The cephalic labial gland secretions of two socially parasitic bumblebees Bombus hyperboreus (Alpinobombus) and Bombus inexpectatus (Thoracobombus) question their inquiline strategy
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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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Short Title: Involvement of CLGS in the inquilinism of *B. hyperboreus* and *B. inexspectatus*

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¶ These first co-authors contributed equally to this work
Abstract-} Socially parasitic Hymenoptera 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 three subgenera independently: in the subgenus *Psithyrus* consisting entirely of parasitic species, in the subgenus *Alpinobombus* with *B. hyperboreus*, and in the subgenus *Thoracobombus* with *B. inexspectatus*. Cuckoo bumblebee males utilize species-specific cephalic labial gland secretions for mating purposes which have been showed as acting in their inquiline strategy. We performed cephalic labial gland secretions comparative analyses of *B. hyperboreus*, *B. inexspectatus* and their hosts. Males of both parasitic species exhibit high species specific levels of cephalic gland secretions, including different main compounds. Our results show 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** Social parasitism, bumblebees, *Bombus hyperboreus, Bombus inexspectatus*, cephalic labial gland secretions, inquiline strategy
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 hetero-specifics 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 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 non-mutual 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 three 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 sub-alpine European species occurs in the Cantabrian Mountains and the Alps from 1200m to 2100m in altitude where it is uncommon (Tkalců 1963; Tkalců 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, 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 pre-mating behavior (Žáček et al. 2009; Lhomme et al. 2012; Ayasse and 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 pre-mating 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 three alternative adaptive patterns: (i) chemical insignificance meaning that males do not produce CLGS for pre-mating 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). Potential other chemical strategies used by other Psithyrus or non-Psithyrus inquiline species is unknown. Here, we performed (i) the first detailed description of the two non-Psithyrus inquiline CLGS and (ii) comparative analyses of CLGS composition between B. hyperboreus, B. inexspectatus and their hosts. We finally discuss the potential chemical strategies involved in these host-inquiline interactions.
Materials and Methods

Sampling

In total, 69 specimens collected belonging to two subgenera were used (Table 1; Supplementary material 1). 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). As a reference group, we used one phylogenetically close species of the host bumblebees of each parasite according to Cameron *et al.* [41]: *B. (Alpinobombus) alpinus* (n=14) (Fennoscandia) and *B. (Thoracobombus) sylvarum* (n=10) (Pyrenees and Massif Central) (Terzo *et al.* 2005).

Concerning *B. alpinus*, the population in Alps have been excluded of the analysis to respect the geographical distribution of the host and parasite. 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 investigation. Exact localities are listed in (Supplementary material 1). 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 pre-mating 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 (Žáček *et al.* 2013) with the same GC conditions to highlight potential common repellent compounds.

GC/MS Analysis- Identification
The composition was determined by Gas Chromatography - Mass Spectrometry (GC/MS) using a Focus GC (Thermo Scientific) with a non-polar 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 quadrupol mass analyzer (Thermo Scientific) with 70 eV electron impact ionization. We used a splitless injection mode (220°C) and helium as a carrier gas (one ml/min). The temperature program of the oven was set to 70°C for two minutes and then heated up at a rate of 10°C/min to 320°C. The temperature was then held at 320°C for five minutes. 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, U.S.A). Double bond positions (C=C) were determined by dimethyl disulfide (DMDS) 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 non-polar 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) 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 one individual of the taxon. The data matrix (Supplementary material 2) 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 1983; 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 - I.C.), 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 indicator compound (threshold of 0.01) was evaluated with a randomization procedure.

**RESULTS**

*Identified Compounds Within Cephalic Labial Gland Secretions*

*Alpinobombus*
In total, we have detected 117 compounds for *Alpinobombus* subgenus (69 from *B. hyperboreus*, 72 from *B. polaris* and 53 from *B. alpinus*) in the CLGS (Supplementary material 2). The results showed that all species were differentiated in their CLGS composition (23 compounds shared by all species inside the *Alpinobombus* group). 34 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* (Supplementary material 2). 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 indicator compounds (I.C.) with high statistical significance (IndVal > 0.8) for these *Alpinobombus* taxa (Supplementary material 2): *B. alpinus* (I.C.= 8) excluding its main compound (hexadec-9-en-1-ol) which is shared with *B. polaris*; *B. hyperboreus* (I.C.= 34) including its main compound (octadec-11-en-1-ol); *B. polaris* (I.C.= 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*) (Supplementary material 2). The results showed that all species were differentiated in their CLGS composition (12 compounds shared by all species inside *Thoracobombus* group). 16 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*.
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 indicator compounds (I.C.) with high statistical significance (IndVal > 0.8) for these *Thoracobombus* taxa (Supplementary material 2): *B. inexspectatus* (I.C.= 15) including the main compound octadec-11-enyl acetate; *B. ruderarius* (I.C.= 3) including the main compound hexadec-9-en-1-ol and *B. sylvarum* (I.C.= 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 (Fig. 2A, 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*. 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 a selection of the cuckoos for similarity to their hosts to improve their ability of 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. inexpectatus. These results are convergent 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; Lecocq et al. 2013; Lecocq et al. 2015a; Lhomme et al. 2015).

Other previous studies have shown that CLGS play a major role the attractiveness of females during pre-nuptial 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 pre-nuptial 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 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 their respective parasite (B. hyperboreus), which is a sister lineages to B. alpinus and B. polaris (Fig. 2A). In the Thoracobombus group, the composition of B. inexpectatus 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*.

**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 one 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 repellant effect.

**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, we can underline that previous study showed that only the quantitative composition of the CLGS varies between intra-nest 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, just few people succeeded to breed *B. polaris* and *B. ruderarius* (Sladen 1912; Wojtowski 1963; Berezin 1995). Moreover, from a total of 988,187 data recorded in Rasmont et al. 2015, only 370 specimens of *B. hyperboreus* (0.03%) and 275 specimens of *B. inexspectatus* (0.02%) were recorded including them as the rarest West-Palaearctic bumblebees.

In bumblebees, the chemical cue is not only due to 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, neuro-physiological 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 simply impossible to realize 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

The authors declare that they have no conflict of interest.

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TABLES

Table 1. Sampling sites and number (N) of specimens collected for B. hyperboreus, B. polaris, B. alpinus, B. inexpsectatus, B. ruderarius, and B. sylvarum. All sampling information in supplementary material 1.

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Table 2. List of main compounds (Bold) identified in cephalic labial gland secretions of *Bombus hyperboreus*, *B. polaris*, and *B. alpinus*. MW: the molecular weight, M: median of relative concentration of compound (%). All identified compounds in Supplementary material 2.
Table 3. List of main compounds (Bold) identified in cephalic labial gland secretions of *Bombus inexspectatus*, *B. ruderarius*, and *B. sylvarum*. MW: the molecular weight; M: median of relative concentration of compound (%). All identified compounds in *Supplementary material 2.*

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<tr>
<th>Compounds</th>
<th>MW</th>
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<th><em>B. ruderarius</em></th>
<th><em>B. sylvarum</em></th>
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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), D) B. ruderarius male from Pyrenees, France (photo: P. Rasmont).
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, B. alpinus (A); B. inexpectatus, B. ruderarius and B. sylvarum (B). The values near nodes represent multiscale bootstrap resampling values (only values >90 are given). It = Italy; Fr = France and Ch = Switzerland.