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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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1 The cephalic labial gland secretions of two socially parasitic bumblebees *Bombus*
2 *hyperboreus* (*alpinobombus*) and *Bombus inexpectatus* (*thoracobombus*) question their
3 inquiline strategy

4 Nicolas Brasero^{1*¶}, Baptiste Martinet^{1*¶}, Thomas Lecocq^{1,2}, Patrick Lhomme³, Paolo Biella⁴,
5 Irena Valterova⁵, Klara Urbanova^{5,6}, Maurizio Cornalba⁷, Heather Hines³, Pierre Rasmont¹

6 1: *University of Mons, Research institute of Biosciences, Laboratory of Zoology, Place du*
7 *Parc 20, 7000 Mons, Belgium*

8 2: *Research Unit Animal and Functionalities of Animal Products (URAFPA), University of*
9 *Lorraine – INRA, 2 Avenue de la Forêt de Haye, BP 172, 54505, Vandoeuvre-lès-Nancy,*
10 *France*

11 3: *The Pennsylvania State University, Department of Biology, 208 Mueller Labs, University*
12 *Park, PA 16802, United States of America (USA)*

13 4: *University of South Bohemia, Department of Zoology, Faculty of Science, Branišovská 31,*
14 *37005, České Budějovice, Czech Republic; Biology Centre of the Academy of Sciences of the*
15 *Czech Republic, v.v.i., Institute of Entomology, Branišovská 31, 37005, České Budějovice,*
16 *Czech Republic*

17 5: *Academy of Sciences of the Czech Republic, Institute of Organic Chemistry and*
18 *Biochemistry, Flemingovo nám 2, CZ-166 10 Prague, Czech Republic*

19 6: *Czech University of Life Sciences, Faculty of Tropical AgriSciences, Department of*
20 *Sustainable Technologies, Kamýcká 129, CZ-165 21 Prague, Czech Republic*

21 7: *University of Pavia, Department of Mathematics, via Ferrata 5, I-27100 Pavia, Italy*

22 Short Title: Involvement of CLGS in the inquilinism of *B. hyperboreus* and *B. inexpectatus*

23 * **Co-first authors and Corresponding authors; E-mail:** nicolas.brasero@umons.ac.be

24 (NB) 0032(0)65373435; baptiste.martinet@umons.ac.be (BM) 0032(0)65373405

25 ¶ These first co-authors contributed equally to this work

26 **Running title:** CLGS in social parasite

27 **Abstract-** Socially parasitic Hymenoptera have evolved morphological, chemical, and
28 behavioral adaptations to overcome the sophisticated recognition and defense systems of their
29 social host to invade host nests and exploit their worker force. In bumblebees, social
30 parasitism appeared in at least three subgenera independently: in the subgenus *Psithyrus*
31 consisting entirely of parasitic species, in the subgenus *Alpinobombus* with *B. hyperboreus*,
32 and in the subgenus *Thoracobombus* with *B. inexpectatus*. Cuckoo bumblebee males utilize
33 species-specific cephalic labial gland secretions for mating purposes which have been showed
34 as acting in their inquiline strategy. We performed cephalic labial gland secretions
35 comparative analyses of *B. hyperboreus*, *B. inexpectatus* and their hosts. Males of both
36 parasitic species exhibit high species specific levels of cephalic gland secretions, including
37 different main compounds. Our results show no chemical mimicry in the cephalic gland
38 secretions between inquilines and their host and we did not identify the repellent compounds
39 already known in other cuckoo bumblebees.

40 **Key Words** Social parasitism, bumblebees, *Bombus hyperboreus*, *Bombus*
41 *inexpectatus*, cephalic labial gland secretions, inquiline strategy

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INTRODUCTION

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51 Brood care is an energy-consuming activity for social insects ranging from nest
52 construction to offspring breeding (Smisteh et al. 2012). This high energetic cost has favored
53 the evolution of cheating species that exploit brood care behavior of hetero-specifics for their
54 own offspring rearing (Roldan & Soler 2011). This adaptive strategy involves the exploitation
55 of host social behavior by specialized species (so called social parasites) (Lenoir et al. 2001).
56 Social parasites have evolved morphological, chemical, and behavioral adaptations
57 (usurpation behaviors) to overcome the sophisticated recognition system (based on species-
58 specific pheromonal and cuticular signatures) and defense systems of their social host (Fisher
59 & Sampson 1992; Martin et al. 2010).

60 Among bumblebees (Hymenoptera, Apidae, *Bombus*), the cuckoo species exemplify
61 such a social parasitic strategy. Their life cycle relies on a non-mutual symbiotic relationship
62 (called inquilinism) with their hosts (Lhomme et al. 2012). This interaction leads to a
63 decrease or, in some cases, to a complete loss of host reproductive success (Lhomme et al.
64 2013). These “cheaters” infiltrate host colonies to take advantage of their food resources and
65 workers (Nash & Boomsma 2008). In bumblebees, obligate inquilinism evolved in at least
66 three subgenera independently (Hobbs 1967; Wilson 1971; Michener 1974): in the subgenus
67 *Psithyrus* consisting entirely of parasitic species (Williams 1998), in the subgenus
68 *Alpinobombus* with *B. hyperboreus* (Milliron & Oliver 1966; Richards 1973) (Fig. 1A), and in
69 the subgenus *Thoracobombus* with *B. inexpectatus* (Yarrow 1970; Williams 1998; Müller
70 2006) (Fig. 1C).

71 All *Psithyrus* species are completely dependent on their host because these socially
72 parasitic bumblebees do not produce a worker caste, do not have pollen collection baskets and
73 produce little to no wax to build cells (Benton 2006; Sramkova & Ayasse 2008). In the
74 *Alpinobombus* subgenus, the circum arctic *B. (Alpinobombus) hyperboreus* is mainly known

75 as an inquiline of *B. (Alpinobombus) polaris* (Fig. 1B) at least in some parts of its range
76 (Milliron & Oliver 1966; LØken 1973; Richards 1973; Pape 1983; Stenström & Bergmann
77 1998; Rampini *et al.* 2012), but nests of *B. (Alpinobombus) balteatus* and *B. (Pyrobombus)*
78 *jonellus* have been found to be infiltrated by *B. hyperboreus* in Norway (Bergwall 1970;
79 Gjershaug 2009). In the Eurasian Arctic and in North America, some females were reported
80 harvesting pollen (Gjershaug 2009), suggesting a facultative inquilinism for this species. In
81 the *Thoracobombus* subgenus, Yarrow (1970) and Müller (2006) have highlighted a number
82 of peculiar biological and morphological traits of *B. (Thoracobombus) inexpectatus* (i.e.:
83 absence of worker caste, loss of wax production and reduction of the hair-like structures of
84 the hind leg used to gather pollen) that likely are adaptations to parasitic life. *Bombus*
85 (*Thoracobombus*) *runderarius* (Fig. 1D) was described as the only host of *B. inexpectatus*
86 (Müller 2006). This sub-alpine European species occurs in the Cantabrian Mountains and the
87 Alps from 1200m to 2100m in altitude where it is uncommon (Tkalčú 1963; Tkalčú 1965;
88 Yarrow 1970; Amiet 1996; Rasmont *et al.* 2015). Compared to their respective hosts, the
89 geographical distribution of *B. inexpectatus* and *B. hyperboreus* and their ecological niches
90 are restricted (Rasmont & Iserbyt 2014) .

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

99 For male offspring of inquiline species, avoiding host recognition is especially
100 challenging because of their early production of large amounts of the species-specific
101 Cephalic Labial Gland Secretions (CLGS) involved in attracting conspecific females during
102 the pre-mating behavior (Žáček et al. 2009; Lhomme et al. 2012; Ayasse and Jarau 2014).
103 Indeed, CLGS are a complex mixture of (mainly aliphatic) compounds (Calam 1969) derived
104 from saturated fatty acids (Luxová et al. 2003) involved in pre-mating behavior (Bergström et
105 al. 1981). The species-specific CLGS are synthesized *de novo* by cephalic labial glands in the
106 head of bumblebee males (Žáček et al. 2013). This highly concentrated specific signature are
107 discriminated by host workers and potentially lead to male offspring destruction (Lhomme et
108 al. 2012). Consequently, a strong selective pressure (beside the sexual selection) on the
109 inquiline male CLGS should likely lead to adaptive changes. Similarly to alternative chemical
110 infiltration strategies, one could expect three alternative adaptive patterns: (i) chemical
111 insignificance meaning that males do not produce CLGS for pre-mating behavior or produce
112 CLGS only after leaving the host nest or even males produce undetected compounds; (ii)
113 chemical mimicry meaning that the inquiline actively produce the same compounds as its
114 host; or (iii) chemical repellency meaning that specific repellent compounds are produced
115 within the CLGS and act as "chemical shield" to avoid host worker aggressiveness. A
116 previous study on the cuckoo bumblebee *B. (Psithyrus) vestalis* demonstrated a chemical
117 repellent effect of CLGS on host workers triggered by tetradecyl acetate and (Z)-11-
118 hexadecenyl acetate (Lhomme et al. 2015). Potential other chemical strategies used by other
119 *Psithyrus* or non-*Psithyrus* inquiline species is unknown. Here, we performed (i) the first
120 detailed description of the two non-*Psithyrus* inquiline CLGS and (ii) comparative analyses of
121 CLGS composition between *B. hyperboreus*, *B. inexpectatus* and their hosts. We finally
122 discuss the potential chemical strategies involved in these host-inquiline interactions.

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Materials and Methods

125 *Sampling*

126 In total, 69 specimens collected belonging to two subgenera were used (Table 1;
127 Supplementary material 1). The test group comprised *B. hyperboreus* (n=15) (Fennoscandia
128 and Alaska), and its main host *B. polaris* (n=13) (Fennoscandia and Alaska), and *B.*
129 *inexpectatus* (n=9) (Alps), and its host *B. ruderarius* (n=8) (Alps and Pyrenees). As a
130 reference group, we used one phylogenetically close species of the host bumblebees of each
131 parasite according to Cameron *et al.* [41] : *B. (Alpinobombus) alpinus* (n=14) (Fennoscandia)
132 and *B. (Thoracobombus) sylvarum* (n=10) (Pyrenees and Massif Central) (Terzo *et al.* 2005).
133 Concerning *B. alpinus*, the population in Alps have been excluded of the analysis to respect
134 the geographical distribution of the host and parasite. For *Alpinobombus*, we chose to follow
135 the taxonomy of Milliron (1971) and Løken (1973) as recent updates of Williams *et al.* (2015)
136 still require further investigation. Exact localities are listed in (Supplementary material 1). All
137 bumblebee specimens were killed by freezing at -20° C.

138 *Extraction of Bumblebee Male Cephalic Labial Gland Secretions*

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

142 The CLGS were extracted from dissected whole cephalic labial glands using 400 µl of
143 n-hexane -see protocol in (De Meulemeester *et al.* 2011). Samples were stored at - 40° C prior
144 to the analyses. The composition of CLGS was compared to that established by (Žáček *et al.*
145 2013) with the same GC conditions to highlight potential common repellent compounds.

146 *GC/MS Analysis- Identification*

147 The composition was determined by Gas Chromatography - Mass Spectrometry (GC/MS)
148 using a Focus GC (Thermo Scientific) with a non-polar DB-5 ms capillary column [5%
149 phenyl (methyl) polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm;
150 film thickness 0.25 μm] coupled to DSQ II quadrupol mass analyzer (Thermo Scientific) with
151 70 eV electron impact ionization. We used a splitless injection mode (220°C) and helium as a
152 carrier gas (one ml/min). The temperature program of the oven was set to 70°C for two
153 minutes and then heated up at a rate of 10°C/min to 320°C. The temperature was then held at
154 320°C for five minutes. Compounds were identified in Xcalibur™ using the retention times
155 (t_r) and mass spectra of each peak, in comparison to those at National Institute of Standards
156 and Technology library (NIST, U.S.A). Double bond positions (C=C) were determined by
157 dimethyl disulfide (DMDS) derivatization (Cvacka *et al.* 2008).

158 *GC-FID Analysis- Quantification*

159 All samples were quantified (quantitative composition) with Gas Chromatography with a
160 Flame Ionization Detector (GC-FID) Shimadzu GC-2010 system equipped with a non-polar
161 SLB-5 ms capillary column [5% phenyl (methyl) polysiloxane stationary phase; column
162 length 30 m; inner diameter 0.25 mm; film thickness 0.25 μm]. The chromatographic
163 conditions were the same as described above (cf. GC/MS Analysis). We quantified the peak
164 areas of compounds in GC solution post-run (Shimadzu Corporation) with automatic peak
165 detection and noise measurement. The relative areas (RAs, expressed in %) of compounds in
166 each sample were calculated by dividing the peak areas of compounds by the total area of all
167 compounds. All compounds for which RA were recorded as less than 0.1% for all specimens
168 were excluded from the analysis (De Meulemeester *et al.* 2011). We identified the main
169 compound as the compound that has the highest relative proportion (RA) among all
170 compounds of CLGSs at least in one individual of the taxon. The data matrix (Supplementary
171 material 2) for each taxa was based on the alignment of each relative proportion of compound

172 between all samples performed with GCAAligner 1.0 (Dellicour & Lecocq 2013a). To facilitate
173 the alignment of compounds, before each sample injection, a standard (Kovats) were injected
174 containing a mix of pure hydrocarbons (alkanes) from C 10 (decane) to C 40 (tetracontane).
175 Kovats indices were calculated with GCKovats 1.0 according to the method described by
176 (Dellicour and Lecocq 2013b).

177 *Statistical Analysis*

178 Each pair of parasite-host complex was considered independently. We performed statistical
179 comparative analyses based on the CLGS matrix using R (R Development Core Team 2013)
180 to detect chemical differentiations between hosts and parasites. A clustering method,
181 computed with the unweighted pair-group method with average linkage (UPGMA) based on
182 Pearson correlation distance matrices (RA of each compound) (Legendre & Legendre 1983;
183 Paradis *et al.* 2004) was used to detect the divergence between taxa in the CLGS composition.
184 We assessed the uncertainty in hierarchical cluster analysis using p-values calculated *via*
185 multiscale bootstrap resampling with 100 000 bootstrap replications (R package pvcust)
186 (Suzuki & Shimodaira 2011). Only bootstrap values greater than 90% were considered
187 significant. To determine specific compounds of each taxon (indicator compounds - I.C.), the
188 indicator-value (IndVal) method was used (Dufrêne & Legendre 1997). This value is the
189 product of relative concentration and relative occurrence frequency of a compound within a
190 group. The statistical significance of an indicator compound (threshold of 0.01) was evaluated
191 with a randomization procedure.

192 RESULTS

193 *Identified Compounds Within Cephalic Labial Gland Secretions*

194 *Alpinobombus*

195 In total, we have detected 117 compounds for *Alpinobombus* subgenus (69 from *B.*
196 *hyperboreus*, 72 from *B. polaris* and 53 from *B. alpinus*) in the CLGS (Supplementary
197 material 2). The results showed that all species were differentiated in their CLGS composition
198 (23 compounds shared by all species inside the *Alpinobombus* group). 34 compounds are
199 shared between the parasite *B. hyperboreus* and the host *B. polaris*, 39 between *B. polaris* and
200 *B. alpinus* and 27 between *B. hyperboreus* and *B. alpinus* (Supplementary material 2). In
201 addition to qualitative differences in minor compounds, other differences are also present in
202 main compounds. In *Alpinobombus* group, octadec-11-en-1-ol (23.90% - 56.21%, Table 2) is
203 the main compound in the *B. hyperboreus* CLGS. While the CLGS of *B. polaris* is dominated
204 by hexadec-9-enal (20.8% - 43.03%) and hexadec-9-en-1-ol (26.87% - 46.68%). The main
205 compound of *B. alpinus* is hexadec-9-en-1-ol (63.95% - 89.43%) (Table 2). Thus the main
206 compound of *B. alpinus* (hexadec-9-en-1-ol) is shared by its sister species *B. polaris* and not
207 its social parasite (Table 2).

208 The IndVal method revealed several indicator compounds (I.C.) with high statistical
209 significance (IndVal > 0.8) for these *Alpinobombus* taxa (Supplementary material 2): *B.*
210 *alpinus* (I.C.= 8) excluding its main compound (hexadec-9-en-1-ol) which is shared with *B.*
211 *polaris*; *B. hyperboreus* (I.C.= 34) including its main compound (octadec-11-en-1-ol); *B.*
212 *polaris* (I.C.= 22) including one of its main compounds (hexadec-9-enal).

213 *Thoracobombus*

214 We detected 78 compounds in the CLGS of the species belonging to *Thoracobombus*
215 subgenus (36 from *B. inexpectatus*, 52 from *B. ruderarius* and 39 from *B. sylvarum*)
216 (Supplementary material 2). The results showed that all species were differentiated in their
217 CLGS composition (12 compounds shared by all species inside *Thoracobombus* group). 16
218 compounds were shared between the parasite *B. inexpectatus* and the host *B. ruderarius*, 24
219 between *B. ruderarius* and *B. sylvarum* and 16 between *B. inexpectatus* and *B. sylvarum*

220 (Supplementary material 2). In addition to qualitative differences in minor compounds, other
221 differences were also present in main compounds. In the *Thoracobombus* group, the main
222 compounds of *B. inexpectatus* were hexadec-7-en-1-ol (17.23% - 36.14%), octadec-11-en-1-
223 ol (12.25% - 32.57%) and octadec-11-enyl acetate (9.99% - 23.44%) while those of *B.*
224 *runderarius* and *B. sylvarum* were respectively hexadec-9-en-1-ol (16.49% - 70.64%) and
225 hexadec-7-enyl acetate (48.74% - 65.34%) (Table 3).

226 The IndVal method revealed several indicator compounds (I.C.) with high statistical
227 significance (IndVal > 0.8) for these *Thoracobombus* taxa (Supplementary material 2): *B.*
228 *inexpectatus* (I.C.= 15) including the main compound octadec-11-enyl acetate; *B. ruderarius*
229 (I.C.= 3) including the main compound hexadec-9-en-1-ol and *B. sylvarum* (I.C.= 9)
230 including the main compound hexadec-7-enyl acetate.

231 *Interpretation of Clusters*

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

235 DISCUSSION

236 *Comparison of the Parasite CLGS Composition With Past*

237 *Identifications*

238 Compared with Svensson and Bergström (1979), we have identified the same main
239 compounds for *B. hyperboreus*. However, thanks to improved methods of chemical analyses,
240 we identified many more compounds in the CLGS composition especially with double bounds
241 information. Concerning *B. inexpectatus*, the description of CLGS composition is here
242 reported for the first time.

243 *Evidence for Host Evasion Strategies in Parasitic Males*

244 *Chemical Mimicry Hypothesis*

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

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

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

263 The interspecific CLGS comparisons (Rasmont *et al.* 2005; Lecocq *et al.* 2015a;
264 Brasero *et al.* 2015) show higher composition similarities between phylogenetically closely
265 related bumblebee species. For *Alpinobombus* group, the cluster analysis indicates that the
266 CLGS composition of the hosts (*B. polaris*) is more similar to its sister-species (*B. alpinus*)
267 than to their respective parasite (*B. hyperboreus*), which is a sister lineages to *B. alpinus* and
268 *B. polaris* (Fig. 2A). In the *Thoracobombus* group, the composition of *B. inexpectatus* is

269 more similar to the reference species (*B. sylvarum*) than its own host (*B. ruderarius*) (Fig.
270 2B). Hines and Cameron (2010) showed that *B. inexpectatus* is phylogenetically closer to *B.*
271 *sylvarum* than its host *B. ruderarius*.

272 *Chemical Insignificance Hypothesis*

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

276 *Chemical Repellency Hypothesis*

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

285 *Limitations and Future Prospects*

286 Since we sampled only extra-nest males, we cannot exclude potential mimicry or chemical
287 insignificance strategies during the early age of parasite males. However, we can underline
288 that previous study showed that only the quantitative composition of the CLGS varies
289 between intra-nest and mature emerged males (Žáček *et al.* 2009). Detailed assessment of
290 these hypotheses requires *in natura* observations of nest infestations or bioassays on
291 laboratory-reared specimens that will be challenging to obtain: wild nest infestation
292 observations are uncommon (Lecocq *et al.* 2011) and species-specific year-round breeding

293 methods needed for bioassays (Lhomme *et al.* 2012; Lhomme *et al.* 2013) are available for
294 only few species (Hasselrot 1960). Moreover, in recent decades, *B. hyperboreus* and *B.*
295 *inexpectatus* have become scarce especially in Europe (Rasmont *et al.* 2015) increasing the
296 difficulty of developing such a protocol with a suitable sampling. Indeed, as far as we know,
297 just few people succeeded to breed *B. polaris* and *B. ruderarius* (Sladen 1912; Wojtowski
298 1963; Berezin 1995). Moreover, from a total of 988.187 data recorded in Rasmont *et al.* 2015,
299 only 370 specimens of *B. hyperboreus* (0.03%) and 275 specimens of *B. inexpectatus*
300 (0.02%) were recorded including them as the rarest West-Palearctic bumblebees.

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

306 To test these hypotheses, neuro-physiological experiments could be useful (i.e. gas
307 chromatography coupled with Electro-Antennographic Detector (Lhomme *et al.* 2015) or a
308 glomerular function test (Devaud *et al.* 2015)). Unfortunately, the lack and the difficulty to
309 breed these specimens make experiments simply impossible to realize for now.

310 CONCLUSIONS

311 In this study we find that the independently evolving social parasites *B. hyperboreus*
312 and *B. inexpectatus* display a species-specific mating chemistry in their cephalic labial
313 glands typical of bumblebees. Although these mating secretions could make them vulnerable
314 to detection and exclusion from the host nest, our results do not support shifts in chemistry
315 expected from major hypothesized host evasion strategies. These species are not devoid of
316 sexual cephalic labial attractive compounds so are not chemically undetectable. These CLGS
317 are also not more similar to their hosts than would be expected phylogenetically so are also

318 not involved in chemical mimicry. Finally, we have not identified the repellent compounds
319 highlighted by Lhomme *et al.* (2015) in *B. vestalis*. Other repellent compounds in these or
320 other glands could, however, be important for host evasion.

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336

DISCLOSURE

337 The authors declare that they have no conflict of interest.

338

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516

TABLES

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

Taxa	Collecting sites	Coordinates	N	Code
<i>Alpinobombus</i>				
<i>B. hyperboreus</i>	Sweden, Bjorklieden	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
<i>B. polaris</i>	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
<i>B. alpinus</i>	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
<i>Thoracobombus</i>				
<i>B. inexpectatus</i>	Italy, Val Brembana	45°58'12"N 09°47'53"E	3	NBRA0918 NBRA0919 NBRA0920
	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
<i>B. ruderarius</i>	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
<i>B. sylvarum</i>	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

520

521 *Table 2. List of main compounds (Bold) identified in cephalic labial gland secretions of*
522 *Bombus hyperboreus, B. polaris, and B. alpinus. MW: the molecular weight, M: median of*
523 *relative concentration of compound (%). All identified compounds in Supplementary material*
524 *2.*

Compounds	MW	<i>B. alpinus</i>			<i>B. polaris</i>			<i>B. hyperboreus</i>		
		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

525

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

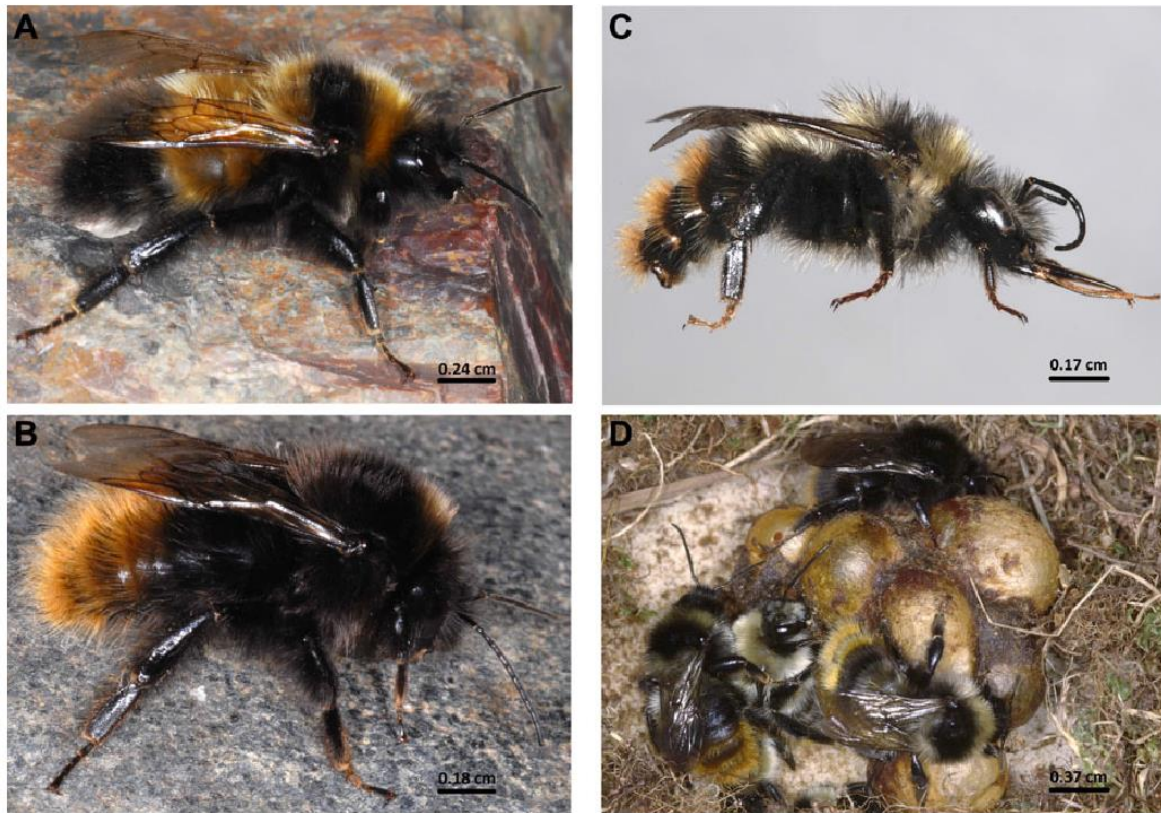
Compounds	MW	<i>B. inexpectatus</i>			<i>B. ruderarius</i>			<i>B. sylvarum</i>		
		Min	M	Max	Min	M	Max	Min	M	Max
Hexadec-7-en-1-ol	240	17.23	24.99	36.14	0.00	0.00	0.00	23.54	28.65	32.14
Hexadec-9-en-1-ol	240	4.94	7.47	8.32	16.49	37.26	70.64	0.00	0.00	0.00
Octadec-11-en-1-ol	268	12.25	17.66	32.57	3.97	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

530

531

532

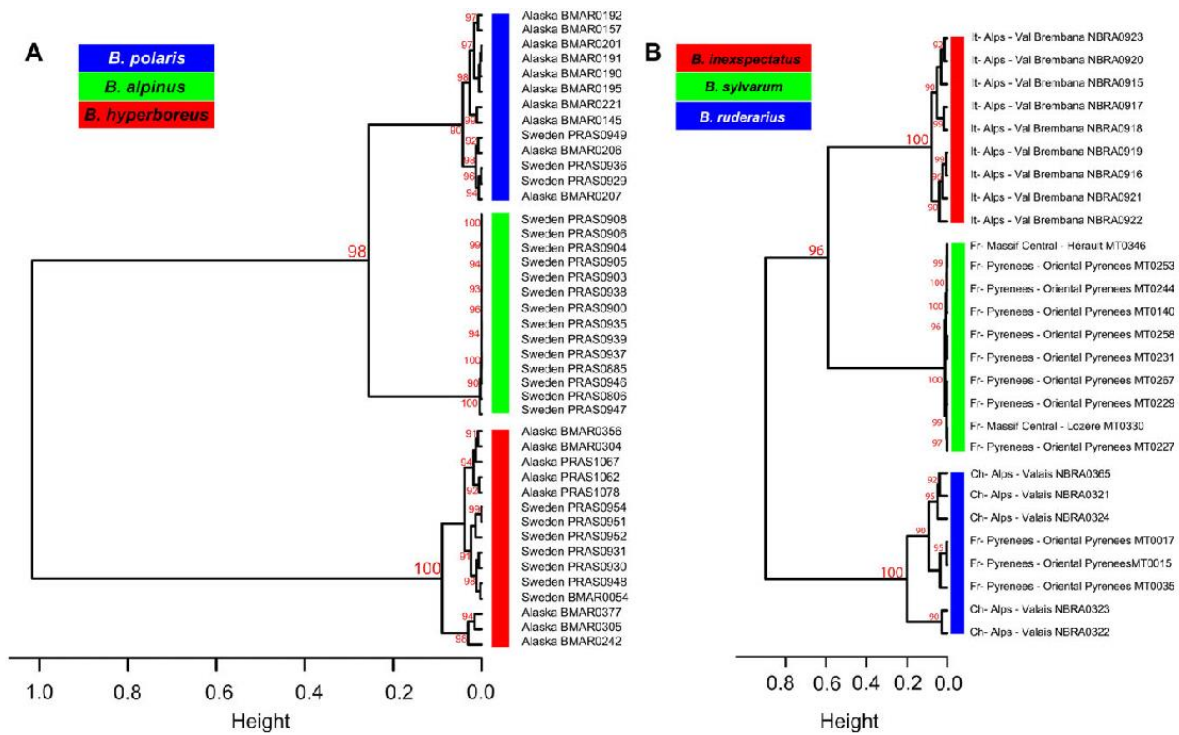
FIGURES



534

535 **Fig. 1:** Photos of the studied bumblebee species: A) *Bombus hyperboreus* male (PRAS1067)
536 from Alaska, USA (photo: P. Rasmont), B) *B. polaris* male (PRAS0936), from Norway (photo:
537 P. Rasmont), C) *B. inexpectatus* male from Alps, Italy (photo: P. Rasmont), D) *B. ruderarius*
538 male from Pyrenees, France (photo: P. Rasmont).

539



540

541 **Fig. 2** Unweighted pair group method with arithmetic mean (UPGMA) cluster based on a
 542 correlation matrix calculated from the composition of cephalic labial gland secretions of
 543 *Bombus hyperboreus*, *B. polaris*, *B. alpinus* (A); *B. inexpectatus*, *B. ruderarius* and *B.*
 544 *sylvarum* (B). The values near nodes represent multiscale bootstrap resampling values (only
 545 values >90 are given). It= Italy; Fr= France and Ch= Switzerland.

546