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CHEMICAL CHARACTERIZATION OF YOUNG VIRGIN QUEENS AND MATED EGG-LAYING QUEENS IN
THE ANT *Cataglyphis cursor*

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Abstract- Social insects are well known for their extremely rich chemical communication, yet their sex pheromones remain poorly studied. We analysed the cuticular hydrocarbon profiles and Dufour's gland contents of queens of different ages and reproductive statuses (sexually immature gynes, sexually mature gynes, mated and egg-laying queens) and of workers in the thermophilic and thelytokous ant *Cataglyphis cursor*. Random forest classification analyses show that the four groups of individuals were well separated for both chemical sources, except mature gynes that clustered with queens for cuticular hydrocarbon and with immature gynes for Dufour's gland. Analyses carried out with two groups of females only allowed identifying candidate chemicals for queen signal and for sexual attractant. In particular, gynes produce more undecane in the Dufour's gland. This chemical is both the sex pheromone and the alarm pheromone of the ant *Formica lugubris*. It may therefore

possibly act as sex pheromone in *C. cursor*, and/or be involved in the restoration of monogyny that occurs rapidly following colony fission. Indeed, new colonies often start with several gynes and all but one are rapidly culled by workers, and this process likely involves chemical signals between gynes and workers. These findings open novel opportunities for experimental studies of inclusive mate choice and queen choice in *C. cursor*.

Key Words- Queen pheromone, sex pheromone, cuticular hydrocarbons, Dufour's gland, random forest.

Acknowledgments- None

INTRODUCTION

Mate attraction and mate choice are essential features of living organisms (Clutton-Brock and Mcauliffe 2009, Jennions and Petrie 1997; Stearns and Hoekstra 2005;). They rely on different modalities of communication depending on species. For instance, birds predominantly rely on acoustic and visual signals (songs, plumage and courtship dances), mammals use acoustic, visual and chemical cues, and insects and arachnids mostly rely on tactile, acoustic and chemical signals. Social insects are well known for their extremely rich chemical communication. In ants, chemical trails often underline foraging, and cuticular hydrocarbon (CHC) profiles allow nestmate recognition (Hefetz 2007; van Zweden and d’Ettorre 2010) and the signalling of queen fertility (Holman et al. 2010; Holman et al. 2013; Le Conte and Hefetz 2008; Monnin 2006; van Oystaeyen et al. 2014). However, although ant semiochemicals are well studied in many regards their sex pheromones remain poorly studied (Ayasse et al. 2001).

Young, virgin, queens (hereafter gynes) attract males and advertise their sexual receptivity with volatile signals produced by the Dufour’s gland, the venom gland or the pygidial glands (reviewed in Ayasse et al. 2001; Hölldobler and Wilson 1990). To our knowledge, only two ant sex pheromones have been identified, in *Formica lugubris* (Walter et al. 1993) and in *Polyergus breviceps* (Greenberg et al. 2007). The chemical bouquet produced by a queen changes once she is mated, as reflected in modifications of the relative abundances of cuticular hydrocarbons within hours or a few days from mating in several ant species (Hora et al. 2008; Monnin 2006; Oppelt and Heinze 2009) and in the honeybee (Babis et al. 2014). This is because mated queens never re-mate (Boomsma 2013) hence need not attract males anymore whereas they need to advertise their fertility to colony members (Foitzik et al. 2011; Hannonen et al. 2002; Holman et al. 2010; Holman et al. 2013; Keller and Nonacs 1993; Le Conte and Hefetz 2008; Monnin 2006; Ruel et al. 2013; van Oystaeyen et al. 2014).

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65 We address this scarcity of knowledge of ant sex pheromones by examining the production
66 of chemicals by queens of different ages and reproductive statuses in the thermophilic and
67 thelytokous ant *Cataglyphis cursor*. Identifying queen signals would be particularly rewarding in this
68 species where inclusive sexual selection and culling of supernumerary queens occur and likely involve
69 chemical signals. In *C. cursor*, gynes do not fly. They mate at the nest entrance, where foreign males
70 gather (Cronin et al. 2011; Lenoir et al. 1988). Gynes show no mate choice, but workers harass males
71 seeking to mate (Helft et al. 2015; Helft et al. 2016). How males are attracted to the nest entrance
72 and how they identify sexually receptive gynes are unknown in *C. cursor*, but they likely rely on
73 chemical signals. Newly mated gynes then disperse with workers to found new colonies (dependent
74 colony foundation, Chéron et al. 2011a; Cronin et al. 2013; Lenoir et al. 1988). Many new colonies
75 are temporarily polygynous, but this state is transitory and monogyny is restored within a few days
76 (Chéron et al. 2011a; Cronin et al. 2012). How monogyny is restored is unknown, but it likely entails
77 some degree of competitive chemical signalling of newly-mated queens vying for colony ownership.
78 Identifying potential queen signals would therefore allow designing precise key bioassays and studies
79 on inclusive sexual selection and queen competition in this interesting ant species.

80

81 Queen chemistry has not been studied in *C. cursor* and very little is known from other
82 *Cataglyphis* species (Dahbi and Lenoir 1998; van Oystaeyen et al. 2014), even though worker
83 chemistry has been studied in *C. cursor* (Hefetz and Lenoir 1992; Nowbahari et al. 1990) and in other
84 *Cataglyphis* species (Ali et al. 1988; Dahbi et al. 2008; Gökçen et al. 2002; Hefetz and Lenoir 1992).
85 The CHC profile of three mature, egg-laying, *C. iberica* queens was compared with that of their
86 workers. It revealed no qualitative differences but higher relative abundances of three linear alkanes
87 ($n\text{-C}_{27}$, $n\text{-C}_{28}$, $n\text{-C}_{29}$) and one dimethylalkane (dime C_{29}) and a lower relative abundance of two
88 methylalkanes (11- and 13-me C_{27}) in queens (Dahbi and Lenoir 1998). The treatment of orphaned
89 workers with a mixture of linear alkanes and methylalkanes ($n\text{-C}_{27}$, $n\text{-C}_{29}$ and 3-me C_{29}) repressed their

ovarian development, suggesting that these chemicals function as queen pheromones (van Oystaeyen et al. 2014).

To our knowledge, no gyne (i.e. young virgin queen) has been studied in *Cataglyphis*. We analyse here the cuticular hydrocarbon (CHC) profiles and Dufour's gland (DG) contents of egg-laying queens, sexually receptive gynes, gynes too young to be sexually receptive and workers of *C. cursor*. Our hypothesis is that chemicals over-expressed in egg-laying queens and in sexually receptive gynes may be candidate components of queen pheromones and sexual pheromones, respectively.

METHODS AND MATERIALS

Sixteen complete colonies of *C. cursor* were collected in Argelès-sur-Mer, Southern France (42.5722508°N, 3.043670°E), on the beginning of March 2014 (1 colony) and March 2015 (15 colonies) (Table 1). This is right after the end of hibernation and immediately prior the onset of sexual production in April-May, with mating taking place in May-June (Chéron et al. 2011a; Cronin et al. 2011). Colonies contained 642 ± 496 workers (mean \pm SD, range 168-1857) and were set up in the laboratory within a week of the collection. Each colony was housed in a plaster nest contained in a 35 x 25 x 12 cm box as foraging arena, kept under a 12:12 day:night light cycle and 25-28°C, provided with sugar and water *ad libitum*, and fed mealworm and crickets twice to thrice a week. Three colonies were queenless by late April, when we started monitoring sexual production (Table 1). We retained them because *C. cursor* workers produce new queens by thelytokous parthenogenesis when orphaned (Cagniant 1979; Chéron et al. 2011b).

Only four colonies, including the three orphaned colonies, produced sexuals in the laboratory (Table 1). These four colonies produced gynes, but only two colonies (one queenright and one

orphaned) produced males. Note that orphaned colonies mainly produce diploid males who behave like haploid males and can mate and fertilise the queen (Doums et al. 2013b). Males were removed upon emergence to prevent them from dispersing to other colonies and mate, hence gynes remained virgin. We sampled 25 gynes between 11th of May and 5th of June 2015. Twelve were sampled when they were one to five days old (mean age of three days, hereafter gynes G0) while the remaining 13 were sampled at eight to 11 days old (mean age of 10 days, gynes G1). Gynes G0 and G1 are sexually immature and sexually mature, respectively, and this was confirmed by the shedding of wings and dissections (see results). They originated from four and three sexuals-producing colonies, respectively (Table 1). This introduces some pseudo replication in our dataset. This is common in chemical studies of social insect sexuals because of the difficulties for obtaining samples from many independent colonies. Our study focuses on chemicals involved in behaviours that are not expected to differ in any consistent way between colonies (mate attraction, competition between young queens for colony “ownership”) hence these chemicals should not be affected by colony effects and our result should be robust. Gynes were immediately stored at -80°C when collected, until later dissection and chemical extraction. In addition, 13 queens (Q) and 16 workers (W) were collected on the 9th of June hence totalling 54 individuals (Table 1).

Chemical extraction and dissections were carried out at the end of June. Each ant was individually retrieved from the freezer immediately before its chemical extraction and dissection. We recorded whether gynes were winged or dealated as a cue of sexual maturity independent from our age-based groups of Gynes G0 and G1. Wing loss is indicative of mating in the field where 83.5% of virgin gynes vs. 16% of mated gynes were winged (Doums et al. 2013a). We then separated the gaster from the rest of the body. The gasterless body was immersed in 200 µl of pentane for 5 min in order to extract the cuticular hydrocarbons. At the same time, we dissected the gaster to collect the Dufour’s gland, which was extracted in 100 µl of pentane. Lastly, we dissected the reproductive apparatus and recorded mating status and ovarian development.

Chemical Analysis

Samples were analysed by gas chromatography-mass spectrometry (GC-MS). Each sample was evaporated at ambient temperature under an extractor hood and re-diluted with 70 µl of pentane. Two µl of the solution were injected using an Agilent G4513A Automatic Liquid Sampler into an Agilent Technologies 7890A gas chromatograph (capillary column: Agilent HP-5MS, 30 m × 0.25 mm × 0.25 µm; split–splitless injector; carrying helium gas at 1 mL/min) coupled with an Agilent 5975 C mass spectrometer with 70 eV electron impact ionization. The injector was set at 280°C and the temperature programme was as follows: an initial hold at 70°C for 1 min, then 70°C to 175°C at 10°C.min⁻¹, and up to 320°C at 5°C.min⁻¹ and hold for 3 min.

Peak areas were integrated with MSD ChemStation software version E.02.01.1177 (Agilent technologies). We used the best profile for each chemical source (CHC, DG) and each group of individuals (W, G0, G1, Q) to select and identify the peaks to include in our analyses. We identified chemical compounds by their retention time, fragmentation pattern and comparisons with published results on *Cataglyphis* species including *C. cursor* (Ali et al. 1988; Dahbi et al. 2008; Gökçen et al. 2002; Hefetz and Lenoir 1992; Keegans et al. 1992; Nowbahari et al. 1990).

Statistical Analysis

The results were analysed separately for each chemical source (CHC, DG). We used random forest classification analysis (RF) (Liaw and Wiener 2002) to see whether the four groups (egg-laying queens, sexually mature gynes, sexually immature gynes, workers) could be discriminated and to identify peaks that best discriminate groups. RF is a method increasingly used in chemical studies, including the chemical ecology of social insects (Jansen et al. 2016; Mitra et al. 2014; Oliveira et al.

2016). It is a tree-based machine learning tool for analysing multivariate dataset with more variables than individuals (Goldstein et al. 2011), and this is typically the case in chemical analyses. In addition, RF deals well with multicollinearity, assumes no specific distribution of the variables and does not require transformation of constrained data (such as chemical compounds) (Ranganathan and Borges 2010).

We used R v3.2.2 (R Core Team 2016) with Rstudio v1.0.44 (RStudio Team 2016). We used the relative abundances of peaks (i.e. proportions each peak represents relative to the sum of all peaks) computed from the raw dataset, including all the zero values. RF were ran using the package randomForest (Liaw and Wiener 2002). We performed RF using 10,000 trees to determine the importance of each peak in classifying the individuals in the four groups. We used the default value for the number of input variables randomly selected to build each node of the tree (denoted by *mtry*), that is the squared root of the number of variables (seven in our case). We also used the default value (33%) for defining the set of observations not used for building the current tree, i.e. the out of bag sample (OOB sample). This OOB sample is used to generate the confusion matrix and OOB error rate (i.e. rate of unsuccessful assignment of the OOB samples to the correct group). A low OOB error rate indicates a high ability of the indicator variables to predict the group of origin of the individual. The separation of the groups was visualized using a multidimensional scaling plot based on a proximity matrix of the individuals on the RF (i.e. the number of times they end up on the same leaf in the trees) (function MDSplot).

For each chemical source, as a second step, we tried to identify the peaks that are the most important for queen recognition by comparing queens vs. workers, and for sexual attraction by comparing gynes G1 vs. gynes G0 and gynes G1 vs. queens. To do so, we ran three additional RF analyses, each contrasting only the two groups of interest, and used the package VSURF (Genuer et al. 2015) to identify the peaks important in discriminating the two groups. The measure of variable

importance is based on the decrease of classification accuracy when the observed values of the given variable are randomly permuted in the OOB samples (Genuer et al. 2015). The selection procedure is based on three steps. The first (thresholding step) ranks variables according to a score of importance, and eliminates variables with a score lower than a threshold value. The second selects the variables needed to best discriminate the groups of interest. Embedded RF models are grown, starting with a RF build with only the most important variable and finishing with a RF with all the variables retained in the previous step, and the best model is selected on the basis of the means OOB error. The third step refines the previous selection for a predictive purpose and was therefore not used here. As above, the RF models were run with 10,000 trees and a sample of seven peaks for each node. The selection procedure for each comparison was run 20 times to assess the stability of the variable selection process. For each comparison, the chemical compounds were therefore ranked by their level of importance in discriminating the two groups and we averaged their ranks over the 20 runs of the variable selection process.

RESULTS

The dissections confirmed that all gynes were virgin, as expected since males were removed from colonies to prevent mating. Gynes G0 were all winged and had inactive ovaries without oocytes or with a few small and non-yolky oocytes at most. Seven gynes G1 were dealated (three partially and four fully) and six were winged. Gynes G1 had slightly more developed ovaries than gynes G0: five had small non-yolky oocytes while the remaining eight had small but yolky oocytes. This supports our hypothesis that gynes G1 were sexually mature because dealation is a good indicator that a gyne is mated in nature (Doums et al. 2013a). All queens were mated and had active ovaries with yellow bodies, except the queen of colony 1268 which showed regressing ovaries indicative of aging.

Table S1 lists the compounds identified from the cuticle (60 peaks) and Figure S1 provides representative chromatograms for each group of individuals. Similarly, Table S2 lists the compounds from the Dufour's gland (36 peaks) and Figure S2 shows representative chromatograms. The compounds identified include alkanes, methylalkanes and alkenes. In addition, some acetates and alcohols were also found in the DG.

Cuticular Hydrocarbons

The RF analysis with the four groups of individuals shows that workers were clearly separated from sexual females (gynes and queens) along the first axis, while the three groups of sexual females were separated along the second axis (Figure 1). Gynes G0 were clearly separated from queens and most gynes G1 clustered with queens, although two clustered with gynes G0. That is, there was a gradual transition of the CHC profile from G0 to G1 and queens. The OOB error rate (i.e. individuals not correctly assigned to their group) was 12.96% and most misclassifications occurred between gynes G1 and either gynes G0 (2 errors) or queens (4 errors, Table S3).

In order to identify the compounds most important for differentiating two given types of females (Q vs. W; G1 vs. G0; G1 vs. Q), we ran three additional RF analyses considering only two groups of individuals at a time, for both CHC and DG. We selected the variables (compounds) of importance for the discrimination using the package VSURF and ran each variable selection process 20 times to assess the stability of the selection process (see Methods and Materials). The results show that the importance of chemical compounds was stable over the 20 runs. The first step of the variable selection procedure (thresholding step) retained 45 to 51 peaks out of 60 peaks (i.e. 75-85%). Most compounds were consistently ranked with the same importance, and when variation occurred it was small. The variable selection procedure was reliable for the three paired

comparisons, and thus for the sake of clarity we present the 20 peaks with the highest averaged ranks over the 20 runs (Figure 2).

The Q/W comparison aimed at identifying chemicals that may be queen signals. The OOB error rate was much lower (3.45%) than when considering the four groups. The 20 peaks with the highest averaged rank of importance show a clear queen/worker difference in terms of the weight of their CHCs, with the heavier compounds being more abundant in queens than in workers (Figure 2A). The second step of the variable selection procedure retained a single compound as needed for obtaining the best discrimination (Peak 26 = 3-meC₂₇). However, this chemical was more abundant in workers than in queens (Table S1) hence is unlikely to be a queen signal.

The comparison gynes G1 vs. gynes G0 aimed at identifying compounds differentiating sexually mature and sexually immature gynes. In agreement with the results obtained when considering the four groups of individuals (Figure 1), gynes G1 and G0 were well differentiated (OOB error rate of 8%). The second step of the selection procedure identified three chemicals as necessary for best discrimination (P35 = *n*-C₂₉, P44 = 14-meC₃₀ + 8,12-dimeC₃₀ and P50 = 11,13-dimeC₃₁), among which one was more abundant in gynes G1 and two were more abundant in gynes G0 (Figure 2B).

Last, we compared gynes G1 and queens. Again, in agreement with the results when considering the four groups of individuals (Figure 1), they were rather poorly differentiated (OOB error rate of 15%). Nine of the 20 peaks with the highest averaged rank of importance were more abundant in queens than in gynes G1, and these comprised the four heaviest and the five lightest compounds (Figure 2C). Three peaks were required for the best discrimination (P53 = *n*-C₃₃, P47 = *n*-C₃₁ and P54 = 15+17-meC₃₃), and they were more abundant in queens and among the four heaviest compounds.

Dufour's Gland

The RF analysis with the four groups of individuals shows that the first axis separates workers from sexual females, and that the second axis separates sexual females (Figure 1). Gynes G0 and G1 clustered together and were well separated from queens. That is, there was a gradual transition of the DG content from G0 and G1 to queens, but while gynes G1 clustered with queens for CHC they clustered with gynes G0 for DG. The OOB error rate was 11.11%, and most misclassification occurred between gynes G0 and G1 (DG: 5 errors, Table S3).

We performed the same variable selection procedure than for CHC, using RF to identify the compounds most important in differentiating two given types of females. As for CHC, the variable selection procedure was stable over the 20 runs. The first step of the procedure (thresholding step) retained 24 to 35 peaks out of 36 peaks (67-97%) and for the sake of clarity we present the 20 peaks with the highest averaged ranks over the 20 runs (Figure 3).

The RF comparing queens and workers yielded a low OOB error rate of 3.45%, in agreement with the results obtained when including the four groups. As for CHC, there was a very clear queen/worker difference in terms of the weight of chemicals, with all but one of the heavier compounds being more abundant in queens than in workers (Figure 3A). The second step retained one compound only as needed for best discrimination, but this compound varied across runs (it was either peak 27 = $C_{27:1}$, P31 = $C_{29:1}$ or P33 = $C_{31:2}$). This is because these three compounds were correlated hence the information they provide for discriminating the two groups was redundant. It is worth noticing that the three compounds are structurally related (alkene) and more abundant in queens than in workers (Table S2).

The comparison of gynes G1 and G0 yielded a high OOB error rate (20%), which is also in agreement with the results obtained when considering the four groups of individuals where G1 and G0 clustered together (Figure 1). The second step of the selection procedure showed that three peaks are needed for the best discrimination (P15 = C_{19:2}, P12 = me-C₁₇ and P21 = C_{23:1}), including two more abundant in gynes G1 and one more abundant in gynes G0 (Figure 3B).

Last, we compared gynes G1 and queens. They were perfectly differentiated (OOB error rate of 0%), in agreement with the comparison using the four groups of individuals (Figure 1). Most of the peaks more abundant in queens were heavy compounds (Figure 3C). The second step selected three peaks (P8 = tridecanol, P7 = dodecanyl acetate and P1 = *n*-C₁₁). All were more abundant in gynes G1 and among the light compounds (Figure 3C), which is consistent with their putative use as volatile attractants of foreign males for mating.

DISCUSSION

We examined the changes in chemical production that *C. cursor* sexual females undergo as they age and mate, by comparing virgin and sexually immature queens (gynes G0), virgin and sexually mature queens (gynes G1) and mated and egg-laying queens. We included workers as a control and analysed the chemical compounds from the cuticle and the Dufour's gland. As expected, the four groups of individuals were overall well discriminated, for the two chemical sources. Subsequent analyses contrasting two types of females each (queens vs. workers, gynes G1 vs. gynes G0 and gynes G1 vs. queens) pinpointed the chemical compounds that are good candidate as chemical signals. Compounds that were of high importance for discriminating queens from workers and that were more abundant in the former may be queen signals. Similarly, compounds that were important in

discriminating gynes G1 from G0 or from queens and that were more abundant in gynes G1 may be male attractants or chemicals involved in the restoration of monogyny.

The branched alkane 3-meC₂₇ was important in discriminating queen and worker CHC profiles. This is in apparent agreement with previous studies that have found 3-methylalkanes to be characteristic of queen vs. worker CHC profiles in several social insect species (3-meC₂₉ in *C. iberica*: Oystaeyen et al. 2014; from 3-meC₂₅ to 3-meC₃₅ in several *Lasius* species: Holman et al. 2013; Oi et al. 2015). However, 3-meC₂₇ was less abundant in *C. cursor* queens than workers, whereas in contrast 3-methylalkanes are more abundant in *C. iberica* and *Lasius* queens than workers (Holman et al. 2013; van Oystaeyen et al. 2014). It is therefore doubtful that 3-meC₂₇ could be (a component of) the queen signal in *C. cursor*. Other compounds that were both important in discriminating queen and worker CHC profiles and more abundant in the former may constitute the queen signal. These include linear alkanes (*n*-C₃₁ and *n*-C₃₃), methylalkanes (13+15-meC₃₁ and 15+17-meC₃₃) and alkenes (C_{23:1}, C_{31:1} and C_{33:1}) (Figure 2 and Table S1). In addition, *C. cursor* queens also differed from workers by producing more alkenes (C_{27:1}, C_{29:1} and C_{31:2}) in the Dufour's gland. Chemicals of these types have been identified as putative queen signals in several other social insect species (e.g. Le Conte and Hefetz 2008; Oi et al. 2016; van Zweden et al. 2014) including the congeneric *C. iberica* (Dahbi and Lenoir 1998; van Oystaeyen et al. 2014). It is now relatively consensual that queen signals are honest (Heinze and d'Ettorre 2009; Keller and Nonacs 1993), but whether they are largely conserved across social insect species (Oi et al. 2016; van Oystaeyen et al. 2014) or variable (Amsalem et al. 2015; Evison et al. 2012; Smith et al. 2016) remains debated. The activity of the queen-characteristic compounds presently identified in *C. cursor* should be determined with an adequate bioassay, such as whether they inhibit (delay or decrease) egg-laying by orphaned workers (e.g. Oi et al. 2016). The queen is multiply mated in *C. cursor* and it would also be worth investigating the sensitivity of worker patriline to queen pheromones, to test for instance whether it correlates negatively with their

propensity to reproduce in presence of their queen (i.e. behave as cheaters, Doums et al. 2013b) or to enter foreign colonies and reproduce (i.e. behave as social parasite, Chéron et al. 2011b).

CHC profiles also discriminated well gynes G0 from gynes G1, and this likely stems from the fact that gynes G0 had a callow-like chemical profile while gynes G1 had already started producing a queen profile given that they had started developing their ovaries. In contrast, DG contents did not differentiate between the two categories of gynes, which suggests that CHC and DG contents mature at different speeds. Complementary studies are necessary to get a better understanding of the maturation of gynes, for instance by using additional age groups of gynes to cover a larger range of age differences, or by sampling gynes performing sexual calling in the field (as in Walter et al. 1993).

Although the comparison between gynes G1 and gynes G0 did not allow identifying putative sex pheromones, the comparison of gynes G1 with queens was more informative. CHC profiles did not discriminate well these two types of females. This may be because gynes G1 had started developing their ovaries and producing queen pheromones. However, the Dufour's gland content allowed a perfect discrimination of gynes G1 and queens, in agreement with our expectation that virgin and sexually mature gynes G1 produce male attractants whereas mated and egg-laying queens do not. Undecane ($n\text{-C}_{11}$) was one of three components allowing this discrimination and it was more abundant in gynes G1 than in queens. Undecane is the main component of the sex pheromone of the ant *Formica lugubris* (Walter et al. 1993), opening up the possibility that it may be (a component of) the sex pheromone of *C. cursor*. The effect of undecane could be experimentally tested by recording males' responses to synthetic chemical deposited on filter paper, dummies (e.g. glass beads or dead individuals) or live individuals (workers or non-receptive queens). *C. cursor* workers are involved in inclusive sexual selection (Helft et al. 2015; Helft et al. 2016) and it would be interesting to test their possible contribution to male attraction. Workers use DG secretions for marking the surroundings of the nest entrance (Mayade et al. 1993) and by doing so they may release undecane and contribute to

attract males. Indeed, although each worker harbours little undecane in her DG (Table S2), workers may collectively deposit significant amounts.

Another possibility is that undecane is involved in the restoration of monogyny during the process of colony fission. Indeed, undecane has two functions in *F. lugubris*: sex pheromone in queens and alarm pheromone in workers (Walter et al. 1993). In *C. cursor* new colonies are founded by colony fission. They often start with several gynes but monogyny is restored within a few days (Chéron et al. 2011a) by fights between young queens and by attacks by workers (pers. obs.). Undecane could be a signal allowing physiologically more fertile gynes to mark less fertile gynes and thereby target worker attacks against the latter, as occurs in some other species in comparable contexts (de Souza et al. 2017; Monnin et al. 2002; Smith et al. 2012). The involvement of undecane in intra-colonial aggressions could be experimentally tested by recording workers' response to the deposit of synthetic chemical on live individuals (workers or non-receptive queens). A preliminary study indeed suggests that undecane triggers worker aggressions against male. When a worker and a male from another colony were placed together in a small box containing a filter paper, the worker attacked the male in 6 out of 18 replicates when the filter paper contained undecane whereas she did not when the filter paper was blank (0 out of 18 trials, Fisher exact test: $p = 0.019$).

Our results show that the complex chemical bouquet of *C. cursor* queens changes according to age, mating status and reproductive activity, as occurs in other species where mating triggers chemical switches (Babis et al. 2014; Hora et al. 2008; Oppelt and Heinze 2009). These changes are related to mate attraction and to queen signalling towards workers, and the identification of candidate chemicals is a first step towards identifying pheromones. These would in turn allow exciting studies of the chemical determinants of mating site selection by males, restoration of monogyny (i.e. queen choice) by workers, and the link between sensitivity to queen pheromone and cheating and social parasitism.

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Fig. 1 MDS plots of the proximity distance estimated from the Random Forest analysis including the four groups of individuals. The first two dimensions of the multidimensional distance matrix are shown for cuticular hydrocarbons and Dufour's gland content.

Fig. 2 Random forest analyses for cuticular hydrocarbons, comparing queens and workers (a), gynes G1 and gynes G0 (b), and gynes G1 and queens (c). For clarity, each plot only shows the 20 peaks with the highest averaged rank of importance. The plot shows average ranks, as well as minimal and maximal ranks when some variation occurred. The colour of the dots indicates the group of female in which the peak was more abundant (values from Table S1). Peaks importance for discriminating the two groups of females decrease from rank 1, the most important peak, to rank 20, the least important peak. Peaks needed to obtain the best discrimination (i.e. retained by the second selection step, see M&M) are encircled. Peak identification is given in Table S1.

Fig. 3 Random forest analyses for Dufour's gland content, comparing queens and workers (a), gynes G1 and gynes G0 (b), and gynes G1 and queens (c). Same legend than Figure 2. Peak identification and abundance are given in Table S2.

568 **Table 1** Colony origin of the 54 individuals analysed. Three colonies were queenless (QL) at the time
 569 of gyne production, and two colonies produced males in addition to gynes
 570

COLONY CODE	COLONY SIZE	MALES PRODUCED	INDIVIDUALS SAMPLED			
			QUEEN	GYNES G0	GYNES G1	WORKER
1206 QL*	606			2		1
1250	254		1			1
1251	1561		1			1
1252	877		1			1
1254	217		1			1
1257	430		1			1
1258 QL	459	4		5	6	1
1259 QL	1857			2	4	1
1260	168		1			1
1262	867		1			1
1263	299		1			1
1264	912		1			1
1265	185		1			1
1266	361		1			1
1267	883		1			1
1268	340	23	1	3	3	1
TOTAL			13	12	13	16

571 * collected in March 2014

572

Fig. 1

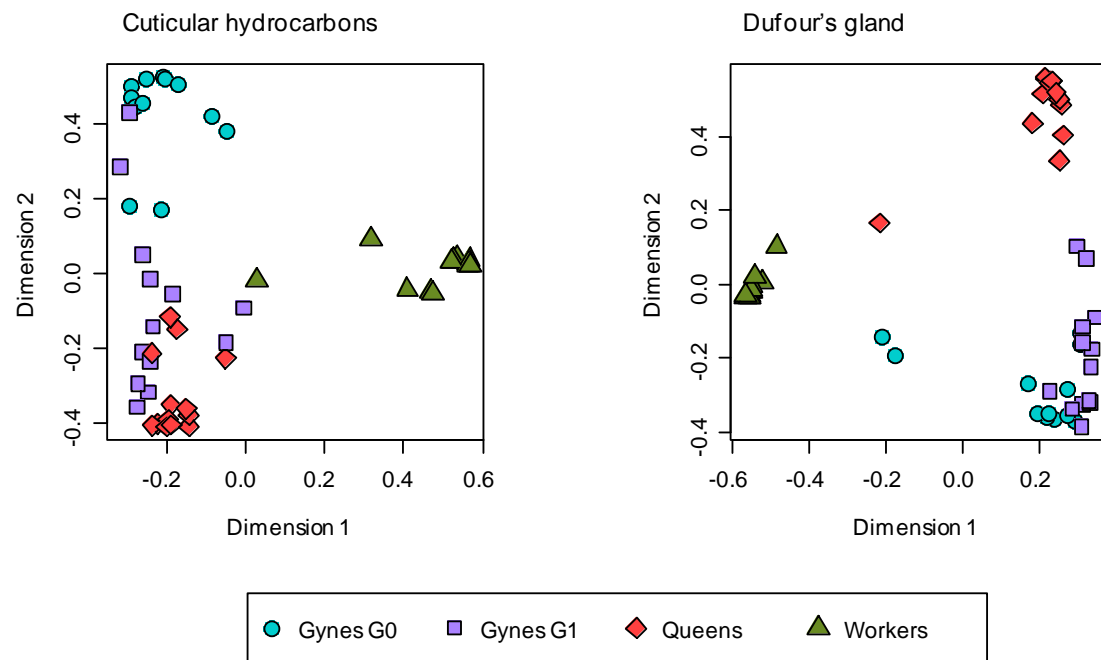


Fig. 2

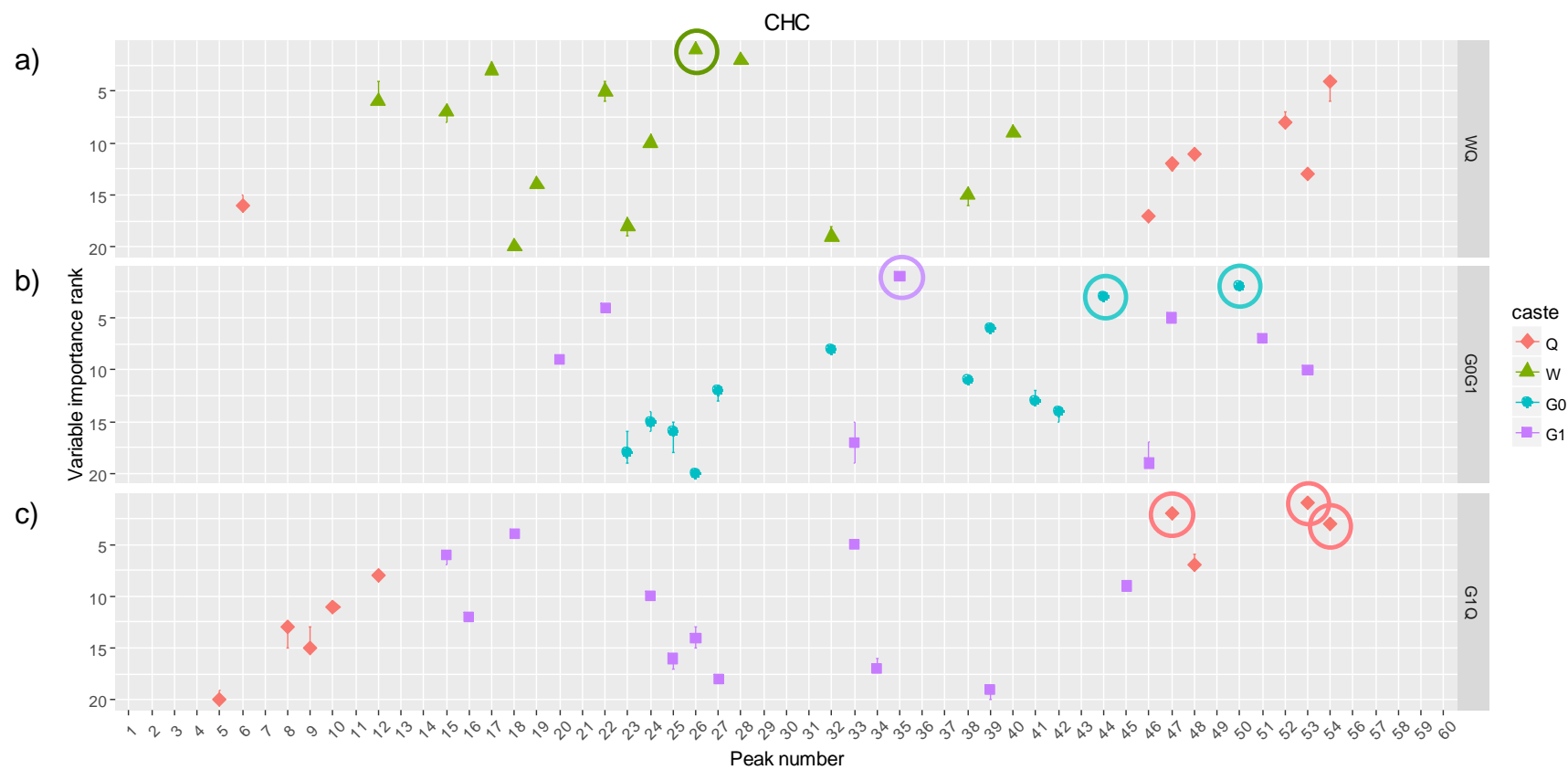
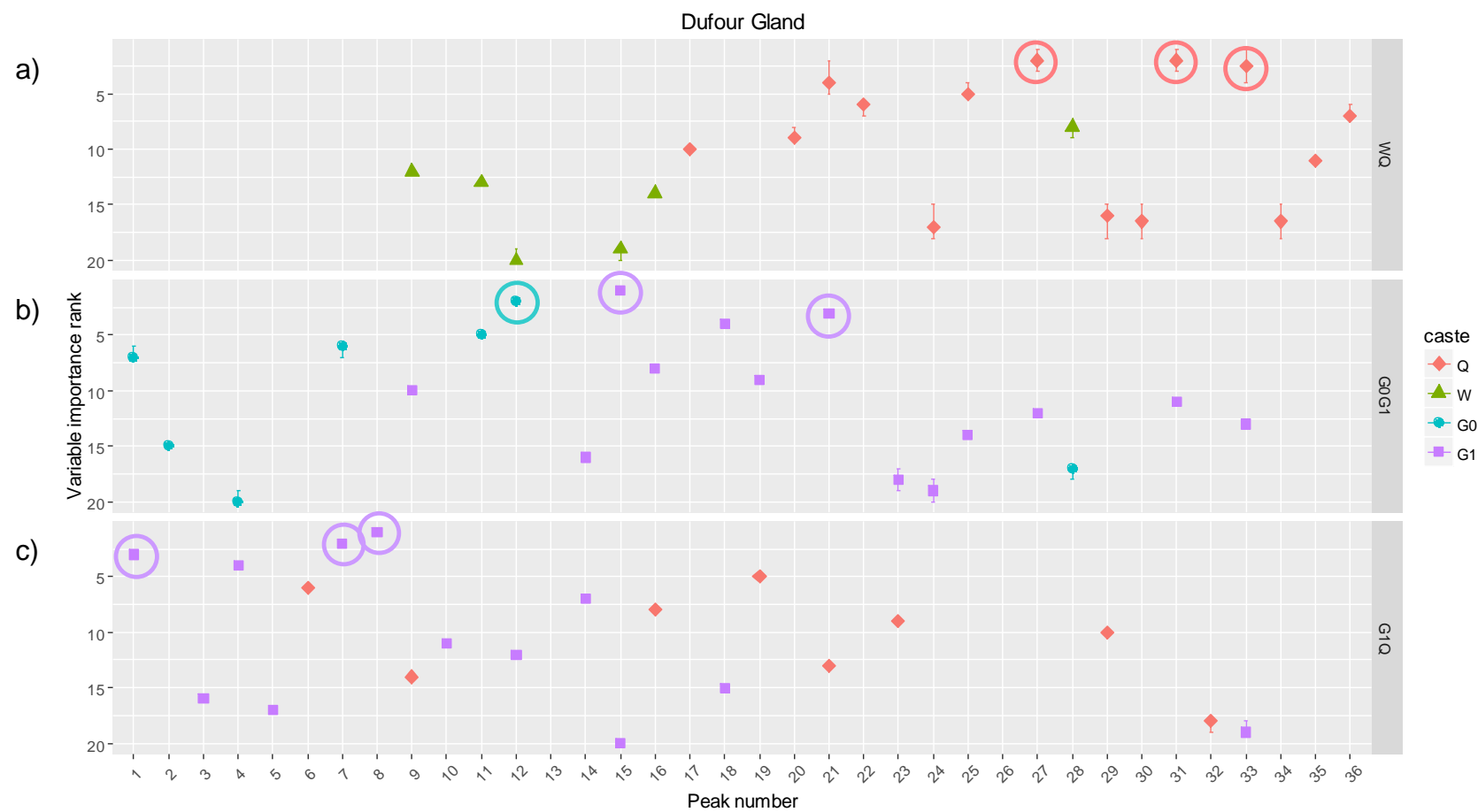


Fig. 3



Supplementary materials

Table S1 Relative abundance of peaks (percentage of total area) in extracts of cuticular hydrocarbons (CHC) of workers, gynes G0 and G1, and queens

PEAK NUMBER	PEAK IDENTIFICATION	RETENTION TIME	PEAK RELATIVE ABUNDANCE (MEAN % \pm SD)			
			WORKERS	GYNES G0	GYNES G1	QUEENS
P1	C _{21:1}	19.36	0.12 \pm 0.44	0.04 \pm 0.07	0.18 \pm 0.41	0.58 \pm 0.96
P2	<i>n</i> -C ₂₁	19.78	0.06 \pm 0.04	0.05 \pm 0.09	0.15 \pm 0.26	0.35 \pm 0.61
P3	x,y-dimeC ₂₁	20.40	1.01 \pm 0.44	0.17 \pm 0.10	0.41 \pm 0.34	1.43 \pm 2.05
P4	x,y-dimeC ₂₁	21.06	2.01 \pm 0.82	0.35 \pm 0.18	0.65 \pm 0.31	1.27 \pm 0.70
P5	<i>n</i> -C ₂₂	21.37	0.31 \pm 0.12	0.03 \pm 0.05	0.04 \pm 0.07	0.15 \pm 0.12
P6	C _{23:1}	22.56	0.01 \pm 0.03	0.09 \pm 0.14	0.42 \pm 1.07	1.05 \pm 2.01
P7	<i>n</i> -C ₂₃	22.97	0.14 \pm 0.13	0.14 \pm 0.18	0.32 \pm 0.63	0.74 \pm 1.60
P8	x,y-dimeC ₂₃	23.77	0.62 \pm 0.29	0.10 \pm 0.07	0.20 \pm 0.09	0.37 \pm 0.18
P9	x,y-dimeC ₂₃	24.41	1.35 \pm 0.61	0.21 \pm 0.14	0.43 \pm 0.18	0.85 \pm 0.49
P10	<i>n</i> -C ₂₄	24.60	0.73 \pm 0.23	0.12 \pm 0.08	0.16 \pm 0.10	0.57 \pm 0.81
P11	C _{25:1}	25.65	1.16 \pm 2.41	0.97 \pm 0.26	1.68 \pm 1.48	1.71 \pm 3.33
P12	<i>n</i> -C ₂₅	26.04	9.22 \pm 6.01	1.57 \pm 0.33	2.27 \pm 1.06	1.17 \pm 0.28
P13	11+13-meC ₂₅	26.54	0.51 \pm 0.23	0.42 \pm 0.06	0.37 \pm 0.10	0.34 \pm 0.16
P14	5-meC ₂₅	26.79	0.42 \pm 0.24	0.30 \pm 0.08	0.28 \pm 0.14	0.26 \pm 0.14
P15	3-meC ₂₅	27.11	2.04 \pm 0.90	1.51 \pm 0.46	1.30 \pm 0.43	0.69 \pm 0.19
P16	5,9-dimeC ₂₅	27.25	0.29 \pm 0.33	0.39 \pm 0.07	0.29 \pm 0.10	0.12 \pm 0.08
P17	<i>n</i> -C ₂₆	27.51	1.50 \pm 0.63	0.19 \pm 0.11	0.27 \pm 0.09	0.22 \pm 0.19
P18	3,7-dimeC ₂₅	27.63	0.98 \pm 0.49	1.57 \pm 0.60	0.98 \pm 0.34	0.41 \pm 0.23
P19	4-meC ₂₆	28.34	0.69 \pm 0.19	0.64 \pm 0.22	0.48 \pm 0.20	0.26 \pm 0.15
P20	C _{27:1}	28.60	2.57 \pm 1.45	3.55 \pm 1.15	6.97 \pm 2.15	4.44 \pm 2.60
P21	4,8-dimeC ₂₆	28.81	1.54 \pm 3.47	1.48 \pm 0.40	1.06 \pm 0.43	0.58 \pm 0.21
P22	<i>n</i> -C ₂₇	28.94	10.35 \pm 4.07	1.48 \pm 0.31	3.58 \pm 1.11	2.29 \pm 0.74
P23	11+13-meC ₂₇	29.40	2.68 \pm 0.40	4.09 \pm 0.43	2.83 \pm 0.98	1.81 \pm 0.72
P24	5-meC ₂₇	29.65	4.43 \pm 1.60	3.93 \pm 1.10	2.85 \pm 1.01	1.43 \pm 0.65
P25	11,15-dimeC ₂₇	29.80	0.40 \pm 0.74	0.58 \pm 0.18	0.39 \pm 0.19	0.13 \pm 0.14
P26	3-meC ₂₇	29.97	3.68 \pm 0.92	2.09 \pm 0.69	1.67 \pm 0.45	0.89 \pm 0.46
P27	5,9-dimeC ₂₇	30.08	3.43 \pm 1.17	5.47 \pm 1.16	3.82 \pm 1.32	2.12 \pm 0.99
P28	<i>n</i> -C ₂₈	30.34	1.04 \pm 0.77	0.62 \pm 1.28	0.20 \pm 0.09	0.17 \pm 0.12

P29	3,11-dimeC ₂₇	30.48	4.39 ± 1.71	8.45 ± 3.28	6.00 ± 2.54	3.40 ± 1.50
P30	12-meC ₂₈	30.77	0.79 ± 0.34	1.47 ± 0.36	0.92 ± 0.35	0.63 ± 0.31
P31	8,12-dimeC ₂₈	30.82	0.96 ± 0.46	2.40 ± 0.74	1.60 ± 0.80	0.85 ± 0.65
P32	4-meC ₂₈	31.13	0.58 ± 0.23	0.40 ± 0.06	0.25 ± 0.12	0.19 ± 0.19
P33	C _{29:1}	31.37	1.75 ± 0.76	2.44 ± 0.54	3.40 ± 0.77	2.23 ± 1.06
P34	4,12-dimeC ₂₈	31.53	1.70 ± 1.63	3.20 ± 0.67	2.26 ± 0.64	1.28 ± 0.55
P35	<i>n</i> -C ₂₉	31.68	5.55 ± 1.16	0.75 ± 0.27	3.18 ± 1.04	4.61 ± 1.55
P36	11+13+15-meC ₂₉	32.09	3.45 ± 1.08	7.28 ± 2.25	5.45 ± 1.60	5.08 ± 1.98
P37	7-meC ₂₉	32.21	0.56 ± 0.20	0.77 ± 0.11	0.59 ± 0.25	0.46 ± 0.24
P38	5-meC ₂₉	32.34	1.32 ± 0.44	0.84 ± 0.22	0.65 ± 0.17	0.47 ± 0.20
P39	7,11-dimeC ₂₉	32.60	0.36 ± 0.19	0.81 ± 0.14	0.43 ± 0.16	0.20 ± 0.19
P40	3-meC ₂₉	32.64	0.89 ± 0.47	0.40 ± 0.19	0.35 ± 0.09	0.22 ± 0.18
P41	5,9-dimeC ₂₉	32.74	2.40 ± 0.84	4.26 ± 0.62	2.75 ± 0.99	1.67 ± 0.87
P42	3,13-dimeC ₂₉	33.04	2.15 ± 0.79	4.50 ± 0.88	2.64 ± 1.08	1.65 ± 0.90
P43	3,7-dimeC ₂₉	33.09	1.17 ± 0.39	1.36 ± 0.27	0.10 ± 0.43	0.71 ± 0.25
P44	14-meC ₃₀ + 8,12-dimeC ₃₀	33.44	1.91 ± 0.77	3.59 ± 0.42	2.06 ± 0.58	1.80 ± 0.61
P45	C _{31:2}	33.77	0.82 ± 0.35	1.06 ± 0.40	0.85 ± 0.38	0.52 ± 0.16
P46	C _{31:1}	34.00	1.55 ± 0.58	2.65 ± 0.79	4.70 ± 1.66	4.47 ± 2.00
P47	<i>n</i> -C ₃₁	34.26	2.10 ± 0.83	0.20 ± 0.13	1.82 ± 1.09	7.62 ± 2.86
P48	13+15-meC ₃₁	34.62	2.27 ± 0.98	4.92 ± 2.68	4.17 ± 0.74	7.12 ± 3.22
P49	7-meC ₃₁	34.76	0.50 ± 0.43	0.29 ± 0.11	0.40 ± 0.12	0.55 ± 0.34
P50	11,13-dimeC ₃₁	35.55	1.65 ± 0.53	2.42 ± 0.37	1.27 ± 0.41	0.99 ± 0.74
P51	C _{33:2}	36.17	1.54 ± 1.30	3.11 ± 2.81	6.62 ± 3.97	6.93 ± 3.84
P52	C _{33:1}	36.42	2.56 ± 1.24	5.00 ± 1.90	6.42 ± 1.45	8.84 ± 2.65
P53	<i>n</i> -C ₃₃	36.68	0.57 ± 0.59	0.07 ± 0.14	1.00 ± 0.59	4.52 ± 2.03
P54	15+17-meC ₃₃	37.00	0.45 ± 0.25	1.15 ± 0.83	0.92 ± 0.31	1.90 ± 0.79
P55	C _{34:1}	37.44	0.07 ± 0.11	0.42 ± 0.28	0.24 ± 0.29	0.22 ± 0.52
P56	4, <i>y</i> -dimeC ₃₃	37.98	0.50 ± 1.15	0.40 ± 0.18	0.32 ± 0.47	0.33 ± 0.80
P57	C _{35:2}	38.45	0.55 ± 0.38	0.65 ± 0.48	1.26 ± 1.27	1.41 ± 1.00
P58	C _{35:1}	38.67	1.36 ± 0.46	1.99 ± 0.62	2.03 ± 1.46	2.58 ± 2.03
P59	<i>x,y</i> -dimeC ₃₅	40.12	0.28 ± 0.76	0.24 ± 0.17	0.07 ± 0.15	0.07 ± 0.18
P60	C _{37:1}	40.77	0	0.27 ± 0.28	0.18 ± 0.21	0.04 ± 0.13

Table S2 Relative abundance (percentage of total area) of peaks in extracts of Dufour's gland (DG) of workers, gynes G0 and G1, and queens

PEAK NUMBER	PEAK IDENTIFICATION	RETENTION TIME	PEAK RELATIVE ABUNDANCE (MEAN % \pm SD)			
			WORKERS	GYNES G0	GYNES G1	QUEENS
P1	<i>n</i> -C ₁₁	5.72	0.08 \pm 0.22	5.39 \pm 2.63	2.79 \pm 0.94	0.64 \pm 0.49
P2	<i>n</i> -C ₁₂	7.15	0.01 \pm 0.02	0.26 \pm 0.18	0.13 \pm 0.05	0.11 \pm 0.07
P3	<i>n</i> -C ₁₃	8.54	4.53 \pm 3.80	15.84 \pm 6.29	12.24 \pm 3.42	10.41 \pm 3.48
P4	Dodecyl acetate	9.98	0.02 \pm 0.03	0.29 \pm 0.24	0.23 \pm 0.14	0.00 \pm 0.00
P5	Dodecanol	10.82	0.27 \pm 0.43	0.93 \pm 1.45	0.31 \pm 0.35	0.09 \pm 0.13
P6	<i>n</i> -C ₁₅	11.09	2.56 \pm 1.68	2.13 \pm 1.32	2.10 \pm 0.42	3.24 \pm 0.67
P7	Dodecanyl acetate	12.46	21.76 \pm 8.46	20.72 \pm 5.23	14.41 \pm 3.63	5.21 \pm 3.32
P8	Tridecanol	12.78	0.20 \pm 0.20	0.36 \pm 0.23	0.41 \pm 0.10	0.08 \pm 0.06
P9	<i>n</i> -C ₁₇	13.67	6.78 \pm 1.23	1.49 \pm 0.96	2.02 \pm 0.36	2.69 \pm 0.95
P10	? (fatty acid)	13.80	0.32 \pm 0.16	0.89 \pm 0.47	1.10 \pm 0.19	0.76 \pm 0.32
P11	meC ₁₇	13.84	0.72 \pm 1.13	0.97 \pm 2.03	0.05 \pm 0.05	0.06 \pm 0.04
P12	meC ₁₇	14.44	1.29 \pm 2.05	1.77 \pm 3.46	0.21 \pm 0.31	0.09 \pm 0.07
P13	Tetradecanol acetate	15.22	1.42 \pm 0.66	3.25 \pm 2.21	4.21 \pm 1.35	4.29 \pm 2.22
P14	Farnesol acetate	15.75	0	0.14 \pm 0.15	0.26 \pm 0.11	0.06 \pm 0.06
P15	C _{19:2}	16.14	2.90 \pm 0.98	0.19 \pm 0.18	1.10 \pm 0.82	0.93 \pm 0.43
P16	C _{19:1}	16.25	44.39 \pm 9.89	4.90 \pm 2.83	9.86 \pm 3.10	17.62 \pm 6.49
P17	<i>n</i> -C ₁₉	16.60	1.32 \pm 0.31	2.92 \pm 1.80	3.71 \pm 1.04	3.59 \pm 1.01
P18	C _{21:2}	19.27	1.20 \pm 0.55	0.51 \pm 0.49	1.94 \pm 1.34	1.88 \pm 0.52
P19	C _{21:1}	19.36	2.76 \pm 0.96	1.17 \pm 0.75	2.44 \pm 0.80	4.41 \pm 0.87
P20	<i>n</i> -C ₂₁	19.77	0.07 \pm 0.11	2.41 \pm 1.48	2.87 \pm 1.33	2.13 \pm 0.70
P21	C _{23:1}	22.57	0.23 \pm 0.21	4.30 \pm 1.11	5.29 \pm 0.71	6.15 \pm 1.54
P22	<i>n</i> -C ₂₃	23.00	0	4.85 \pm 2.98	5.31 \pm 2.05	4.21 \pm 1.41
P23	Eicosenol	24.29	2.67 \pm 0.89	0.91 \pm 1.02	2.37 \pm 1.45	5.52 \pm 1.83
P24	Eicosyl acetate	24.68	0.02 \pm 0.05	0.38 \pm 0.37	0.56 \pm 0.25	0.83 \pm 0.43
P25	C _{25:1}	25.81	0	6.39 \pm 1.79	7.56 \pm 1.00	7.98 \pm 2.26
P26	<i>n</i> -C ₂₅	26.03	0.08 \pm 0.13	0.59 \pm 0.53	0.48 \pm 0.35	0.42 \pm 0.29
P27	C _{27:1}	28.60	0.01 \pm 0.04	3.24 \pm 1.68	4.41 \pm 0.55	4.69 \pm 0.80

P28	Dodecyl myristate	29.91	4.24 ± 2.578	0.89 ± 1.23	0.28 ± 0.28	0.31 ± 0.27
P29	Tetracosanal	30.22	0	0.32 ± 0.23	0.53 ± 0.18	0.83 ± 0.32
P30	Tetracosyl acetate	30.55	0	0.60 ± 0.54	0.86 ± 0.41	0.74 ± 0.38
P31	C _{29:1}	31.45	0	3.36 ± 1.98	4.27 ± 1.09	3.56 ± 0.95
P32	C ₃₀	32.96	0.09 ± 0.24	0.28 ± 0.25	0.49 ± 0.25	0.74 ± 0.43
P33	C _{31:2}	34.00	0	0.51 ± 0.37	1.00 ± 0.54	0.62 ± 0.24
P34	?	35.44	0	0.40 ± 0.30	0.63 ± 0.25	0.93 ± 0.45
P35	?	37.79	0.06 ± 0.25	1.81 ± 0.88	2.07 ± 0.65	2.66 ± 1.44
P36	?	40.11	0	4.60 ± 11.07	1.48 ± 0.51	1.51 ± 0.95

Table S3 confusion matrices of the random forests performed with the four groups of individuals

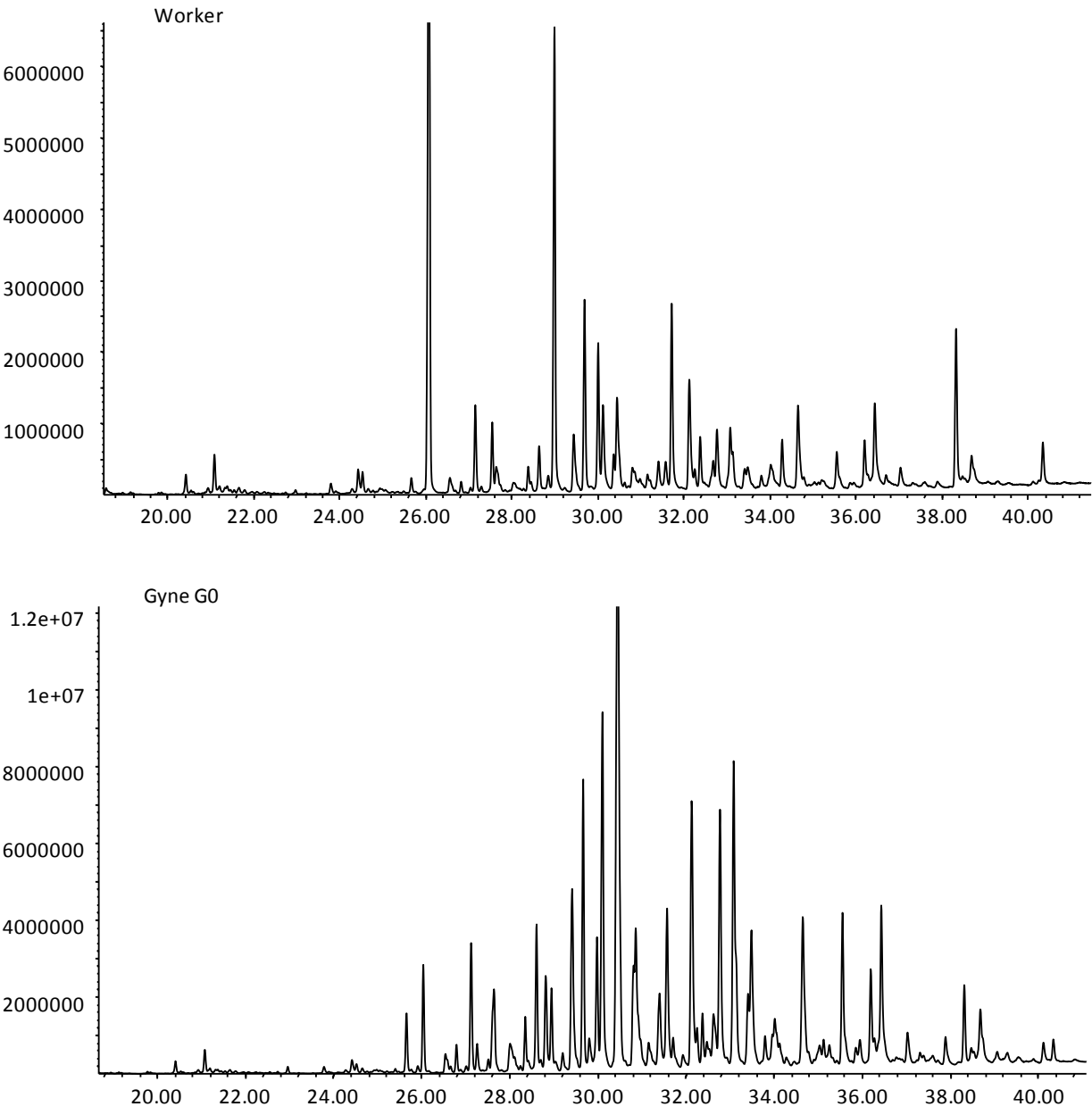
Cuticular hydrocarbons:

	G0	G1	Q	W	Classification error
G0	12	0	0	0	0
G1	2	10	1	0	0.231
Q	0	3	10	0	0.231
W	0	1	0	15	0.062

Dufour's gland:

	G0	G1	Q	W	Classification error
G0	9	3	0	0	0.250
G1	2	11	0	0	0.154
Q	0	0	12	1	0.077
W	0	0	0	16	0

Fig. S1 Representative chromatograms of cuticular hydrocarbon extracts for the four groups of individuals. X and y-axes are retention time and abundance, respectively.



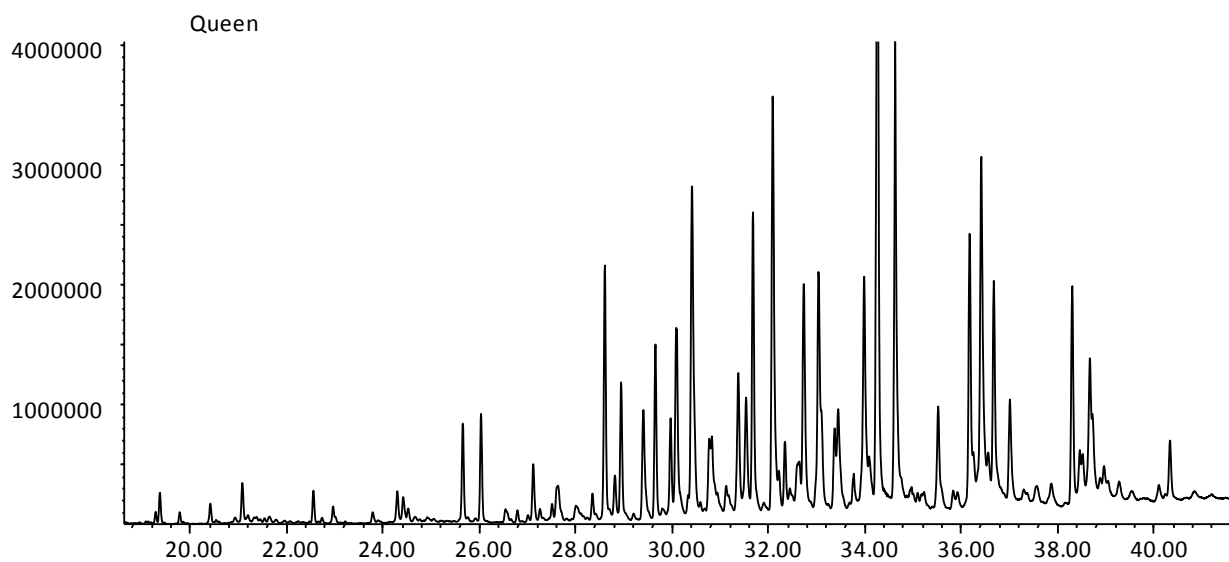
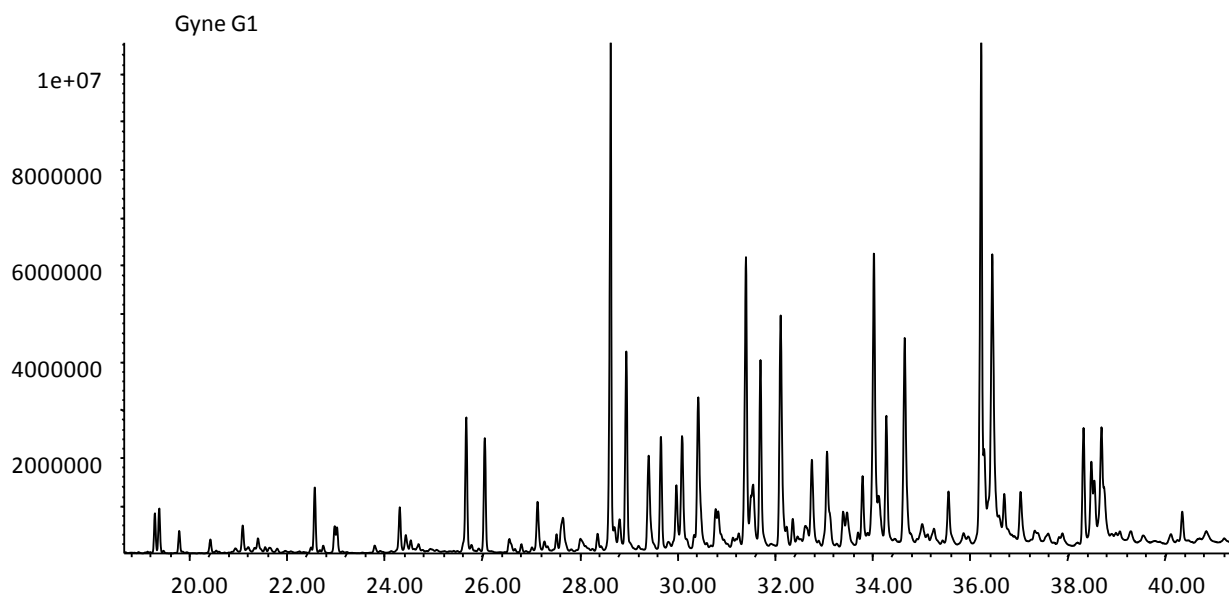


Fig. S2 Representative chromatograms of Dufour’s gland extracts for the four groups of individuals. X and y-axes are retention time and abundance, respectively.

