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The cephalic labial gland secretions of two socially parasitic bumblebees *Bombus hyperboreus* (*Alpinobombus*) and *Bombus inexspectatus* (*Thoracobombus*) question their inquiline strategy

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Abstract Social parasitic Hymenopterans have evolved morphological, chemical, and behavioral adaptations to overcome the sophisticated recognition and defense systems of their social host to invade host nests and exploit their worker force. In bumblebees, social parasitism appeared in at least 3 subgenera independently: in the subgenus *Psithyrus* consisting entirely of parasitic species, in the subgenus *Alpinobombus* with *Bombus hyperboreus*, and in the subgenus *Thoracobombus* with *B. inexspectatus*. Cuckoo bumblebee males utilize species-specific cephalic labial gland secretions for mating purposes that can impact their inquiline strategy. We performed cephalic labial gland secretions in *B. hyperboreus*, *B. inexspectatus* and their hosts. Males of both parasitic species exhibited high species specific levels of cephalic gland secretions, including different main compounds. Our results showed no chemical mimicry in the cephalic gland secretions between inquilines and their host and we did not identify the repellent compounds already known in other cuckoo bumblebees.

Key words bumblebees; *Bombus hyperboreus*; *Bombus inexspectatus*; cephalic labial gland secretions; inquiline strategy; social parasitism

Introduction

Brood care is an energy-consuming activity for social insects ranging from nest construction to offspring breeding (Smisteh *et al.*, 2012). This high energetic cost has favored the evolution of cheating species that exploit brood care behavior of heterospecifics for their own offspring rearing (Roldan & Soler, 2011). This adaptive strategy involves the exploitation of host social behavior by specialized species (so called social parasites) (Lenoir *et al.*, 2001). Social parasites have evolved

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morphological, chemical, and behavioral adaptations (usurpation behaviors) to overcome the sophisticated recognition system (based on species-specific pheromonal and cuticular signatures) and defense systems of their social host (Fisher & Sampson, 1992; Martin *et al.*, 2010).

Among bumblebees (Hymenoptera: Apidae: *Bombus*), the cuckoo species exemplify such a social parasitic strategy. Their life cycle relies on a nonmutual symbiotic relationship (called inquilinism) with their hosts (Lhomme *et al.*, 2012). This interaction leads to a decrease or, in some cases, to a complete loss of host reproductive success (Lhomme *et al.*, 2013). These "cheaters" infiltrate host colonies to take advantage of their food resources and workers (Nash & Boomsma, 2008). In bumblebees, obligate inquilinism evolved in at least 3 subgenera independently (Hobbs, 1967; Wilson, 1971; Michener, 1974): in the subgenus *Psithyrus* consisting entirely of parasitic species (Williams, 1998), in the subgenus *Alpinobombus* with *B. hyperboreus* (Milliron & Oliver, 1966; Richards, 1973) (Fig. 1A), and in the subgenus *Thoracobombus* with *B. inexspectatus* (Yarrow, 1970; Williams, 1998; Müller, 2006) (Fig. 1C).

All *Psithyrus* species are completely dependent on their host because these socially parasitic bumblebees do not produce a worker caste, do not have pollen collection baskets and produce little to no wax to build cells (Benton, 2006; Sramkova & Ayasse, 2008). In the *Alpinobombus* subgenus, the circum arctic *B.* (*Alpinobombus*) *hyperboreus* is mainly known as an inquiline of *B.* (*Alpinobombus*) *polaris* (Fig. 1B) at least in some parts of its range (Milliron & Oliver, 1966; Løken, 1973; Richards, 1973; Pape, 1983; Stenström & Bergmann, 1998; Rampini *et al.*, 2012), but nests of *B.* (*Alpinobombus*) *balteatus* and *B.* (*Pyrobombus*) *jonellus* have been found to be infiltrated by *B. hyperboreus* in Norway (Bergwall, 1970; Gjershaug, 2009). In the Eurasian Arctic and in North America, some females were reported harvesting pollen (Gjershaug, 2009), suggesting a facultative inquilinism for this species. In the *Thoracobombus* subgenus, Yarrow (1970) and Müller (2006) have highlighted a number of peculiar biological and morphological traits of *B.* (*Thoracobombus*) *inexspectatus* (i.e., absence of worker caste, loss of wax production and reduction of the hair-like structures of the hind leg used to gather pollen) that likely are adaptations to parasitic life. *Bombus* (*Thoracobombus*) *ruderarius* (Fig. 1D) was described as the only host of *B. inexspectatus* (Müller, 2006). This subalpine European species occurs in the Cantabrian Mountains and the Alps from 1200 to 2100 m in altitude where it is uncommon (Tkalců, 1963, 1965; Yarrow, 1970; Amiet, 1996; Rasmont *et al.*, 2015). Compared to their respective hosts,

the geographical distribution of *B. inexspectatus* and *B. hyperboreus* and their ecological niches are restricted (Rasmont & Iserbyt, 2014).

The infiltration strategies of parasitic females have been the focus of abundant research (Nash & Boomsma, 2008): some bumblebee species produce very low amounts of cuticular hydrocarbons (chemical insignificance) and/or acquire their host's chemical profile (chemical mimicry) (Dronnet *et al.*, 2005; Martin *et al.*, 2010), whereas others use repellents (allomones) to defend themselves against the attacks of host workers (Zimma *et al.*, 2003; Martin *et al.*, 2010). More recently, a few studies have started to investigate the strategies used by bumblebee inquiline offspring to be tolerated by host workers (Lhomme *et al.*, 2012; Lhomme *et al.*, 2015).

For male offspring of inquiline species, avoiding host recognition is especially challenging because of their early production of large amounts of the species-specific Cephalic Labial Gland Secretions (CLGS) involved in attracting conspecific females during the premating behavior (Žáček *et al.*, 2009; Lhomme *et al.*, 2012; Ayasse & Jarau, 2014). Indeed, CLGS are a complex mixture of (mainly aliphatic) compounds (Calam, 1969) derived from saturated fatty acids (Luxová *et al.*, 2003) involved in premating behavior (Bergström *et al.*, 1981). The species-specific CLGS are synthesized *de novo* by cephalic labial glands in the head of bumblebee males (Žáček *et al.*, 2013). This highly concentrated specific signature are discriminated by host workers and potentially lead to male offspring destruction (Lhomme *et al.*, 2012). Consequently, a strong selective pressure (beside the sexual selection) on the inquiline male CLGS should likely lead to adaptive changes. Similarly to alternative chemical infiltration strategies, one could expect 3 alternative adaptive patterns: (i) chemical insignificance meaning that males do not produce CLGS for premating behavior or produce CLGS only after leaving the host nest or even males produce undetected compounds; (ii) chemical mimicry meaning that the inquiline actively produce the same compounds as its host; or (iii) chemical repellency meaning that specific repellent compounds are produced within the CLGS and act as "chemical shield" to avoid host worker aggressiveness. A previous study on the cuckoo bumblebee *B.* (*Psithyrus*) *vestalis* demonstrated a chemical repellent effect of CLGS on host workers triggered by tetradecyl acetate and (*Z*)-11-hexadecenyl acetate (Lhomme *et al.*, 2015). The chemical strategies used by other *Psithyrus* or non-*Psithyrus* inquiline species are unknown. Here, we perform (i) the first detailed description of the 2 non-*Psithyrus* inquiline CLGS and (ii) comparative analyses of CLGS composition between *B. hyperboreus*, *B. inexspectatus* and their hosts. We finally
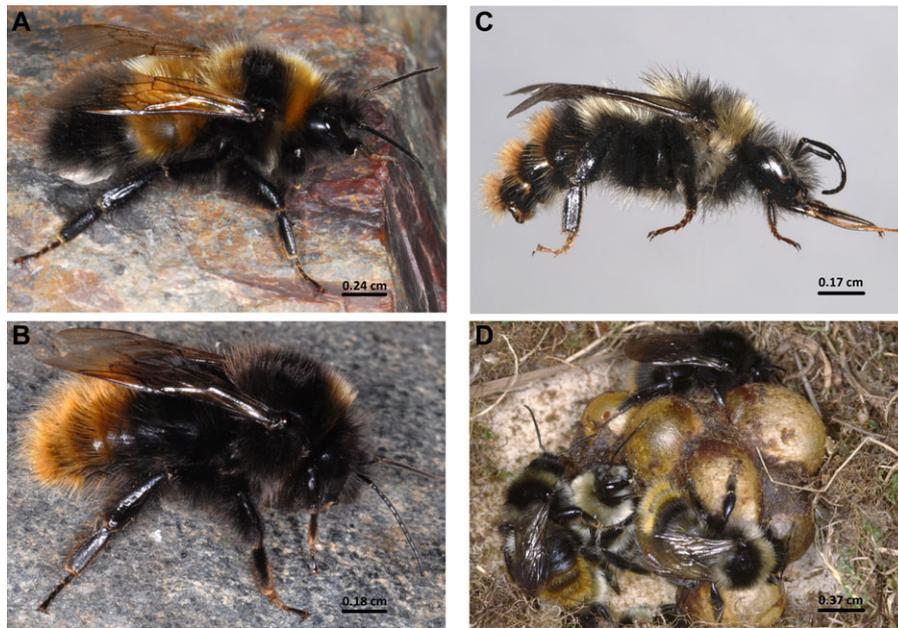
Fig. 1 Photos of the studied bumblebee species: (A) *Bombus hyperboreus* male (PRAS1067) from Alaska, USA (photo: P. Rasmont), (B) *B. polaris* male (PRAS0936) from Norway (photo: P. Rasmont), (C) *B. inexspectatus* male from Alps, Italy (photo: P. Rasmont), and (D) *B. ruderarius* male from Pyrenees, France (photo: P. Rasmont).

discuss the potential chemical strategies involved in these host-inquiline interactions.

Materials and methods

Sampling

In total, 69 specimens (extra-nest males) collected in the field with a net belonging to 2 subgenera were used (Tables 1 and S1). The test group comprised *B. hyperboreus* (*n* = 15) (Fennoscandia and Alaska), and its main host *B. polaris* (*n* = 13) (Fennoscandia and Alaska), and *B. inexspectatus* (*n* = 9) (Alps), and its host *B. ruderarius* (*n* = 8) (Alps and Pyrenees). For each parasite we used as reference group a species which is phylogenetically close, according to Cameron *et al.* (2007), to the respective host bumblebees. *B.* (*Alpinobombus*) *alpinus* (*n* = 14) (Fennoscandia) and *B.* (*Thoracobombus*) *sylvarum* (*n* = 10) (Pyrenees and Massif Central) (Terzo *et al.*, 2005). Concerning *B. alpinus*, individuals from the Alps have been excluded from the analysis to fit with the geographical distribution of *B. hyperboreus*. For *Alpinobombus*, we chose to follow the taxonomy of Milliron (1971) and Løken (1973) as recent updates of Williams *et al.* (2015) still require further investigations. Exact localities are listed in Table S1. All bumblebee specimens were killed by freezing at –20 °C.

Extraction of bumblebee male cephalic labial gland secretions

We focused on the most studied reproductive trait involved in the bumblebee premating recognition (Ayasse *et al.*, 2001; Baer, 2003; Ayasse & Jarau, 2014; Lecocq *et al.*, 2015b): the Cephalic Labial Gland Secretions (CLGS).

The CLGS were extracted from dissected whole cephalic labial glands using 400 μL of n-hexane—see protocol in De Meulemeester *et al.* (2011). Samples were stored at –40 °C prior to the analyses. The composition of CLGS was compared to that established by Lhomme *et al.* (2015) with the same GC conditions to highlight potential common repellent compounds.

Gas chromatography–mass spectrometry analysis—identification

The composition was determined by gas chromatography–mass spectrometry (GC–MS) using a Focus GC (Thermo Scientific) with a nonpolar DB-5 ms capillary column (5% phenyl (methyl) polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm; film thickness 0.25 μm) coupled to DSQ II

Table 1 Sampling sites and number (*N*) of specimens collected for *Bombus hyperboreus*, *B. polaris*, *B. alpinus*, *B. inexpectatus*, *B. ruderarius*, and *B. sylvarum*.

Taxa	Collecting sites	Coordinates	*N*	Code
Alpinobombus				
B. hyperboreus	Sweden, Björkliden	68°24′22″N 18°40′26″E	1	BMAR054
	Sweden, Tarfala	67°53′55″N 18°37′57″E	1	PRAS0951
	Sweden, Tarfala	67°53′55″N 18°37′57″E	1	PRAS0954
	Sweden, Tarfala	67°54′42″N 18°36′36″E	1	PRAS0948
	Sweden, Tarfala	67°54′15″N 18°37′15″E	1	PRAS0952
	Norway, Narvik	68°06′23″N 17°29′20″E	2	PRAS0930
				PRAS0931
	USA, Alaska, Galbraith lake	68°25′31″N 149°21′32″W	1	PRAS1067
	USA, Alaska, Galbraith lake	68°27′21″N 149°28′56″W	2	PRAS1062
				BMAR0242
	USA, Alaska, Toolik lake	68°37′37″N 149°35′46″W	1	PRAS1078
	USA, Alaska, Toolik lake	68°38′22″N 149°34′35″W	2	BMAR0304
				BMAR0305
	USA, Alaska, Toolik lake	68°37′37″N 149°35′46″W	1	BMAR0356
	USA, Alaska, Sag River	68°46′46″N 148°51′19″W	1	BMAR0377
B. polaris	USA, Alaska, Toolik Field station	68°37′40″N 149°35′07″W	10	BMAR0191
				BMAR0157
				BMAR0145
				BMAR0190
				BMAR0192
				BMAR0195
				BMAR0201
				BMAR0206
				BMAR0207
				BMAR0221
	Norway, Narvik	68°06′23″N 17°29′20″E	1	PRAS0929
	Norway, Narvik	68°04′55″N 17°27′02″E	1	PRAS0936
	Sweden, Tarfala	67°54′42″N 18°36′36″E	1	PRAS0949
B. alpinus	Norway, Narvik	68°06′57″N 17°33′13″E	7	PRAS0939
				PRAS0937
				PRAS0938
				PRAS0903
				PRAS0904
				PRAS0905
				PRAS0906
	Norway, Narvik	68°09′06″N 17°29′45″E	1	PRAS0900
	Norway, Narvik	68°07′48″N 17°29′37″E	1	PRAS0935
	Norway, Narvik	68°07′59″N 17°28′52″E	1	PRAS0908
	Sweden, Tornehamn	68°26′28″N 18°37′45″E	1	PRAS0806
	Sweden, Kiruna	68°13′15″N 19°42′46″E	1	PRAS0885
	Sweden, Tarfala	67°54′42″N 18°36′36″E	2	PRAS0946
				PRAS0947
Thoracobombus				
B. inexpectatus	Italy, Val Brembana	45°58′12″N 09°47′53″E	3	NBRA0918
				NBRA0919
				NBRA0920

(to be continued)

Table 1 Continue.

Taxa	Collecting sites	Coordinates	N	Code
	Italy, Val Brembana	45°58′13″N 09°47′58″E	1	NBRA0915
	Italy, Val Brembana	45°58′12″N 09°47′57″E	2	NBRA0916
	Italy, Val Brembana	45°58′11″N 09°47′52″E	3	NBRA0921
				NBRA0922
				NBRA0923
B. ruderarius	Switzerland, Valais, Motôt	46°05′21″N 07°24′13″E	4	NBRA0321
				NBRA0322
				NBRA0323
				NBRA0324
	Switzerland, Valais, Mâssmatte	46°28′37.5″N 08°23′14.6″E	1	NBRA0365
	France, Oriental Pyrenees, Saillagouse	42°28′N 02°02′E	2	MT0015
				MT0017
	France, Oriental Pyrenees, Err	42°25′N 02°03′E	1	MT0035
B. sylvarum	France, Oriental Pyrenees, Llo	42°27′N 02°04′E	1	MT0140
	France, Oriental Pyrenees, Dorres	42°28′N 01°45′E	3	MT0229
				MT0231
				MT0244
	France, Oriental Pyrenees, Eyne	42°29′N 02°05′E	4	MT0253
				MT0258
				MT0267
				MT0277
	France, Lozère, Les Salces	44°33′N 03°07′E	1	MT0330
	France, Hérault, Romiguières	43°48′N 03°14′E	1	MT0346

Note: All sampling information in Table S1.

quadrupol mass analyzer (Thermo Scientific, Austin, Texas, USA) with 70 eV electron impact ionization. We used a splitless injection mode (220°C) and helium as a carrier gas (1 mL/min). The temperature program of the oven was set to 70°C for 2 min and then heated up at a rate of 10°C/min to 320°C. The temperature was then held at 320°C for 5 min. Compounds were identified in Xcalibur™ using the retention times (t_r) and mass spectra of each peak, in comparison to those at National Institute of Standards and Technology library (NIST, USA). Double bond positions (C=C) were determined by dimethyl disulfide derivatization (Cvacka *et al.*, 2008).

GC-FID analysis—quantification

All samples were quantified (quantitative composition) with gas chromatography with a Flame Ionization Detector (GC-FID) Shimadzu GC-2010 system equipped with a nonpolar SLB-5 ms capillary column (5% phenyl (methyl) polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm; film thickness 0.25 μm). The chromatographic conditions were the same as described above (cf. GC–MS analysis). We quantified the peak areas of compounds in GC solution postrun (Shimadzu Corporation, Kyoto, Japan) with automatic peak detection and noise measurement. The relative areas (RAs, expressed in %) of compounds in each sample were calculated by dividing the peak areas of compounds by the total area of all compounds. All compounds for which RA were recorded as less than 0.1% for all specimens were excluded from the analysis (De Meulemeester *et al.*, 2011). We identified the main compound as the compound that has the highest relative proportion (RA) among all compounds of CLGSs at least in 1 individual of the taxon. The data matrix (Table S2) for each taxa was based on the alignment of each relative proportion of compound between all samples performed with GCAligner 1.0 (Dellicour & Lecocq, 2013a). To facilitate the alignment of compounds, before each sample injection, a standard (Kovats) were injected containing a mix of pure hydrocarbons (alkanes) from C 10 (decane) to C 40 (tetracontane). Kovats indices were calculated with GCKovats 1.0 according to the method described by Dellicour and Lecocq (2013b).

Statistical analysis

Each pair of parasite–host complex was considered independently. We performed statistical comparative analyses based on the CLGS matrix using R (R Development Core Team, 2013) to detect chemical differentiations between hosts and parasites. A clustering method, computed with the unweighted pair-group method with average linkage (UPGMA) based on Pearson correlation distance matrices (RA of each compound) (Legendre & Legendre, 2004; Paradis *et al.*, 2004), was used to detect the divergence between taxa in the CLGS composition. We assessed the uncertainty in hierarchical cluster analysis using *P* values calculated via multiscale bootstrap resampling with 100,000 bootstrap replications (R package pvclust) (Suzuki & Shimodaira, 2011). Only bootstrap values greater than 90% were considered significant. To determine specific compounds of each taxon (indicator compounds [ICs]), the indicator-value (IndVal) method was used (Dufrêne & Legendre, 1997). This value is the product of relative concentration and relative occurrence frequency of a compound within a group. The statistical significance of an IC (threshold of 0.01) was evaluated with a randomization procedure.

Result

Identified compounds within cephalic labial gland secretions

Alpinobombus In total, we have detected 117 compounds for the *Alpinobombus* subgenus (69 from *B. hyperboreus*, 72 from *B. polaris*, and 53 from *B. alpinus*) in the CLGS (Table S2). The results showed that all species were differentiated in their CLGS composition (23 compounds shared by all species inside the *Alpinobombus* group). Thirty-four compounds are shared between the parasite *B. hyperboreus* and the host *B. polaris*, 39 between *B. polaris* and *B. alpinus*, and 27 between *B. hyperboreus* and *B. alpinus* (Table S2). In

addition to qualitative differences in minor compounds, other differences are also present in main compounds. In *Alpinobombus* group, octadec-11-en-1-ol (23.90%–56.21%, Table 2) is the main compound in the *B. hyperboreus* CLGS. While the CLGS of *B. polaris* is dominated by hexadec-9-enal (20.8%–43.03%) and hexadec-9-en-1-ol (26.87%–46.68%). The main compound of *B. alpinus* is hexadec-9-en-1-ol (63.95%–89.43%) (Table 2). Thus the main compound of *B. alpinus* (hexadec-9-en-1-ol) is shared by its sister species *B. polaris* and not its social parasite (Table 2).

The IndVal method revealed several ICs with high statistical significance (IndVal > 0.8) for these *Alpinobombus* taxa (Table S2): *B. alpinus* (IC = 8) excluding its main compound (hexadec-9-en-1-ol), which is shared with *B. polaris*; *B. hyperboreus* (IC = 34) including its main compound (octadec-11-en-1-ol); *B. polaris* (IC = 22) including one of its main compounds (hexadec-9-enal).

Thoracobombus We detected 78 compounds in the CLGS of the species belonging to *Thoracobombus* subgenus (36 from *B. inexspectatus*, 52 from *B. ruderarius* and 39 from *B. sylvarum*) (Table S2). The results showed that all species were differentiated in their CLGS composition (12 compounds shared by all species inside *Thoracobombus* group). Sixteen compounds were shared between the parasite *B. inexspectatus* and the host *B. ruderarius*, 24 between *B. ruderarius* and *B. sylvarum*, and 16 between *B. inexspectatus* and *B. sylvarum* (Table S2). In addition to qualitative differences in minor compounds, other differences were also present in main compounds. In the *Thoracobombus* group, the main compounds of *B. inexspectatus* were hexadec-7-en-1-ol (17.23%–36.14%), octadec-11-en-1-ol (12.25%–32.57%), and octadec-11-enyl acetate (9.99%–23.44%) while those of *B. ruderarius* and *B. sylvarum* were, respectively, hexadec-9-en-1-ol (16.49%–70.64%) and hexadec-7-enyl acetate (48.74%–65.34%) (Table 3).

The IndVal method revealed several ICs with high statistical significance (IndVal > 0.8) for these *Thoracobombus* taxa (Table S2): *B. inexspectatus* (IC = 15) including

Table 2 List of main compounds (bold) identified in cephalic labial gland secretions of *Bombus hyperboreus*, *B. polaris*, and *B. alpinus*.

Compounds	MW	*B. alpinus*			*B. polaris*			*B. hyperboreus*		
		Min	M	Max	Min	M	Max	Min	M	Max
Hexadec-9-enal	238	0.00	0.07	1.27	**20.80**	**28.18**	**43.03**	0.00	0.00	0.00
Hexadec-9-en-1-ol	240	**63.95**	**83.16**	**89.43**	26.87	36.33	46.68	0.00	0.00	0.00
Octadec-11-en-1-ol	268	0.00	0.03	0.19	0.00	0.00	0.00	**23.90**	**38.39**	**56.21**

Note: MW, the molecular weight; M, median of relative concentration of compound (%). All identified compounds in Table S2.

Table 3 List of main compounds (bold) identified in cephalic labial gland secretions of *Bombus inexspectatus*, *B. ruderarius*, and *B. sylvarum*.

Compounds	MW	B. inexspectatus			B. ruderarius			B. sylvarum		
		Min	M	Max	Min	M	Max	Min	M	Max
Hexadec-7-en-1-ol	240	**17.23**	**24.99**	**36.14**	0.00	0.00	0.00	23.54	28.65	32.14
Hexadec-9-en-1-ol	240	4.94	7.47	8.32	**16.49**	**37.26**	**70.64**	0.00	0.00	0.00
Octadec-11-en-1-ol	268	**12.25**	**17.66**	**32.57**	3.97	7.57	11.25	0.00	0.00	0.00
Hexadec-7-enyl acetate	282	3.27	5.35	11.97	0.00	0.00	0.00	**48.74**	**56.52**	**65.34**

Note: MW, the molecular weight; M, median of relative concentration of compound (%). All identified compounds in Table S2.

the main compound octadec-11-enyl acetate; *B. ruderarius* (IC = 3) including the main compound hexadec-9-en-1-ol and *B. sylvarum* (IC = 9) including the main compound hexadec-7-enyl acetate.

Interpretation of clusters

For both groups (*Alpinobombus* and *Thoracobombus*), the statistical analyses of the CLGS composition confirmed the interspecific differentiations and are supported by high values of multiscale bootstrap resampling (Figs. 2A and B).

Discussion

Comparison of the parasite CLGS composition with past identifications

Compared with Svensson and Bergström (1979), we have identified the same main compounds for *B. hyperboreus*. Concerning *B. sylvarum* and *B. ruderarius*, we have also identified the same main compounds determined by Bergström *et al.* (1985). However, thanks to improved methods of chemical analyses, we identified many more compounds in the CLGS composition especially with double bounds information. Concerning *B. inexspectatus*, the description of CLGS composition is here reported for the first time.

Evidence for host evasion strategies in parasitic males

Chemical mimicry hypothesis In an older study investigating bumblebee taxonomic relationship based on their CLGS (Bellés *et al.*, 1987), both cuckoos (*Bombus Psythirus rupestris*) and hosts (*Bombus Melanobombus lapidarius*) appeared mixed together in the cluster analysis. According to the authors, there is selection in cuckoos for similarity to their hosts to improve parasitization. These data were in accordance with observations made by Cederberg (1979) showing that *B. rupestris* recognizes the specific marking secretion of its host *B. lapidarius*.

While chemical mimicry is a widespread strategy in social parasites (Dettner & Liepert, 1994; Akino, 2008), our CLGS composition-based comparative analyses seem to rule out the chemical mimicry hypothesis for both *B. hyperboreus* and *B. inexspectatus*. These results are consistent with previous studies on CLGS of other parasitic bumblebees (namely subgenus *Psithyrus*) that do not show similarities between inquiline and host species (Lecocq *et al.*, 2011, 2013, 2015a; Lhomme *et al.*, 2015).

Other previous studies have shown that CLGS play a major role in the attractiveness of females during prenuptial behaviors (Lhomme *et al.*, 2012; Ayasse & Jarau, 2014). These secretions are very specific even in cryptic species (Bertsch *et al.*, 2005; Brasero *et al.*, 2015). Thus sharing the same recognition signals could affect the intrinsic role of CLGS in prenuptial behaviors. This may explain why chemical signals from these glands are maintained as distinct from their host species.

The interspecific CLGS comparisons (Rasmont *et al.*, 2005; Lecocq *et al.*, 2015a; Brasero *et al.*, 2015) show higher composition similarities between phylogenetically closely related bumblebee species. For the *Alpinobombus* group, the cluster analysis indicates that the CLGS composition of the hosts (*B. polaris*) is more similar to its sister species (*B. alpinus*) than to its parasite (*B. hyperboreus*), which is a sister lineage to *B. alpinus* and *B. polaris* (Fig. 2A). In the *Thoracobombus* group, the composition of *B. inexspectatus* is more similar to the reference species (*B. sylvarum*) than its own host (*B. ruderarius*) (Fig. 2B). Hines and Cameron (2010) showed that *B. inexspectatus* is phylogenetically closer to *B. sylvarum* than its host *B. ruderarius*.
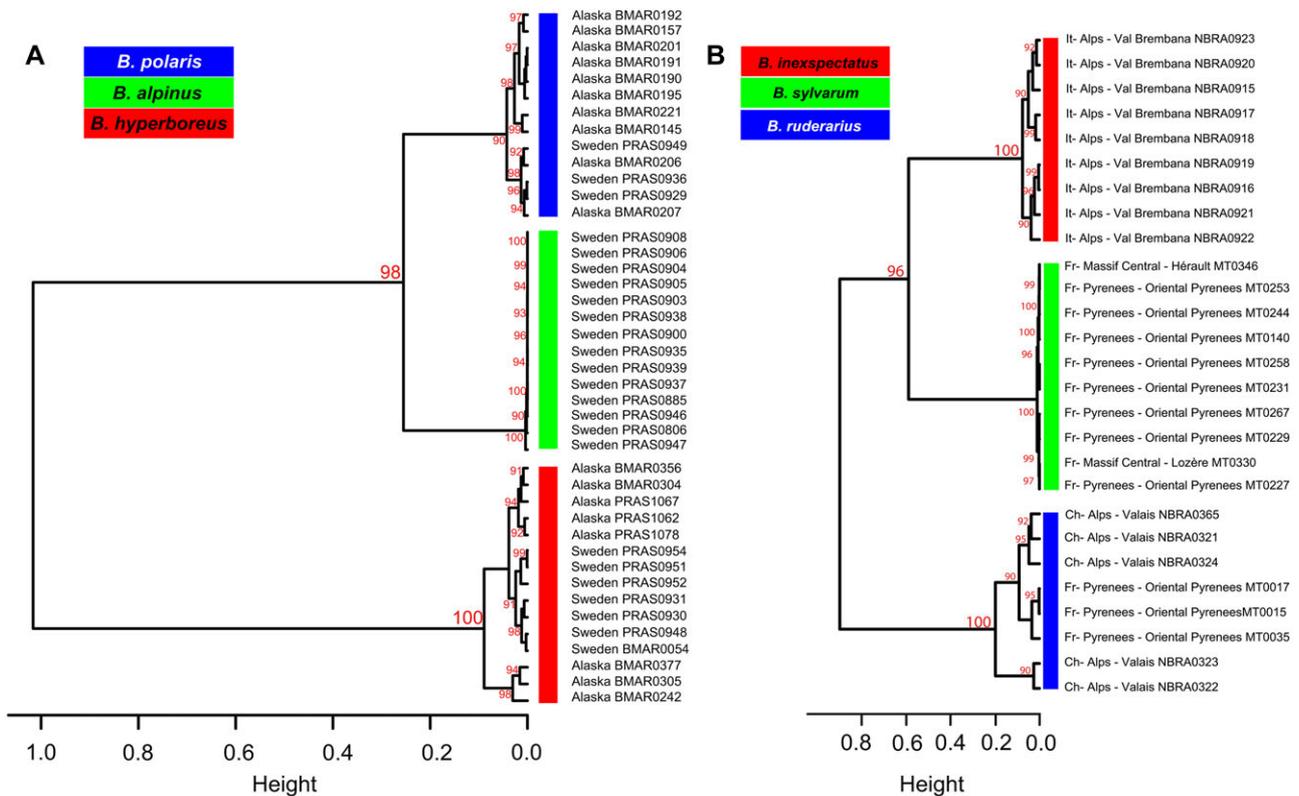
Fig. 2 Unweighted pair group method with arithmetic mean (UPGMA) cluster based on a correlation matrix calculated from the composition of cephalic labial gland secretions of *Bombus hyperboreus*, *B. polaris*, and *B. alpinus* (A); *B. inexspectatus*, *B. ruderarius*, and *B. sylvarum* (B). The values near nodes represent multiscale bootstrap resampling values (only values >90 are given). It, Italy; Fr, France; Ch, Switzerland.

Chemical insignificance hypothesis Our results showed that mature male parasites emit a specific mixture of compounds from their cephalic labial glands so they are not using an absence of species-specific chemicals to avoid host detection (i.e., chemical insignificance).

Chemical repellency hypothesis Since our results suggest that chemical insignificance and chemical mimicry strategies are unlikely in *B. hyperboreus* and *B. inexspectatus*, an alternative hypothesis seems to be the production of 1 or more repellent substances within CLGS by males. However, the repellent compounds identified by Lhomme *et al.* (2015) and Zimma *et al.* (2003) in other cuckoo bumblebees (dodecyl acetate, tetradecyl acetate, and (*Z*)-11-hexadecenyl acetate) have not been found in the CLGS extraction of *B. hyperboreus* and *B. inexspectatus*. Other compounds in these glands or other glands that produce species-specific cuticular hydrocarbons could instead have a repellent

effect but future studies are needed to confirm this hypothesis.

Limitations and future prospects

Since we sampled only extra-nest males, we cannot exclude potential mimicry or chemical insignificance strategies during the early age of parasite males. However, a previous study showed that only the quantitative composition of the CLGS varies between intranest and mature emerged males (Žáček *et al.*, 2009). Detailed assessment of these hypotheses requires *in natura* observations of nest infestations or bioassays on laboratory-reared specimens that will be challenging to obtain: wild nest infestation observations are uncommon (Lecocq *et al.*, 2011) and species-specific year-round breeding methods needed for bioassays (Lhomme *et al.*, 2012; Lhomme *et al.*, 2013) are available for only few species (Hasselrot, 1960). Moreover, in recent decades, *B. hyperboreus* and *B. inexspectatus* have become scarce

especially in Europe (Rasmont *et al.*, 2015) increasing the difficulty of developing such a protocol with a suitable sampling. Indeed, as far as we know, only a few people have succeeded in breeding *B. polaris* and *B. ruderarius* (Sladen, 1912; Wojtowski, 1963; Berezin, 1995). Moreover, from a total of 988,187 specimens recorded in Rasmont *et al.* (2015), only 370 *B. hyperboreus* (0.03%) and 275 *B. inexspectatus* (0.02%) were recorded including them as the rarest West-Palearctic bumblebees.

In bumblebees, chemical communication is not only through the CLGS. Cuticular compounds should also play an important role in the recognition systems between host and parasite (Martin *et al.*, 2010). Therefore, it would be interesting to investigate the composition of this cuticular signature to improve our knowledge on the intrusion and escape mechanisms of these non-*Psithyrus* parasites.

To test these hypotheses, neurophysiological experiments could be useful (i.e., gas chromatography coupled with electro-antennographic Detector (Lhomme *et al.*, 2015) or a glomerular function test (Devaud *et al.*, 2015). Unfortunately, the lack and the difficulty to breed these specimens make experiments challenging for now.

Conclusions

In this study, we find that the independently evolving social parasites *B. hyperboreus* and *B. inexspectatus* display a species-specific mating chemistry in their cephalic labial glands typical of bumblebees. Although these mating secretions could make them vulnerable to detection and exclusion from the host nest, our results do not support shifts in chemistry expected from major hypothesized host evasion strategies. These species are not devoid of sexual cephalic labial attractive compounds so are not chemically undetectable. These CLGS are also not more similar to their hosts than would be expected phylogenetically so are also not involved in chemical mimicry. Finally, we have not identified the repellent compounds highlighted by Lhomme *et al.* (2015) in *B. vestalis*. Other repellent compounds in these or other glands could, however, be important for host evasion.

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Disclosure

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 All sampling information of specimens collected for *Bombus hyperboreus*, *B. polaris*, *B. alpinus*, *B. inexspectatus*, *B. ruderarius*, and *B. sylvarum*.

Table S2 All identified compounds identified in cephalic labial gland secretions of *Bombus hyperboreus*, *B. polaris*, and *B. alpinus*.