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# Surface lipids of queen-laid eggs do not regulate queen production in a fission-performing ant

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**Abstract** In animal societies, most collective and individual decision making depends on the presence of reproductive individuals. The efficient transmission of information among reproductive and non-reproductive individuals is therefore a determinant of colony organization. In social insects, the presence of a queen modulates multiple colonial activities. In many species, it negatively affects worker reproduction and the development of diploid larvae into future queens. The queen mostly signals her presence through pheromone emission, but the means by which these chemicals are distributed in the colony are still unclear. In several ant species, queen-laid eggs are the vehicle of the queen signal. The aim of this study was to investigate whether queen-laid eggs of the ant *Aphaenogaster senilis* possess queen-specific cuticular hydrocarbons and/or Dufour or poison gland compounds, and whether the presence of eggs inhibited larval development into queens. Our results show that the queen- and worker-laid eggs shared

cuticular and Dufour hydrocarbons with the adults; however, their poison gland compounds were not similar. Queen-laid eggs had more dimethylalkanes and possessed a queen-specific mixture of cuticular hydrocarbons composed of 3,11+3,9+3,7-dimethylnonacosane, in higher proportions than did worker-laid eggs. Even though the queen-laid eggs were biochemically similar to the queen, their addition to experimentally queenless groups did not prevent the development of new queens. More studies are needed on the means by which queen ant pheromones are transmitted in the colony, and how these mechanisms correlates with life history traits.

**Keywords** Social insect · *Aphaenogaster senilis* · Egg marking · Cuticular hydrocarbons · Queen pheromones

## Introduction

In animal societies, collective behaviors and individual decision making rely on an efficient transmission of information among colony members. Identifying reproductive potentials of other individuals is essential in mediating conflicts over reproduction and maintaining social cohesion. In social hymenopterans, queens have a central role in colony-level reproductive decisions. Their presence inhibits worker ovarian development and the production of numerous haploid male eggs (Hoover et al. 2003; Bhadra et al. 2010; Holman et al. 2010). Moreover, in several species, the queen prevents diploid larvae from developing into future queens (Vargo and Fletcher 1986; Vargo and Passera 1991), either directly by interacting with larvae or by affecting worker nursing behavior (Vargo and Fletcher 1986; Jarau et al. 2010; Penick and Liebig 2012). This phenomenon is manifest in species that reproduce by colony fission like the honeybee *Apis mellifera* and the gypsy ant *Aphaenogaster senilis* in which almost the entire diploid brood is reared into

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the worker caste. In both species, queens develop only on rare occasions, i.e., when the colony is large enough to fission or when the current queen dies or is experimentally removed (Winston et al. 1989; Boulay et al. 2007).

The means by which the queen signals her presence is still unclear for many species, but the size of the colony seems critical. In a small group, the queen may control workers and brood through physical suppression (Brian 1970; Bartels 1988; Peeters 1989; Retana and Cerdá 1990; Vargo and Passera 1991). However, as colony size increases, direct behavioral interactions rapidly become inefficient, and chemical communication prevails (Keller and Nonacs 1993). In the honeybee, the queen pheromones that inhibit worker ovarian development and queen rearing are secreted mostly by the mandibular glands and are composed of 9-oxo-2-decenoic acid (Barbier and Lederer 1960) and other minor components (Slessor et al. 1988; Katzav-Gozansky et al. 2001; Wessler 2002; Hoover et al. 2003). These secretions have a low volatility and are disseminated by physical contact. Messenger workers acquire the queen signal through extensive contact with the queen and redistribute it throughout the hive, thus informing the colony of the queen presence (Seeley 1979; Naumann et al. 1991).

The identity and function of queen pheromones is less well known in ants than in the honeybees. Yet, most studies point to cuticular hydrocarbons (HCs) being the main queen signal (Le Conte and Hefetz 2008). In the ant *Camponotus floridanus*, whose colonies contain several thousands of workers, the queen's eggs bear HCs that are specifically found on her cuticle. Workers presumably transport queen-laid eggs to the multiple nest chambers and, by so doing, inform their nestmates of the queen's presence. Hence, workers coming in contact with the queen-laid eggs refrain from reproducing (Endler et al. 2004, 2006). A queen-specific HC, 3-methylhentriacontane, was also found on queen-laid eggs in *Lasius niger* (Holman et al. 2010). This unique compound limits ovarian development in workers. In addition to informing the colony of the queen's presence, egg marking with queen-specific HCs allows workers to discriminate between queen-laid and worker-laid eggs, and thus conditionally destroy the latter (Ratnieks 1995; Monnin and Peeters 1997; D'Ettorre et al. 2004a; Shimoji et al. 2012). Egg marking by the queen is an important process in the social regulation of worker reproduction.

Few studies have tested whether the presence of queen-laid eggs also inhibits the production of new reproductive females. In *Solenopsis invicta* and *Linepithema humile*, the queen pheromones prevent diploid brood from developing into queens. These pheromones also trigger the workers to attack larvae oriented toward queens. However, the daily addition of eggs to queenless groups of workers and larvae did not limit the production of queens in either species (Vargo and Fletcher 1986; Vargo and Passera 1991).

We used the gypsy ant *A. senilis* as a model system to test whether the queen-laid eggs may be used as vehicle to distribute the queen signal within colonies and prevent larvae from developing into future queens. First, we analyzed queen-laid and worker-laid egg surface chemicals and compared them to adult cuticular compounds and secretions from two abdominal glands (the Dufour and poison glands). We hypothesized that some compounds are queen specific and are transmitted to the surface of queen-laid eggs. Second, we tested whether the frequent addition of fresh queen-laid eggs inhibited the production of queens from diploid larvae. If the queen-laid eggs prevent the production of queens, then we may expect the queen pheromone to be present on the egg surface.

## Methods

### Model system

*Aphaenogaster senilis* is a monogynous ant species that is distributed from Southern France to Morocco. Colonies contain an average of 1,260 workers (range, 120–3,900; Boulay et al. 2007). Nests are complex structures of chambers and tunnels excavated down to a depth of 70 cm in the ground. Although the brood and workers occupy all the chambers, the single queen is usually found in the deepest one.

Colonies were collected in Doñana National Park (South western Spain) and housed in the laboratory in  $\varnothing 10 \times 10$  cm circular boxes connected to  $\varnothing 2 \times 20$  cm test tubes. They were maintained in total darkness at  $28 \pm 1$  °C and  $50 \pm 10$  % humidity and fed three times a week with sliced mealworms (*Tenebrio molitor*), honey, and fruits.

Pictures of queen- ( $n=233$ ) and worker-laid eggs ( $n=78$ ) were taken with a stereomicroscope (Zeiss-Stemi2000) equipped with a digital camera (Axiocam lcc1 Zeiss). Egg volume was obtained using the formula:  $V = 1/2 \times \pi \times a \times b \times c$ , where  $a$ ,  $b$ , and  $c$  are the length of three perpendicular egg axis measured using the program AXIOVISION 2010 v.4.8.2.

### Chemical analyses

The queens of seven colonies were housed with 100 nestmate workers for 5 days. Eggs were collected after the 1st and the 5th day. Egg samples were prepared by pooling 10 eggs from the same queen. However, two queens apparently stopped laying eggs after 24 h. As a result, we were able to prepare seven samples of 10 1-day-old eggs but only five samples of 4-day-old eggs. The seven queens were then dissected to collect their thoraces, Dufour glands, and poison glands. The workers started laying eggs 8 days after queen removal. When 10 worker-laid eggs appeared in a queenless group, they were collected.

The chemical compounds contained on the ant thoraces, in their glands, and on the surface of the eggs were extracted in 50  $\mu\text{L}$  of dichloromethane and the extracts stored at 4 °C until chemical analyses. First, the HC profiles of the queens' thoracic cuticles were compared to those of two whole queenright workers per colony and the pools of 10 queen- or worker-laid eggs. Samples were injected into a gas chromatograph (GC 2010 Shimadzu) equipped with a flame ionization detector. Oven temperature was programmed to run from 130 to 240 °C at 15 °Cmin<sup>-1</sup>, and then from 240 to 300 °C at 3 °Cmin<sup>-1</sup>. Second, the profiles of eggs ( $n=10$  for queen-laid eggs and  $n=4$  for workers-laid eggs) were compared with those of adults for Dufour and poison gland secretions ( $n=6$  for queens and  $n=7$  foreign workers). The GC column temperature was programmed to run from 60 to 210 °C at 5 °Cmin<sup>-1</sup>, and then from 210 to 300 °C at 15 °Cmin<sup>-1</sup>. Two hundred nanograms of eicosane was added to each sample as an internal standard. Compound identification was achieved by performing mass spectrometry (GC-MS Perkin-Elmer) on 50 eggs, 1 queen, and 10 workers from 2 colonies under the same chromatographic conditions, and compared to previously published identifications (Lenoir et al. 2001; Boulay et al. 2007; Lenoir et al. 2011).

Compound proportions were standardized by subtracting the mean proportion of a compound across samples to each individual proportion and dividing by the result by standard deviation of the mean (see Boulay et al. 2007). By so doing, all the variables have a zero mean and unit standard deviation. The differences in chemical profiles were analyzed by means of multivariate analyses. The 18 and 12 major compounds present in cuticular and Dufour gland extracts, respectively, were selected. The proportions of these compounds, in at least half of the ants in one group, were more than 2 and 3 % of the total quantity of compounds. A principal component analysis was performed in order to reduce the number of variables. The factor scores of the first components explaining most of the variance in our data (45.9, 22.9, and 8.9 % for cuticular hydrocarbons, and 32, 21.1, 13.7, and 10.1 % for Dufour gland compounds) were used in a discriminant analysis to test whether the predefined groups (queen's eggs, workers' eggs, queen, and workers) could be distinguished according to their chemical profiles. Mann–Whitney tests were performed to compare the total amounts of compounds found on queens versus eggs, and the proportion of the single HCs found on adults versus eggs, between the adults, and between queen- and worker-laid eggs. The sequential Holm–Bonferroni correction was used to control for family-wise error across the multiple comparisons.

#### Effects of queen-laid eggs on larval fate

Thirty-three colonies were each divided into three groups of 200 workers collected inside the nest and 15 first-instar

larvae. Of the three identified larval instars, only the first was shown to be totipotent (Boulay et al. 2009). Each group was subjected to a different treatment. One group contained the mother queen (QR, hereafter) while the other two groups were queenless (QL and QL-egg). All the eggs produced in the QR group were carefully transferred with smooth forceps every second day to the corresponding QL-egg group. After 20 days, the queen and the eggs were removed from the QR and the QL-egg groups, respectively. Given that egg incubation time in this species is approximately 30 days (Ruel, personal observation), the eggs added to the QL-egg groups did not have time to hatch. Larvae were monitored every day observing throughout the test tube to record their gender, caste, survival rate, and development time until all surviving larvae had pupated. We also measured the egg-laying rate of 18 queens by counting the eggs transferred from the QR to the QL groups every second day during the first 16 days of experiment.

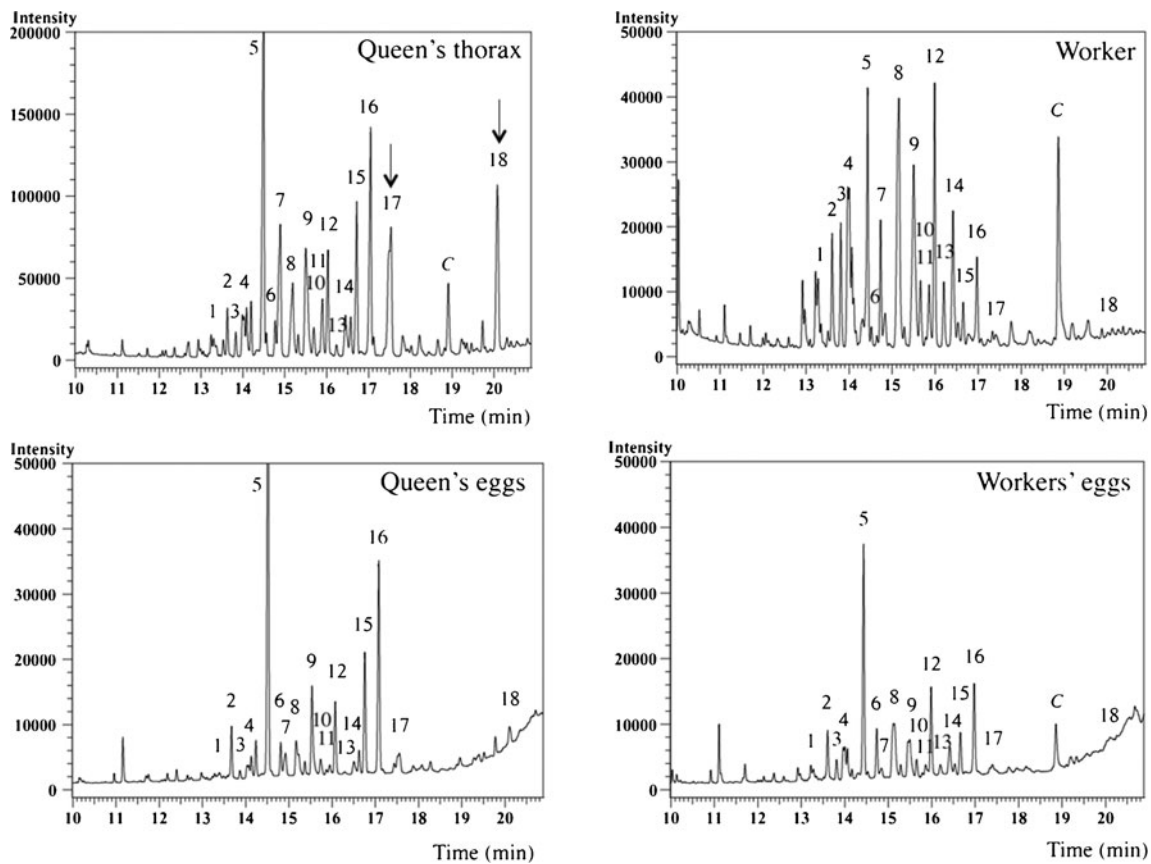
Data were analyzed with the R-package software v. 2.7.2 (R Core Team, Vienna, Austria). Two generalized linear mixed models (lme4 package) were fitted to compare the probability of producing at least one queen during the 40 days of experiment and the proportion of surviving larvae among the QR-, QL-, and QL-egg treatments. Both models were fitted using the binomial error distribution and logit link function. Colony was included as a random factor. For groups that produced at least one queen, a mixed model (nlme package) was fitted to compare the time to production of the first queen in each group among treatments. The response variable was square-root transformed to fit model assumptions.

## Results

### Adult long-chain cuticular hydrocarbons found on the egg surface

Forty-three long-chain saturated HCs (from 25 to 32 carbons) or mixtures of HCs were found on queens' and workers' cuticles and on fresh eggs, albeit in different amounts and proportions (Fig. 1). Discriminant analysis highlighted major differences between the profiles of both castes and their eggs (Fig. 2a). All samples types were well classified by the model. The first axis, which explained 86 % of the variance, distinguished the workers from the queens and their eggs. The second axis, which explained 13 % of the variance, separated the queens from their eggs.

Queen and worker profiles differed mainly in their percentage of eight compounds (Table 1). In particular, two mixtures of long dimethylalkanes, 3,11+3,9+3,7-dimethylnonacosane and 3,9+3,11-dimethylhentriacontane (peaks 17 and 18 marked with an arrow in Fig. 1), represented



**Fig. 1** Four examples of cuticular HC profiles of a queen's thorax, a nestmate worker, and 10 of their respective eggs. Numbers are given to the major compounds: 1=8,12+6,10DiMeC26, 2=C27, 3=4,8,12Tri-MeC26, 4=7+9+11+13MeC27, 5=3MeC27, 6=C28, 7=3,7+3,9DiMeC27, 8=x,yDiMeC28+10MeC28, 9=4MeC28, 10=

6,10DiMeC28, 11=4,8DiMeC28, 12=C29, 13=4,8,12TriMeC28, 14=11MeC29, 15=5MeC29, 16=3MeC29, 17=3,11+3,9+3,7DiMeC29, 18=3,9+3,11DiMeC31. C cholesterol. A complete list of cuticular HCs is given in Lenoir et al. (2001)

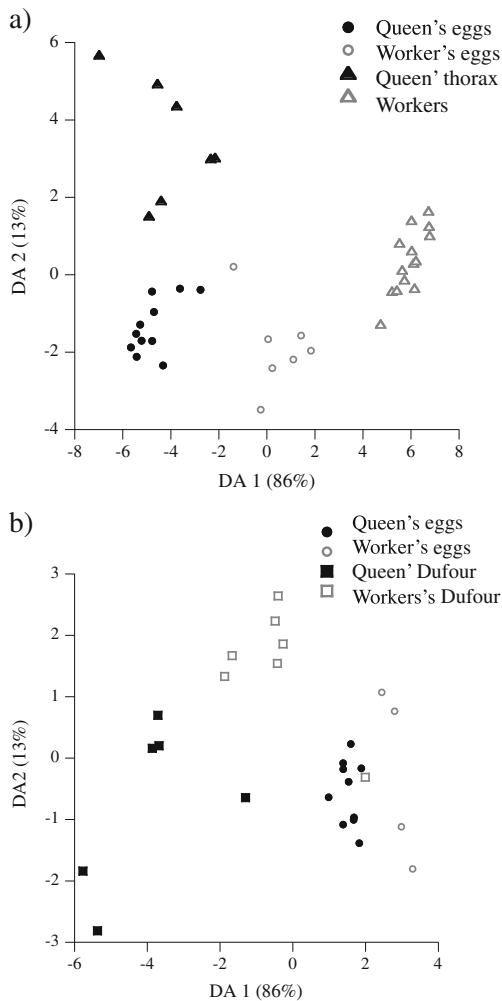
13.1±1.3 % (means ± SE) and 10.1±2.3 % of the queens' profiles, respectively, and were almost absent in workers (0.6±0.1 % and 0.5±0.1 %, respectively). More generally, a queens' cuticle contained relatively more dimethylalkanes and fewer monomethylalkanes than a worker's cuticle.

The HC profiles of 1- and the 4-day-old queen-laid eggs were similar and were consequently pooled together. The profiles of queens' and workers' eggs were dominated by 3-methylheptacosane (24±1.1 %; peak 5 in Fig. 1). Overall, queens' eggs contained significantly more long-chain dimethylalkanes and fewer linear alkanes than workers' eggs. The mixtures of 3,11+3,9+3,7-dimethylnonacosane (peak 17) and 3,9+3,11-dimethylhentriacontane (peak 18) were present in relatively low proportions on eggs, but the proportion of the former was significantly higher on queens' than workers' eggs. The total amount of HCs did not differ significantly between queens' and workers' eggs (87±9 ng vs 73±13 ng for volumes of 31.7±0.3 μm<sup>3</sup> vs 25.9±0.4 μm<sup>3</sup>; Mann–Whitney test:  $U=51$ ,  $N_1=12$ ,  $N_2=7$ ,  $P=0.482$ ).

Adult abdominal gland compounds found on the egg surface

The queens' and workers' Dufour glands contained 33 saturated and unsaturated HCs (from 13 to 19 carbons) plus small amounts of an aldehyde, hexadecanal (Appendix 1; Table 2). Twenty HCs were specific to the Dufour gland, as they were never present in the poison gland.

Thirteen of these Dufour-specific HCs were detected on the egg surface. The discriminant function analysis correctly classified 88.9 % of the samples and revealed extensive overlaps among the four groups (Fig. 2b). The first axis mainly separated the eggs, irrespective of their origin, from the adults. Hence, queen- and worker-laid eggs did not differ significantly with respect to major Dufour gland compounds. The total amount of Dufour HCs did not differ significantly between queens' and workers' eggs (20±3 ng vs 10±2 ng; Mann–Whitney test:  $U=28$ ,  $N_1=10$ ,  $N_2=4$ ,  $P=0.304$ ). The egg surface diverged from the adult glands by seven HCs. The eggs had higher proportions of tridecane, 5-methyltridecane, 9-methylnonadecane, an unidentified HC, and an unidentified terpene than the adults (Table 1).



**Fig. 2** Discriminant analysis of **a** cuticular HC profiles of queens' thoraces (*filled triangle*), entire workers (*empty triangle*), queen-laid eggs (*filled circle*), and worker-laid eggs (*empty circle*); **b** Dufour HC and alkaloids profiles of queens' glands (*filled square*), workers' glands (*empty square*), queen-laid eggs (*filled circle*), and worker-laid eggs (*empty circle*). The percentage of variance explained by the first two discriminants is given for each axis

Adults' Dufour glands, on the other hand, contained more pentadecane and nonacosene. The second axis of the discriminant function analysis mostly differentiated workers and queens. This difference was due to a higher proportion of 5- and 3-methylpentadecane in the former (in bold in Table 1).

Finally, 17 compounds, including HCs and three alkaloids (anabasine, anabaseine, and an unidentified alkaloid), were found in queens' and workers' poison glands (Appendix 2; Table 2). Specific poison gland compounds were absent from the surface of queen- and worker-laid eggs. Alkaloids were overrepresented in workers' and queens' poison glands, reaching  $66\pm 12.7$  and  $66.6\pm 8.4$  % of the total content, respectively. Queens and workers did not differ in any of the poison gland compounds.

### Effects of queen-laid eggs on larval fate

Out of 15 larvae initially including in the group,  $6.9\pm 0.7$  survived until the pupal stage in the QL-egg treatment. Larval survival was significantly lower in the QR treatment ( $5.2\pm 0.7$ ;  $Z=2.9$ ,  $P=0.004$ ). The QL treatment did not differ significantly from the two others (QL-egg vs QL:  $6.9\pm 0.7$  vs  $5.8\pm 0.5$ ,  $Z=1.861$ ,  $P=0.063$ ; QR vs QL:  $5.2\pm 0.6$  vs  $5.8\pm 0.5$ ,  $Z=1.05$ ,  $P=0.294$ ).

The queens laid a mean of  $24\pm 9.6$  eggs per day, showing large interindividual variation. The addition of queen-laid eggs to QL groups neither inhibited nor delayed queen production (Fig. 3). At least one larva developed into a queen in 44.4 % of the QL-egg groups. Their probability of producing at least one queen did not differ from that of the QL groups ( $Z=-0.237$ ,  $P=0.813$ ), in which queens were produced in 38.9 % of the time. As expected, the production of queens in the QR groups was low (13.9 %) and differed significantly from the QL-egg groups ( $Z=2.344$ ,  $P=0.019$ ).

The time to queen production did not differ between the QL and the QL-egg treatments ( $20\pm 1$  vs  $23\pm 2$  days respectively;  $t_{13}=1.556$ ,  $P=0.144$ ). However, they both produced queens significantly earlier than the QR treatment ( $35\pm 2$  days to the first queen; QL-egg vs QR,  $t_{13}=4.811$ ,  $P<0.001$ ; QL-egg vs QR,  $t_{13}=3.926$ ,  $P=0.002$ ), since queens started to be produced 10 days after removing the mother queen at the end of the treatment.

### Discussion

Ant queens advertise their presence by producing pheromones that have profound effects on the social harmony, reproduction, and resource allocation of their colonies. Depending on the species, the presence of a mated queen may inhibit worker reproduction and/or the development of larvae into future queens. So far, most studies point to long-chain cuticular HCs acting as the main ant queen pheromones. These compounds were shown to correlate with her fertility and are recognized by workers, or affect their reproduction (Monnin and Peeters 1997; Liebig et al. 2000; D'Etorre et al. 2004b; Endler et al. 2004; Holman et al. 2010). However, the non-volatility of these compounds at ambient temperatures raises the question of their transmission within the colony. Queen-laid eggs, which are transported by workers to various nest chambers, have been hypothesized to vehicle the queen signal. However, our results show that in *Aphaenogaster senilis*, larvae still develop into queens despite the biochemical similarity between the queen and her eggs.

The analysis of cuticular HC secretions highlighted several compounds that may be part of the queen signal. Queens' cuticles contained great amounts of long dimethylalkanes,

**Table 1** Percentage (mean  $\pm$  SE) of the major cuticular hydrocarbons, classified by chemical nature, for queens, workers, and their respective eggs ( $n=7, 14, 12,$  and  $7,$  respectively)

Peak number in Fig. 1	Compounds	Queen vs workers	Queen's eggs vs workers' eggs
2	C27	3.6 $\pm$ 2.3 vs 2.3 $\pm$ 0.2 ns	<b>3<math>\pm</math>0.2 vs 7.5<math>\pm</math>3.4</b> <b>&lt;0.006</b>
6	C28	0.9 $\pm$ 0.1 vs 2.4 $\pm$ 0.4 ns	<b>1.8<math>\pm</math>0.1 vs 3.9<math>\pm</math>0.4</b> <b>&lt;0.004</b>
12	C29	2.4 $\pm$ 0.4 vs 4.8 $\pm$ 0.6 ns	<b>4.5<math>\pm</math>0.3 vs 7<math>\pm</math>0.4</b> <b>&lt;0.004</b>
	Total linear alkanes	9.3 $\pm$ 3.7 vs 11.1 $\pm$ 1.3 ns	<b>10.3<math>\pm</math>0.5 vs 19.9<math>\pm</math>2.9</b> <b>&lt;0.025</b>
4	7+9+11+13MeC27	<b>3.7<math>\pm</math>0.7 vs 11.9<math>\pm</math>1</b> <b>&lt;0.005</b>	<b>2.5<math>\pm</math>0.2 vs 5.2<math>\pm</math>0.6</b> <b>&lt;0.005</b>
5	3MeC27	10.1 $\pm$ 0.1 vs 8.2 $\pm$ 0.6 ns	25.4 $\pm$ 0.9 vs 21.6 $\pm$ 2.2 ns
9	4MeC28	5.6 $\pm$ 0.9 vs 10.8 $\pm$ 0.9 ns	6.2 $\pm$ 0.1 vs 5.3 $\pm$ 0.4 ns
14	11MeC29	2.5 $\pm$ 0.3 vs 3.8 $\pm$ 0.7 ns	<b>1.5<math>\pm</math>0.1 vs 3.5<math>\pm</math>0.3</b> <b>&lt;0.003</b>
15	5MeC29	3.8 $\pm$ 0.6 vs 2.2 $\pm$ 0.4 ns	<b>7.5<math>\pm</math>0.4 vs 4.5<math>\pm</math>0.3</b> <b>&lt;0.003</b>
16	3MeC29	5 $\pm$ 0.9 vs 3.3 $\pm$ 0.4 ns	9.8 $\pm$ 1.8 vs 9.7 $\pm$ 0.6 ns
	Total methylalkanes	<b>44.3<math>\pm</math>4 vs 64.5<math>\pm</math>0.9</b> <b>&lt;0.017</b>	65.6 $\pm$ 2.3 vs 66.3 $\pm$ 3.7 ns
1	8,12+6,10DiMeC26	<b>1.1<math>\pm</math>0.2 vs 2.4<math>\pm</math>0.2</b> <b>&lt;0.004</b>	<b>2.7<math>\pm</math>0.8 vs 7.1<math>\pm</math>1.1</b> <b>&lt;0.006</b>
7	3,7+3,9DiMeC27	<b>7.6<math>\pm</math>1 vs 1.5<math>\pm</math>0.1</b> <b>&lt;0.004</b>	<b>2.9<math>\pm</math>0.2 vs 1.3<math>\pm</math>0.1</b> <b>&lt;0.003</b>
8	x,yDiMeC28+10MeC28	<b>5.8<math>\pm</math>0.6 vs 14.1<math>\pm</math>0.6</b> <b>&lt;0.003</b>	<b>3<math>\pm</math>0.3 vs 7.8<math>\pm</math>0.5</b> <b>&lt;0.004</b>
10	6,10DiMeC28	<b>1.3<math>\pm</math>0.1 vs 2.3<math>\pm</math>0.1</b> <b>&lt;0.003</b>	1.2 $\pm$ 0.1 vs 1.5 $\pm$ 0.1 ns
11	4,8DiMeC28	3.3 $\pm$ 0.5 vs 2.8 $\pm$ 0.1 ns	1.4 $\pm$ 0.2 vs 1.6 $\pm$ 0.1 ns
17	3,11+3,9+3,7DiMeC29	<b>13.1<math>\pm</math>1.3 vs 0.6<math>\pm</math>0.1</b> <b>&lt;0.003</b>	<b>4.7 <math>\pm</math>0.4 vs 1<math>\pm</math>0.2</b> <b>&lt;0.003</b>
18	3,9+3,11DiMeC31	<b>10.1<math>\pm</math>2.3 vs 0.5<math>\pm</math>0.1</b> <b>&lt;0.004</b>	5.1 $\pm$ 0.8 vs 1.6 $\pm$ 0.9 ns
	Total dimethylalkanes	<b>53.7<math>\pm</math>3.1 vs 26.2<math>\pm</math>0.5</b> <b>&lt;0.025</b>	<b>48.5<math>\pm</math>1.4 vs 32.7<math>\pm</math>1.3</b> <b>&lt;0.017</b>
3	4,8,12TriMeC26	<b>1.2<math>\pm</math>0.2 vs 3.9<math>\pm</math>0.2</b> <b>&lt;0.003</b>	0.6 $\pm$ 0.1 vs 1.2 $\pm$ 0.1 ns
13	4,8,12TriMeC28	1.6 $\pm$ 0.5 vs 2.5 $\pm$ 0.1 ns	<b>0.4<math>\pm</math>0.1 vs 1.4<math>\pm</math>0.2</b> <b>&lt;0.005</b>

Cuticular HCs of queens versus workers and queen- versus worker-laid eggs are compared (Mann–Whitney test with Holm–Bonferroni correction). Absent or undetected compounds were given the minimum threshold detected by the gas chromatograph. The dimethylalkane x,yDiMeC28 was not identified. The corresponding peak number in Fig. 1 is given for each compound

3,11+3,9+3,7-dimethylnonacosane and 3,9+3,11-dimethylhentriacontane, that were almost absent in workers. A recent

study showed that these compounds are also present in virgin queens, but in lower proportions than in mated queens (Ruel et



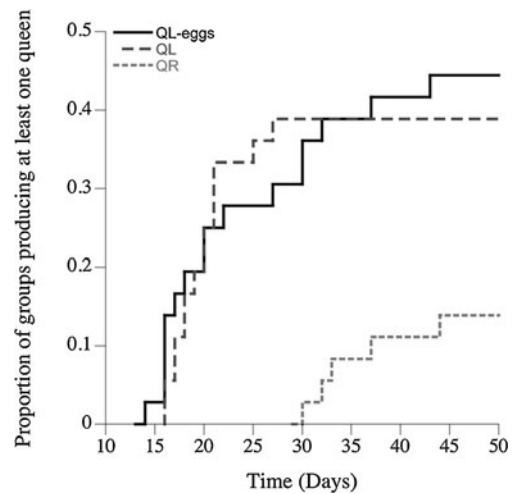
**Table 2** Percentage (mean  $\pm$  SE) of Dufour and poison glands compounds in queens ( $n=6$ ), workers ( $n=7$ ), and on the surface of 10 queen- and worker-laid eggs ( $n=10$  and  $n=4$ , respectively)

Retention time	Compound names	Queen-laid eggs	Worker-laid eggs	Queen's Dufour gland	Worker's Dufour gland	Queen's poison gland	Worker's poison gland
12.5	C13:1		1.7 $\pm$ 0.4	0.3 $\pm$ 0.2	1.3 $\pm$ 1.1		
12.8	C13	8.9 $\pm$ 3.9	8.4 $\pm$ 4.3	1 $\pm$ 0.2	2.9 $\pm$ 1	4.9 $\pm$ 2.9	5 $\pm$ 2
13.7	5MeC13	5.7 $\pm$ 1	7.2 $\pm$ 2.3	0.8 $\pm$ 0.4	0.8 $\pm$ 0.3	0.9 $\pm$ 0.2	1.3 $\pm$ 0.6
14.3	Unidentified HC 1		6 $\pm$ 2.1	0.3 $\pm$ 0.2	3.6 $\pm$ 3.3	5.1 $\pm$ 3.2	4.6 $\pm$ 4
14.5	3MeC13	4.7 $\pm$ 1.6	1.6 $\pm$ 0.3	0.7 $\pm$ 0.3	2.4 $\pm$ 1.3		
14.7	C14:1					1.1 $\pm$ 0.5	0.7 $\pm$ 0.3
15.3	C14	2.4 $\pm$ 0.3	1.5 $\pm$ 0.1	0.6 $\pm$ 0.1	1.6 $\pm$ 0.5	1 $\pm$ 0.4	2.1 $\pm$ 1.1
16.8	3MeC14	2.3 $\pm$ 0.3	1.2 $\pm$ 0.1	0.4 $\pm$ 0.1	0.6 $\pm$ 0.2		
17	C15:2	2.6 $\pm$ 0.2		0.4 $\pm$ 0.1	0.8 $\pm$ 0.3		
17.5	C15:1	4 $\pm$ 1.1	3 $\pm$ 0.8	2.2 $\pm$ 1.6	14 $\pm$ 3.3		
17.7	C15		1.8 $\pm$ 0.2	22.3 $\pm$ 4.5	14.9 $\pm$ 4.9		
17.9	Unidentified terpene	4.5 $\pm$ 0.8	7.6 $\pm$ 2.8	0.8 $\pm$ 0.4	0.6 $\pm$ 0.1		
18.15	Anabasine					23.8 $\pm$ 4.4	19.9 $\pm$ 4.3
18.8	7MeC15		1.3 $\pm$ 0.1	0.6 $\pm$ 0.1	0.6 $\pm$ 0.2		
18.9	5MeC15	2.3 $\pm$ 0.3		9.2 $\pm$ 2.6	1.1 $\pm$ 0.2		
19	Anabaseine					25.6 $\pm$ 9.5	34.5 $\pm$ 10
19.4	3MeC15	2.2 $\pm$ 0.2	1.2 $\pm$ 0.1	7.8 $\pm$ 1.8	1.4 $\pm$ 0.4		
19.5	Unidentified alkaloid					17.2 $\pm$ 5.5	11.6 $\pm$ 2.7
19.8	C16:1			0.8 $\pm$ 0.1	0.4 $\pm$ 0.2		
20	C16	3.4 $\pm$ 0.7	1.6 $\pm$ 0.2	1.6 $\pm$ 0.2	1.5 $\pm$ 0.5		
20.3	8MeC16			0.5 $\pm$ 0.1	0.6 $\pm$ 0.2		
20.5	3,7MeC15			0.3 $\pm$ 0.2	0.3 $\pm$ 0		
21.2	5Me C16			0.5 $\pm$ 0.2	1.5 $\pm$ 0.2		
21.5	4MeC16			0.4 $\pm$ 0.2	1.5 $\pm$ 0.6		
21.5	C17:2		1.2 $\pm$ 0.1	2.7 $\pm$ 0.7	2 $\pm$ 0.7		
21.1	C17:2					3.1 $\pm$ 2.1	2.6 $\pm$ 1.1
21.4							
21.75	3MeC16	2.7 $\pm$ 0.3	3.3 $\pm$ 0.6	1.4 $\pm$ 0.2	3.4 $\pm$ 0.7		
21.6	C17:1					1.7 $\pm$ 0.5	2.2 $\pm$ 1.1
21.9							
22.2	C17	2.8 $\pm$ 0.3	4.6 $\pm$ 2.7	8 $\pm$ 1.3	8.6 $\pm$ 5.6	2.2 $\pm$ 0.8	1.3 $\pm$ 0.5
23.2	7MeC17		1.2 $\pm$ 0.1	1 $\pm$ 0.2	1.5 $\pm$ 0.5		
23.3	5MeC17			0.7 $\pm$ 0	0.3 $\pm$ 0		
23.6	C18:2	3.1 $\pm$ 0.3	4.6 $\pm$ 2.8	1.6 $\pm$ 1.1	1 $\pm$ 0.5		
23.75	Unidentified HC 2					0.7 $\pm$ 0.1	2.3 $\pm$ 1.1
23.8	C18:1		1.3 $\pm$ 0	0.6 $\pm$ 0.1	0.8 $\pm$ 0		

**Table 2** (continued)

Retention time	Compound names	Queen-laid eggs	Worker-laid eggs	Queen's Dufour gland	Worker's Dufour gland	Queen's poison gland	Worker's poison gland
24.3	C18		2.4±0.7	1.2±0.5	1.3±0.5	0.8±0.3	4±1
24.6	Unidentified HC 3					1.2±0.4	0.8±0.4
24.6	4MeC18			0.4±0.1	0.4±0.1		
25.2	Hexadecanal	2.1±0.3	2.8±0.7	0.8±0.4	0.7±0.4		
25.8	C19:2	6.3±1.6	13±6.8	6.4±1	14.3±2.6	4.1±1.1	4.5±1.4
25.9	C19:1		1.2±0.1	18±4.7	12.2±3.1	5.9±3.4	1.8±0.7
26.3	C19			2.9±1.9	0.6±0.1	0.8±0.3	0.7±0.3
27.2	9MeC19	6.6±0.2	8.6±5.1	2.5±1.7	0.4±0		

The blanks indicate that a compound is absent or undetected. These compounds were given the minimum threshold detected, explaining why the total does not add up to 100%. Three hydrocarbons, one terpene, and one alkaloid could not be identified. Gas chromatographs profiles of the Dufour and poison glands are published in Boulay et al. (2007) and Lenoir et al. (2011), respectively



**Fig. 3** Time to queen production when queen-laid eggs were present (QL-eggs, solid black line), the queen was present (QR, larger dashed red line), or the queen was absent (QL, smaller dashed blue line). For each treatment,  $n=36$

al., submitted). These dimethylalkanes are associated with caste and mating status. They may reflect an individual's fertility. In this case, they would be expected to increase in orphan egg-laying workers. A similar pattern was observed in the ponerine ants *Harpegnathos saltator* and *Pachycondyla inversa*, in which egg-laying individuals had higher amounts of, respectively, 13,23-dimethylheptatriacontane and 3,11-dimethylheptacosane than non-reproductive workers (Liebig et al. 2000; D'Ettorre et al. 2004b). Our results also indicate caste dimorphism in Dufour secretions, with the queen's gland containing a higher proportion of 5- and 3-methylpentadecane. This result corroborates Smith et al.'s (2012) findings that the *Aphaenogaster cockerelli* queen's Dufour gland contained more methylalkanes than those of the workers. Given that the proportion of Dufour gland compounds did not differ between virgin and mated queens, the Dufour gland may be the source of caste-related cues rather than fertility signals (Ruel et al., submitted). The composition of the poison gland did not differ between castes, indicating that this gland is unlikely to be involved in queen signaling. Finally, other compounds with higher polarity such as small peptides that are not extracted in dichloromethane might also be involved in queen signaling.

Cuticular and Dufour gland HCs were present on the eggs surface. As when the queen was compared to the workers, queen-laid eggs had more dimethyl alkanes than worker-laid eggs. The proportion of one of the two queen-specific cuticular HCs, 3,11+3,9+3,7-dimethylnonacosane, was greater in queen-laid eggs than in worker-laid eggs. However, HC composition differed between the queen and her eggs. Although the composition of cuticular HCs of *C. floridanus* adults and their eggs diverged somewhat, Endler et al. (2004) also found some similarities in their profiles, as was the case here with *A. senilis*. Several queen-specific

compounds were present on the surface of queen-laid eggs but absent from worker-laid eggs. Our results also showed that proportions of two Dufour gland compounds in worker and queen differ significantly. However, their eggs have similar proportions of Dufour gland-deriving HCs. If a signal of caste or fertility is present in the Dufour gland, it is absent from the eggs. The queen did not mark her eggs with the poison gland, implying that the opening of the abdominal glands is independent in queens of *A. senilis*.

Daily egg addition to the experimental QL-egg groups did not inhibit queen production. In contrast, the control QR groups from which the eggs were removed every second day did not produce any queens until day 35. A previous study showed that emission of queen pheromones prevents the development of the larvae into queens (Boulay et al. 2007). Additionally, the queen's body shape or queen's behavior toward workers or larvae might also prevent the production of queens. Vargo and Passera (1991) showed that adding queen-laid eggs to queenless groups did not inhibit queen production in the ant *L. humile*. They suggested that the queens produced could have developed from the added eggs. This proposition is unlikely in the present study, since the first queen pupa appeared at day 16 and egg incubation in *A. senilis* lasts about 30 days (Boulay et al. 2009). The presence of eggs, however, affected larval survival. More larvae reached the pupa stage in the groups to which queen-laid eggs were added than in the QR groups. Cannibalism of eggs could affect brood survival, as it can provide additional nutrition (Brian and Rigby 1978). Several lines of evidence supported oophagy by larvae in a recent study of the effect of worker-laid eggs on larval development (Ruel et al. 2012).

Our results, like those of Vargo and Passera (1991), suggest that queen-laid eggs do not bear the queen signal that inhibits larvae from developing into queens. However, in *C. floridanus*, queens' eggs were shown to limit worker ovarian development even in absence of the queen (Endler et al. 2004), possibly through queen-specific hydrocarbons. We did not test whether the eggs inhibited worker reproduction in *A. senilis*. If we had done so, we might have found that the amount of queen-specific HCs on eggs was sufficient to limit worker ovarian development but not enough to affect larval development. Alternatively, two different signals may affect worker reproduction and larval development, and the former might be absent from the eggs. Queen-laid eggs bear queen pheromones affecting reproductive decisions in *C. floridanus* but not in *A. senilis* (our results; Endler et al. 2004), which belong to different subfamilies. It may also differ between species of the same subfamily. Transmission of queen pheromones could additionally depend on the chemical nature and properties of the queen signal or on the life history traits of each species. The mean colony size of *C. floridanus* is larger than that of *A. senilis*, which limits contact between queen, workers, and brood. Additionally, the production of queens is a crucial

process in *A. senilis* colony life since it allows the colony to survive after queen disappearance (Cheron et al. 2009). Therefore, the queen signal in this species may have been selected to disappear rapidly after queen death in order to transmit up-to-date information on the presence of a reproductive queen and to allow the colony to proceed more quickly. Direct transmission by the queen would reduce the time required for message transmission. As sociality evolved, the formation of colonies and the increasing group size may have selected for efficient transmission of information, such that the colony could rapidly and adequately adjust to its current state. The use of chemical communication in organizing colonial activities has been found to be prevalent; however, much remains to be understood about the transmission of pheromones utilized in colonies of social insects.

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