s gland would be the main source of egg-marking compounds, the overlap among samples of cuticle, eggs and *Dufour's* gland was explored. For this set of analysis, only samples of queens and reproductive workers were used (we did not have samples of reproductive workers for *V. crabro*, but we present a heatmap and analysis in the supplementary material, Figure S1). To check whether eggs were chemically similar to CHCs or *Dufour's* gland secretion the compounds were log2-transformed using relative abundances and compared by ANOVA followed by Tukey test with FDR correction. A heatmap using the mean relative abundance was prepared to visualize the hydrocarbon composition of the three different groups of samples (CHCs x eggs x *Dufour's* gland). For that, the row z scores calculated per group were used. Clustering was based on a UPGMA hierarchical clustering using Euclidean distance as the distance metric. The chemical variation of the previously published queen pheromones in *V. vulgaris* and *D. saxonica* was inspected (Van Oystaeyen et al. 2014; Oi et al. 2016) in our samples. A similar approach was used to indicate the fertility cues in the study species. Additionally, to check if egg-marking compounds are species-specific or if there exist similarity among the chemical compounds released by queens and reproductive workers of Vespinae species a PERMANOVA test (9999 permutations) was performed, followed by a PCA plot for visualization (using ggplot2 – Wickham et al. 2016) and finally a SIMPER analysis in egg-marking compounds data for

queen-laid and worker-laid eggs. For the second analysis, the PERMANOVA test was used to verify whether samples were similar according to their species. The PCA was used to visualize whether samples would group together (chemical similarity among compounds released during oviposition) or if they would group separately (existence of a species-specific egg-marking). The SIMPER was used to highlight which chemical compounds would be specific to females (species-specific). All the statistical analyses were performed using RStudio (version 1.4.1717, R core team 2021).

Results

We summarized the chemical characterization in the four species *Dolichovespula media, Dolichovespula saxonica, Vespa crabro*, and *Vespula germanica.* The results are shown per group of females (workers, virgin queens and queens) and chemical source (CHCs, eggs and *Dufour's* gland), combining the chemical compounds in class of compounds as linear alkanes, methyl alkanes, dimethyl alkanes and unknown compounds (Table 2). The results are presented separately per species.

Table 2: General chemical characterization of *Dolichovespula media, Dolichovespula saxonica, Vespa crabro*, and *Vespula germanica* (Number of compounds (n), range of compounds and percentage found for each class of compounds). Type were CHC = cuticular hydrocarbons, DF = *Dufour*'s gland and eggs. Class of compounds were linear = linear alkanes, methyl = methyl alkanes, dimethyl = dimethyl alkanes and unk = unknown compounds.

Dolichovespula saxonica							
type	n	range	% linear	% methyl	% dimethyl	% alkenes	% unk.
CHC	45	*n*-C23 - C33:1	17.77	44.44	22.22	11.11	4.44
eggs	54	*n*-C20 - *n*-C33	22.22	38.88	18.51	20.37	-
DG	49	*n*-C23 - *n*-C35	22.44	38.77	18.36	20.4	-
Dolichovespula media							
type	n	range	% linear	% methyl	% dimethyl	% alkenes	% unk.
CHC	45	*n*-C18 - 17-;15-;13-;11-MeC35	31.11	46.66	6.66	15.55	-
eggs	25	C21:1 - 15-;13-;11-;9-MeC31	20.00	44.00	8.00	28.00	-
DG	37	C21:1 to 17-;15-;13-;11-MeC35	24.32	40.54	8.10	27.02	
Vespa crabro							
type	n	range	% linear	% methyl	% dimethyl	% alkenes	% unk.
CHC	33	C21:1 - 3-MeC29	27.27	42.42	6.06	12.12	12.12
eggs	37	*n*-C18 - 15-;13-;11-;9-MeC29	32.43	48.64	7.7	13.51	2.7
DG	53	*n*-C21 - *n*-C35	26.41	54.71	11.32	7.54	-
Vespula germanica							
type	n	range	% linear	% methyl	% dimethyl	% alkenes	% unk.
CHC	31	*n*-C22 - 7,x-diMeC33	25.8	51.61	12.9	9.67	-
eggs	27	*n*-C21 - *n*-C31	33.33	44.44	11.11	11.11	-
DG	23	*n*-C23 - 13,17-diMeC33	30.43	56.52	4.34	8.69	-

Dolichovespula saxonica

We found that the proportion of linear alkanes was higher in queens and reproductive workers, whereas virgin queens were most represented by alkenes, and workers did not express any class in a higher proportion when compared to queens and virgin queens (Figure 1). According to the permutation analysis, overall, all groups (queens, virgin queens, reproductive workers, and sterile workers) were statistically different based on their CHCs (Table 3). However, based on the post-hoc permutation analysis, only three pairs were statistically different (queens x workers / virgin queens x workers / reproductive workers x workers) (Table 3). Based on the SIMPER analysis, the most representative compounds that contributed to group differentiation included two linear alkanes (*n*-C31 and *n*-C29), two alkenes (C31:1 and C29:1), two methyl-alkanes (15-;13-;11-;9-MeC29 and 15-;13-MeC31), and two dimethyl-alkanes (5,x-diMeC31 and x,y-diMeC28) (Table S4). On the eggs, the proportion of methyl-alkanes was higher in queen-laid eggs, whereas worker-laid eggs were best represented by linear alkanes and alkenes (Figure 2). According to the permutation analysis, queen-laid and worker-laid eggs of *D. saxonica* were not different based on their hydrocarbons (Table 3). Among the five most representative compounds that contributed to group differentiation according to SIMPER, only 3-MeC29 was statistically different, and the average was higher in queen-laid eggs (Table S4). In the *Dufour's* gland, the proportion of methyl-alkanes was higher in the queen *Dufour*'s gland if compared with reproductive worker and worker samples, but not if compared to virgin queens. Virgin queen *Dufour*'s glands were most represented by alkenes, whereas reproductive workers and workers *Dufour*'s gland had a higher proportion of linear alkanes (Figure 3). According to the permutation analysis, overall, all groups (queens, virgin queens, reproductive workers, and workers) were statistically different in their *Dufour*'s glands composition (Table 3). However, based on the post-hoc permutation analysis, neither of the pairs was statistically different. Based on the SIMPER analysis, the most representative compounds that contributed to group differentiation included two linear alkanes (*n*-C28 and *n*-C31), one alkene (C27:1-1), two methyl-alkanes (15-;13-;11-;9-MeC29 and 3-MeC29) (Table S4).

Dolichovespula media

In the CHC, the proportion of linear alkanes was higher in queens, and the proportion of alkenes was higher in virgin queens. Reproductive workers, and workers, on the other hand, were best represented by methyl-alkanes (Figure 1). According to the permutation analysis, the global and pair-wise comparisons were statistically different (Table 3). Based on the SIMPER analysis, the most representative compounds that contributed to group differentiation included three linear alkanes (*n*-C27, *n*-C29 and *n*-C31), one alkene (C29:1), and four branched alkanes (3-MeC27 + 5,9-;5,13-diMeC25, 15-;13-;11-;9-MeC29, 7-MeC29 and 3-MeC29) (Table S8). Regarding eggs, queen-laid eggs were represented by a higher proportion of methyl-alkanes, whereas worker-laid eggs were most represented by linear alkanes and alkenes (Figure 2). The permutation analysis revealed that queen-laid and worker-laid eggs are chemically distinct based on their hydrocarbons (Table 3). According to the SIMPER analysis, two linear alkanes (*n*-C27 and *n*-C29), and three methyl-alkanes (3-MeC27, 3-MeC29 and 15-;13-;11-;9-;7-MeC29) were the most important to promote group differentiation (Table S8). Queen *Dufour*'s glands had a higher proportion of methyl-alkanes, virgin queen *Dufour*'s glands were more represented by linear alkanes and alkenes, and worker *Dufour*'s glands did not have a higher proportion of any compound class when compared to queens and

virgin queens (Figure 3). The global permutation analysis revealed that *Dufour*'s gland samples are chemically different, which was consistent with the pairwise comparisons, with the exception of the pair queen versus worker, which had a marginal *p*-value ($p = 0.053$) (Table 3). Based on the SIMPER analysis, the most representative compounds that contributed to group differentiation included one alkene (C29:1-1), five methyl-alkanes (13-;11-;9-;7-MeC27, 14-;13-;12-;11-MeC28 and 15-;13-;11-;9-;7-MeC29), and one mixture containing a linear alkane and one alkene (C29:1-2 + n-C29) (Table S8).

Vespa crabro

In the CHC of *V. crabro*, the proportion of linear alkanes was higher in queens, whereas workers were best represented by alkenes (Figure 1). According to the permutation analysis, queens, and workers of *V. crabro* are statistically different based on their CHCs (Table 3). Based on the SIMPER analysis, the most representative compounds that contributed to group differentiation included two linear alkanes (*n*-C25 and *n*-C27), two alkenes (C25:1 and C27:1), and one methyl-alkane (3-MeC27) (Table S12). In the queen-laid eggs, the proportion of alkenes was higher, while on the worker-laid eggs the proportion of linear alkanes was higher (Figure 2). The permutation analysis indicated that queen-laid and worker-laid eggs are chemically distinct (Table 3). According to the SIMPER analysis, three linear alkanes (*n*-C25, *n*-C27 and *n*-C29), and one alkene (C25:1) were the most important for promoting group differentiation (Table S12). In the *Dufour's* gland, linear alkanes were found in higher proportions in queens, and alkenes were the class that best represented worker *Dufour*'s glands (Figure 3). Based on the permutation analysis, queen *Dufour*'s gland and worker *Dufour*'s gland were chemically distinct (Table 3). Based on the SIMPER analysis, the most representative compounds that contributed for group differentiation included one alkene (*n*-C27), one alkene (C25:1), and two methyl-alkanes (3-MeC25 and 3-MeC27) (Table S12).

Vespula germanica

In the CHCs, the proportion of linear alkanes was higher in queens and the proportion of methyl-alkanes was higher in workers, both reproductive and sterile (Figure 1). According to the permutation analysis, queens and workers were statistically different (Table 3). Based on the SIMPER analysis, the most representative compounds that contributed for group differentiation included three linear alkanes (*n*-C25, *n*-C26 and *n*-C27), and two methyl-alkanes (13-;11-;9-;7-MeC25 and 13-;11-;9-MeC27) (Table S16). Queen-laid eggs were best represented by methyl-alkanes, whereas worker-laid eggs were more represented by linear alkanes (Figure 2). According to the permutation analysis, queen-laid and worker-laid eggs are not different (Table 3). According to the SIMPER analysis, only 3-MeC27 was statistically different among the five most important compounds, which was found in a higher proportion in queen-laid eggs (Table S16). Queen *Dufour*'s gland were best represented by linear alkanes, whereas methyl-alkanes were found in higher proportions in worker *Dufour*'s gland (Figure 3). According to the permutation analysis, the *Dufour*'s gland content of queens and workers was statistically different (Table 3). Based on the SIMPER analysis, the most representative compounds that contributed to group differentiation included one alkane (n-C26) and a mixture containing a linear and a dimethyl-alkane (n-C28 + 3,9-;3,11-diMeC27) (Table S16).

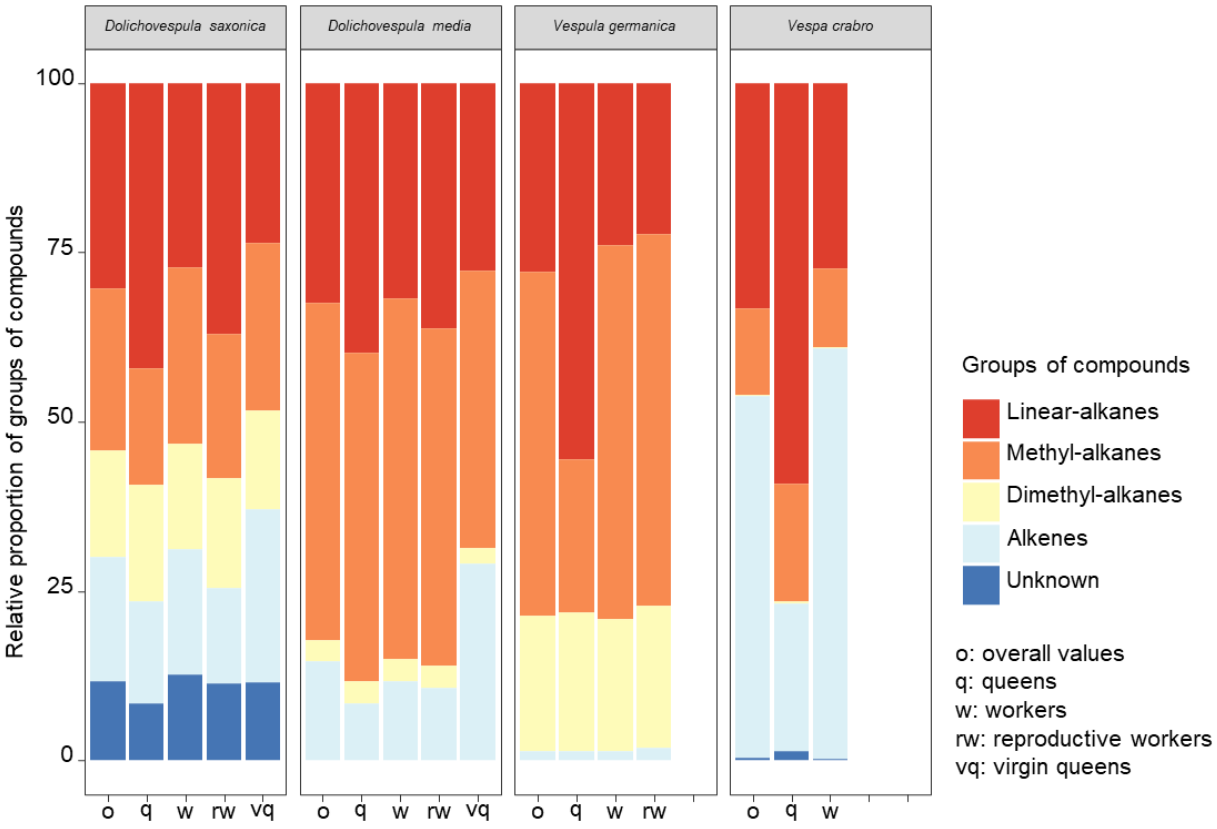

Figure 1: Queens (q), virgin queens (vq), reproductive workers (rw) and sterile workers (w) expressed different proportions of cuticular hydrocarbons (CHCs). Schematic representation of CHC classes identified in *Dolichovespula saxonica*, *Dolichovespula media*, *Vespula germanica* and *Vespa crabro*, grouped by compound classes: linear alkanes, methyl alkanes, dimethyl-alkanes, alkenes and unknown compounds. Each bar plot represents the relative proportion of groups and the overall proportion of the species.

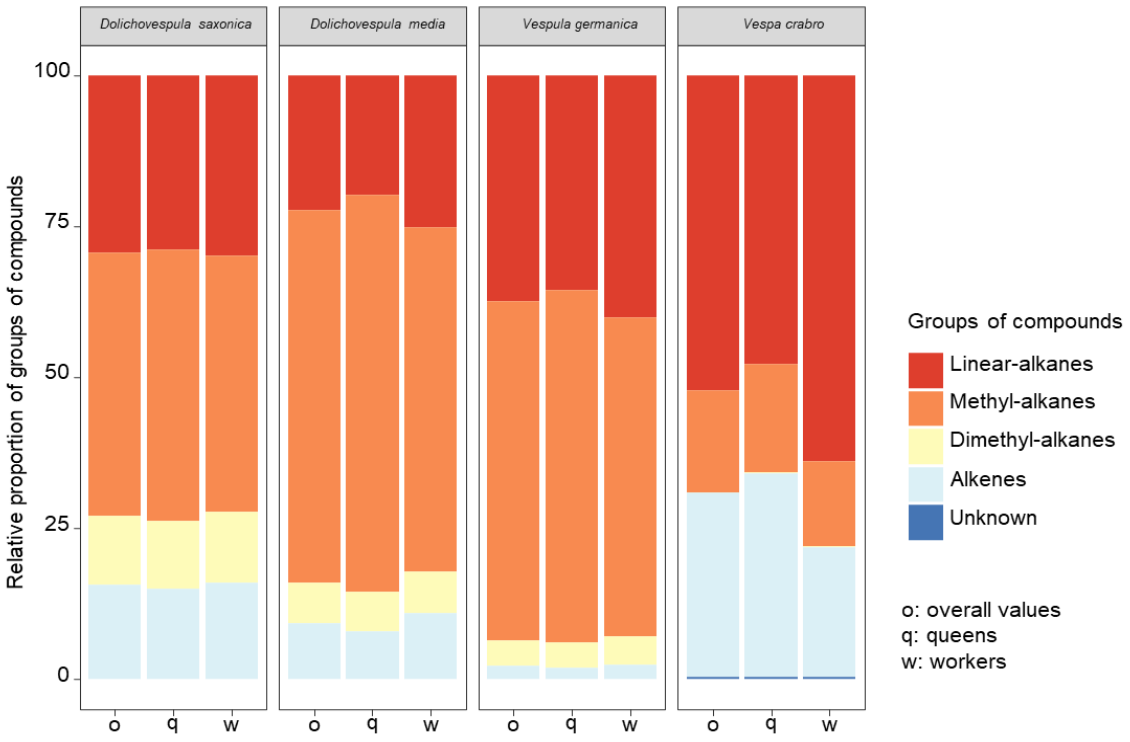

Figure 2: Queen-laid eggs (q) and worker-laid eggs (w) of *Dolichovespula saxonica*, *Dolichovespula media*, *Vespula germanica* and *Vespa crabro* expressed different proportions of hydrocarbons in their surface. Each bar plot represents the relative proportion of compound classes: linear alkanes, methyl alkanes, dimethyl-alkanes, alkenes and unknown compounds.

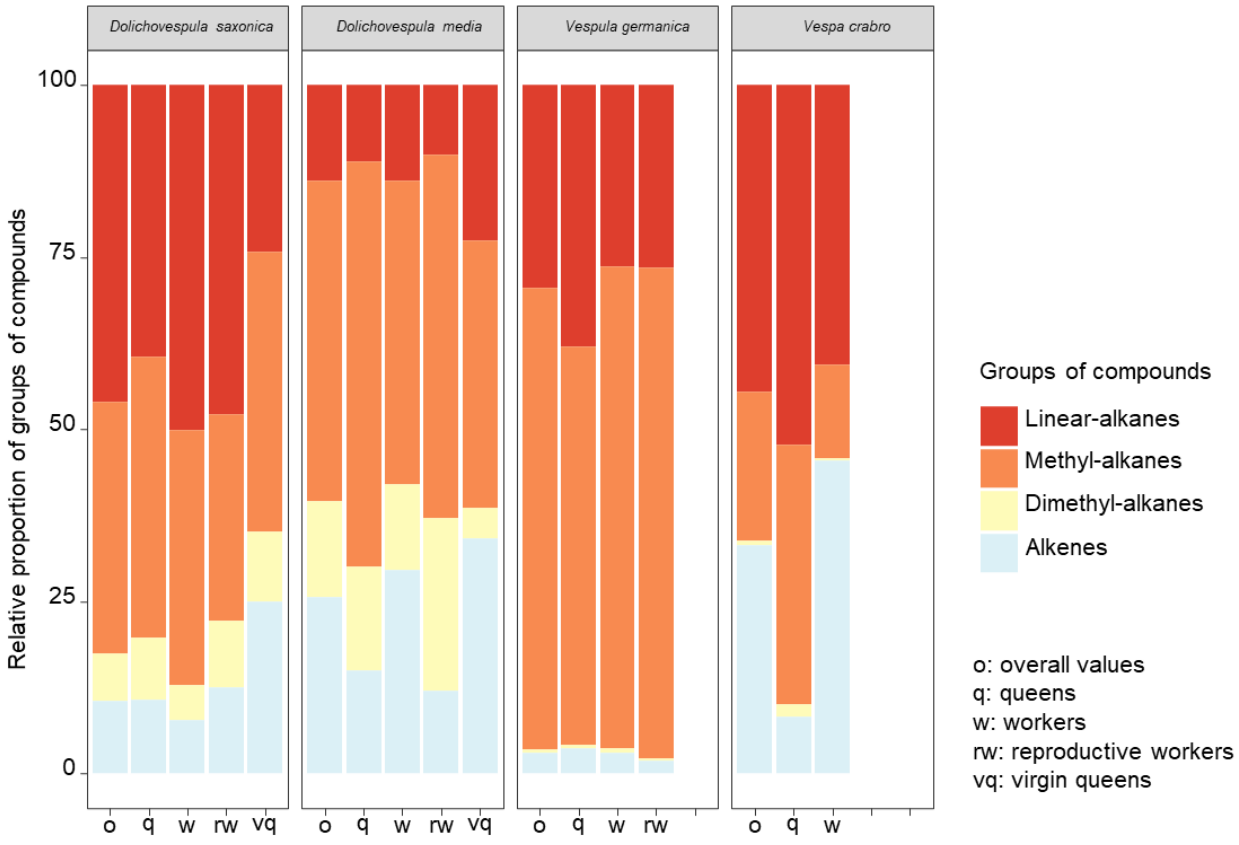

Figure 3: The *Dufour*'s gland of queens (q), virgin queens (vq), reproductive workers (rw) and sterile workers (w) of *Dolichovespula saxonica*, *Dolichovespula media*, *Vespula germanica* and *Vespa crabro* express different proportions of hydrocarbons. Classes of compounds were linear alkanes, methyl alkanes, dimethyl-alkanes, alkenes and unknown compounds. Each bar plot represents the relative and the overall proportion (o) of the species.

Table 3: Permutation analysis (PERMANOVA) per species and group of samples (CHCs, eggs, and *Dufour's* gland). Significance levels * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

***Dolichovespula saxonica* - CHCs**				
	F value	R^2	*p* value	Sig. Level
all groups	8.707	0.501	<0.001	***
queens x virgin queens	6.802	0.531	0.474	n.s
queens x reproductive workers	1.707	0.196	0.948	n.s
queens x workers	11.579	0.378	<0.01	**
virgin queens x reproductive workers	21.890	0.757	0.054	n.s
virgin queens x workers	9.661	0.337	<0.01	**
reproductive workers x workers	7.701	0.278	<0.01	**
***Dolichovespula media* - CHCs**				
	F value	R^2	*p* value	Sig. Level
all groups	25.297	0.617	<0.001	***
queens x virgin queens	48.090	0.787	<0.01	**
queens x reproductive workers	21.478	0.682	<0.05	*

queens x workers	28.622	0.472	<0.01	**
virgin queens x reproductive workers	23.454	0.609	<0.01	**
virgin queens x workers	35.727	0.491	<0.01	**
reproductive workers x workers	2.341	0.064	0.498	n.s
***Vespa crabro* - CHCs**				
	F value	R^2	*p* value	Sig. Level
queens x workers	139.600	0.772	<0.001	***
***Vespula germanica* - CHCs**				
	F value	R^2	*p* value	Sig. Level
all groups	31.368	0.699	<0.001	***
queens x reproductive workers	50.147	0.862	<0.05	*
queens x workers	61.269	0.735	<0.01	**
reproductive workers x workers	0.772	0.031	1.000	n.s
***Dolichovespula saxonica* - eggs**				
	F value	R^2	*p* value	Sig. Level
queens x workers	1.843	0.068	0.131	n.s
***Dolichovespula media* - eggs**				
	F value	R^2	*p* value	Sig. Level
queens x workers	5.507	0.113	<0.001	***
***Vespa crabro* - eggs**				
	F value	R^2	*p* value	Sig. Level
queens x workers	10.207	0.169	<0.001	***
***Vespula germanica* - eggs**				
	F value	R^2	*p* value	Sig. Level
queens x workers	1.701	0.038	0.156	n.s
***Dolichovespula saxonica – Dufour*'s gland**				
	F value	R^2	*p* value	Sig. Level
all groups	3.141	0.385	<0.001	***
queens x virgin queens	6.722	0.770	1.000	n.s
queens x reproductive workers	1.139	0.275	1.000	n.s
queens x workers	4.998	0.294	0.066	n.s
virgin queens x reproductive workers	2.067	0.407	1.000	n.s
virgin queens x workers	2.648	0.180	0.318	n.s
reproductive workers x workers	2.870	0.180	0.180	n.s
***Dolichovespula media – Dufour*'s gland**				
	F value	R^2	*p* value	Sig. Level
all groups	4.860	0.267	<0.001	***
queens x virgin queens	6.668	0.454	<0.05	*
queens x reproductive workers	1.609	0.138	1.000	n.s
queens x workers	3.266	0.098	0.060	n.s

virgin queens x reproductive workers	8.637	0.463	<0.01	**
virgin queens x workers	6.395	0.175	<0.01	**
reproductive workers x workers	4.586	0.125	<0.05	*
***Vespa crabro* – *Dufour*'s gland**				
	F value	R^2	*p* value	Sig. Level
queens x workers	40.890	0.650	<0.001	***
***Vespula germanica* – *Dufour*'s gland**				
	F value	R^2	*p* value	Sig. Level
all groups	2.790	0.166	<0.01	**
queens x reproductive workers	4.942	0.291	<0.01	**
queens x workers	4.346	0.158	<0.01	**
reproductive workers x workers	0.222	0.010	1.000	n.s

Identification of putative fertility cues

Several hydrocarbons differed significantly when we compared all the queen and reproductive worker samples (CHCs, eggs and *Dufour*'s gland) compared to sterile workers (Figure 4, Table S20, Table S21 and Table S22). The heatmap UPGMA cluster analysis of the three species revealed that samples clustered primarily based on their group (CHCs, eggs or *Dufour*'s gland) regardless of their caste. Secondarily, samples of *D. saxonica* formed a separate cluster including eggs and *Dufour*'s gland (Figure 4a). Samples of *D. media* and *V. germanica*, on the other hand, formed a cluster including *Dufour*'s gland and another one including both CHCs and eggs (Figure 4b, 4c). In summary, these results indicate that each source (CHCs, eggs and *Dufour*'s gland) has its own relative proportion for the three species, since samples did not cluster based on their correspondent caste. We indicate some putative fertility cues that are interesting for each of the species, although in our comparisons, most of them were not significative. In *D. saxonica*, the linear alkanes n-C29 and n-C31 stand out as queen pheromones (Oi et al. 2016), they are highly produced by both queens and reproductive workers and their levels do not differ between the two groups ($p > 0.05$). Interestingly, the methyl alkane 3-MeC29 and the linear alkane n-C29 were found in higher amounts in eggs and in the *Dufour*'s gland of queens and reproductive workers of *D. saxonica*, however the differences were not significant ($p > 0.05$). In *D. media*, the only CHC previously identified as queen pheromone was the alkane n-C28, which was not different between queens and reproductive workers ($p > 0.05$). The eggs of *D. media* have higher levels of 3-MeC27 and 3-MeC29, but neither were statistically different between queen and reproductive workers ($p > 0.05$). *Dufour*'s gland samples did not have similar proportions of 3-MeC27 and 3-MeC29 compared to eggs. In *V. germanica*, the putative queen pheromone is n-C27. We found high levels of 3-MeC27 covering the surface of eggs and *Dufour*'s gland content, but neither n-C27 nor 3-MeC27 differed between queens and reproductive workers ($p > 0.05$).

Is Dufour's gland the source of egg compounds in Vespinae wasps?

Our data suggests that chemical compounds that cover the surface of eggs are more similar to the chemical composition of *Dufour's* gland only for *D. saxonica*. For *D. media* and *V. germanica*, we found a different pattern, with the chemical composition of eggs being chemically closer to those found over the cuticle (CHCs) (Figure 4).

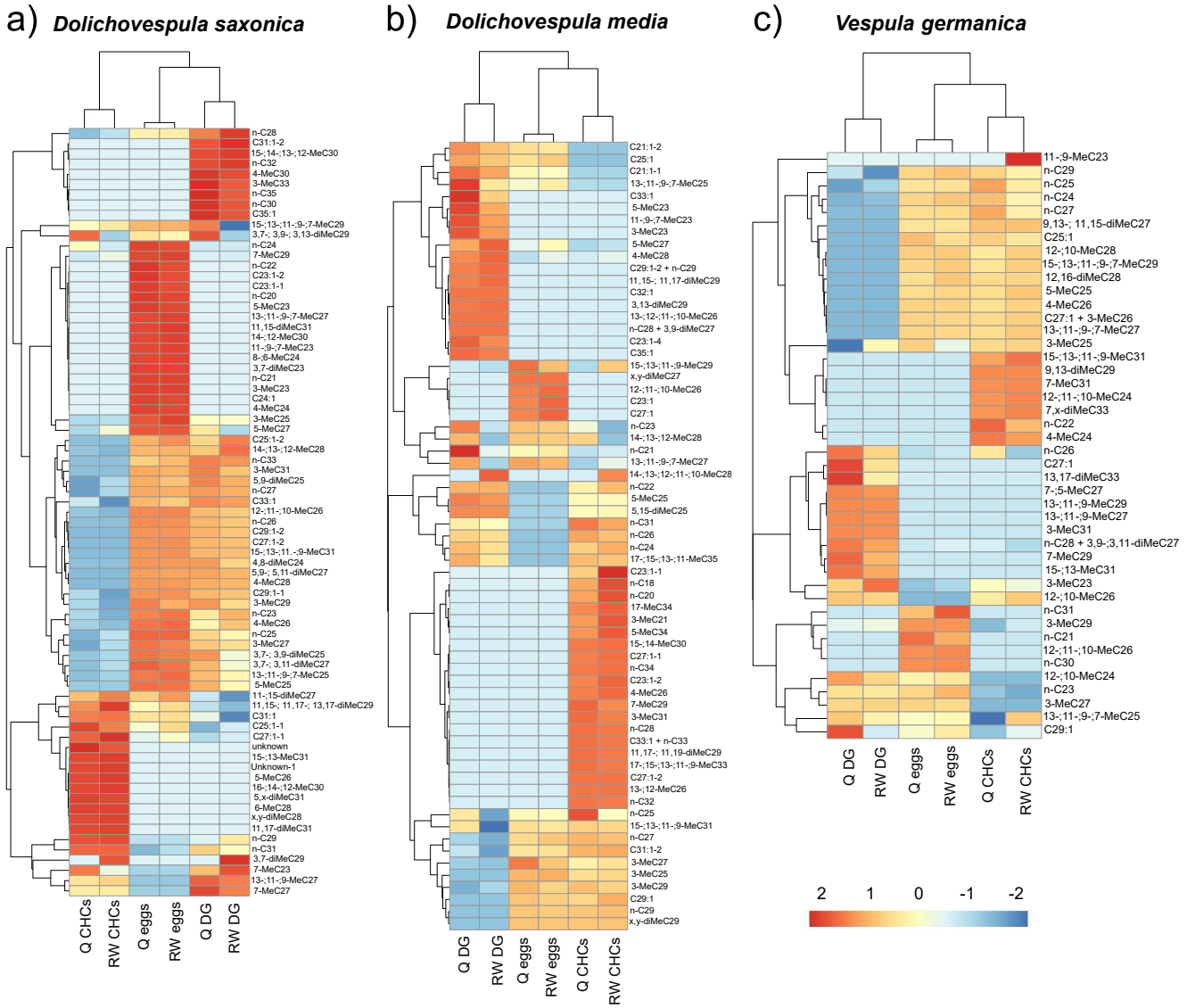

Figure 4: Heatmaps representing the differences in relative abundance of compounds (log-transformed) in the chemical profile of the cuticle (CHCs), egg surface (eggs) and in *Dufour*'s gland (DG) secretion for the three species (a) *Dolichovespula saxonica*, (b) *Dolichovespula media* and (c) *Vespula germanica*. Colours represent the mean fold difference in relative abundance of each hydrocarbon found for each of the three groups of samples. Compounds were clustered based on a UPGMA clustering using Euclidean distance as the distance metric. Q = queen; RW = reproductive worker; Q eggs = queen-laid eggs, RW eggs = reproductive worker-laid eggs.

Are egg-marking cues species-specific?

According to the PERMANOVA analysis, egg-marking cues varied significantly among queen-laid eggs from different species ($p = 0.006$ among the six pairwise comparisons) and among worker-laid eggs from different species ($p = 0.006$ among the six pairwise comparisons). The PCA analysis revealed that the combination of the first two PCs explained a total of 80.58% of the overall variation among egg hydrocarbons found over queen-laid eggs (Figure 5a), and a total of 62.79% of the overall variation was detected among egg hydrocarbons found over worker-laid eggs (Figure 5b). In both cases (queen-laid and worker-laid eggs) samples clustered primarily according to their species (Figure 5a and 5b). According to the SIMPER analysis, the top five compounds from each pairwise comparison based on queen-laid egg samples included three linear alkanes (n-C25, n-C27 and n-C29), four alkenes (C29:1, C29:1 – 1, C29:1 – 3 and C31:1), and five methyl-alkanes (3-MeC25, 13-;11-;9-;7-MeC27, 3-MeC27, 15-;13-;11-;9-;7-MeC29 and 3-MeC29). Whereas the top five compounds from each pairwise comparison based on worker-laid egg samples included three linear alkanes (n-C25, n-C27 and n-C29), three alkenes (C29:1 – 1, C29:1 – 3 and C31:1) and three methyl-alkanes (13-;11-;9-;7-MeC27, 15-;13-;11-;9-;7-MeC29 and 3-MeC27) (Table S24). Interestingly, a certain chemical convergence was detected between queen-laid and worker-laid eggs from different species. Eggs laid by *D. media* females were represented by 3-MeC27 and 15-;13-;11-;9-;7-MeC29; eggs laid by *D. saxonica* females were represented by C29:1 1 and C31:1; eggs laid by *V. germanica* females were represented by n-C27 and 13-;11-;9-;7-MeC27; eggs laid *V. crabro* females were represented by n-C25 and C25:1 - 3. The fact that these hydrocarbons were conserved between queen-laid and worker-laid eggs from each species indicates that they could potentially be species-specific. The egg-marking cues from *V. crabro* were the most divergent for both queen-laid and worker-laid eggs (Figure 5). Even though being statistically different, the egg-marking cues from *D. media* and *D. saxonica* were the most similar (Figure 5).

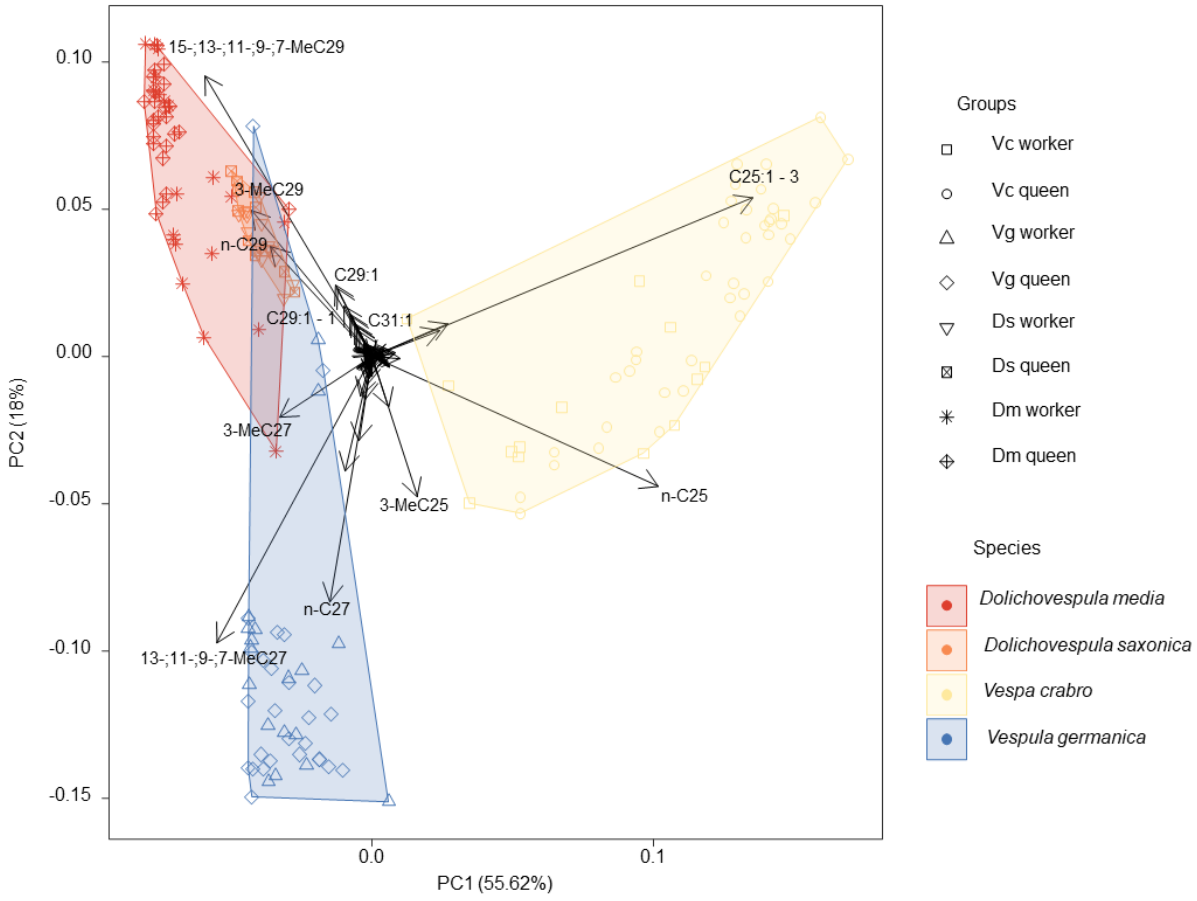

Figure 5: Principal component analysis (PCA) plots based on the chemical information identified in the surface of (a) queen-laid and (b) worker-laid eggs. Compounds represent the top five hydrocarbons highlighted by the SIMPER analysis (the ones the most likely explain the variation among samples from different species). Vc = *Vespa crabro*, Vg = *Vespula germanica*, Ds = *Dolichovespula saxonica* and Dm = *Dolichovespula media*.

Discussion

Our results demonstrate that the chemical composition of cuticular hydrocarbons (CHCs), egg surface, and *Dufour*'s gland differs according to caste in the four Vespinae wasps, *D. saxonica*, *D. media*, *V. germanica* and *V. crabro*. The CHC samples were the most chemically divergent for the four species. Moreover, for egg samples a significant difference was found in the permutations across two species *D. media* and *V. crabro*, whereas for the other two species *D. saxonica* and *V. germanica*, only a subtle variation was detected. In the eggs, the most representative compounds, based on SIMPER analysis, were the 3-MeC29 for *D. saxonica*, which was found in higher proportions in queen-laid eggs; while for *V. germanica* it was the compound 3-MeC27. For *Dufour's* gland samples, the majority of the pairwise comparisons were statistically different based on the overall chemical composition. In *D. saxonica*, variation was driven by specific compounds, queens had higher proportions of 15-;13-;11-;9-MeC29 and *n*-C31, and virgin queens had a higher proportion of an alkene C27:1. We also found that the chemical composition varies primarily according to their sources (CHCs, eggs and *Dufour'* gland) rather than caste. In samples of *D. saxonica* we detected a higher chemical resemblance between eggs and *Dufour*'s gland. In samples of *D. media* and *V.*

germanica, although there is an overlap among compounds of eggs and *Dufour*'s gland, a higher chemical resemblance was detected between samples of CHCs and eggs. The queen-linked egg-maternity compounds 3-MeC27 and 3-MeC29 previously reported in *V. vulgaris* (ref?) potentially reflect fertility/maternity status in the species explored here. In *D. saxonica* and *D. media*, we found that the same methylated hydrocarbons are overproduced in queen and reproductive worker samples (egg and *Dufour*'s gland samples). In *V. germanica*, we detected the 3-MeC27 in similar proportions in egg and *Dufour*'s gland of queen and reproductive worker samples. Finally, we found that the compounds covering the eggs differed between species, for both queen-laid and worker-laid eggs. These results suggest that in addition to carry information regarding egg maternity (queen-laid or worker-laid), the compounds released in the eggs during oviposition also carry species-specific information.

The CHCs that were most specific to queens based SIMPER analysis were *n*-C29, n-C31, and 5,x-diMeC31 for *D. saxonica*; 3-MeC29, 7-MeC29, and *n*-C31 for *D. media*; *n*-C25, *n*-C27, and 3-MeC27 for *V. crabro*; and *n*-C25, *n*-C26, and *n*-C27 for *V. germanica*. Among these compounds, *n*-C25, *n*-C26, *n*-C27, 3-MeC27, *n*-C29, 3-MeC29, *n*-C31 were previously reported to be queen-specific in Vespinae wasps (Butts et al. 1991; Van Oystaeyen et al. 2014; Oi et al. 2016; van Zweden et al. 2014; Derstine et al. 2018; da Silva et al. 2021). The correlation among certain CHCs and castes is linked to the fact that these chemical molecules are overproduced as a consequence of female fertility (=ovary activation) in social insects (Izzo et al. 2010; Oi et al. 2015a; Holman 2018; Honorio et al. 2019). Here, we show that the chemical variation is not only restricted to CHCs in *D. saxonica*, *D. media*, *V. crabro*, and *V. germanica*, but rather extended to other sources (e.g. *Dufour*'s gland and hence eggs). The overproduction of certain CHCs is also common in queens of Polistinae wasps (Santos et al. 2018; Soares et al. 2021; Kelstrup et al. 2014; Oi et al. 2019; Tannure-Nascimento et al. 2008; Layton et al. 1994).

The hydrocarbon cues that cover social insect eggs are used in policing behavior. Specifically, in Vespinae wasps, in the common wasp *V. vulgaris* the hydrocarbon 3-MeC29 signals egg maternity and enables egg policing (Oi et al. 2015b). The same compound was identified in higher proportions on the egg surface and in the *Dufour*'s gland of queens in three out of the four species *D. saxonica*, *D. media*, and *V. germanica*. In *V. crabro*, however, 3-MeC29 was found only in a higher proportion in CHC and *Dufour*'s gland samples. Queens that live in large colonies can potentially use egg-marking as an alternative strategy of communication, by advertising their presence through compounds deposited onto the eggs and nest surface, without requiring direct contact with the workers (some examples include *Myrmica rubra* - Brian and Rigby 1978 and *Aphaenosgaster cockerelli* - Hölldobler and Carlin 1989). In the relatively small colonies, where individuals frequently meet, as in the nests of *Polistes* wasps, females use the same strategy to selectively detect and destroy eggs based on their egg maternity (Liebig et al. 2005). In addition to possibly containing information that reflects maternity, we found that eggs also carry information that is specific to their species. We did not find evidence for chemical similarity among compounds covering the egg surface of queens of different species, implying the non-existence of a specific compound responsible for informing egg maternity in the different Vespine wasps. However, compounds that could be potentially tested in bioassay for each species would be 3-MeC27 and 15-;13-;11-;9-;7-MeC29 (*D. media*), C29:1 1 and C31:1 (*D. saxonica*), n-C27 and 13-;11-;9-;7-MeC27 (*V. germanica*) and n-C25 and C25:1 – 3 (*V. crabro*).

The link between hydrocarbons and species is not only restricted to the body surface in Vespinae wasps but is also extended to other components such as eggs and *Dufour'*s gland (Derstine et al. 2018). The *Dufour*'s gland is proposed to be a homologue of the colleterial gland, which then helps to attach the eggs (Mitra 2013). In social insects besides releasing compounds that may have similar functions, the secretions of the *Dufour*'s gland may have acquired secondary functions (social functions) (Sarmiento 2020; Ross & Matthews 2018). One of these functions could be related to egg protection and egg-marking (Sarmiento 2020). Our results show chemical similarities between the *Dufour*'s gland and egg source, so this could potentially indicate that compounds present in the gland can somehow cover the surface of eggs (egg-marking pheromones) in *D. saxonica.* For the other two species, *V. germanica* and *D. media, Dufour*'s gland can potentially work as the source of compounds that partially compose egg-marking surface, as we detected some overlap between eggs and *Dufour'*s gland. The fact that the chemical composition of *Dufour*'s gland and eggs do not fully match is not surprising, as eggs can acquire compounds through the direct contact with adults and nest surface. For instance, queens touch the eggs with their antennae from time to time (personal observation), furthermore, it is possible that during this process queens release chemical compounds from other sources such as cephalic glands. In fact, trophallactic fluids exchanged by ants of the species *Camponotus floridanus* contain several chemical molecules, among them several long-chain hydrocarbons (LeBoeuf et al. 2016). Similarly, the cephalic salivary gland in honeybees stores several hydrocarbons and oleic acids (Martin et al. 2018). Further research should consider checking functions of exocrine glands in social wasps, as many are still unknown (Ross & Mathew 2018). For example whether cephalic fluids released by queens of Vespinae can be deposited into the eggs and how *Dufour'*s gland compounds can be transferred to the eggs.

Overall, chemical quantitative variation was detected across samples in the four Vespinae species studied. This means that females from different castes have a similar chemical composition of CHCs, hydrocarbons deposited on their eggs, and also *Dufour*'s gland content, but the CHC proportions are not the same. The only exceptions are for CHC samples of *D. saxonica*, and *V. germanica* and also for *Dufour*'s gland samples of *V. crabro*, in which females from different castes differed either quantitatively or qualitatively. In conclusion, our results reinforce the hypothesis that hydrocarbons must play a central role in the chemical communication system of Vespinae wasps, once they are caste-linked. Additionally, we demonstrate that the chemical variation associated with caste exists across different components – cuticular surface, eggs' surface, and *Dufour*'s gland content. These differences likely contribute synergically to the communication between castes within the same nest and between females from different nests. The connection between *Dufour*'s gland composition and eggs' surface suggests that this gland is potentially used by queens to mark their eggs. Further research is necessary to determine empirically whether one or more of the hydrocarbons present in higher quantities in queen samples (CHCs and egg-marking compounds) act indeed as queen signals in *D. saxonica*, *D. media*, *V. crabro*, and *V. germanica*. Lastly, it will be important to address in the future whether other exocrine glands, as cephalic glands, also contribute to the chemical surface of eggs.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Rafael Carvalho da Silva and Cintia Akemi Oi contributed to the study conception and design, material preparation, data collection and analysis. Cintia Akemi Oi, Tom Wenseleers and Fabio Santos do Nascimento were responsible for obtaining funding. Cintia Akemi Oi, Tom Wenseleers and Fabio Santos do Nascimento were responsible for project administration. Rafael Carvalho da Silva wrote the first draft of the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript version.

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