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Characterization of queen-specific components of the fluid released by fighting honey bee queens

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Summary. Swarming honey bee (*Apis mellifera* L.) colonies rear supernumerary young queens that compete for the limited resources (workers) necessary for founding a new colony. Young queens often fight to death. During fights, queens often release rectal fluid with a strong smell of grapes, after which they temporarily stop fighting. This potentially reduces the risk of deadly injury. The fluid and one of its components, *ortho*-aminoacetophenone, were previously found to have a pheromonal effect on workers. Recently, it has been suggested that the effects of this substance may be context- or concentration-specific. We performed semi-quantitative gas-chromatography mass spectrometry (GC/MS) analysis of the fluid (i) released by queens during their first fight, (ii) released during a subsequent fight, and (iii) obtained by dissecting the hindgut of queens and (iv) of workers. Following preliminary results by Page *et al.* 1988 (Experientia 44:270–271), we scored presence/absence of eight substances. Five substances (*ortho*-aminoacetophenone, decanoic acid, dodecanoic acid, octyl decanoate, and decyl decanoate) were characteristic of queens only. *ortho*-Aminoacetophenone was detected in all queens and in none of the workers, in agreement with previous findings that worker rectal contents do not have pheromonal effect. The fluid released by queens on their second fight also contained *ortho*-aminoacetophenone, but in smaller quantities. This study confirms the unique presence in queens of five compounds, demonstrates their rectal origin, and estimates the amount of *ortho*-aminoacetophenone released during fights, as required to design experiments addressing the function and adaptive significance of fluid release behaviour.

Key words. conflict – fighting behaviour – pheromone – gas chromatography – *ortho*-aminoacetophenone – insects – Hymenoptera – Apis – honeybee

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Introduction

Conflicts among queens in insect societies often take place during colony reproduction (Enquist & Leimar 1990; Visscher 1993). For instance, in some ant species unrelated queens first cooperate in colony founding, and then fight to the death to monopolize the colony's future reproduction (Bernasconi & Strassmann, 1999; Choe & Perlman 1997). In the honey bee (*Apis mellifera* L.), colonies reproduce by swarming and fatal fights often occur among young queens who compete for the opportunity to head a daughter colony (Winston 1987; Visscher 1993; Tarpy & Fletcher 1998). The mother queen leaves the nest with some of the workers (prime swarm) while 10–20 young queens are reared in the colony (Winston 1987). The queen that is first to emerge attempts to kill immature queens still inside their cells (Boch 1979), and queens emerging at the same time often fight to death (Winston 1987, p. 188). Depending on the strength of the worker force, the surviving queen(s) inherit either the entire colony, or a portion of the workers which depart with her in an afterswarm (Winston 1987). A sufficient number of workers are required for winter survival, and usually only one to three daughter colonies are produced (Winston 1987). Thus, survival to become an egg-laying queen has several components (individual survival through development, aggression from other queens, predation risk during mating flight, and colony survival), of which fighting success is important in direct queen-queen competition (Tarpy & Fletcher 1998).

Virgin honey bee queens show several adaptations to fighting. For instance, short developmental time (Winston 1987) and early onset of venom production (Bachmeyer *et al.* 1972; Owen & Bridges 1976; Owen *et al.* 1977) suggest that selection has acted through age-related fighting advantage. During fights one of the queens often releases 10–30 µl of fluid with an intense grape smell (Page & Erickson 1986; Post *et al.* 1987; Page *et al.* 1988; Breed *et al.* 1992; Tarpy & Fletcher 1998), after which queens usually release each other and temporarily interrupt fighting (Bernasconi *et al.*, 1999). This "spraying" behaviour is specific to aggressive interactions between young queens (Post *et al.* 1987). Its

function is unresolved, but has been proposed to benefit the sprayer by (i) causing costs to the queen that is contaminated (*e.g.*, toxicity or reduced feeding rates by the workers; Post *et al.* 1987; Breed *et al.* 1992), (ii) modifying worker behaviour (Page & Erickson 1986; Post *et al.* 1987; Page *et al.* 1988; Tarpy & Fletcher 1998), or (iii) interrupting queen-queen fights (Page & Erickson 1986; Post *et al.* 1987; Bernasconi *et al.* 1999). Queens potentially face several fighting encounters, each bearing a substantial risk of fatal injury. Interruption of fights may allow the sprayer to escape and hide until other queens first kill each other or have departed with an afterswarm, and so increase her probability of becoming an egg-layer. We expect only one of the queens to spray if benefits differ between queens, *e.g.* if spraying reverses a vulnerable relative fighting position (Butz & Dietz 1994), or if it allows to escape a stronger competitor if queens are able to assess their relative fighting ability. This fluid is unlikely to be a digestive product (Page *et al.* 1988; Breed *et al.* 1992), as it has been observed to have a pheromonal effect causing autogrooming behaviour in workers independently of queen diet (Post *et al.* 1987). It is known to contain *ortho*-aminoacetophenone (Post *et al.* 1987; Page *et al.* 1988), a volatile component reported in a few other invertebrates (mandibular gland secretions of the fungus-growing ant, *Mycocepurus goeldii*: Blum *et al.* 1981; sex pheromone of the larch sawfly, *Cephalacia laricifila*: Baker *et al.* 1983) and vertebrates (mustelids: Mason *et al.* 1991). The pheromonal effects of the fluid, and in particular of *ortho*-aminoacetophenone, may provide a mechanism for the observed cessation of aggressive behaviour. Alternatively, interruption of fights may result if spraying large amounts of fluid on the opponent interferes with its ability to orientate and to fight effectively, and/or to maintain a less vulnerable fighting position (Butz & Dietz 1994).

In this study, we extend work by Page *et al.* (1988) by presenting semi-quantitative gas chromatography/mass spectrometry analysis of this fluid. Because of evidence that the fluid repels (Post *et al.* 1987; Page *et al.* 1988) or attracts workers (Tarpy & Fletcher 1998), it has recently been suggested that the effect may depend on context or on the amount released (Tarpy & Fletcher 1998). Post *et al.* (1987, p. 587) report that honey bee workers are attracted by small amounts of queen faeces, and repelled by the large amounts released by fighting queens. Interestingly, in the ant *Mycocepurus goeldii*, workers are also attracted to low and repelled by high *ortho*-aminoacetophenone concentrations (Blum *et al.* 1981). Thus, estimates of the amount of *ortho*-aminoacetophenone released during fighting are required for the design of experiments aimed at elucidating both the mechanism and adaptive significance of spraying behaviour. In addition, we compared the composition of the fluid released by queens during fights with the hindgut content from dissected queens and workers, to identify components potentially specific to the context of fighting, and of queens only.

Material and methods

European race honey bee queens (*Apis mellifera* L.) were reared in August 1998 by grafting female larvae (aged ≤ 24 hours) from worker cells into artificial queen cells following standard apicultural procedures (Laidlaw & Page 1997, Ratnieks & Nowogrodzki 1987, Tarpy & Fletcher 1998). After the cells were capped, we individually transferred them to vials ($\varnothing$ 1.5 cm, height 5 cm) and placed them in an incubator (31–34°C) until emergence of the adult queen. In the vial we put sugar candy as a source of food for emerged queens. We recorded time of emergence as the day when the queen opened the cell cap, and transferred emerged queens individually to mesh cages kept in the source colony. Queens were sisters, as occurs naturally (Winston 1987).

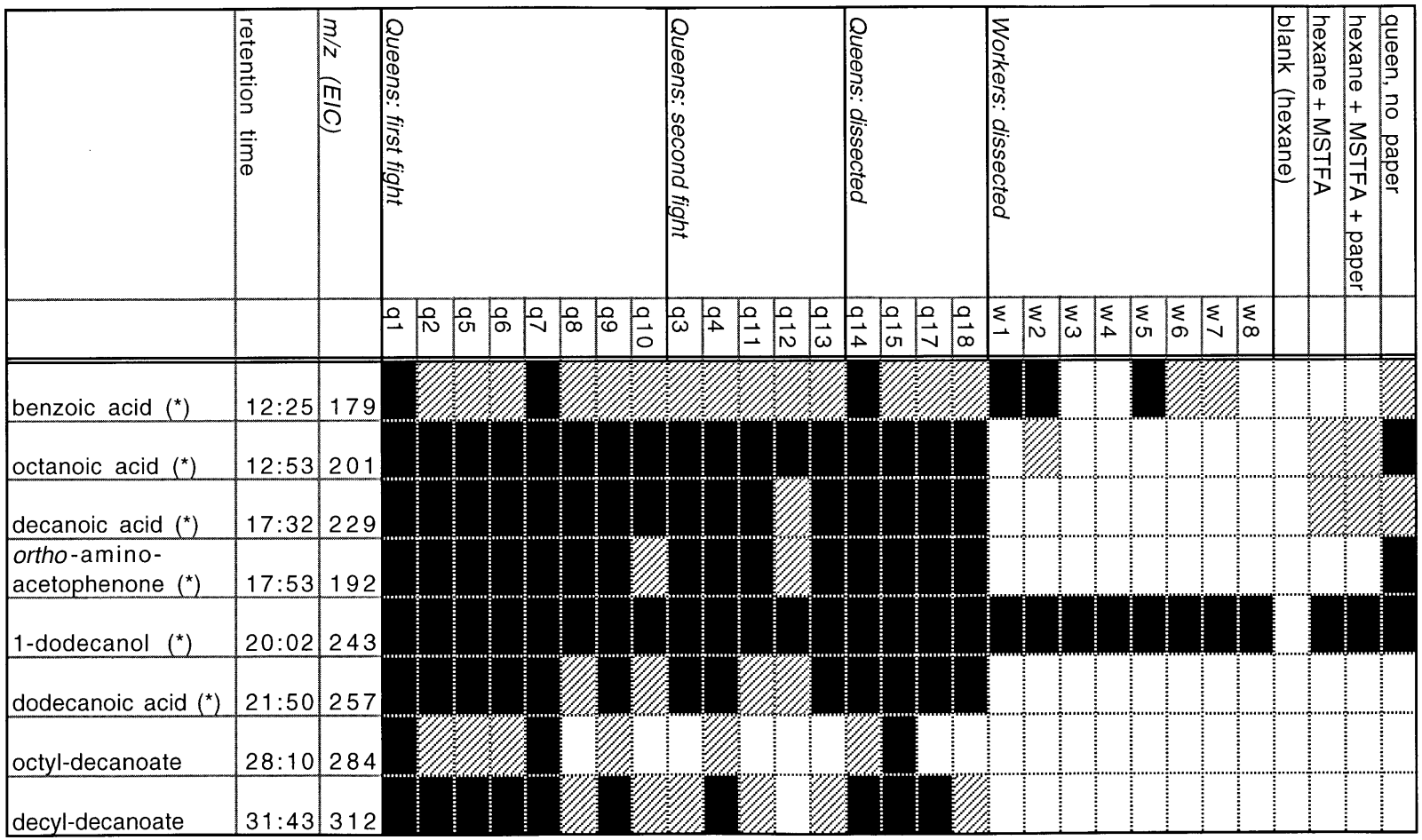
We staged fights between eight pairs of queens aged 3 days (± 1 day) in stoppered glass vials ($\varnothing$ 1.5 cm, height 7.5 cm) pre-cleaned with hexane (*puriss*, BDH Laboratory Supplies, Poole, UK). Queens were introduced sequentially to the vial. Most of the vial's inner surface was lined with a filter paper (LS 14, Schleicher & Schuell, Dassel, D). By leaving a small part of the vial without filter paper we could observe queens fighting. Fights in which one of the queens sprayed were interrupted. We then removed the filter paper, cut out the portion covered by fluid using pre-cleaned scissors, and transferred it to a glass vial. The fluid was extracted from the filter paper with 1000 µl hexane. The advantage of collecting the fluid with filter paper is that it does not prevent or interfere with natural fighting behaviour. A limitation is that it only allows for semi-quantification (see below) because not all of the fluid released can be captured on the filter paper, *e.g.* when some material remains on the queen body.

To investigate whether depletion of some critical substance, in particular *ortho*-aminoacetophenone (Page *et al.* 1988), occurs during fighting, we immediately (< 5 minutes) refought the queens from spraying fights against a different (but equally experienced) opponent and collected five samples. Because of the filter paper lining most of the vial, we could not always identify individually which queen sprayed. Thus, possibly, not all queens tested for depletion had indeed sprayed in the first fight. If the probability of spraying is independent of queen identity, but, for instance, results from random relative position during fight (Butz & Dietz 1994), then in at least 25% of the cases the same queen should spray twice in subsequent fights. This probability will be higher, if queens of inherently lower fighting ability are more likely to spray; and lower, if the same queen is unlikely to spray in both subsequent fights. Thus, this method provides conservative information on whether depletion occurs.

To investigate whether the fluid released contains substances specific to the fighting context and to queens only, we collected samples of the hindgut content of eight workers and four queens that had not fought before. Indeed, previous reports suggested that the fluid released during fights is derived from the hindgut (Post *et al.* 1987). For this, the bees were dissected under CO_2-anaesthesia and low magnification, to expose their intact hindgut, which was then ruptured onto a clean glass slide. The liquid was collected from the slide with a micropipette and placed into vials with 200 µl hexane. The results take into account the different amounts of solvent used. All samples were kept at 4°C until chemical analysis.

For the derivatization, 50 µl of each sample was mixed with 50 µl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA, Fluka, *puriss*). The solution was then heated to 60°C for 20 minutes and injected into a GC/MS instrument consisting of a Varian 3400 (Walnut Creek, CA, USA) gas chromatograph and a Finnigan MAT (San Jose, CA, USA) model SSQ 700 single stage quadrupole mass spectrometer. The splittless injector was at 250°C. The GC capillary column was DB5MS (J&W Scientific, Folsom, CA, USA; 30 m × 0.25 mm i.d., 0.25 µm film thickness) and was operated from 50 to 220°C in 30 min and held there for 10 min. Helium was used as carrier gas. The transfer line was at 250°C. Mass spectra were recorded under electron impact at 70 eV, over the range 40 to 550 m/z at one scan/1.2 s for the full scan mode and over the range 192.15 ± 0.15 m/z at one scan/0.5 s for the single ion monitoring (SIM) mode. The chromatographic peaks were identified by injection of pure compounds. Benzoic, octanoic, decanoic, and dodecanoic acid (*puriss*), 1-dodecanol (*puriss*) and *ortho*-aminoacetophenone (*purum*) were purchased from Fluka. Octyl decanoate and decyl decanoate were synthesized in our laboratories and their structures confirmed by NMR spectroscopy (unpublished results).

Table 1 Presence of eight compounds in samples of queens and workers (based on extracted ion chromatograms). Black = detectable; dashed = traces; white = no traces



(*) detected as trimethylsilyl derivative

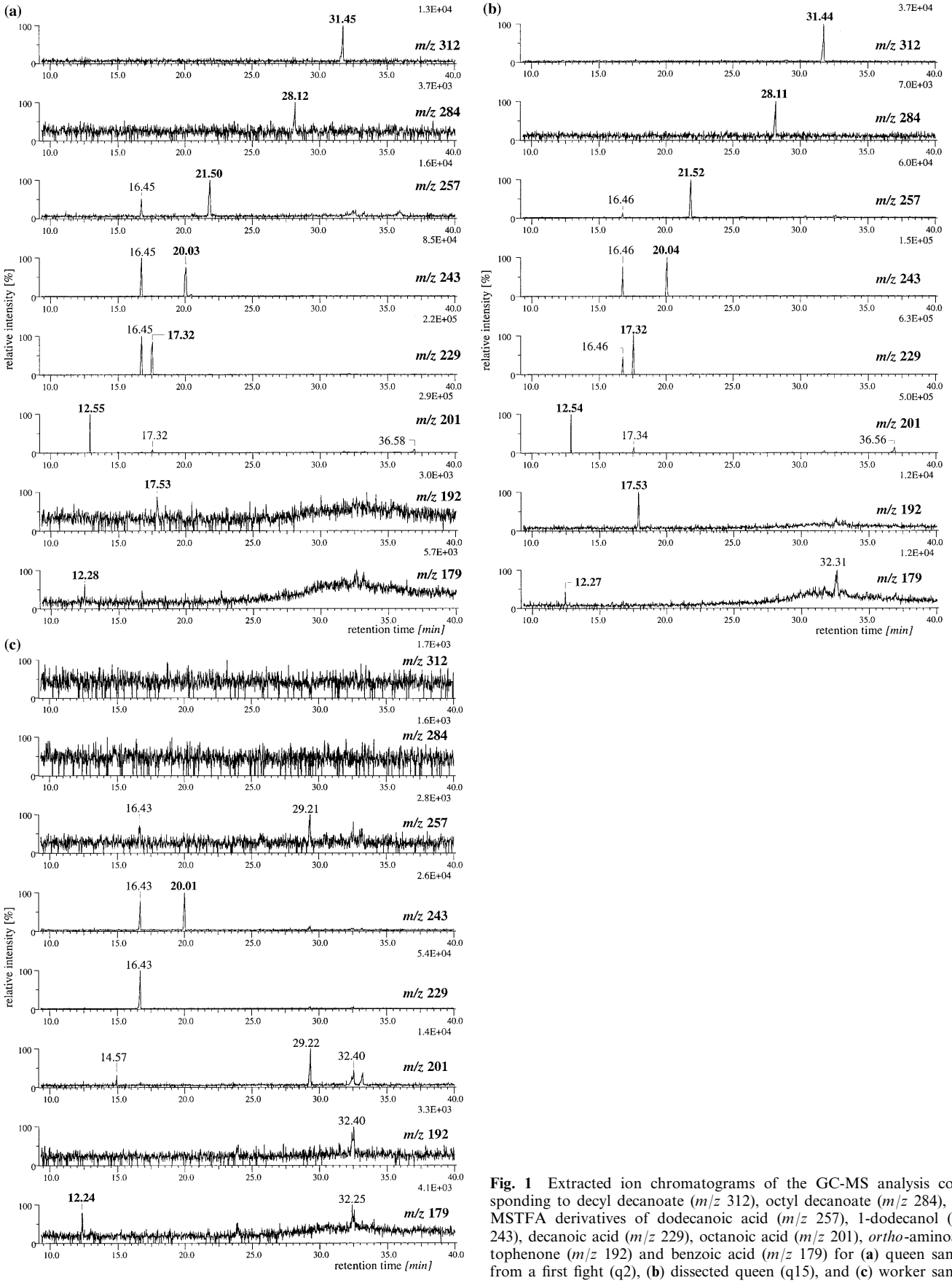


Fig. 1 Extracted ion chromatograms of the GC-MS analysis corresponding to decyl decanoate (m/z 312), octyl decanoate (m/z 284), and MSTFA derivatives of dodecanoic acid (m/z 257), 1-dodecanol (m/z 243), decanoic acid (m/z 229), octanoic acid (m/z 201), *ortho*-aminoacetophenone (m/z 192) and benzoic acid (m/z 179) for (**a**) queen sample from a first fight (q2), (**b**) dissected queen (q15), and (**c**) worker sample (w5). An unidentified impurity ($R_t = 16{:}45$) was detected in MSTFA

Table 2 Estimates of the amount of *ortho*-aminoacetophenone released during a fight, or obtained through dissection of hindgut (single-ion monitoring: m/z 192 ± 0.15; R_t = 17:53 ± 2); t = traces

	Queens: first fight[1]								Queens: second fight[1]					Queens: dissected[2]				Workers: dissected[2]							
	q1	q2	q5	q6	q7	q8	q9	q10	q3	q4	q11	q12	q13	q14	q15	q17	q18	w1	w2	w3	w4	w5	w6	w7	w8
<20 ng								t				t						×	×	×	×	×	×	×	×
20–50 ng						×			×	×	×														
>50 ng	×	×	×	×	×		×						×	×	×	×	×								

[1] = ng/spray; [2] = ng/individual

The results are presented as extracted-ion chromatograms (EIC) of the GC-MS analysis. This allows the detection of substances present in very different concentrations in the samples. The amount of *ortho*-aminoacetophenone in the samples was estimated semi-quantitatively. The peak area obtained by GC-MS in the SIM mode was compared with that of a known amount of a standard solution of *ortho*-aminoacetophenone in hexane (20, 50, 100, 250 ng/ml). Estimates are expressed as ng/spraying event (if collected with filter paper) or, respectively, as ng/queen and ng/worker (if collected through dissection) and aim to estimate the order of magnitude of *ortho*-aminoacetophenone potentially released during fights. As controls, we analyzed one sample of each (i) hexane only (= blank), (ii) hexane/MSTFA 1:1, and (iii) filter paper left during 48 h/RT in hexane and subsequently derivatized with MSTFA.

Results

There was a marked difference between queen and worker samples in that five of the substances identified were restricted to queens. *ortho*-Aminoacetophenone, decanoic acid, dodecanoic acid, octyl decanoate and decyl decanoate were detected in queen samples (Table 1) but not in worker samples, as illustrated by the extracted ion chromatograms (EIC; Fig. 1). One of the compounds mentioned by Page *et al.* (1988), 1-dodecanol, was detected in all samples except the hexane-only control. That is, 1-dodecanol was detected in the controls containing MSTFA (Table 1). Thus, it is not possible to establish with certainty whether 1-dodecanol occurs naturally in both queen and worker samples. Benzoic acid also was found both in workers and in queens (Table 1). One worker sample had traces of octanoic acid. Figure 1 also illustrates how the single fluid components varied greatly in their relative concentrations (peak height compared to background).

The chemical composition of the fluid obtained by dissecting the hindguts of unfought queens corresponded to that of the fluid released during queen-queen fights (Table 1). This demonstrates that the fluid released in fights directly stems from the hindgut. Both in fights between previously unfought queens and in the hindgut of dissected queens, we found the highest amounts of *ortho*-aminoacetophenone (> 50 ng; Table 2); values as high as approximately 250 ng were recorded among these samples. This also indicates close similarity in the amount of *ortho*-aminoacetophenone obtained from the hindgut content before fights, and the amount present in the fluid released during fighting.

The estimated amount of *ortho*-aminoacetophenone exceeded 50 ng in 10 out of 12 samples of previously unfought queens (*i.e.*, either dissected queens or first fights), and in only 1 out of 5 second fight samples (Table 2), suggesting that depletion takes place in subsequent fighting encounters. *ortho*-Aminoacetophenone was not detectable in worker samples. Neither were traces detected using GC/MS in the single-ion monitoring mode (Table 2).

Discussion

By comparing queen and worker hindgut content and the fluid released by fighting queens, we identified five substances specifically characteristic of queens: *ortho*-aminoacetophenone, decanoic acid, dodecanoic acid, octyl decanoate, and decyl decanoate. This difference cannot be ascribed to a different collection method, because we found these queen-specific substances in liquid from hindgut dissection, but not from similar dissection of workers. The controls also confirm that these five queen-specific substances do not stem from either the filter paper, the solvent, or the derivatization medium. These compounds characteristic of queens had been previously listed by Page *et al.* (1988; as an unpublished result); our data reveal that two other substances indicated by Page *et al.* as components of the queen rectal fluid are also found in workers (benzoic acid, octanoic acid). Because the commercially-available derivatization agent we used (MSTFA) contained traces of 1-dodecanol, we were unable to study its natural occurrence in queen and worker samples. For *ortho*-aminoacetophenone, pheromonal effects are established (Page *et al.* 1988). If other substances in Page *et al.*'s list have a biological effect (Page *et al.* 1988; Breed *et al.* 1992), they are likely to be among those specific to queens. Thus, decanoic acid, dodecanoic acid, octyl decanoate, and decyl decanoate potentially deserve further investigation. Decyl decanoate may be especially interesting, because it has been identified in the ethanol extract of the tergite gland of 4-day-old queens, which was found to attract workers (Espelie *et al.* 1990).

That the hindgut content of queens of fighting age contains specific substances absent in workers is in agreement with previous behavioural bioassays, which are now substantiated by our results. Post *et al.* (1987) observed that when queens released hindgut fluid dur-

ing biting and stinging behaviour, the workers usually moved away. By contrast, faeces of older queens (> 2 weeks) or of workers did not elicit any avoidance response.

It is as yet unknown where *ortho*-aminoacetophenone and the other queen-specific substances are produced, both anatomically and in terms of metabolic pathways. We found close similarity between the fluid obtained from dissecting unfought queens and the fluid collected in the queens' first fight: the same substances were identified, and samples obtained in first fights and through queen dissection contained similar amounts of *ortho*-aminoacetophenone. This suggests that the fluid released in fights corresponds with the content of the hindgut. Post *et al*. (1987) found that the response of workers to fluid release was independent of queen diet, suggesting that the active substances are not a normal product of digestion.

Queens in their second fight had lower *ortho*-aminoacetophenone amounts in the fluid they released than inexperienced queens (*i.e*., queens in their first fight, or dissected, unfought queens). This potentially suggests depletion, in agreement with observations that recently-fought queens are less likely to survive a subsequent fight (FLW Ratnieks, unpublished). If the release of rectal liquid lowers the chance that a queen dies in a fight, then we would expect that queens will release a large amount, and subsequently be depleted, either if refighting is rare (*e.g*., if the first fight is fatal in many cases) or if survival during a fight is positively affected by how much is released. However, our estimates are conservative because we cannot establish with certainty that second-fight samples with low *ortho*-acetoaminophenone concentration stem from queens that sprayed in the first fight (see Methods). It is unknown whether the probabilities that the same individual queen sprays in subsequent fight are statistically independent, or whether queens differ in their probability to spray, for instance because of inherent differences in fighting ability. Assuming that depletion occurs, any protective function of *ortho*-aminoacetophenone for spraying queens will probably be highest when queens are inexperienced. Depletion of protective substances, in addition to high risk of fatal injury, may constrain queen behaviour, *i.e*., vulnerable, or recently-fought queens may do best by hiding until their fluid stores has increased again, or until other queens first eliminate each other. Our finding that the fluid released in fights corresponds to hindgut dissection liquid will allow to directly address depletion by examination of the hindgut content of queens before and after fights.

In conclusion, we found five queen-specific substances potentially involved in eliciting the behavioural responses observed during fights when one of the queens releases fluid with an intense grape smell. These substances are equally present in samples obtained by dissecting the queen hindgut and released during fights, suggesting that the fluid released stems directly from the hindgut. The fact that hindgut dissection liquid has a similar chemical composition will greatly facilitate further behavioural studies, because the relevant substances for experimentation can be easily obtained through dissection. We estimated the amount of *ortho*-aminoacetophenone in the fluid of previously unfought and recently-fought queens to vary between 20–250 ng per queen. Whether *ortho*-aminoacetophenone alone or a given combination of the queen-specific substances are relevant to fighting behaviour needs to be clarified by future studies.

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