

Review article

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Sexual attraction: a review of bumblebee male pheromones

<https://doi.org/10.1515/znc-2019-0003>

Received January 11, 2019; revised May 28, 2019; accepted July 31, 2019

Abstract: Males of many bumblebee species exhibit a conspicuous pre-mating behavior with two distinct behavioral components: scent marking and patrol flying. The marking pheromone is produced by the cephalic part of the labial gland (CLG). As far as is known, the CLG secretion is species specific, and it usually consists of two types of compounds: (i) straight-chain aliphatic alcohols, aldehydes or esters, and (ii) acyclic mono-, sesqui- and diterpenes (alcohols or acetates). Here, we summarize data from the literature reporting chemical composition of the CLG secretions of more than 80 bumblebee species. Similarities and differences within and between subgenera are discussed in the context of biosynthetic pathways and evolution.

Keywords: *Bombus*; bumblebee males; chemical composition; marking pheromone; sex communication.

1 Introduction to bumblebees

Organization and communication in social bees have interested researchers for a long time. The most studied among them is the domesticated Western honeybee, *Apis mellifera*. However, less is known about the primitive eusocial species (i.e. species having a solitary phase in their life cycle), such as bumblebees, but in the last

two decades, the attention of scientists turned toward this group. Many scientists worldwide study the biology, social organization, chemical communication, genetics and evolution of the bumblebee species. Thus, our knowledge on all aspects of the bumblebee life increased substantially. All bumblebees are included in the tribe Bombini [1]. This tribe forms a monophyletic group of over extant 250 species and a few fossil ones [2, 3]. A system of subgenera has been widely used for nearly a century to communicate ideas of relationships among bumblebee species. However, with 38 subgenera, the system was too complicated. Therefore, using a new strongly supported estimate of phylogeny for almost all bumblebee species, the subgeneric system has been reduced to 15 subgenera [4]. At the species level, the tribe Bombini is a taxonomically confused group. Indeed, the morphological homogeneity encountered in bumblebees remains an important obstacle to their identification. In addition, the difficulty is accentuated in the case of cryptic species [5, 6], regional chromatic convergences [7–9] or high intraspecific variability [10].

Bumblebees are large organisms compared to most other species of bees [11]. These endothermic animals are covered with a thick and dense plumose fur, which in addition to capture pollen, allows them to be extremely well adapted to cold regions [12]. They are present on all continents except Antarctica, Oceania and sub-Saharan Africa [2] (Figure 1). However, some parts of the globe, such as New Zealand and Tasmania, have been invaded by *Bombus terrestris*, a species raised and marketed around the world [13].

Bumblebees are haplo-diploid animals, where males (haploids) and queens (diploids) provide the reproductive role and where workers (diploids) maintain the colony in the free-living species [12, 14, 15]. An overwintering queen starts alone the establishment of the nest. She forages on the pollen and nectar to provide resources to the first batch of larvae. At the beginning of the colony cycle, the queen exercises control over all the workers in terms of inhibition of the development of their ovaries [16]. Many attempts were done to find the source and structure of the queen pheromone in bumblebees, e.g. [17, 18]. The

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Figure 1: Original distribution of the *Bombus* genus around the world (yellow). In red, the regions where *B. terrestris* was imported (from Williams [2]).

mandibular gland was long considered a source of inhibiting signal, but a reinvestigation by Bloch and Hefetz [19] has not confirmed the role of any queen's gland in ovary inhibition of workers. The behavior of the queen seems to be crucial at this point [20, 21]. Although *n*-pentacosane was reported as a putative queen pheromone in *B. terrestris* and claimed a conserved signal in bumblebees [22], later studies did not prove the effect of neither this compound nor the body extracts in *Bombus impatiens* [23–25].

After the so-called “switch-point” at the time of the colony's sexual maturation [26–28], new queens and males emerge and leave the colony. The males perform their courtship display and mate with the virgin queens. In the non-tropical areas, the freshly fertilized queens dig and insulate themselves in a hibernaculum for overwintering until the following spring.

The success of social insects largely comes from their ability to accumulate and store large amounts of food resources through a very elaborate division of labor. This considerable energy cost has favored the evolution of parasitic species, which aim to divert this joint effort in their own interest [29]. They exploit their hosts for the rearing of their offspring and, thus, use most of their energy for reproduction [30]. Within bumblebees, some species evolved into nest social parasites called cuckoo bumblebees (or inquilines). With the aim of usurping their guests, these social parasites have adapted physiologically (no worker castes, important number of ovarioles and atrophied wax gland) and morphologically (lack of pollen baskets, larger mandibles, fusion of the intersegment

membranes, a sting connected to more powerful muscles and a larger venom gland) [15, 31–36]. Moreover, they have also managed to overcome the sophisticated recognition systems of their hosts (pheromones and specific cuticular hydrocarbon signatures) [32, 37–39].

2 Pre-mating behavior of bumblebees, chemical communication and pheromones

The encounter between sexual partners is essential in animal reproduction. This encounter and the choice of sexual partners are achieved through a courtship behavior that involves one or more reproductive traits such as secreting semiochemicals [40, 41]. In bumblebees, these secretions are the main signal for pre-copulatory recognition [9, 42]. It is not common to observe mating of bumblebees in the field. However, the pre-copulatory behavior of males is easily observable, and many studies have shown distinct strategies including (i) scent marking, (ii) patrol flying and (iii) nest entrance awaiting [43]. The eco-climatic conditions in which the different species live could explain why one strategy dominates over another. Patrolling behavior is the far most common pre-mating strategy among bumblebee species including cuckoo bumblebees. Males establish flight paths in which they

mark leaves, branches or stones with their cephalic labial gland (CLG) secretions to attract virgin queens. The spatial location of secretions is species specific [44–49]. Males mark their spots in the morning and during the day; they fly from one spot to another and inspect the scent marks.

Perching behavior is less common in bumblebees. This territorial strategy is represented by males waiting at prominent objects (perches) in order to see and approach virgin queens [8]. Species that have adopted this strategy have typically very large eyes [50]. This morphological adaptation does not seem to enable them to distinguish a bumblebee queen from other flying insects [15]. This strategy has been described in several species of subgenera *Bombias*, *Cullumanobombus* and *Mendacibombus*. Beside the optical orientation of males, males use a pheromone, too. They mark their perches (usually 1–3, average: 3.7 per male) [51] with the CLG secretion to increase the efficiency of pre-mating strategy and the probability of encountering a conspecific female. The marking is done in the morning and later in the day; males sit on their marked perches and dart at all passing objects [51]. The composition of the CLG secretion has been described in several perching species and the exclusive optical orientation of males has been disproved.

The third type of pre-mating behavior has been described for males awaiting emerging gynes right at a nest entrance. This strategy was reported for some *Thoracobombus* and *Subterraneobombus* species, occurring in Europe or North America. In late mornings or early

afternoons, males sit on the ground and survey the nest. As soon as a virgin queen comes out of the nest, males fight with each other and some may even chase the queen inside the nest to mate [15]. Aggressive interactions between males have been observed [52]. Recently, a study of genetic divergences (microsatellites) has shown that males around nests came from other colonies [53]. In species using this pre-mating strategy, scent marking has not been observed. However, the males' labial gland produces a secretion in a concentration comparable with patrolling species, and its components resemble those present commonly in CLG secretions [51, 54]. The role of the secretion in this strategy has not been explained yet.

Numerous studies have demonstrated the significant role of chemical signals in intra- and interspecific communication in bumblebees, both inside and outside the colony [55]. All three pre-mating behavioral strategies described above are related to CLG secretion. The secretion plays a role in sexual attraction and species isolation. It functions as a territorial marking pheromone as well as the male sex pheromone. The pheromone-marked places attract conspecific females for mating [56]. The marking pheromone functions both as an attractant and an arrestant for females and, moreover, as a short-term aphrodisiac for males themselves [45]. This pheromone is produced by a paired acinar gland in the head, the cephalic part of the labial gland [57–59]. The glands occupy more than half of the volume of the head (Figure 2A and B). It

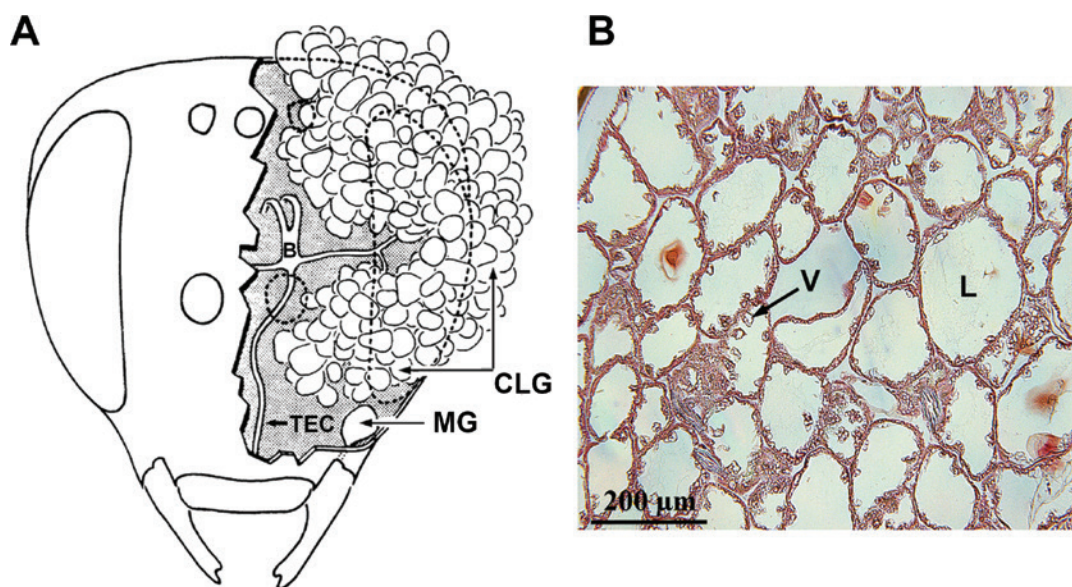


Figure 2: (A) Drawing of a *B. lapidarius*' (*Melanobombus* subgenus) head from the front view. The cuticle and the eye on the left side were removed to reveal the CLG and the mandibular gland (MG). The bursa (B), which receives in particular the secretions of the CLG, as well as the terminal excretory duct (TEC) are visible. (B) Cut in the acini that makes up the CLG. Acini light (L) and secretory vesicles (V) are shown (from Ågren et al. [58] and Terzo et al. [60]).

is secreted at the base of mandibles through an excretory duct [58, 60, 61].

Despite many papers describing the chemical composition of the male CLG secretion, there are only a few reports proving the behavioral role in a bioassay. It has been shown that males deposit the CLG secretion on the vegetation or other prominent objects [51, 56, 59]. In a dual-choice test, Bergman showed that virgin *B. terrestris* queens were able to distinguish between the scent emitted by conspecific and heterospecific males. Lecocq et al. [62] showed that virgin queens were more attracted to CLG secretions of the same subspecies (*B. terrestris dalmatinus*) than to other *B. terrestris* subspecies. The attractiveness of males for virgin females changes with the males' age. Among the individuals 1–30 days old, the 10-day-old males of *B. terrestris* were the most attractive in bioassays [63]. It is still unclear whether the attraction is based on one or several secretion components, or whether the mixture of compounds in specific proportions is the cue for queens. Different volatiles were tested for electroantennographic detection (EAD) responses of queens', males' and workers' antennae. The antennae of workers and males responded equally, while the queens' antennae showed selectivity in perception [64]. Later, active components of the CLG secretion were found by means of gas chromatography (GC)/EAD in *B. terrestris* and *Bombus lucorum* [61, 65]. The queens' antennae responded to the most volatile fraction of the secretion represented by main and medium-abundant components. The role of less-volatile components in communication has not been clarified.

Very little is known about the chemical signals of young females showing their receptivity for mating. Vanhonk et al. [66] reported that the mandibular secretion of young virgin queens contains a sex pheromone that induces mating behavior of conspecific males. The composition of secretions of three different glands of virgin females of five bumblebee species has been published [67], however, without evidence of biological effect of the identified components. More details for *B. terrestris* were given by Krieger et al. [68], who identified several antennally and behaviorally active components of the body surface and cephalic extract of virgin queens. The active compounds were fatty acids and their esters. Geranylgeraniol was the only isoprenoid showing some activity. 3-Oxo- and 3-hydroxydecanoic acids were present in both body and cephalic extracts [68]. (S)-3-hydroxydecanoic acid was later found in the mandibular gland of *B. terrestris* queens as one of the medium-abundant components [69]. Its amount in the secretion changes substantially with the age of queens (as opposed to octadec-9-enoic acid, the main component of the mandibular gland secretion),

reaching a maximum between 5- and 8-day-old queens [69]. Hydroxy- and oxo-acids may, thus, be candidates for further studies on female sex pheromone in bumblebees. According to Krieger et al. [68], testing a mixture of the EAG active compounds in the corresponding concentration did not reach a full effect on males as living queens. Thus, there might also be other cues necessary to stimulate males for mating, such as visual cues.

3 Intraspecific variability of CLG secretions

Several studies highlighted the intraspecific variability of CLG secretions in bumblebees. These studies have focused on the temporal variation of these secretions during the life span of bumblebees [58, 65, 70]. Ågren and co-workers [58] were the first to show variations in the concentration of CLG during the life of some species such as *Bombus (Melanobombus) lapidarius*, *Bombus (Pyrobombus) hypnorum* and *Bombus (Megabombus) hortorum*. The CLG concentration increases up to the fourth day of the male's life. Forty years later, Žáček and co-workers [65] were able to demonstrate that the concentration of secretion reaches its maximum 7 days after the emergence of individuals. In *B. (Bombus) terrestris*, after these 7 days, the concentration decreases very quickly, while it remains fairly stable in *B. (Bombus) lucorum* [65]. These differences were explained by the apoptosis of the secretory cells of the CLGs of males [61]. The cell death begins on the fifth day in *B. terrestris*, whereas in *B. lucorum*, the secretory cells remain functional throughout life [65]. The explanation might be in the type of pheromonal components, among which some compounds such as isoprenoids at high concentration may activate the apoptosis of the secretory cells. This was earlier shown for farnesol, which induces cell apoptosis in different organisms including humans [71–74].

Šobotník and co-workers [61] measured responses of queens to CLG secretions of males of different ages by electroantennography recordings. Although each secretion elicited a response, maximal sensitivity was observed for extracts of glands 2–10 days old. Older gland extracts gradually lose their effectiveness. Coppée and co-workers [63] confirmed the attractiveness in *B. terrestris* females in bioassays. Virgin queens were significantly more attracted to gland extracts of 7-day-old males than to younger or older ones. Although some authors [75, 76] considered male CLG secretions to be invariable between individuals of the same species, local intraspecific differentiations have been observed by other authors among widespread

species such as *Bombus* (*Thoracobombus*) *runderarius* [77], *Bombus* (*Pyrobombus*) *monticola* [78], *Bombus* (*Thoracobombus*) *pascuorum* [10] and *B. (Bombus) terrestris* [70]. In *B. ruderarius*, the CLG secretion was compared for two earlier distinguished subspecies, *B. ruderarius ruderarius* and *B. ruderarius montanus*. These subspecies differ substantially in coloration, but the composition in the CLG secretion is not significantly different [77]. Thus, the two subspecies occurring in the Pyrenees are recommended to be regarded as forms of a single population rather than subspecies [77]. Similarly for *B. pascuorum*, in which 24 subspecies were reported earlier, the taxonomy was simplified to four subspecies groups based on genetic differentiation, color pattern, geographic distribution and analysis of CLG secretions [10]. On the other hand, a revision of the *B. monticola* complex (occurring in the South European mountains and in the Arctic regions) led to its separation into three species, *Bombus conradini*, occurring in the Central Apennine mountains, five subspecies of *B. monticola* distributed in the North Apennine mountains, and *Bombus lapponicus* in the Arctic regions. In *B. terrestris*, six subspecies were compared, including those isolated geographically (e.g. *B. terrestris canariensis*) [70]. Some subspecies could not be distinguished based on the CLG secretion (*B. terrestris terrestris* and *B. terrestris lusitanicus*). The different chemical composition was directly connected to different attractiveness of the CLG extracts for queens [62]. Virgin queens preferred extracts from males of the same subspecies [62].

4 Chemical composition of male marking secretions: interspecific differences

The chemical nature of the males' marking pheromones has been studied extensively. The research in this field started in Scandinavia [44, 75, 79], and it was followed by many studies of the Middle and West European bumblebee species later [77, 80–85]. Recently, reports on CLG secretion of the South and Central American bumblebees [86, 87] as well as of the Japanese species [88, 89] were published. At present, the pheromones of more than 80 bumblebee species are known (i.e. 30% of the described species) [9, 55, 85–87, 90–92] (Table 1 and the Supplementary Table S1).

As far as is known, each bumblebee species produces a specific blend of compounds (reviews [55, 90, 120, 121]). Bergström and co-workers also studied the temporal

and spatial segregation between species and subspecies [93, 110]. Except for the geographical isolation, species patrolling in the same area segregate to some extent in time and space. Species occurring in the same time and habitat differ substantially in the composition of their marking pheromone to avoid interspecies mating. These differences were also used for chemotaxonomical purposes [122]. There is still no report in the literature on the identical composition of the CLG secretions in two different species. Interspecific differentiation is always more important than intraspecific variability.

The gland secretion contains mostly two types of compounds: (i) straight-chain saturated and unsaturated aliphatic alcohols, aldehydes, esters, rarely hydrocarbons with the chain length C_{12} – C_{18} , and (ii) acyclic mono-, sesqui- and diterpenes (alcohols, aldehydes or acetates). The secretions usually contain few main components and a number of lower-abundant or minor components. Beside the main components usually present in milligram quantities per gland of an adult male, unbranched alkanes and alkenes occur in the secretion forming 6%–15% of the mixture, exceptionally in higher proportions. These hydrocarbons are not species specific (odd-numbered chains C_{23} or C_{25} usually prevail with double bonds of alkenes located in position 7 or 9), and they are suspected not to play a role in the communication. When antennal responses of virgin queens to the secretion components were tested, no activity has been found for these alkanes/alkenes [61, 65]. Older males have a higher proportion of hydrocarbons and a lower proportion of antennally active compounds that are anticipated to function as pheromonal components [63].

The main compounds found in the CLG secretions of known bumblebee species are summarized in Table 1. Only those forming a main component in at least one individual of the taxon are listed. Unfortunately, the older literature does not mention numeric values for the concentration of individual compounds. To overcome the diversity in presenting data in the literature, the amounts in Table 1 are marked as “xxx” (relative amount >30%), “xx” (relative amount between 10% and 30%) and “x” (relative amount <10%). A detailed table with all described compounds and original values (either numeric or just semi-quantitative data) is available as Supplementary data (Table S1). Only components higher than 1% are included in this Supplementary file.

Among the CLG secretion main components, the aliphatic compounds prevail. Octadecen-1-ol is the most common, present in 33 species in substantial quantity. The double bonds are located in position 9 or 11; no other position has been found in octadecenol. The second most

Table 1: Abundance of main components in the male CLG secretion.

Subgenus	Bombus sensu stricto																
Bombus Species/compound	terrestris	cryptarum	lucorum	magnus	patagiatus	sporadicus	hypocrita	ignitus	renardi	xanthopus	minshaniola	burjaeticus	moderatus	florilegus	Alpigeno- bombus	Bombias confusus	Kallo- bombus
Isoprenoids																	
Citronellol									x								
Farnesol																	
Farnesyl acetate																	
2,3-Dihydrofarnesol	xxx						xx		xx		xx						
2,3-Dihydrofarnesal							xxx										
Geranylgeraniol		x							x								
Geranylgeranial																	
Geranylgeranyl acetate		x							xx							x	xxx
Geranylcitronellol	xxx															xx	
Geranylcitronellal															xxx		
Geranylcitronellyl acetate																	
Aliphatic alcohols																	
Hexadecan-1-ol	x		x						x						x		
Hexadecen-1-ol																	
Octadecen-1-ol																	
Icosen-1-ol	x	x											x Δ15				
Icosadien-1-ol																	
Aldehydes, ketone																	
Hexadecenal			x Δ7						x Δ7	x							
Octadecenal																	
Icosadienal															xxx		
Heptadecan-2-one																	
Esters																	
Tetradecyl acetate						xxx											
Hexadecenyl acetate																	
Octadecenyl acetate																	xxxΔ9
1,3-Diacetyl-2-dodecan- oylglycerol																	
Ethyl dodecanoate	xxx	xxx	x	xxx	xxx	x	xxx		x		xxx		xxx				
Ethyl tetradecenoate	x Δ9	xΔ9	xxxΔ9						xx Δ9								
Alkene																	
Nonadec-9-ene																	
Literature	[44, 61, 65, 70, 75, 93–95]	[75, 93, 96, 97]	[75, 82, 93, 98]	[44]	[44]	[84]	[88]	[88]	[9]	[9]	[99]	[100]	[100]	[100]	[101]	[80]	[44, 75]

Table 1 (continued)

Subgenus	Cullumanobombus		Melano-bombus		Sibiricobombus	Megabombus		Subterraneo-bombus	
Bombus Species/compound	cullumanus	semeno-viellus	rubicundus	hortulanus	melaucus	morrisoni	rufocinctus	griseo-collis	lapidarius
<i>Isoprenoids</i>									
Citronellol									
Farnesol								xx	xx
Farnesyl acetate	x								
2,3-Dihydrofarnesol									
2,3-Dihydrofarnesal									
Geranylgeraniol	x					xx			
Geranylgeranial									
Geranylgeranyl acetate	xxx	xxx	xxx	xxx	xxx	xxx	xxx		xxx
Geranylcitronellol									
Geranylcitronellal									
Geranylcitronellyl acetate									xxx
<i>Aliphatic alcohols</i>									
Hexadecan-1-ol		x							
Hexadecen-1-ol									
Octadecen-1-ol									
Icosen-1-ol									
Icosadien-1-ol									
<i>Aldehyde, ketone</i>									
Hexadecenal									
Octadecenal									
Icosadienal									
Heptadecan-2-one									
<i>Esters</i>									
Tetradecyl acetate			x					xxx	
Hexadecenyl acetate			x Δ9						
Octadecenyl acetate			xx Δ9						
1,3-Diacetyl-2-dodecan-oylglycerol			x Δ9						
Ethyl dodecanoate									
Ethyl tetradecenoate									
<i>Alkene</i>									
Nonadec-9-ene								xxx	xxx
Literature	[44, 75, 81]	[81]	[86]	[86]	[86]	[102]	[102]	[103]	[44, 75, 104]
									[91]
									[9, 106]
									[88]
									[105]

Table 1 (continued)

Subgenus	Thoracobombus																	
Bombus Species/ compound	pascuorum	humilis	muscorum	rudericus	syllvarum	veteranus	inexpectatus	excellens	atratus	deutero- nymus	exil	filchnerae	diligens	medius	mexi- canus	pensy- lvanicus	trinomi- natus	weisi
Isoprenoids																		
Citronellol																		
Farnesol																		
Farnesyl acetate																		
2,3-Dihydrofarnesol																		
2,3-Dihydrofarnesal																xx		
Geranylgeraniol																		
Geranylgeranial																		
Geranylgeranyl acetate																		
Geranylcitronellol																		
Geranylcitronellal																		
Geranylcitronellyl acetate																		
Aliphatic alcohols																		
Hexadecan-1-ol	xx Δ7	xx Δ9		xxx Δ9	xx Δ7	xxx Δ9	xx Δ7			xxx Δ7	xx Δ9	x						
Hexadecen-1-ol																		
Octadecen-1-ol	x Δ9	xxx Δ9	xx Δ9	x Δ9	x	xx	xx Δ11	xxx Δ11	xxx Δ9	xxx Δ11	x Δ9	xx Δ9	xx Δ11	xxx Δ11	xxx Δ9	xxx Δ11	xx Δ11	xxx Δ11
Icosen-1-ol																	x Δ11	xxx Δ11
Icosadien-1-ol																		
Aldehydes, ketone																		
Hexadecenal	xx Δ7	x Δ9									xxx Δ9							
Octadecenal		xxx Δ11						x Δ11			x Δ9				x Δ9			
Icosadienal																		
Heptadecan-2-one																		
Esters																		
Tetradecyl acetate																		
Hexadecenyl acetate	x	x			xxx Δ7	xx	x Δ7											
Octadecenyl acetate			xxx Δ9			x	xx Δ11	x Δ11	x Δ9			xxx Δ9			xx Δ9			
1,3-Diacetyl-2- dodecan-oylglycerol																		
Ethyl dodecanoate																		
Ethyl tetradecenoate																		
Alkene																		
Nonadec-9-ene																		
Literature	[10, 98, 105, 107]	[92, 105]	[44, 92]	[87, 105, 108]	[87, 105, 108]	[87, 105, 108]	[87]	[86]	[86]	[92]	[92]	[92]	[109]	[109]	[109]	[54, 109]	[109]	[109]

Table 1 (continued)

Subgenus	Pyrobombus					Alpinobombus													
Bombus Species/ compound	monticola	lapponicus	hypnorum	pratorum	huntii	jonellus	cingu- latus	bimacu- latus	konradini	perp- lexus	ardens	impatiens	alpinus	hyper- boreus	balteatus	kirbiellus	neobo- reus	pyrho- pygus	
Isoprenoids																			
Citronellol				x							xxx		x						
Farnesol				xxx	x		xxx												
Farnesyl acetate				xx															
2,3-Dihydrofarnesol					xxx	xxx	xxx				xxx		xx						
2,3-Dihydrofarnesal						xx	x						x						
Geranylgeraniol				x															
Geranylgeranial																xxx			
Geranylgeranyl acetate			x	xx				xxx		xxx			x	xx					
Geranylcitronellol		xxx	xxx																
Geranylcitronellal																			
Geranylcitronellyl acetate			x																
Aliphatic alcohols																			
Hexadecan-1-ol	x		x	xx	x					xx							x Δ9	xxx Δ9	
Hexadecen-1-ol	x Δ9	xx	x Δ9					x Δ7		xx Δ9			xxx Δ9				xxx Δ9		
Octadecen-1-ol				xx Δ11	xxx Δ11									xxx Δ11					
Icosen-1-ol																			
Icosadien-1-ol																		xxx Δ9	
Aldehyde, ketone																			
Hexadecenal																			
Octadecenal																			
Icosadienal																			
Heptadecan-2-one																			
Esters																			
Tetradecyl acetate															xxx				
Hexadecenyl acetate	xxx Δ9							xxx Δ9	xxx Δ9				x						
Octadecenyl acetate																			
1,3-Diacetyl-2-dodecan- oylglycerol																			
Ethyl dodecanoate																			
Ethyl tetradecenoate																			
Alkene																			
Nonadec-9-ene																			
Literature	[75, 78, 110]	[75, 78, 110]	[44, 75, 111]	[44, 75, 111]	[54]	[44, 111]	[111]	[78]	[78]	[112]	[88]	[113]	[87, 114]	[87, 114, 115]	[114]	[115]	[115]	[115]	

Table 1 (continued)

<i>Subgenus</i>	<i>Psithyrus</i>											
<i>Bombus</i> Species/compound	<i>vestalis</i>	<i>perezii</i>	<i>bohemicus</i>	<i>rupestris</i>	<i>campestris</i>	<i>sylvestris</i>	<i>quadricolor</i>	<i>norvegicus</i>	<i>flavidus</i>	<i>insularis</i>	<i>maxillosus</i>	<i>barbutellus</i>
<i>Isoprenoids</i>												
Citronellol			x								x	
Farnesol											xx	xx
Farnesyl acetate												
2,3-Dihydrofarnesol												
2,3-Dihydrofarnesal												
Geranylgeraniol												
Geranylgeranial				x	x							
Geranylgeranyl acetate	x			xxx						xxx	x	x
Geranylcitronellol	xx	xx										
Geranylcitronellal												
Geranylcitronellyl acetate	xxx	xx									x	
<i>Aliphatic alcohols</i>												
Hexadecan-1-ol				x			x			xx		
Hexadecen-1-ol			xxxΔ11	xxx		xxxΔ11		xx	xxx			
Octadecen-1-ol					xΔ11	x	xx	xxx		xxxΔ11	xxΔ11	xxΔ11
Icosen-1-ol	xΔ15		xΔ15		xxΔ11						xΔ13	
Icosadien-1-ol	x		xxΔ11,15	xx								
<i>Aldehydes, ketone</i>												
Hexadecenal			xΔ11			xΔ11	xxx					
Octadecenal	xΔ11		xΔ11		xxΔ11							
Icosadienal	xxΔ11,15	x	xxΔ11,15									
Heptadecan-2-one												
<i>Esters</i>												
Tetradecyl acetate												
Hexadecenyl acetate	xΔ9											
Octadecenyl acetate											xΔ11	
1,3-Diacetyl-2-dodecanoyl glycerol											xx	xx
Ethyl dodecanoate						x						
Ethyl tetradecenoate						xΔ9	x		xx			
<i>Alkene</i>												
Nonadec-9-ene												
Literature	[83, 116, 117]	[118]	[44, 79, 83, 116]	[44, 79, 83]	[44, 79, 83]	[44, 79, 83]	[44, 79]	[79]	[79]	[54]	[83]	[44, 79, 119]

Double bond positions (Δ) are given where ever known.

common component (in 23 species) is hexadecen-1-ol with double bond position 7 or 9, rarely 11. Icosen-1-ol is common, too, with double bond position 11 or 15. From saturated alcohols, hexadecane-1-ol is the most common, but it usually forms a medium or a minor component only. Other saturated alcohols are rather rare or present in very low proportions.

Unlike in alcohols, hexadecenal is by far most common aldehyde in CLG secretions. Similarly to hexadecenol, the double bond position is usually 7 or 9, in some species also 11. Octadecenal is present in seven species, with the double bonds located in position 9 or 11. In one species only, *Bombus wurflenii*, ketones have been found. Heptadecan-2-one and a small amount of pentadecan-2-one are the two main components of this species. *B. wurflenii* is the only species yet analyzed from the subgenus *Alpigenobombus*. Thus, there is no comparison, so far, for the presence of ketones in the CLG secretion of consubgeneric-related species.

Among esters in the CLG extracts, acetates of aliphatic alcohols and ethyl esters of fatty acids occur most frequently. Tetradecyl acetate and the more frequent hexadecenyl acetate are produced by many species. Also, octadecenyl acetate is rather common. The double bond position in hexadecenyl acetate is mostly 9 (exceptionally 7), and in octadecenyl acetate it is 9 or 11. The regioisomers are thus the same as in alcohols and aldehydes. Ethyl esters of many fatty acids occur in the secretions, but only two of them form main components: ethyl dodecanoate and ethyl tetradecenoate. Ethyl dodecanoate is practically exclusively present in species belonging to the subgenus *Bombus sensu stricto*, where it is a “diagnostic” or subgenus-specific component shared by all species. Tetradecenyl acetate, with the double bond exclusively in position 9, occurs in the subgenera *Bombus sensu stricto* and *Psithyrus*.

An interesting subgenus from the chemical point of view is *Megabombus*. Analyses of pheromones of four species were published (Table 1). Three of them produce nonadec-9-ene as the main component. We have analyzed CLG samples of three more *Megabombus* species (*Bombus argillaceus*, *Bombus gerstaeckeri* and *Bombus portchinsky*) and found the same main component (Rasmont, Terzo and Valterová, unpublished results). The only species without nonadecene published so far in this subgenus is *Bombus diversus*. As was mentioned above, hydrocarbons are usually not considered active pheromonal components. However, in this subgenus, the amount of nonadecene in the labial gland secretions is high and corresponds to usual concentrations of the main components in other bumblebee species. Therefore, nonadec-9-ene might play

an important role in the courtship behavior inside this subgenus.

As for isoprenoids, sesqui- and diterpenes usually dominate the CLG secretion. Citronellol was the only monoterpene found, and only four species produced it in a detectable amount. These species belong to different subgenera (*Pyrobombus*, *Alpinobombus* and *Psithyrus*). The low occurrence of monoterpenes might be connected to their higher volatility, which does not suit well to the marking purposes. Marks are usually deposited in the morning and checked by males during the day, but they are not renewed until the next morning [51]. Therefore, less volatile compounds stay on the marks longer and might be advantageous for the marking strategy.

Diterpenic alcohols and their acetates are much more common than mono- or sesquiterpenes among isoprenoids. Geranylgeraniol occurs in 20 species, and so does its acetate. Geranylcitronellol is produced by males of 13 species, and the sesquiterpene 2,3-dihydrofarnesol in 9 species. It is interesting that citronellol, 2,3-dihydrofarnesol [123] and the corresponding aldehyde 2,3-dihydrofarnesal occur in the CLG secretions exclusively as almost pure 3*S*-isomers (enantiomeric purity >98% *S*). Samples of the seven species underwent enantioselective GC, and only traces of 3*R*-enantiomers were detected [113]. Unfortunately, the enantiomeric pairs of diterpenes did not separate on the chiral column; thus, their absolute configuration could not be determined.

When looking at the occurrence of specific pheromonal components within subgenera, we can see a similar composition in some, but a high variability in other subgenera (Table 1). Thus, geranylgeranyl acetate is the main component in all *Cullumanobombus* and *Sibircobombus* subgenera, *Thoracobombus* and *Alpinobombus* are characterized by octadecenol and hexadecenol; however, different double bond positions are present in these alcohols in single species. With one exception (*Bombus pensylvanicus*), no isoprenoids were found in the subgenus *Thoracobombus*. Species of the subgenera *Bombias*, *Cullumanobombus*, *Kallobombus*, *Pyrobombus*, *Megabombus*, *Melanobombus*, *Sibircobombus* and *Subterraneobombus* use no aliphatic aldehydes for communication. No esters occur among the main components in subgenera *Megabombus*, *Sibircobombus*, *Subterraneobombus* and *Kallobombus* (Table 1). Species of the subgenus *Psithyrus* usually produce more than one main/medium component; the CLG secretion is more complex than that in other subgenera. In *Bombus sensu stricto*, a “diagnostic” (subgenus-specific) component of the CLG secretion is ethyl dodecanoate, present in all species studied so far (except for the Japanese *Bombus ignitus*

and the Corsican *Bombus xanthopus*). This compound dominates in some *Bombus sensu stricto* species, while in others, it is a medium or minor component; additional compound(s) distinguish the species. It seems that closely related species belonging to one subgenus share the same enzymes for producing one subgenus-specific component, and the species that are not separated geographically or otherwise evolved new enzymes and pathways for producing additional pheromonal component(s).

A very particular structure and chemistry of CLGs were found in a small monophyletic group (formerly *Rhodobombus*) of the *Thoracobombus* subgenus. By comparing CLGs of four species belonging to this group with those of a well-known species (i.e. *B. terrestris*), it was found that CLG secretions of these four species were unusual and proportionally reduced [60, 85, 124]. The CLG extracts contain mainly hydrocarbons, which are also found on the cuticle [85]. No volatile compounds commonly identified in other species are present in this group [55]. Histological studies have revealed that the CLGs of these species are atrophied and probably non-functional. In addition, morphological structures such as barbae mandibularis, needed for depositing secretions on the substrate, are absent in these species [51, 60]. Thus, males of these species certainly do not use their CLG to attract conspecific females at a distance.

The three basic pre-mating strategies used by bumblebee males are not clearly correlated with the structure of the pheromonal components. While the patrolling behavior is far most common in bumblebees, the number of species whose males perch or wait at the nest entrance is low. No cuckoo bumblebee species has been reported to use any other strategy than patrolling. The perching behavior was described for 11 species of the subgenera *Bombias* (3 species), *Mendacibombus* (3 species), *Cullumanobombus* (2 species), *Sibiricobombus* (1 species), *Melanobombus* (1 species) and *Alpigenobombus* (1 species) [55, 56]. Within other subgenera, no perching species has been reported. The nest waiting strategy has been observed for six species of the genera *Thoracobombus* (five species) and *Subterraneobombus* (one species) [56, 125]. Since these species are not frequent and only a few were reported from the chemical point of view compared to the number of patrolling species, it is not possible to see any chemical pattern that would be connected to certain behavioral strategy. Brasero and co-workers [92] came with a hypothesis that patrolling species often use more volatile pheromonal components (C_{16} derivatives), while nest waiting species produce less volatile components (C_{18} derivatives). This was, however, observed in the *Thoracobombus* subgenus only, but it cannot be generalized

or simply extended to other subgenera. A similarity in pheromonal components can be seen in related species belonging to certain subgenera (see above), but there is no obvious correlation between the behavioral strategy and chemical composition of the CLG secretion.

5 Biosynthesis of the male marking pheromone components

Based on analysis of compound patterns isolated from 22 bumblebee species, Lanne and co-workers suggested that CLG compounds are produced from saturated fatty acids by the action of specific glandular desaturases [94]. This hypothesis was, however, formulated without any experimental evidence. Later, experiments with 2H -, ^{13}C - and ^{14}C -labeled acetate and fatty acids applied in vitro and in vivo were done [126–128]. The model species was mainly *B. terrestris*, species that can be reared in the laboratory, and thus, material of defined age and physiological state can be obtained for experiments. The in vitro experiments proved the formation of both aliphatic and isoprenoid pheromonal components in the CLG [127]. The interpretation of in vivo applications was, however, not unambiguous. There exist two hypotheses on the biosynthesis of aliphatic pheromonal components: (i) de novo formation in the labial gland from acetate units, or (ii) by modification of fatty acids stored in the fat body after their hemolymph transport to the CLG. A mass balance of labeled compounds in feeding experiments supported the transport hypothesis [128]. Thus, the biosynthetic system of the marking pheromone in bumblebee males seems to be very flexible, and the particular active pathway is likely to be controlled by regulatory mechanisms or to depend on the availability of particular substrates.

A transcriptomic approach was used for clarification of the biosynthetic pathways leading to pheromonal components [129]. Next-generation sequencing and quantitative real-time polymerase chain reaction were used to identify and quantify transcript abundances of genes from the isoprenoid biosynthetic pathway in *B. terrestris* and *B. lucorum*. Genes coding the whole set of enzymes needed for isoprenoid synthesis were present in the CLG of both *B. terrestris* and *B. lucorum* males, but their expressions differed dramatically. This explains the difference in the pheromone composition. While in *B. terrestris*, 2,3-dihydrofarnesol is the main component, *B. lucorum* CLG does not contain any isoprenoids. The expression results, thus, indicate that the biosynthesis of isoprenoids is regulated at the transcriptional level [130].

To generalize, the transcriptional regulation might be the reason why closely related bumblebee species often differ in the main components of the male marking pheromones, and this process might be involved in speciation.

6 Integrated taxonomy of bumblebees

Closely related species are often difficult to distinguish using morphological traits. In bumblebees, some species complexes are especially challenging (e.g. *B. lucorum* complex) [131]. There have been many attempts to clarify bumblebee taxonomy by using alternative features such as wing shape, DNA or eco-chemical traits [9]. Recent studies have used a multisource approach to gather different lines of evidence in order to draw a strongly supported taxonomic hypothesis in bumblebee species status. Yet, the resulting taxonomic status is not independent of selected evidence and of consensus methodology. Lecocq and co-workers [132] developed integrated methods including geometric morphometry of wing shape, definition of private haplotypes, sequence-based (nuclear/mitochondrial) species delimitation methods (e.g. bGMYC) and diagnostic composition of the CLG secretions. The usefulness of this method has been shown on several taxa such as *Bombus barbutellus* [119], *B. lapidarius* group [104], *B. monticola* [78, 133], *B. pascuorum* [10], *B. terrestris* [134] and the subgenus *Alpinobombus* [115], in which the taxonomy of species and subspecies has been clarified. Thus, the chemical composition of male CLG secretions is of a great help to taxonomists and forms an irreplaceable part of the method of integrated taxonomy of bumblebees.

7 Phylogenetic consideration: does CLG chemistry meet phylogeny?

Data based on CLG secretions associated with a well-resolved phylogeny offer a possibility to study the evolution and the diversification of these compounds. However, as already shown in other insects such as beetles [135], unpublished and preliminary results suggest that there is no clear phylogenetic pattern in bumblebee pheromones, taking into account only main components. Although some types of compounds are diagnostic for some subgenera (i.e. acetates in *Cullumanobombus*), all these compounds are also found in other subgenera. While some characteristic phylogenetic patterns can be found in

several subgenera so far studied, it does not seem to reflect wider relationships. Besides, if the eco-climatic constraint of the environment, which could interact with compounds deposited by bumblebee males, plays a role in the composition or the detection of the CLG secretions, additional character mapping analyses of these reproductive traits would enable us to detect any evolutionary convergences in similar eco-climatic regions (biomes). It is particularly interesting because bumblebees live in most of the biomes defined in our planet. The effect of eco-climatic conditions has been demonstrated in traits directly associated with reproduction in Lepidoptera [136] and also in birds [137, 138]. Several studies are in progress to explore these hypotheses in bumblebees.

According to the latest phylogenetic study based on transcriptomic analyses, bumblebees (Bombini) are closest to stingless bees (Meliponini) [139]. Males of stingless bees are known to aggregate around the nest entrance of conspecific colonies, but no other similarity with bumblebees in behavior or in chemical communication has been reported. Meliponini drones are not known to release any pheromone attractive for conspecific gynes [140].

8 Comparison of bumblebee pre-mating system with other Hymenoptera

The pre-mating behavior of bumblebee males is not unique in insects; it occurs in other Hymenoptera too. Males of the solitary bee *Colletes cunicularius* wait at the nest entrance to mate the emerging females immediately. In their mandibular gland, both males and females produce (+)-S-linalool, which was shown to be a mate attractant [141]. Males of several *Andrena* bees patrol areas marked with secretions of their mandibular gland. A broad spectrum of terpenes was identified in different *Andrena* species. *Andrena wilkella* produces a specific compound, 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, that was proved to be of high enantiomeric purity, having (2S,6R,8S) configuration [142]. Male territorial marking has been also described for other solitary bees (genus *Centris*, monoterpenic alcohols as marking pheromones) [143] and carpenter bees (genus *Xylocopa*, straight-chain hydrocarbons and fatty acid esters used as marking pheromone, and also, e.g. sesqui- and diterpenic alcohols and aldehydes in *Xylocopa varipuncta* mesosomal gland) [144].

The digging wasps and bee wolves mark their territories too. Males of several species of decorator wasps

(genus *Eucerceris*) display abdomen-dragging behavior on plants surrounding their nest [145]. The source of marking signal is the mandibular gland, from which the secretion spreads to the brush of hairs present on the clypeus and from there is passed onto hairs arranged along the ventral part of the abdomen. The main secretion component is (Z)-3-hexenyl 3-hydroxybutanoate. Males gather at certain spots to compete for females, which can be characterized as lek behavior. The male beewolves, solitary hunting wasps (genus *Philanthus*) use their postpharyngeal gland for territorial marking [146]. The gland has different functions in males and females, and it also differs in the secretion composition, however mostly on the quantitative level.

Males of the stenogastrine wasps, the most primitive among the social wasps, perform rapid flights, stopping on perches where they exhibit special behavior indicating a mark deposited from their abdominal part [147]. The chemistry has not been studied for these insects. In the social wasp *Ropalidia marginata*, it was believed that cuticular hydrocarbons were involved in sex communication, but the bioassay has not proved this hypothesis [148]. This species does not seem to use any long-distance mate attraction cues. On the other hand, in the paper wasps of the genus *Polistes*, the existence of the male marking pheromone has been proved in bioassays, but the chemical nature has not been reported [149, 150].

To summarize, one can say that despite a relatively conserved mating tactics in different Hymenoptera families, where male territorial marking is most common, the diversity of glands used by males for marking is broad as well as the chemodiversity of the marking/sex signals.

9 Remarks to the significance of the research

The conservation of native plant communities, as well as of the wider biodiversity, is dependent on pollinators that are currently under threat and often declining. Recent research has focused on losses in managed honeybee colonies and on declines in wild pollinators. Among other factors, the chemodiversity of male marking pheromones ensures the separation of bumblebee species, and thus, it helps the monitoring of biodiversity. It is important to care for healthy and diverse pollinator communities and for the biodiversity of natural species at their natural area of distribution. Furthermore, the similarities/differences in the CLG secretions may also be an inspiration for further research on the evolution of biosynthetic pathways and enzymes participating in the formation of pheromonal

components. Specific insect enzymes with unique properties have great biotechnological potential, as shown by Tupec and co-workers [151].

Acknowledgments: We acknowledge all people who contributed to the data acquisition through the different missions in the field. Special thanks go to D. Evrard (University of Mons) and K. Urbanová (University of Life Sciences, Prague) for the maintenance of GC mass spectrometry and GC flame ionization detection and evaluation of the samples. B.M. is a Ph.D. student of F.R.S.-FNRS (Fonds de la Recherche Scientifique). The authors also thank P. Kyjaková (Institute of Organic Chemistry and Biochemistry Prague) for her help with the literature database.

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Supplementary Material: The online version of this article offers supplementary material (<https://doi.org/10.1515/znc-2019-0003>).