

Reduced cephalic labial glands in the male bumblebees of the subgenus *Rhodobombus* Dalla Torre (Hymenoptera: Apidae: *Bombus* Latreille)

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Abstract. Until now, all males of bumblebees are known to attract conspecific females by marking places with a chemical signal secreted by their cephalic labial gland. The specific combination of patrolling flight and scentmarking is of utmost importance to bumblebees as it is their main species specific recognition system. We report here the lack of that recognition system in species of the subgenus *Rhodobombus* by comparing the morphology and histology of the cephalic labial glands of *Bombus* (*Rhodobombus*) *mesomelas* with those of a well known species, *B. (Bombus) terrestris* (L.) The cephalic labial glands are much smaller in *B. mesomelas* than in other bumblebees species and most likely non-functional. This morphology is also observed in *B. pomorum* and *B. brodmanni* which also belong to the subgenus *Rhodobombus*. Our morphological observations are consistent with the chemical analyses of the secretions in *B. mesomelas* and *B. pomorum* which are very limited and of most unusual composition for a bumblebee. In addition, whereas other species are thought to use their *barbae mandibularis* to spread their secretion onto the substrate, these structures are absent in all *Rhodobombus*. All these observations would mean that the males of *Rhodobombus* do not attract females from far away by the use of their cephalic labial gland secretions.

Résumé. Glandes labiales céphaliques réduites chez les mâles de bourdons du sous-genre *Rhodobombus* Dalla Torre (Hymenoptera : Apidae : *Bombus* Latreille). Jusqu'à présent, tous les mâles de bourdons sont connus pour attirer leurs femelles conspécifiques à l'aide d'un marquage odorant sécrété par les glandes labiales céphaliques. Les particularités du vol nuptial et de la composition de ce marquage odorant forment une combinaison d'une extrême importance puisqu'elle constitue le principal système de reconnaissance spécifique chez les bourdons. Dans ce travail, nous rapportons l'absence de ce système de reconnaissance spécifique chez les espèces du sous-genre *Rhodobombus*. En comparant l'ultrastructure et l'histologie des glandes labiales céphaliques de *Bombus* (*Rhodobombus*) *mesomelas* avec celles bien connues d'une autre espèce, *B. (Bombus) terrestris* (L.) Nous constatons que les glandes labiales céphaliques de *B. mesomelas* sont extrêmement réduites et qu'elles sont très probablement non fonctionnelles. Cette morphologie est également observée chez *B. pomorum* et *B. brodmanni*, deux autres espèces du sous-genre *Rhodobombus*. Ces observations morphologiques corroborent les analyses chimiques des sécrétions de ces glandes chez *B. mesomelas* et *B. pomorum*, lesquelles sont très réduites et de composition très inhabituelle pour des bourdons. De plus, alors que les autres espèces de bourdons utilisent leurs *barbae mandibularis* pour déposer leurs sécrétions sur les substrats, ces organes sont absents chez tous les *Rhodobombus*. Tout ceci impliquerait que les mâles de *Rhodobombus* ne se servent pas de leurs glandes labiales céphaliques pour attirer les femelles à distance.

Keywords: *Bombus mesomelas*, *Bombus terrestris*, ultrastructure, courtship behaviour, sexual pheromones.

In most bumblebee species, males attract females by marking places with a chemical signal. This behaviour has been described by Darwin (1886, in Freeman 1968). These markings are highly specific (Calam 1969) and substances are secreted by the labial cephalic glands (Ågren *et al.* 1979; Kindl *et al.* 1999).

The species specificity of the labial gland secretions has been confirmed for over thirty species [see review by Bergström (1980) and by Terzo *et al.* (2003)]. The prenuptial marking behaviour has been observed in most West-Palaearctic species (Bringer 1973; Kullenberg *et al.* 1973; Kindl *et al.* 1999) and in many species in N. America (O'Neill *et al.* 1991) and Himalaya (Williams 1991). The use of chemical marking during prenuptial behaviour may therefore be regarded as general among male bumblebees.

The prenuptial behaviour of male bumblebees includes first the olfactory marking of some places,

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followed by an exploration of their territory in search of a female. Such behaviours may occur along three main patterns.

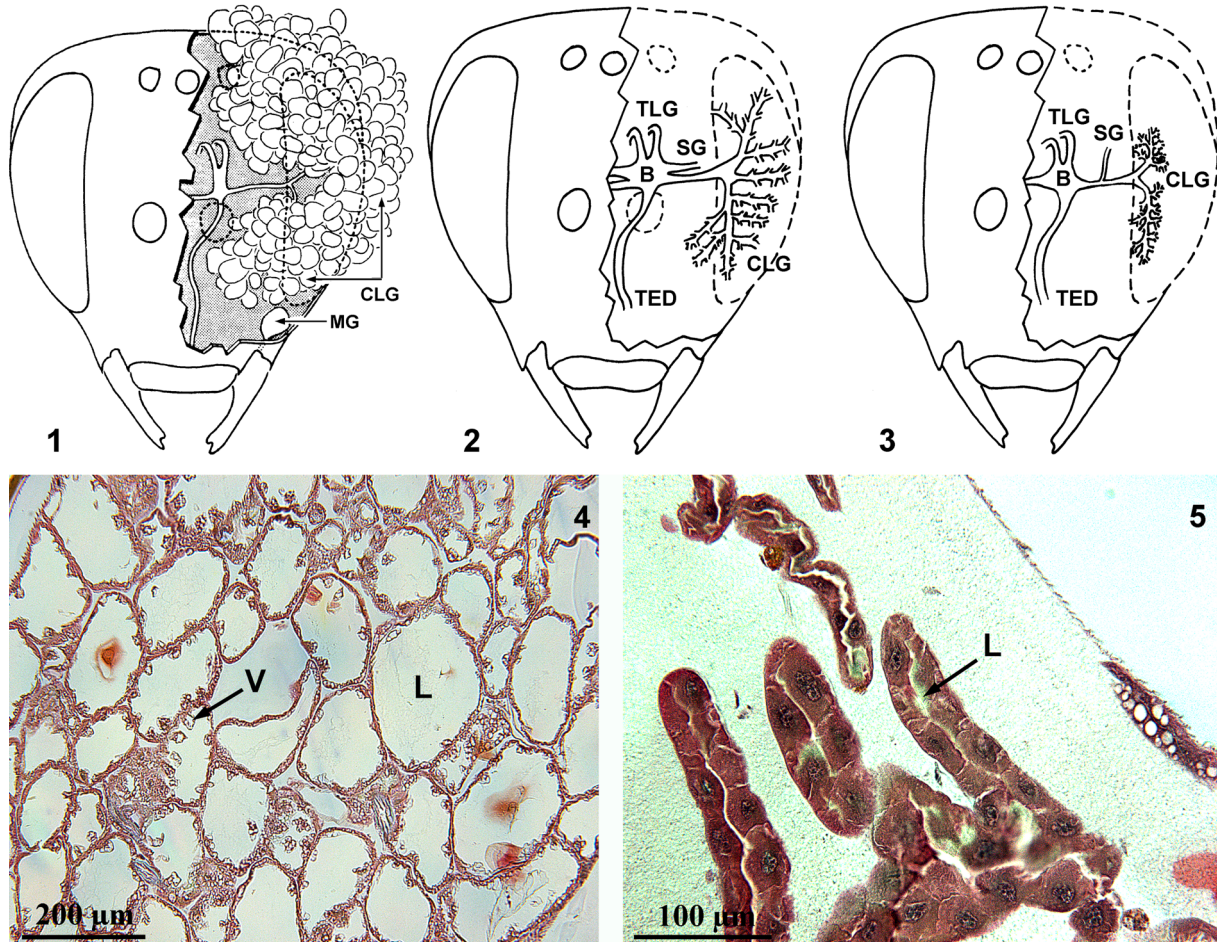
During the “patrolling behaviour”, males scentmark separate places of a circuit, along which they keep flying in search of females. Svensson (1979) has given a thorough description of this behaviour for the bumblebees of N. Scandinavia. This behavioural pattern is common to many species, especially in forests or along wood edges.

Courtship behaviour as described by Haas (1946) in *B. mendax* and by Schremmer (1972) in *B. confusus* corresponds to the “Perching behaviour”. In this case, males scentmark low plants in a territory restricted to a few square meters. They perch in a suitable place of this territory and take off whenever an insect flies by.

Eventually, Williams (1991) described several patterns of the “racing behaviour”. They have in common that the males swiftly circuit at a low altitude, a few tens of centimetres above the ground. This behaviour seems to be adapted to open surroundings.

The specific combination of patrolling flight and scentmarking is of utmost importance to bumblebees as it is the first and perhaps the main specific recognition system (Svensson 1980; Bergman & Bergström 1997). This system fits with the recognition concept of species as given by Paterson (1985).

In *Bombus (Rhodobombus) pomorum* (Panzer), no marking behaviour has ever been observed (Haas 1949). Valterová *et al.* (2001) and Terzo *et al.* (2007) analysed the labial cephalic glands secretions of *B. pomorum* and *B. mesomelas* Gerstaecker respectively, and showed



Figures 1–5

1, semidiagrammatic drawing of a head of *B. lapidarius* male (after Ågren *et al.*, 1979); 2, idem *B. (Bombus) terrestris*; 3, idem *B. (Rhodobombus) mesomelas*, frontal view. The left side cuticle and eye have been removed in order to show the cephalic labial gland (CLG) and the mandibular gland (MG) in fig. 1 and the connections of the cephalic labial gland (CLG), the thoracic labial gland (TLG), the salivary gland (SG) and the terminal excretory duct (TED) with the bursa (B) in figs 2 and 3. 4, acini of the cephalic labial gland of a *B. (Bombus) terrestris* male. L = lumen of the acinus; V = cellular vesicle. 5, acini of the cephalic labial gland of a *B. (Rhodobombus) mesomelas* male. L = lumen of the acinus.

very limited secretions. Their compositions are most unusual for a bumblebee species specific pheromone since they include mostly aliphatic hydrocarbons, but no terpenes and no compounds with oxygenated functional group, with one exception in *B. pomorum* (hexadecanol). Indeed, the most volatile and species specific compounds of other bumblebee species are all absent.

Based on these observations, the present research aims to test the possibility of sexual olfactory marking by males of the subgenus *Rhodobombus* Dalla Torre through a histological study of their cephalic labial glands.

Materials and methods

Material

Twenty three males of *B. (Rhodobombus) mesomelas mesomelas* Gerstaecker were collected in Cerdagne (France, Pyrénées-Orientales) from August 9th to 22nd 2002, between 1600 and 2400 m altitude, in Dorres (WGS: 42°29'N 1°56'E), Err (WGS: 42°26'N 2°02'E), Eyne (WGS: 42°28'N 2°04'E) and Formiguères (WGS: 42°37'N 2°06'E); the observations of prenuptial behaviour were made at Formiguères and Dorres during the same period. Five males of *B. (Rhodobombus) pomorum pomorum* (Panzer) were collected on September 1st 2001 in France, Massif Central, Lozère, Salces (WGS: 44°35'N 03°06'E, 1300 m), feeding on *Cirsium eriophorum* (L.) Scop. and *Cirsium acaule* Scop. One single male of *B. (Rhodobombus) brodmanni* Vogt was collected on August 5th 2002 in Turkey, Erzinçan, between Yeniyöl and Ahmetli (WGS: 39°53'N 39°22'E, 2120 m), feeding on *Cephalaria* sp. In Anatolia, we have not been able to catch even a single male of the fourth known species: *B. (Rhodobombus) armeniacus* Radoszkowski.

While all collected species were dissected to observe the morphology of the cephalic organs *in situ*, the histological preparations were only made on *B. mesomelas* samples and, comparatively, on *B. (Bombus) terrestris* samples. Males of *B. terrestris* were provided by Biobest Ltd, Westerloo, Belgium.

Some prenuptial behaviour has been observed on *B. mesomelas* only.

Morphological study of the cephalic organs

The head was glued to the bottom of a dish with wax and flooded with 95 % ethanol. The cuticle was removed over the whole face with a scalpel, under a binocular microscope.

Histological proceeding

It has been preferred to section the whole head rather than to isolate the organs. This procedure allowed us to observe all the connections under the binocular microscope and to detect even reduced or poorly visible organs. The technique of thick sectioning after polymer embedding is ill adapted to the large size (about 4 mm) of a bumblebee's head. Therefore, we preferred classical paraffin embedding. However, a particular technique had to be devised to soften the

hard cuticle.

Fixation. The heads were immersed for 48 hrs in Dubosq-Brazil fluid: 26 % formalin (40 %), 7 % acetic acid glacial, 45 % picric acid (1 % in 95° alcohol), 22 % distilled water. They were further kept in 95 % alcohol. A part of the eye or the vertex was removed to facilitate the penetration of the embedding fluids.

Softening the cuticle. To soften the cuticle and render sectioning possible, specimens were immersed for 24 to 48 hrs in a bath of equal parts w/w of phenol and chloral hydrate. This had been obtained by moderate warming (40 to 50 °C).

Embedding and sectioning. Specimens were dehydrated for 24 hrs in a mixture of equal parts of butyl alcohol and diethyl ether. They were first immersed for 24 hrs in a solution of 1 % celloidin in a butanol-ether mixture, then, for 48 hrs, in a 2 % solution. The specimens were put in paper moulds (2 × 1 × 1.5 cm), in a 3 % celloidin solution, kept for ten minutes in a low-pressure incubator, then kept in open air until the celloidin had taken a cartilage-like consistency. The preparations were finally embedded in paraffin and sectioned at 5 µm.

Staining. The sections were stained with haematoxylin, fuchsin and Heidenhain blue.

Results

Histology and comparative anatomy of the cephalic labial glands

Bombus terrestris has a pair of voluminous labial glands. These glands extend from the occipital region, between the cerebroid ganglions and the eye to the mandibular glands.

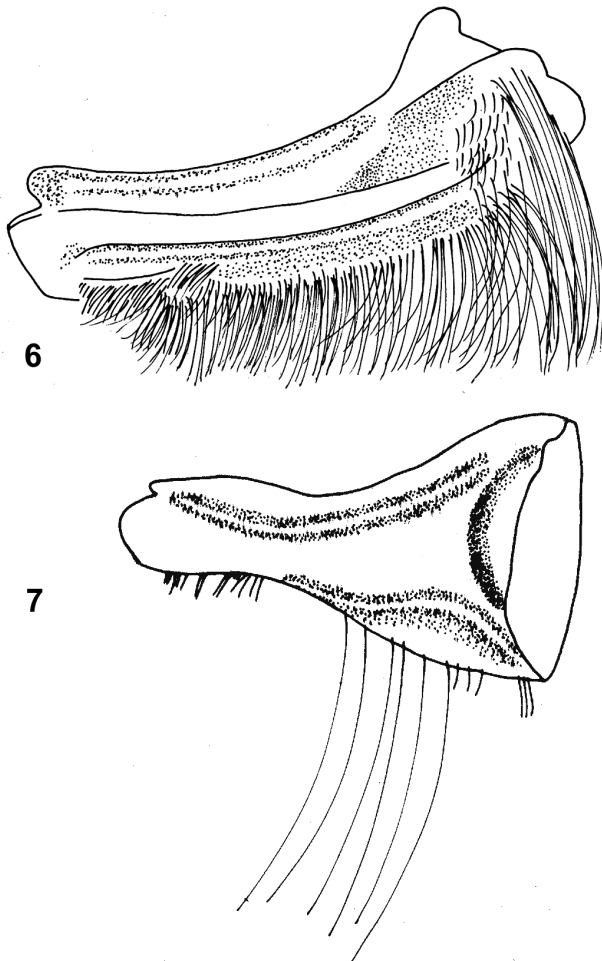
The histology of the cephalic labial glands of *B. terrestris* fits with the observations of Ågren *et al.* (1979) on *B. lapidarius* (L.) (fig. 1). The cephalic labial glands are acinar (fig. 4). The diameter of the acini ranges from 150 to 250 µm. They are constituted of polygonal epithelial cells, with a thin apical cuticular intima. The acini are contiguous, with a large lumen, as may be expected in intensely secreting glands.

The outer diameter of the excretory ducts ranges from 15 to 110 µm. Their wall is a simple squamous epithelium, with a cuticular intima 3 to 4 µm thick. A thin epicuticle lines the inside of the ducts. Outgrowths of the intima distort the epicuticle and protrude into the lumen.

We noticed that the size of the acini is variable, which suggests that they do not secrete at the same time. The more voluminous ones are packed together and their cells are strongly vacuolised. By counting under the binocular microscope (70×), we estimated the total number of acini at 700 for each gland. Assuming that the acini are approximately spherical, we may estimate the gland volume of the pair of cephalic labial glands at 5.90 mm³ (= 4/3 × π × 200³ µm × 700 acini × 2 glands).

Bombus mesomelas also has a pair of labial glands. Their location and histological structure are similar to those of *B. terrestris*, but they are much smaller. Each gland includes about 150 acini, which are flattened, often with a bumpy surface (fig. 5) and not contiguous. Their diameter ranges from 100 to 150 μm and the cells are poorly vacuolised. Assuming that the acini are approximately hemispherical, we may estimate the gland volume of the pair of cephalic labial glands at 0.15 mm^3 ($= 1/2 \times 4/3 \times \pi \times 125^3 \mu\text{m} \times 150$ acini $\times 2$ glands). This volume, about $1/40^{\text{th}}$ that of the *B. terrestris* glands, suggests a very reduced, if not absent, secretory activity.

In both species, the secretory ducts of each gland converge into a main duct. Both these labial gland ducts open laterally into a median bursa (figs 2, 3).



Figures 6–7
Drawing of the left mandible of 6, *B. (Bombus) terrestris* male and 7, *B. (Rhodobombus) mesomelas* male, showing the *barbae mandibularis* on the ventral ridge in *B. terrestris* and its absence in *B. mesomelas*.

Independently of these ducts and more medially, the bursa also collects a pair of ducts draining the thoracic labial glands. Just above the outlets of the cephalic labial glands, the bursa also collects the salivary ducts (in *B. mesomelas*, it may occur that the salivary ducts and the ducts of the labial cephalic glands join before their outlet in the bursa). A long excretory duct leads from the base of the bursa to the base of the glossa. The bursa is located in the middle of the head, beneath all the other organs of the cephalic capsule, between the dorsal wings of the tentorium. As all the ducts connected to the bursa are in a same plane, the general shape of this crossroads is a seven-pointed star.

The diameters of the cephalic labial duct and the cephalic salivary duct are clearly smaller in *B. mesomelas* than in *B. terrestris*. The thoracic labial duct and the common excretory duct are similar in both species.

In the two other *Rhodobombus* species dissected here: *B. pomorum* and *B. brodmanni*, the size and aspect of the cephalic labial gland are similar to what we described for *B. mesomelas*.

In all the males of 61 West-Palaeartic and North American other species, including those collected together with the males of *Rhodobombus* and thus submitted to the same environmental conditions, the morphology and size of the cephalic labial glands were similar to that observed in *B. terrestris*. No one male has shown flattened acini or reduced glands except these *Rhodobombus* species.

Compared morphology of the mandibles

There is one important morphological difference between the mandibles of *B. mesomelas* and those of *B. terrestris*. In the latter, as in the males of all the bumblebee subgenera, except *Rhodobombus*, the ventral margin of the mandible bears a dense and long fringe of setae: the *barbae mandibularis* (fig. 6). This is completely absent in *Rhodobombus* (fig. 7).

Field observations

Part of the prenuptial behaviour of *B. mesomelas* males took place at Dorres in steppe-like surroundings, on dry grassland interspersed with flowering heather (*Calluna vulgaris* Salisb.) and at Formiguères in subalpine grassland clustered with flowering *Trifolium alpinum* L. Both locations were quite different from the foraging sites where the other males were collected. The prenuptial behaviour took place in an open landscape at the top of the hill or of the mountain while feeding sites were found at lower altitude and mostly at the edge of the forests.

In both locations where prenuptial behaviour have been observed, males were seen swiftly flying from one

heather tuft to another. These flights were very low: half a meter above the ground and a few centimetres above or around the flowers. We have seen males flying again and again along the same circuit, never landing to feed nor to mark. As we did not find the nests of the species, we have been unable to notice the behaviour of males at their entrance.

The males of the other *Rhodobombus* species were encountered only while feeding and we have not observed their prenuptial behaviour.

Discussion

The cephalic labial glands of the male *B. (Bombus) terrestris* are acinous and take a very large volume of the cephalic capsule. The same morphology had been observed in *B. (Melanobombus) lapidarius* (L.), *B. (Pyrobombus) hypnorum* (L.) and *B. (Psithyrus) rupestris* (Fabricius) by Ågren *et al.* (1979). This morphology is common to the males of at least 61 bumblebee species from these regions (Terzo, personal observations), except for those of the subgenus *Rhodobombus*. Only the connections of the salivary glands to the bursa have not been observed and described by Ågren *et al.* (1979).

In all the *Rhodobombus* males studied here (*B. brodmanni*, *B. pomorum*, *B. mesomelas*), the cephalic labial glands are very small, almost invisible in the dissection. The histological study of *B. mesomelas* shows that the lumen of the acini as well as the vacuoles of the cells are almost absent. This gives an indication that the secretory activity of the gland is strongly reduced, maybe absent.

It could be suggested that the reduced secretory activity in *Rhodobombus* males is due to the low age of the sampled specimen. According to Ågren *et al.* (1979), in *B. lapidarius* and *B. hypnorum*, the glands start secreting immediately after emergence and are fully functional after four days when males leave their parental nest. This is reflected in the repleted aspect of the acini.

All the bumblebee males examined here have been collected during their prenuptial flight or while feeding. If their cephalic labial secretions are in any way related to the sexual attraction, they should have been active at this time, with repleted acini. On the contrary, in all other bumblebee species flattened acini have never been reported nor observed (Terzo, personal observations) among more than one thousand dissected samples. It is therefore unlikely that the aspect of the *Rhodobombus* cephalic labial glands is due to depletion, to environmental conditions or related to infertility.

Another notable morphological difference for

the males of *Rhodobombus* being the absence of *barbae mandibularis* while they are present in all other bumblebee subgenera (Richards 1968). *Barbae mandibularis* are a dense brush of setae on the lower side of the male mandibles in most bumblebees (fig. 6) and honey bees (Snodgrass 1956). The domestic honey-bee copulates during flight and the females are attracted to the males by the sexual pheromones secreted by the mandibular glands (Lensky *et al.* 1985).

It is more probable that bumblebees' *barbae mandibularis* are associated to the spreading of its sexual pheromones than related to the secretions of the mandibular glands. Indeed, Haas (1946), Krüger (1951), and Bringer (1973) have long described the "chewing behaviour" of bumblebee males. Svensson (1979) observed that this behaviour is related with scentmarking. Eventually, Kindl *et al.* (1999) have determined that the scent deposited onto the substrate during chewing behaviour is identical to the secretion of the labial cephalic glands.

The males of *Rhodobombus* have active mandibular glands (Terzo, personal observations) but its *barbae mandibularis* are reduced to few setae (fig. 7). We suggest that the bumblebees' *barbae mandibularis* are structures dedicated to the application of the scentmarking and if true, the absence of *barbae mandibularis* in male *Rhodobombus* may correlate with the strongly reduced size of their labial glands.

Our morphological and histological observations are also consistent with the chemical analyses of the secretions of *B. pomorum* (Valterová *et al.* 2001) and *B. mesomelas* (Terzo *et al.* 2007) which are very limited and of most unusual composition for a bumblebee species specific pheromone. Indeed, the secretions of their cephalic labial gland do not contain the volatile compounds that are usually met with males of other species (Bergström 1980; Terzo *et al.* 2003). In the latter, major compounds are always either terpenes or aliphatic alcohols, aldehydes or esters while they are very low concentration of only aliphatic hydrocarbons and wax esters in studied species of *Rhodobombus*. Moreover, these latter substances are the same as those detected on the cuticle of many bumblebee species (Oldham *et al.* 1994) as in *B. pomorum* it self (Valterová *et al.* 2001). According to Bergman (1997), aliphatic hydrocarbons are not attracting virgin females of conspecific bumblebee species.

Of course, one cannot exclude that these few secretions of hydrocarbons are used as a pheromone. Hydrocarbons are implicated in intraspecific communication, including premating behaviour but they are usually acting at very short distance or by contact (Ginzel *et al.* 2003; Dronnet *et al.* 2005).

Attracting females from far away with a so small amount of compounds seems to be at least improbable in bumblebees.

Another point in favour of the strongly reduced activity of the cephalic labial glands in male *Rhodobombus* is the probable absence of marking behaviour. The prenuptial behaviour of *B. mesomelas* is of the "patrolling" type (Haas 1949). As this author, we observed the males circuiting swiftly just above the ground, without any evidence of marking by chewing objects.

Frey-Gessner (1899; but no more recent accounts) clearly observed numerous males of *B. mesomelas* entering nests which is not a normal behaviour in other bumblebees: „Die Nester [...] waren stets sehr tief in der Erde, gross und volkreich und verriethen sich durch das lebhafteste unaufhörliche Ab- und Zufleigen von Männchen und Arbeiter.“ [The nests were deeply hidden in the ground, large, populous, and betrayed by incessant comings and goings of males and workers]. It is therefore not unlikely that males do so, in quest of sexual partners. In this hypothesis, marking with pheromones would be pointless.

This hypothesis is related to observations of the mating behaviour of some closely related species of bumblebees. *Fervidobombus* Skorikov is the most closely related subgenus to the *Rhodobombus* (Skorikov 1922; Richards 1968; Rasmont 1983), the only notable morphological difference for the males being the absence of *barbae mandibularis* in the latter (Richards 1968). Although males of *Fervidobombus* do scent mark, they often wait for females emerging from the nest (Villalobos & Shelly 1987; Bergman 1997). The same behaviour has also been recorded by Bergman (1997) in two other subgenera, *Thoracobombus* and *Subterraneobombus*, also related to *Rhodobombus* (Ito 1985).

Conclusion

The morphological study of the cephalic labial glands, which produce sexual marking pheromones in other bumblebees, showed that in *B. mesomelas*, these glands are strongly reduced in size. This reduction has been observed in the three *Rhodobombus* species that we could study. The absence of vacuoles in the glandular cells and the reduction of the lumen suggest that the secretory activity is very low. This is consistent with the chemical analyses of the secretion in *B. pomorum* and *B. mesomelas* we made previously. The absence of *barbae mandibularis* and the fact that no marking behaviour has ever been observed contribute to the hypothesis that males of these species do not scent mark during prenuptial activity. As in other species, scentmarking plays a major role in the specific sexual recognition,

we stay with the hypothesis that copulation might take place within the nests since males of *Rhodobombus* have been observed entering nests. This kind of behaviour may originate in that of the related subgenus *Fervidobombus*, the males of which wait for the females emerging from the nests.

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