SUMMARY

The patrolling behaviours of male Megabombus sylvarum (L.) and M. ruderarius (Müller) have been observed. Their labial gland secretions were analysed chemically. The volatile materials, which are probably used as a marking pheromone, were isolated by extraction and then identified by capillary gas chromatography, mass spectrometry and microchemical derivations. The main components of the labial glands in M. sylvarum are Z-7-hexadecenyl acetate (65%) and Z-7-hexadeceno (26%); while the major components in M. ruderarius are Z-9-hexadecenol (67%) and Z-9-octadecenol (9%).

INTRODUCTION

In some species of bumblebees, the males exhibit patrolling behaviour (Haas, 1949), i.e. approaching objects along a flight route and marking them with a pheromone produced in the cephalic part of the labial gland (Ågren et al., 1979; Svensson, 1980a). In woodland habitats, the species may patrol at different levels above the ground while in treeless areas this spatial segregation is more difficult to achieve. The pheromone-marked spots are regularly patrolled by the males throughout the day and attract virgin females for mating purposes (Awram, 1970; Free, 1971; Svensson, 1980a).
Since many bumblebee species may co-exist within a limited area, one might ask what premating species-isolating mechanisms have evolved to reduce the risk of interspecific matings. In bumblebee species where the male patrols a flight route, marking pheromones play an important role in premating species isolation. In this investigation, we have studied two bumblebee species: *Megabombus sylvarius* (L.) and *M. ruderarius* (Müller). They are morphologically similar, patrol at the same time of the year and at the same level above the ground, and are therefore expected to use recognition substances for reproductive isolation.

We report herein the chemistry of compounds found in the labial glands of the two species. Observations on patrolling behaviour in the field are also presented. This report is a continuation of a larger study of marking pheromones in north European bumblebee species (Svensson, 1980 b; Bergström et al., 1981). The chemical analyses and behavioral studies were performed as a co-operative effort between scientists in France and Sweden.

**MATERIALS AND METHODS**

**Biological material**

*Megabombus* males were collected on the Swedish island of Öland in the Baltic Sea. The cephalic portions of the labial glands were dissected, placed individually into vials containing 0.3 ml hexane for 24 h and stored at —20°C. Bumblebee species were determined through genitalia studies (Löken 1973 and Alford, 1975). The nomenclature follows Reinig (1981). Individual bumblebees were labelled and placed in a reference collection.

**Gas chromatography/Mass spectrometry**

GC analyses were performed on a Hewlett-Packard 5880 A (for the gland extracts and their ozonisation products) and a Hewlett-Packard 588 A (for the epoxides) fitted both with a Superox FA WCOT capillary column (30 m; 0.3 mm id.) operating at 150°C during 5 min after injection, followed by programming up to 225°C at 5°C/min.

GC/MS analyses were performed either on a LKB 2091 GC/MS or a Finnigan GC/MS with INCOS 2000 data system. GC capillary columns were the same, but longer (40 m) and were operated under the same conditions.

1 - 3 µl of the extract or 0.1 µg of the standard were injected.

Direct identifications were made by comparisons with reference compounds for mass spectra and GC retention times. These reference compounds were synthetized in the «Laboratoire des Médiateurs Chimiques» by original procedures (M. Lettere Ph. D thesis in preparation).

**Ozonisations and epoxidations**

Ozonisations were carried out on a microscale according to Beroza and Bieri (1967).

Epoxidations of natural compounds were also carried out on a microscale according to Klun et al. (1980) with m. chloroperbenzoic acid in hexane.
Standards of cis and trans 7,8-epoxy hexadecanols, cis and trans 7,8-epoxy hexadecanyl acetates, cis and trans 9,10-epoxy hexadecanols and cis 9,10-epoxy hexadecanyl acetate were prepared according to the following procedure:

In a three-necked round bottomed flask fitted with a magnetic stirrer, a dropping funnel and a thermometer, we introduced $2\times10^{-3}$ mole of the corresponding ethylenic precursor dissolved in 50 ml of anhydrous methylene chloride. A solution of $3\times10^{-3}$ mole of m. chloroperbenzoic acid in 25 ml methylene chloride was added dropwise at room temperature. After 3 h of stirring and one night at room temperature, the reaction mixture was washed with a 10% sodium hydroxide solution, water and brine. Drying on magnesium sulfate and evaporation of the solvent gave a residue which was distilled or crystallized according to the chemical structure of the required epoxide.

RESULTS

Chemical analysis

The GC and GC/MS analyses of extracts of male labial glands were conducted from a single individual of either *M. sylvarum* or *M. ruderarius*.

Typical gas chromatograms are shown in fig. 1-2. In both species, the total amount of volatile material obtained was about 1 mg and consisted mainly of straight chain alcohols, acetates and hydrocarbons.

![Capillary gas chromatogram](image)  
**Fig. 1.** — Capillary gas chromatogram of a part of a hexane extract of the labial gland from one *M. sylvarum* male. Analytical conditions given in Material and Methods section, Numbering of peaks according to Table 1.
For *M. vyliaruni*, GC analysis showed two main components at the retention times of Z-7 hexadecenyl acetate peak 1 (1) (65 %) and Z-7 hexadecenol peak 2 (2) (26 %), with a third polar compound peak 3 (2 %) — probably an octadecenol — and various hydrocarbons (7 %) including tricosane peak 4 and pentacosane peak 6 (Table 1).

For *M. sylvarum*, GC analysis showed two main components at the retention times of Z-7 hexadecenyl acetate peak 1 (1) (65 %) and Z-7 hexadecenol peak 2 (2) (26 %), with a third polar compound peak 3 (2 %) — probably an octadecenol — and various hydrocarbons (7 %) including tricosane peak 4 and pentacosane peak 6 (Table 1).

**Fig. 2. — Capillary gas chromatogram of a part of a hexane extract of the labial gland from one *M. ruderarius* male. Analytical conditions given in Material and Methods section. Numbering of peaks according to Table 2.**

<table>
<thead>
<tr>
<th>No</th>
<th>Compound</th>
<th>M</th>
<th>RV</th>
<th>% T</th>
<th>% LP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Z-7-hexadecenyl acetate</td>
<td>282</td>
<td>2325</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Z-7-hexadecenol</td>
<td>240</td>
<td>2409</td>
<td>26</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>an octadecenol</td>
<td>268</td>
<td>2635</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>tricosane</td>
<td>324</td>
<td>2300</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>tricosanes</td>
<td>322</td>
<td>2318</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>pentacosane</td>
<td>352</td>
<td>2500</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>pentacosanes</td>
<td>350</td>
<td>2519</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

*° = retention value refers to largest unsaturated hydrocarbon.
M = molecular weight.
RV = retention value relative to straight chain saturated hydrocarbons.
% T = % of total secretion (components larger than 1 %).
% LP = % of largest peak.*
By GC/MS, we confirm presence of a C₁₆ monounsaturated acetate (M⁺ = 222 (M⁺ — 60); 109; 96; 82; 67; 55; 43) and alcohol (Mᵣ = 222 (M⁺ — 18); 109; 96; 82; 67; 55; 43). Other minor compounds are a C₁₈ unsaturated alcohol (Mᵣ = 250 (M⁺ — 18)), tricosane (M⁺ = 324), pentacosane (M⁺ = 352), isomeric tricosenes (M⁺ = 322) and pentacosenes (M⁺ = 350). The two C₁₆ unsaturated compounds were then separated by thin layer chromatography (Kieselgel 60F-254 plates (Merck) eluted with CHCl₃/C₆H₆ : 50/50) to determine position and geometrical isomerism of the double bond. Ozonolysis of the acetate fraction and subsequent GC/MS analysis give two products: Nonanal (3) (Mᵣ = 142 (M⁺); 114 (M⁺ — 28); 98; 82; 69) and 7-acetoxyheptanal (4) (Mᵣ = 172 (M⁺); 144 (M⁺ — 28); 129 (M⁺ — 43); 112 (M⁺ — 60)) indicating a 7-hexadecenyl acetate.

Epoxidation and comparison of the resulting epoxide (Mᵣ = 238 (M⁺ — 60); 185 (M⁺ — C₈H₁₇); 155 (M⁺ — C₈H₁₅O₂)) retention time 21.28' with the two authentic cis and trans 7, 8 epoxy hexadecanyl acetate (5) (retention times respectively 21.27' and 20.65') by GC and GC/MS indicated a Z-7 double bond in the ethylenic precursor which is in fact Z-7 hexadecenyl acetate as postulated before.

Acetylation of the alcoholic fraction followed by ozonolysis and epoxidation gave the same compound indicating a Z-7 hexadecenol as the natural product. For *M. ruderarius*, GC analysis showed only a major polar compound peak 1 (67 %) at the retention time of Z-9 hexadecenol (6) and a minor polar compound peak 2 (9 %) at the retention time of Z-9 octadecenol (7). Hydrocarbons comprised about 24 % of the volatile material and included also tricosane peak 3 and pentacosane peak 6 (Table 2).

**Table 2.** — **Major compounds (larger than 1% of total volatiles) identified in the labial gland secretion of *M. ruderarius* males.**

<table>
<thead>
<tr>
<th>No</th>
<th>Compound</th>
<th>M</th>
<th>RV</th>
<th>% T</th>
<th>% I.P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Z-9-hexadecenol</td>
<td>240</td>
<td>2413</td>
<td>67</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Z-9-octadecenol</td>
<td>268</td>
<td>2627</td>
<td>9</td>
<td>13</td>
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<tr>
<td>3</td>
<td>tricosane</td>
<td>324</td>
<td>2300</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>tricosenes</td>
<td>322</td>
<td>2318</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>pentacosane</td>
<td>352</td>
<td>2500</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>pentacosanes</td>
<td>350</td>
<td>2520</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>

See legend for Table 1 for explanations.

By GC/MS we confirm presence of a C₁₆ and C₁₈ monounsaturated alcohols. (Mᵣ = 222 (M⁺ — 18) and Mᵣ = 250 (M⁺ — 18)) and the same hydrocarbons as previously found in *M. sylvarum*. 
The two alcohols were also separated by thin layer chromatography and each one was submitted to ozonolysis (Fig. 3).

![Chemical structures](image)

**Fig. 3.** — Chemical formulae of identified products and their ozonolysis and epoxidation derivatives.
Ozonolysis of the C₁₆ unsaturated alcohol gave heptanal (8) (M/e = 114 (M⁺); 86 (M⁺ — 28)) and 9-hydroxy-nonanal (9) (M/e = 158 (M⁺); 140 (M⁺ — 18); 130 (M⁺ — 28); 112 (M⁺ — 46)) indicating a 9-hexadecenol; while ozonolysis of the C₁₈ unsaturated alcohol gave nonanal (3) (M/e = 142 (M⁺); 114 (M⁺ — 28); 98; 82; 69) and the same 9-hydroxy-nonanal M/e = 158 (M⁺) indicating a 9-octadecenol.

Determination of the geometrical isomerism of the double bond was achieved by epoxidation on the corresponding acetates. The C₁₆ acetylated epoxide (M/e = 238 (M⁺ — 60); 213 (M⁺ — C₆H₁₃); 127 (M⁺ — C₁₀H₁₉O₂)) had the same retention time (21.39') and the same mass spectra as authentic cis 9-10 epoxy hexadecanyl acetate (10) (21.41' - 20.76' for the trans isomer). This identified the natural ethylenic precursor as Z-9 hexadecenol. The C₁₈ acetylated epoxide (M/e = 266 (M⁺ — 60); 293 (M⁺ — C₈H₁₇); 155 (M⁺ — C₁₀H₁₉O₂)) had the same retention time (29.04') and the same mass spectra as authentic cis 9-10 epoxy octadecanyl acetate (11) (29.04' - 28.01' for the trans isomer), indicating a Z-9 octadecenol as the natural compound.
For the two species, variation in the quantity of each component and the overall composition of each gland from one individual to the other was very small. This is demonstrated by a series of four individual gas chromatograms presented in fig. 4 a-d for *M. sylvarum* males. Analytical conditions were the same for all four, see Material and Methods section, Numbering of peaks according to Table 2.

For the two species, variation in the quantity of each component and the overall composition of each gland from one individual to the other was very small. This is demonstrated by a series of four individual gas chromatograms presented in fig. 4 a-d for *M. sylvarum*.

**Patrolling behaviour**

Males of *M. sylvarum* and *M. ruderarius* were found patrolling in the same areas at the same times together along öland’s coastal plains. Their patrolling behavior was similar, i.e., they flew quite slowly and approached the pheromone-marked spots in between tussocks or patches with low vegetation. Spots marked by the two species were sometimes just a few meters apart. Close to the *M. sylvarum* and *M. ruderarium* flight areas, males of *Pyrobombus lapidarius* (L.) were also observed patrolling without interacting with the other species. The
latter species was found closer to the shore, approaching dry Rumex stands and single low Rosa bushes. Figure 5 illustrates the distribution of the flight paths for *M. sylvarum*, *M. ruderarius* and *P. lapidarius*. Bombus terrestris (L.) males were also found patrolling in this area.

![Figure 5. Flight path distribution between M. sylvarum, M. ruderarius and P. lapidarius in a coastal area on the Island of Öland.](image)

**CONCLUSIONS**

Previous studies have revealed that the composition of male marking pheromones differs greatly among bumblebee species (BERGSTRÖM et al., 1981). With regards to morphologically similar species, the chemical composition may be distinctly different, as in *Pyrobombus lapponicus* (Fabricius) vs. *P. monticola* (Smith) (BERGSTRÖM and SVENSSON, 1973), or very similar as in *Alpinobombus alpinus* (L.) vs. *A. polaris* (Curtis) (SVENSSON and BERGSTRÖM, 1979). The two species studied here can be regarded as having fairly similar marking pheromones. They fall into a general chemical pattern found throughout the subgenus *Thoracobombus* of *Megabombus* (SVENSSON and BERGSTRÖM, unpublished). They probably operate as species specific signals. It remains to be seen how precise chemical structure (chain, length, functional group, double bond isomerism) and proportion between components affect species specificity.

However, *Megabombus ruderarius* and *Pyrobombus lapidarius* (KULLENBERG et al., 1970) exhibit greater similarity in the composition of their marking pheromones. The major component in both species is Z-9-hexadecenol. These species segregate spatially and they are also morphologically separated. As found earlier (SVENSSON, 1980 a), males of different species which utilize the same environmental space for mating usually differ greatly by virtue of their mechanical (construction of the genitalia) and pheromonal pre-mating species isolating mechanisms. The species studied here also fit into this generalization.

Patrolling behaviour in *M. ruderarius* has been reported previously by many workers (BISCHOFF, 1927; HAAS, 1949; KRÜGER, 1951 and CUMBER, 1953). HAAS (1949), KRÜGER (1951) and BRINGER (1973) have conducted similar investigations with *M. sylvarum*. These authors supply very little data regarding the interactions between the flight-paths of different species. The two species are
sometimes found to patrol together at a similar height above the ground. However, when possible, *Pyrobombus lapidarius* patrols at tree top level, separated spatially from the other species. In our study areas where all three species were found flying near each other, *P. lapidarius* chose a different microhabitat.

Electrophysiological studies on the male and female antennae (workers and queens) of the different species must be done to understand how the specific chemicals present in the male labial secretion are recognized at the peripheral level by the sympatric males and the species-specific workers, virgin and old queens.

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**RÉSUMÉ**

**LES PHÉROMONES DE MARQUAGE CHEZ LES MÂLES DE MEGABOMBUS SYLVARUM (L) ET M. RUDERARIUS (MÜLLER) (HYM. APIDAE)**

La partie céphalique des glandes labiales des mâles de bourdons produit des composés phéromonaux intervenant dans le comportement de marquage d’un territoire : lieu de rencontre des mâles et des jeunes reines. Le mâle patrouille régulièrement son territoire pour déposer sur les végétaux ces substances odorantes.

Des études antérieures ont montré que la composition chimique de ces phéromones de marquage est propre à chaque espèce de bourdons et qu’elles pouvaient agir comme un facteur d’isolement spécifique. Nous avons donc entrepris dans le cadre d’une collaboration Suède/France l’étude des sécrétions de la partie céphalique des glandes labiales de 2 espèces sympatriques de bourdons qui présentent par ailleurs des similitudes morphologiques et biologiques : *Megabombus sylvarum* (L.) et *Megabombus ruderarius* (Müller). Les mâles de ces 2 espèces patrouillent en effet dans leur territoire au même moment de l’année et de la journée, dans des biotopes identiques au même niveau de la strate herbacée.

Les sécrétions des glandes labiales des mâles de ces 2 espèces ont été isolées et les composés majoritaires identifiés par chromatographie en phase gazeuse sur colonne capillaire (Fig. 1, 2, 4), par spectrométrie de masse couplée à la chromatographie en phase gazeuse et par microchimie (ozonolyse et époxidation). La quantité importante de produit contenu dans les glandes (1 mg/mâle) nous a ainsi permis de déterminer la position et la géométrie des doubles liaisons présentes dans les produits identifiés (Fig. 3). Les structures attribuées aux principaux constituants des glandes...
Labiales of *M. sylvarum* are as follows: *Acetoxy-1* hexadecene 7Z (65%) and *hexadecene-7* ol-1 (26%) (Table 1), those of *M. ruderarius*: *hexadecene-9* ol-1 (67%) and octadecene-9Z ol-1 (9%) (Table 2). These composites, different from one another, should intervene to ensure the specificity of the encounter of the sexes in the interior of the territory. By the chemical secretion of the cephalic part, at action intraspecific, would permit the male to distinguish a young reine vierge fertile from a young queen aged and sedentary and would be determinants for the realization of the coupling at proximity of the points marked in the territory. Conversely, the substances of marking of territory to action attractive at distance, the recognition of the status social of the queens by the males would effect at short distance after alighting of the males and contacts antennaries with the eventual partner.

Des études électro-antennographiques au niveau périphérique devraient être poursuivies pour évaluer le degré de reconnaissance des différents constituants des glandes labiales par les différentes castes de la même société de bourdons.

### ZUSAMMENFASSUNG

MARKIERUNGSPHEROMONE BEI *MEGABOMBUS SYLVARUM* (L.) UND *M. RUDERARIUS* (MÜLLER) MÄNNCHEN (HYMENOPTERA : APIDAE)

Die cephale Partie der Labialdrüsen der Hummelmännchen produziert Pheromone, die bei der Markierung von Territorien (dem Ort zur Begegnung der Männchen mit den jungen Königinnen) eine Rolle spielen. Die Männchen patrouillieren regelmäßig in ihren Territorien und setzen dabei auf Grünpflanzen Duftmarken ab.


Die Sekretionen der Labialdrüsen der Männchen dieser beiden Arten sind isoliert worden und die Hauptkomponenten durch Kapillar-Gaschromatographie (Fig. 1, 2, und 4), durch daran gekoppelte Massenspektrometrie und durch Mikrochemie (Ozonolyse und Epoxidation) bestimmt worden. Die meisten Produkte der Drüsen (1 mg/Männchen) erlaubten auch eine Bestimmung der Position und räumlichen Anordnung der Doppelbindungen der einzelnen Substanzen (Fig. 3). Die Hauptkomponenten des Labialdrüsensekrets sind bei *M. sylvarum* (Tab. 1): *(Z)-7-Hexadecen-1-Acetat* (65%) und *(Z)-7-Hexadecen-1-ol* (26%), bei *M. ruderarius* (Tab. 2): *(Z)-9-Hexadecen-1-ol* (67%) und *(Z)-9-Octadecen-1-ol* (9%). Diese Komponenten, die sich von Art zu Art unterscheiden, müssen als Isolationsmechanismus wirken, so daß die Begegnung der Geschlechter einer Art innerhalb des Territoriums gesichert ist. Andererseits ermöglicht die cephal Sekretion der Königin als intraspezifische Aktion den Männchen die Unterscheidung zwischen einer jungen, unbegatteten und einer alten, begatteten Königin und könnte Auslöser sein für den Vollzug der Begattung in der Nähe der Markierungstellen im Territorium. Im Gegensatz zu den Markierungssubstanzen, die auf Distanz wirken, erfolgt die Erkennung des Zustands der Königin durch die Männchen auf kurze Entfernung, und zwar nach der Landung durch Aufnahme von Fühlerkontakt mit dem eventuellen Partner.

Die elektroantennographischen Studien auf peripherem Niveau sollten fortgesetzt werden, um den Grad der Erkennung der Sekrete aus den Labialdrüsen durch die verschiedenen Kasten aus der Sozietät der Männchen zu erfassen.
LITERATURE


