

Egg marking in the facultatively queenless ant *Gnamptogenys striatula*: The source and mechanism

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Abstract

Conflicts over reproductive division of labour are common in social insects. These conflicts are often resolved via antagonistic actions that are mediated by chemical cues. Dominant egg layers and their eggs can be recognized by a specific yet similar cuticular hydrocarbon profile. In the facultatively queenless ant *Gnamptogenys striatula*, a worker's cuticular hydrocarbon profile signals its fertility and this determines its position in the reproductive division of labour. How eggs acquire the same hydrocarbon profile is as yet unclear. Here, we search for glandular sources of egg hydrocarbons and identify the putative mechanism of egg marking. We found that eggs carry the same hydrocarbons as the cuticle of fertile workers, and that these hydrocarbons also occur in the ovaries and the haemolymph. None of the studied glands (Dufour, venom, labial and mandibular gland) contained these hydrocarbons. Our results indicate that hydrocarbons are deposited on eggs while still in the ovaries. The low hydrocarbon concentration in the ovaries, however, suggests they are produced elsewhere and transported through the haemolymph. We also found that fertile workers regularly deposit new hydrocarbons on eggs by rubbing laid eggs with a hairy structure on the abdominal tip from which a non-polar substance is secreted.

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1. Introduction

The primary goal of existence for every living organism is to pass as many genes as possible to the next generation (Darwin, 1859). Alternative to direct reproduction, individuals can increase fitness indirectly by helping close relatives reproduce (Hamilton, 1963). This helping behaviour is explicitly present in social insect colonies, where the majority of individuals, the workers, relinquish their own reproduction in favour of the colony. Some workers, however, act selfishly and attempt to produce their own male offspring, which conflicts with the general interest of the colony. To counteract this behaviour, workers prevent or 'police' selfish workers from reproducing by either eating their eggs or aggressing those workers with

developed ovaries (Ratnieks, 1988; egg eating: Nakata and Tsuji, 1996; Monnin and Peeters, 1997; Kikuta and Tsuji, 1999; D'Ettoire et al., 2004; Endler et al., 2004; Helanterä and Sundström, 2005; aggression: Visscher and Dukas, 1995; Gobin et al., 1999; Kikuta and Tsuji, 1999; Liebig et al., 1999; Tsuji et al., 1999; Dampney et al., 2002; Hartmann et al., 2003; Iwanishi et al., 2003; Dietemann et al., 2005; Kawabata and Tsuji, 2005). In order for policing to take place, fecund workers must be distinguishable from infertile workers, and their eggs must be distinguishable from queen-laid eggs.

Fertility information contained in cuticular chemistry is known from a number of species (Monnin et al., 1998; Liebig et al., 2000; Cuvillier-Hot et al., 2001; Tentschert et al., 2001; Heinze et al., 2002; Dietemann et al., 2003; Cuvillier-Hot et al., 2004; de Biseau et al., 2004; Endler et al., 2004; Lommelen et al., 2006; Monnin, 2006). In *Gnamptogenys striatula*, a New World ectatommine ant,

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the cuticular hydrocarbon profile provides a reliable clue to an individual's fertility (Blatrix and Jaisson, 2001; Lommelen et al., 2006). The nests of *G. striatula* are often queenright, but in absence of a queen, workers, which can mate (named gamergates; Peeters and Crewe, 1984), take over the reproduction for the colony (Blatrix and Jaisson, 2001). The cuticular fertility signal of *G. striatula* plays an important role in the regulation of reproduction, as infertile ants rely on this signal to concede reproduction to their more fertile nestmates (Lommelen et al., unpublished). Similarly, police workers may rely on this signal to immobilize sisters that attempt to produce males (as in Lommelen et al., unpublished) as well as to selectively destroy their eggs if these eggs can be distinguished from gamergate eggs. Thus, there is strong selection for gamergates to prevent police workers from destroying their eggs, and egg marking be a mechanism by which fertile gamergates prevent oophagy.

There is strong evidence in ants that eggs laid by queens and workers are chemically distinguishable (D'Etterre et al., 2004; Endler et al., 2004), as are eggs laid by highly and weakly fertile individuals (Monnin and Peeters, 1997; Endler et al., 2006; Monnin, 2006). Egg surface chemicals typically comprise a hydrocarbon mixture that often contains the same compounds as the adult cuticle (Monnin and Peeters, 1997; D'Etterre et al., 2004; Endler et al., 2004, 2006). As a primary function in adult ants, hydrocarbons prevent desiccation and function as a barrier for micro-organisms (Blomquist et al., 1998; Howard and Blomquist, 2005). For a variety of non-hymenopteran insects, the source of surface hydrocarbons is the oenocytes (among others Diehl, 1975; Lockey, 1988 and references therein; Hepburn et al., 1991; Römer, 1991; Blomquist et al., 1998; Fan et al., 2003; Howard and Blomquist, 2005). On the contrary, the Dufour gland appears to be the source of hydrocarbons in several hymenopteran taxa (among others Downing, 1991; Keegans et al., 1993; Ayasse et al., 1999), although this may be a secondary source that receives hydrocarbons after production by a primary source (Katzav-Gozansky et al., 2000). However, neither oenocytes as a primary source nor transportation of hydrocarbons towards the Dufour gland (as a secondary source) have been confirmed in social Hymenoptera. Furthermore, little is known about the mechanism by which hydrocarbons are ultimately deposited on the egg surface. In the fire ant, *Solenopsis invicta*, the venom gland secretion is deposited from the sting onto the eggs during oviposition (Vander Meer and Morel, 1995), but other studies on the subject in Hymenoptera are lacking.

In this paper, we identify the surface chemicals of eggs of *G. striatula*, as well as the secondary source of these compounds by analysing the contents of glands that open at the abdominal tip (Dufour gland and venom gland), where eggs pass during oviposition. We also analysed the contents of the mandibular and labial glands as other potential means for egg marking pheromone transferred during egg handling. Finally, we examined the ovaries

themselves as a putative secondary source and the haemolymph as a transport system, as haemolymph may carry hydrocarbons to the secondary source. We also describe the hairy structure on the abdomen that *G. striatula* workers rub over previously laid eggs, which may be another way of chemically marking eggs.

2. Material and methods

2.1. Study species

We collected six colonies of *G. striatula* from Santa Rosa National Park, Costa Rica, in October 2003. Colonies were brought to the laboratory in Belgium and housed in a Janet-type nest box with plaster of Paris flooring (Hölldobler and Wilson, 1990). To obtain mated workers (gamergates) in our study colonies, all queens were removed from the nests and workers were allowed to mate. Gamergates were allowed to develop for several months. When brood was again abundant in experimental colonies, we selected gamergates that were in the process of laying eggs for chemical sampling (see below). We verified gamergate status by inspecting the spermathecae for seminal fluid and the ovaries for developed oocytes.

2.2. Chemical sampling and analysis

Piles of six intact eggs were removed from the colonies and their surface chemicals extracted in 5 µl hexane. Glands (Dufour, venom, labial and mandibular) and ovaries were dissected from six gamergates and placed separately into a micro-insert to which 5 µl hexane was added and the contents gently crushed with forceps to release the chemicals. Samples were analysed by gas chromatography [Agilent 6850 Series gas chromatograph (Agilent Technologies, USA) equipped with a capillary column (Agilent HP-1; coated with methyl siloxane; length: 30 m; I.D.: 0.32 mm; film thickness: 0.25 µm; Agilent Technologies, USA) and flame ionization detector] in splitless mode. The injector and detector temperatures were 290 and 300 °C respectively, with an inlet pressure of 6.74 psi and a constant flow of 1.1 ml/min. The oven was programmed from 90 to 180 °C at 20 °C/min, then to 290 °C at 5 °C/min and held for 30 min. We used helium as the carrier gas. Data were analysed using ChemStation (Rev A.09.01, Agilent Technologies).

For compound identification, chemicals were extracted from an additional pile of 10 eggs in 10 µl hexane and from six gamergate ovaries in 5 µl hexane by crushing them as described above. Gas chromatography/mass spectrometry analyses on these egg and ovary samples were carried out on an HP 6890 GC (equipped with an HP-5MS column; length: 30 m; ID: 0.25 mm; film thickness: 0.25 µm) connected to an HP5973 MSD (quadrupole mass spectrometer with 70-eV electron impact ionization). Samples were injected in the splitless mode and the oven was programmed from 60 to 250 °C at 10 °C/min and then from

250 to 320 °C at 5 °C/min and held for 7 min at 320 °C. Helium was used as carrier gas, at a constant flow rate of 1.0 ml/min. Cuticular hydrocarbons (alkanes and mono- and dimethylalkanes) were characterized by the use of standard MS databases and diagnostic ions and by determining Kovats indices by the method of Carlson et al. (1998). The 4-methylalkanes were identified according to their strong ion intensities at $m/z = 70/71$ in the mass spectra (Tissot et al., 2001) and by their Kovats indices.

2.3. Sampling and analysis of haemolymph

In order to sample the haemolymph, the abdomens of four gamergates, which were not submerged in dissecting solution, were opened and a solid phase micro-extraction fibre [SPME; 7 µm polydimethylsiloxane (Supelco)] was rubbed on the inner side of the tegument, where fat bodies, subepithelial glands and oenocytes are located. The fibre was inserted into a gas chromatograph (see above for technical information) and desorbed in splitless mode for 5 min. The injector and detector temperatures were 320 and 300 °C, respectively, with an inlet pressure of 8.28 psi. The oven temperature was programmed to increase from 150 to 200 °C at 20 °C/min, then to 290 °C at 10 °C/min, after which the temperature was held constant for 20 min. We used helium as a carrier gas. Data were analysed using ChemStation (Rev A.09.01, Agilent Technologies). For comparison, we sampled the cuticular hydrocarbons by rubbing the SPME fibre over the body of a gamergate for 3 min and analysed the compounds as described for the haemolymph.

2.4. Scanning electron microscopy on hairy structure on abdomen

We used scanning electron microscopy to examine the fine structures of the abdominal tip. Six ants were kept in 50% ethanol until the abdomens were completely distended (approximately 2 min). Abdomens were then severed, subjected to a graded ethanol dehydration series (70%, 90% and 100%) and air dried overnight. The samples were then covered with gold in a Spi-module Sputter Coater for 2 min and examined with a Jeol JSM-6360 scanning electron microscope.

3. Results

3.1. Chemicals on eggs

The hydrocarbon mixture on the eggs laid by *G. striatula* workers contained two *n*-alkanes, 12 methylalkanes and 10 dimethylalkanes (Fig. 1, Table 1). This chemical pattern was basically similar to the cuticular hydrocarbons of fertile workers (Lommelen et al., 2006). The only striking difference was the concentration of the compounds. Early eluting compounds generally occurred in higher concentrations in egg samples with six eggs compared with cuticle

samples (up to four times higher for 4-methyloctacosane), peaks 5–9 had similar concentrations in cuticle and egg samples, and compounds with a high molecular weight had gradually lower concentrations in the egg samples (up to ten times lower for compounds with the highest molecular weight, Fig. 1, Table 1). In the groups of early peaks, middle peaks and late peaks, the relative proportions of hydrocarbons were similar in both samples, making the typical pattern in both chromatograms similar (Fig. 1). Eggs also contained 3-methyldotriacontane (peak 16), which is absent on the cuticle.

3.2. Chemicals in organs and in haemolymph

Ovaries had the same hydrocarbon pattern as the eggs, but the concentration of the compounds was 5–10 times lower (Fig. 1). Additionally, we found cholesterol (peak 10) exclusively in the ovaries. None of the glands showed this specific hydrocarbon pattern (Fig. 2). The Dufour gland had two striking peaks that eluted early, and some less concentrated peaks that eluted later. The venom gland, labial gland and mandibular gland, on the other hand, generally had minor compounds eluting together with or after nonacosane. In the labial gland and mandibular gland, two additional minor compounds eluted very early. The haemolymph, which was sampled from the inside of the cuticle near the subepithelial glands and the oenocytes, had exactly the same chemical pattern as the cuticular hydrocarbons, but the overall concentration was higher (Fig. 3).

3.3. Abdominal brush

During casual observations, we often observed ants stroking the egg pile with a hairy structure on the last tergite near the abdominal tip (Fig. 4A), by curling their abdomen underneath the body (Fig. 5). As this behaviour was not directly associated with egg laying, the functional group (worker vs. egg layer) to which these ants belonged was unclear in colonies with unmarked workers. However, in colonies with marked egg laying workers, it was clear that egg layers performed this egg stroking behaviour. This abdominal brush was also groomed by the egg layers themselves.

Successive solid phase chemical sampling of head, thorax, abdomen and abdominal brush indicated that the abdominal brush contains the highest concentration of cuticular hydrocarbons. As hydrocarbons were also abundant in the haemolymph underneath the cuticle (see above), we expect that hydrocarbons may somehow pass through the cuticle.

At 4000 × magnifications, it was clear that each hair on the abdomen arises from within a crater formed by the cuticle (Fig. 4B). The diameter of the crater from ridge to ridge is 4 µm; each hair is approximately 2 µm in diameter. A viscous fluid appeared to ooze from these craters (Fig. 4C), and may indicate that these are indeed porous.

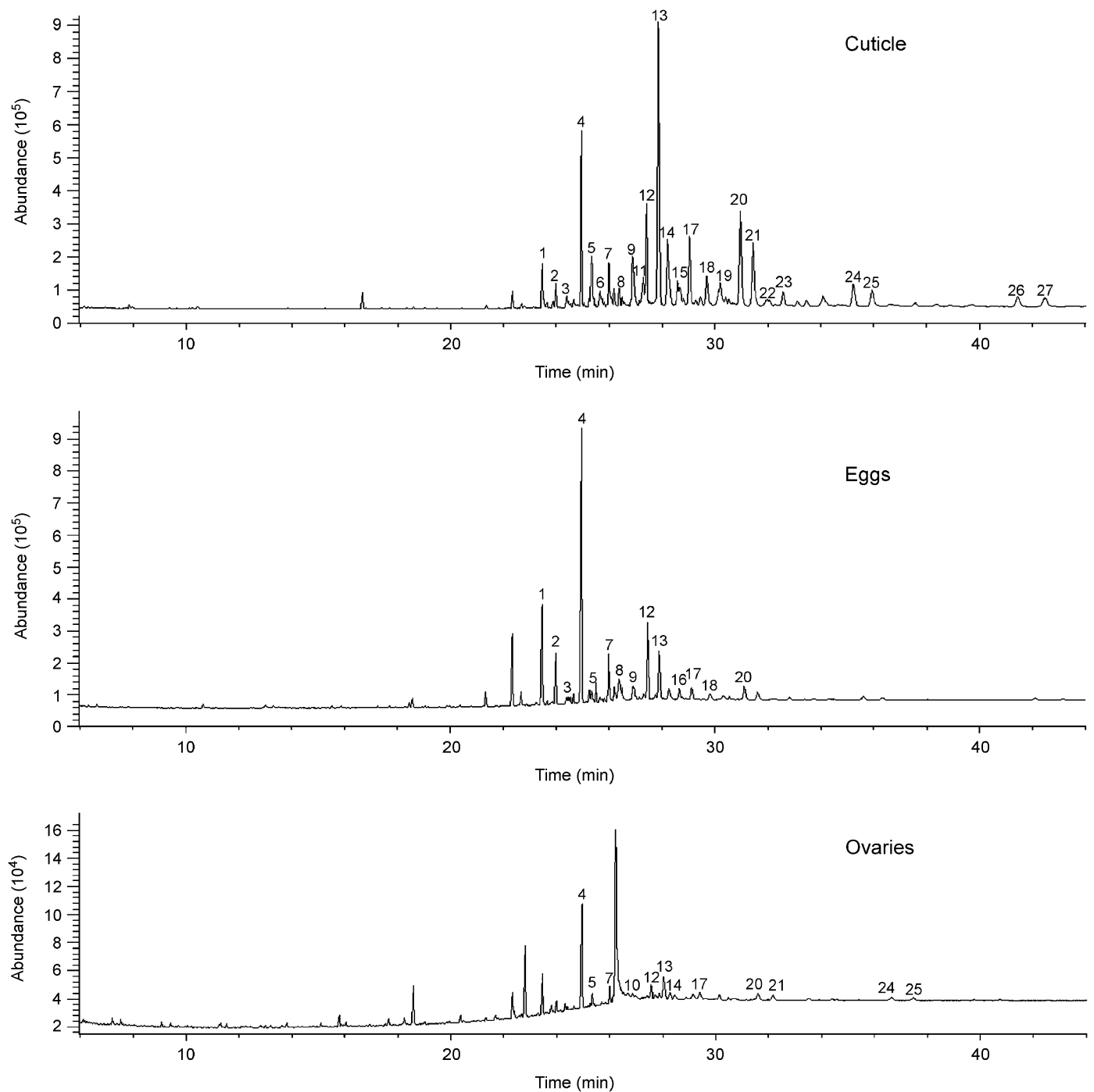


Fig. 1. Representative chromatograms of hexane extractions of cuticular hydrocarbons of a gamergate (adapted from Lommelen et al., 2006), six eggs and ovaries of a gamergate. Peak numbers refer to identified peaks listed in Table 1.

Other sections of the abdominal cuticle showed droplets that had a similar appearance as the secretion emitting from the craters. These secretions did not dissolve with an ethanol rinse suggesting they consist of non-polar compounds. The droplets appeared spread out over the cuticle surface, suggesting an adhesive property with respect to the cuticle. As the pilosity of the abdominal tip is substantially greater than other areas of the body (Fig. 4A), it also contains the densest area of craters and we could thus expect that the

most secretion can be emitted there. This seems to be true, as in two out of three samples analysed by SEM this abdominal tip was completely covered with secretion.

4. Discussion

In social insects with large colonies, the reproductive division of labour tends to conform to the interest of the majority of the individuals, which are usually the infertile

Table 1
Chemical compounds identified by GC–MS on eggs and in gamergate ovaries

Peak	Compound name
1	4-Methyloctacosane
2	Nonacosane
3	11- and 13-Methylnonacosane
4	3-Methylnonacosane
5	3,15-Dimethylnonacosane
6	12- and 18-Methyltriacontane
7	4-Methyltriacontane
8	Hentriacontane
9	9-, 11- and 13-Methylhentriacontane
10	Cholesterol
11	11,19-Dimethylhentriacontane
12	3-Methylhentriacontane
13	3,11-, 3,13- and 3,15-Dimethylhentriacontane
14	12,x-Dimethyldotriacontane
15	4-Methyldotriacontane
16	3-Methyldotriacontane
17	4,10-, 4,12- and 4,14-Dimethyldotriacontane
18	11- and 13-Methyltrtriacontane
19	11,21-Dimethyltrtriacontane
20	3,11-, 3,13- and 3,15-Dimethyltrtriacontane
21	3,11,15-Trimethyltrtriacontane
22	10,14-Dimethyltetracontane
23	4,x-Dimethyltetracontane
24	3,13- and 3,15-Dimethylpentatriacontane
25	3,11,15-Trimethylpentatriacontane
26	3,13- and 3,15-Dimethylheptatriacontane
27	3,11,15-Trimethylheptatriacontane

Peak numbers correspond with the numbers on the chromatograms in Fig. 1.

workers (Alexander and Borgia, 1978). In polygynous species, such as *G. striatula* (Blatrix and Jaisson, 2001), infertile workers, which cannot produce their own offspring, benefit from limiting the number of reproductives to preserve colony efficiency (Monnin and Ratnieks, 2001). Furthermore, as workers are more closely related to their brothers than to their nephews, they attempt to prevent their sisters from producing males either directly by egg destruction or indirectly by aggression (Ratnieks, 1988; Monnin and Ratnieks, 2001). These actions require the recognition of fertile individuals and their eggs, which is possible through the surface hydrocarbon mixture that acts as a signal of chemical fertility (individuals: Monnin et al., 1998; Liebig et al., 2000; Cuvillier-Hot et al., 2001, 2004; Tentschert et al., 2001; Heinze et al., 2002; Dietemann et al., 2003; de Biseau et al., 2004; Endler et al., 2004; Lommelen et al., 2006; Monnin, 2006; eggs: Monnin and Peeters, 1997; D’Ettorre et al., 2004; Endler et al., 2004, 2006; Monnin, 2006). These fertility signals are thought to be honest signals of fertility (Monnin, 2006), thus they are likely to indicate the actual fertility of individuals without any possibility for cheating (Keller and Nonacs, 1993). Cheating by signalling higher fertility could nevertheless benefit individuals of intermediate fertility, as this dishonest signal may rank them within the limited group of

workers that is allowed to reproduce. Signal honesty is generally difficult to verify. However, a signal is more likely to be honest if the source is a chemical by-product of some metabolic pathway associated with egg production, as it is costly for cheaters to imitate such signal (Lachmann et al., 2001). Thus, ovaries are a good potential source of an honest fertility signal.

Studies on Hymenoptera show a variety of glands as being the source for egg hydrocarbons, such as the venom gland in the ant *S. invicta* (Vander Meer and Morel, 1995) and the Dufour gland in several bee and wasp species (among others Downing, 1991; Keegans et al., 1993; Ayasse et al., 1999). In our study species *G. striatula*, none of these glands alone provided the complete battery of compounds found on the eggs. The cuticle and the ovaries, on the other hand, contained the same hydrocarbon pattern as was found on the eggs, which suggests a common hydrocarbon source that provides molecules to both the cuticle and ovaries. This complex hydrocarbon mixture was also abundant in the abdominal haemolymph, underneath the cuticle, which indicates that cuticular hydrocarbons may be produced somewhere in the abdomen and secreted to the cuticle surface. A putative source is the subepithelial glands, which elute their secretion through pores to the outer cuticle. These glands are located throughout the body just beneath the epithelium, and occur in a variety of ant species, among which *G. striatula* (Gobin et al., 2003). Alternatively, cuticular hydrocarbons may be produced in the oenocytes. Oenocytes are glandular cells that initially discharge their secretory products into the haemolymph. They are already known as the source for hydrocarbons in many non-hymenopteran insect species (among others Diehl, 1975; Lockey, 1988 and references therein; Hepburn et al., 1991; Romer, 1991; Blomquist et al., 1998; Fan et al., 2003; Howard and Blomquist, 2005). Oenocytes do not contact the epithelium directly (Lockey, 1988; Roma et al., 2005). Thus a hemolymphic transport system, which requires lipophorin, is likely to bring hydrocarbons to the cuticle surface (Lockey, 1988; Gu et al., 1995; Schal et al., 1998a, b; Fan et al., 2002, 2004). A recent study on hydrocarbon transport towards the cuticle in the termite *Zootermopsis nevadensis* confirmed that this transport indeed requires lipophorin (Fan et al., 2004), revealing it is likely to pass through the haemolymph. There is also evidence that cuticular hydrocarbons are transported to the epithelium (Hepburn et al., 1991; Schal et al., 1998a). How hydrocarbons reach the outer cuticle is nevertheless still poorly understood (Howard and Blomquist, 2005). Early models suggested a transport via ‘pore canals’ (Lockey, 1988 and references therein; Howard and Blomquist, 2005), but more recent studies on the subject focused on hydrocarbon deposition on the cuticle during moulting (Dwyer et al., 1986; de Renobales et al., 1988; Blomquist et al., 1998; Young et al., 1999; Howard and Blomquist, 2005).

Our scanning electron micrographs and the preceding ethanol rinse strongly suggest that a non-polar secretion is

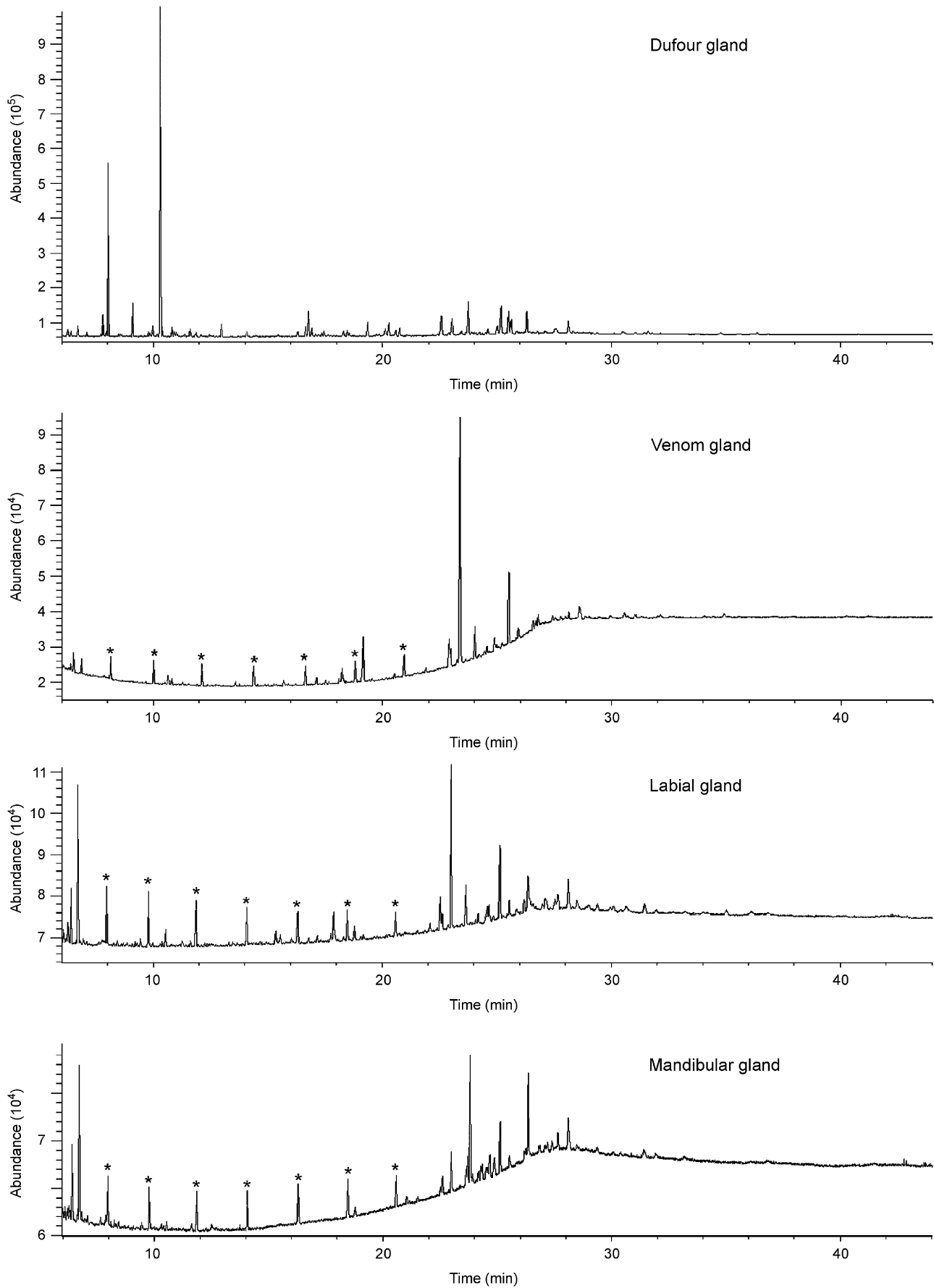


Fig. 2. Representative chromatograms of hexane extractions of a Dufour gland, venom gland, labial gland and mandibular gland. * = contamination.

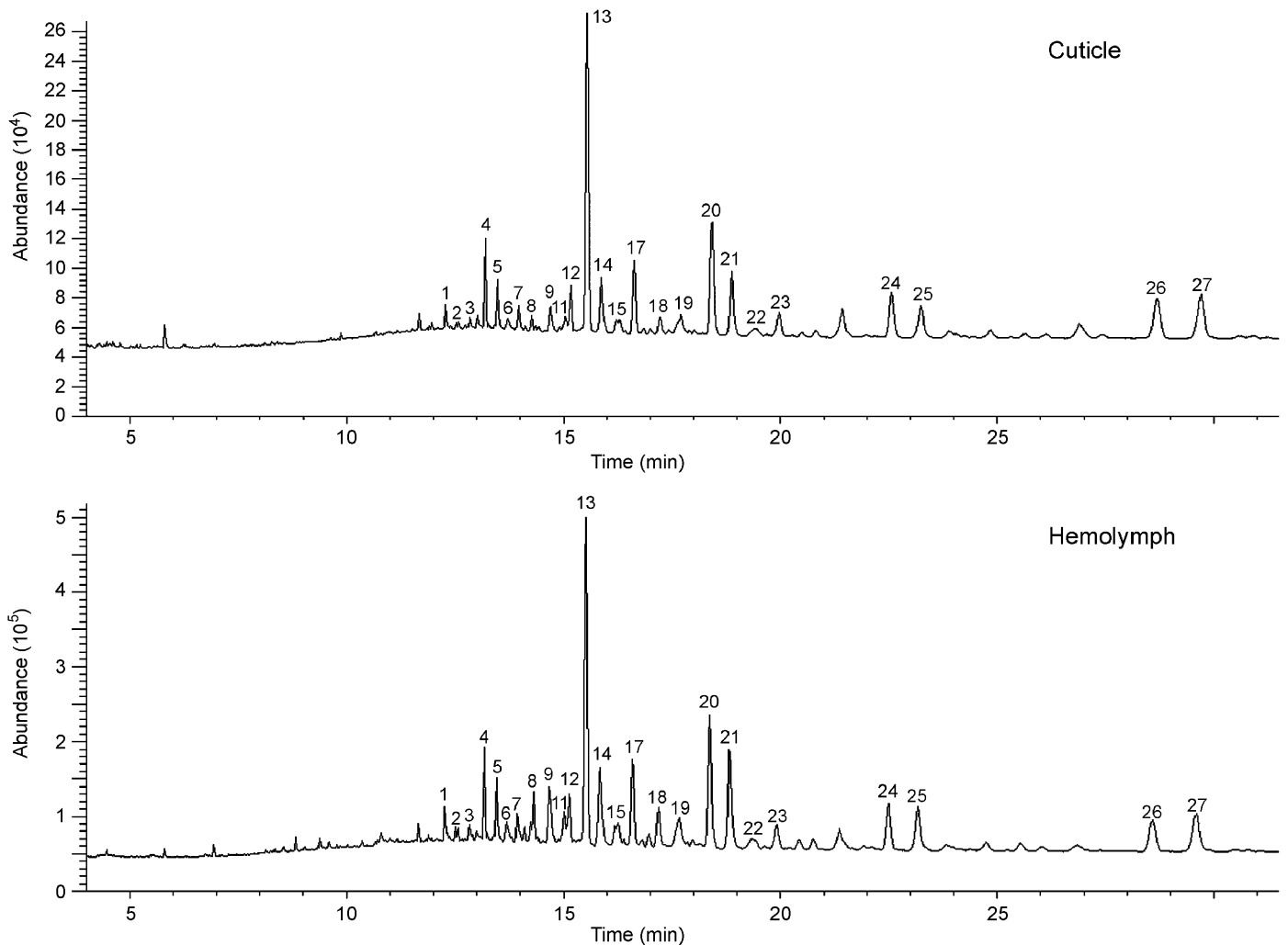


Fig. 3. Representative chromatograms of cuticular hydrocarbons and hemolymph hydrocarbons of a gamergate, sampled by way of solid phase micro-extraction. Peak numbers correspond with numbers listed in Table 1.

eluted via craters that encompass the hairs. Hairs with this secretion site are spread all over the abdomen, and possibly even hairs on other body parts might be associated with these craters, thus we expect that the secretion may be spread out body-wide. The secretion may consist of hydrocarbons that co-elute with cuticular lipids, similar to the hydrocarbons that co-elute with the more solid wax in *Semidalis flinti* (Nelson et al., 2003) and *Diaprepes abbreviatus* (Lapointe et al., 2004). Alternatively, the cuticular hydrocarbon mixture could elute from the pores associated with epithelial glands.

As eggs and cuticle in *G. striatula* are covered with the same complex mixture of hydrocarbons in the same relative proportions, like in other ant species (Monnin and Peeters, 1997; D'Etorre et al., 2004; Endler et al., 2004), egg hydrocarbons could originate from the same source as cuticular hydrocarbons, namely the subepithelial glands or the oenocytes. As ovaries also contain the complex mixture of hydrocarbons, eggs may receive surface hydrocarbons within the ovaries. These hydrocarbons could easily be carried by lipophorin towards the ovaries, as is described

by Schal et al. (1998b) in the cockroach *Blattella germanica*. Here hydrocarbons are produced in the oenocytes, transported through the haemolymph while carried by lipophorin and deposited on the eggs inside the ovaries (Schal et al., 1998b). Fan et al. (2002) confirmed and refined these findings with a detailed study on the uptake of labelled hydrocarbons by the oocytes. Similar pathways of hydrocarbons from the oenocytes through the haemolymph towards a target tissue (i.e. cuticle, ovaries or glands) have been studied in a variety of insect species (among others Lockey, 1988; Gu et al., 1995; Schal et al., 1998a, b; Fan et al., 2002, 2004). These pathways, however, had never been confirmed in social Hymenoptera. Earlier studies tend to show a glandular origin for hydrocarbons (among others Downing, 1991; Keegans et al., 1993; Ayasse et al., 1999). A study that examined the compound biosynthesis of the honeybee in vitro showed that the Dufour gland was unable to produce hydrocarbons despite this gland contained hydrocarbons in vivo (Katzav-Gozansky et al., 2000). Hydrocarbons are therefore likely to be produced elsewhere and transported towards the

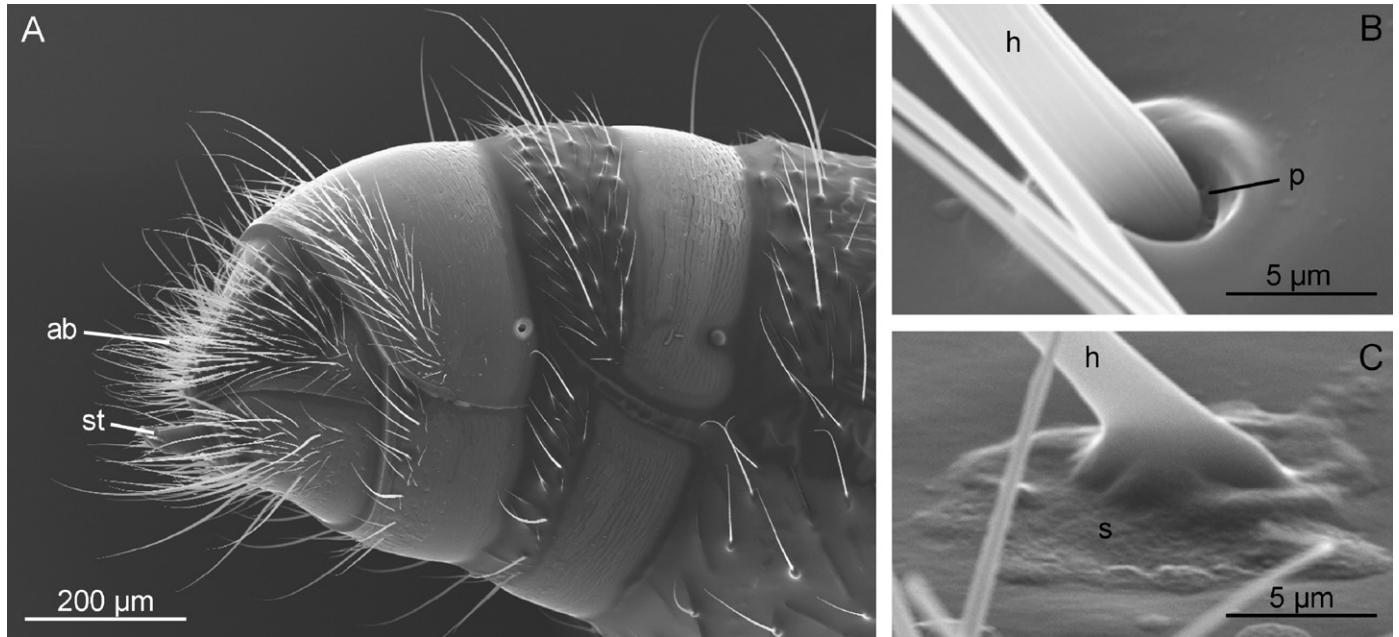


Fig. 4. Scanning electron micrographs of the abdominal brush: (A) overview of the abdominal tip with hairy brush (ab); (B) basis of hair without secretion; (C) basis of hair with secretion. Ab = abdominal brush; h = hair; p = hole (pore) around hair basis; s = secretion eluting from pore; st = sting.

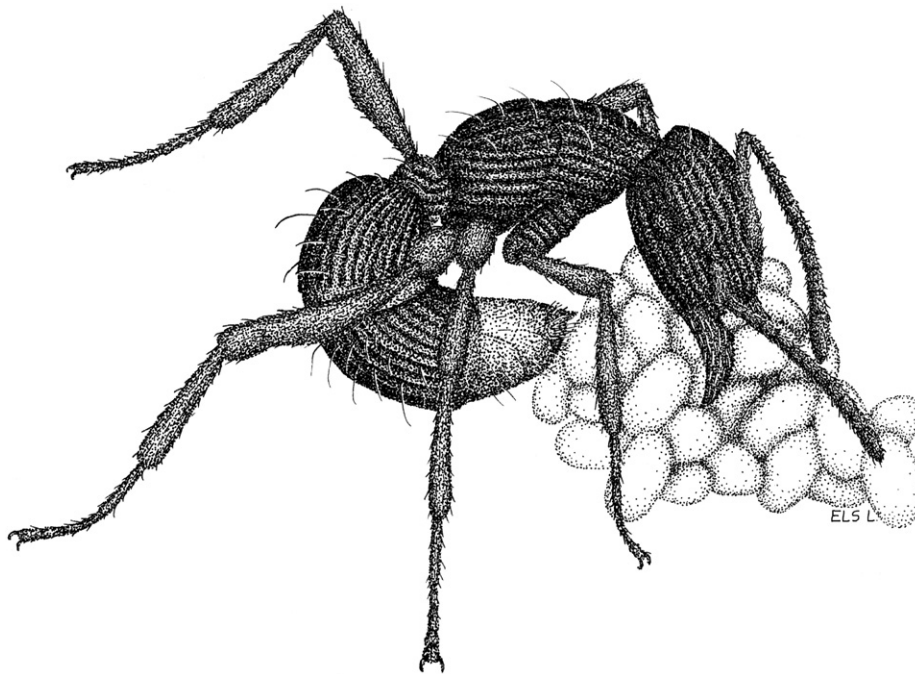


Fig. 5. Illustration of a gamergate rubbing eggs with her abdominal brush.

Dufour gland. It seems thus that also the honeybee might fit into the general model that is found for non-hymenopteran insects, which suggests the oenocytes as a primary source for hydrocarbons. Therefore, other results revealing a glandular origin for hydrocarbons may need to be re-examined.

The regular stroking of eggs by *G. striatula* with the apical hairy structure on the abdomen may be a means to maintain high concentrations of cuticular hydrocarbons.

Gamergates were the only individuals we observed ‘brushing’ eggs. Given that the eggs contained the hydrocarbon profile typical of fertile ants, egg marking is likely to be performed mainly by egg layers themselves. Scanning micrographs show the ant exudes secretion at the basis of the hairs. Here the secretion can easily spread out over the hairs, which provides a good system to deliver hydrocarbons on the eggs. The same ‘egg brushing’ with a cluster of hairs on the abdominal tip was observed in

Gnamptogenys menadensis (Gobin et al., 1998). Here, however, not only gamergates, but also dominant and subordinate workers performed this behaviour (Gobin, unpublished). In *Pachycondyla apicalis*, the front basitarsal brush also spreads hydrocarbons around, though the spread is mainly throughout the ant cuticle and not on the eggs. Moreover, the basitarsal brush accumulates hydrocarbons and provides the postpharyngeal gland with hydrocarbons during self-grooming (Hefetz et al., 2001). Providing the postpharyngeal gland with hydrocarbons could also be a function of the abdominal brush in *G. striatula*, since these ants are often grooming this brush.

Our data indicate that eggs receive a hydrocarbon layer inside the ovaries in *G. striatula*. The source of these hydrocarbons, however, seems to be elsewhere, possibly in the subepithelial glands or the oenocytes. Observations of gamergates ‘brushing’ eggs with an apical abdominal brush further suggest that hydrocarbons secreted through craters associated with the hairs are regularly applied to eggs.

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