

## **Chemical reproductive traits of diploid *Bombus terrestris* males: Consequences on bumblebee conservation**

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1                   **Chemical reproductive traits of diploid *Bombus terrestris* males:**  
2                   **consequences on bumblebee conservation**

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12          **Running title:** Diploid male chemical reproductive traits

13          **Abstract** The current bumblebee decline leads to inbreeding in populations that fosters a loss of allelic diversity  
14          and diploid male production. As diploid males are viable and their offspring are sterile, bumblebee populations  
15          can quickly fall in a vortex of extinction. In this paper, we investigate for the first time a potential pre-mating  
16          mechanism through a major chemical reproductive trait (male cephalic labial gland secretions) that could prevent  
17          monandrous virgin queens from mating with diploid males. We focus our study on the cephalic labial gland  
18          secretions of diploid and haploid males of *Bombus terrestris* (L.). Contrary to initial expectations, our results do  
19          not show any significant differentiation of cephalic labial gland secretions between diploid and haploid  
20          specimens. Queens seem therefore to be unable to avoid mating with diploid males based on their compositions  
21          of cephalic labial gland secretions. This suggests that the vortex of extinction of diploid males could not be  
22          stopped through pre-mating avoidance based on the cephalic labial gland secretions but other mechanisms could  
23          avoid mating between diploid males and queens.

24          **Key Words**

25          Bee decline; Bumblebees; Conservation; Diploid males; Pre-mating recognition; Reproductive traits.

26          **Introduction**

27          About 90% of the world's plant species are pollinated by animals, and mainly bees (Ollerton *et al.*, 2011). This  
28          makes bees a critically important functional group for ecosystem services in wild environments as well as in

29 crops (Free, 1993; Klein *et al.*, 2007). Among bees, females of the social genus *Bombus* (i.e. bumblebees) are  
30 among the most important pollinators of the northern temperate zone (Williams, 1985). However, some  
31 bumblebee species, as well as other wild bee species, are obviously experiencing a strong decline in their  
32 populations (Carvalho *et al.*, 2013; Rasmont *et al.*, 2015). Several factors were proposed to explain this  
33 decline: landscape modifications/fragmentations, intensive use of agrochemicals, pathogen infections,  
34 competition with alien species, climate change, and interactions between all of these factors (review in Winfree  
35 2010; Potts *et al.* 2011). Ultimate consequences are a decrease of population size and an increase of population  
36 fragmentation that leads to a loss of genetic diversity through inbreeding and genetic drift (Zayed, 2009;  
37 Frankham *et al.*, 2010).

38 Loss of genetic diversity is particularly problematic in haplo-diploid species such as bees (Zayed & Packer,  
39 2005; Zayed, 2009). Like most Hymenoptera, bees have a single-locus complementary sex determiner (sl-CSD):  
40 males are hemizygote while females are heterozygote for this locus (Crozier, 1977; Heimpel & de Boer, 2008).  
41 Inbreeding in haplo-diploid species with sl-CSD brings a heavy load through the production of homozygotes  
42 which show male phenotype (Duchateau *et al.*, 1994; Duchateau & Marien, 1995). Diploid male larvae can be  
43 recognized and killed by workers in social bee species like *Apis mellifera*, (Woyke, 1963) but they can develop  
44 into viable adults in bumblebee species (e.g. *Bombus terrestris* (L.); Duchateau *et al.* 1994). The production of  
45 diploid bumblebee males can be substantial: half of the diploid eggs, which normally develop into females, result  
46 in males after brother/sister mating (Duchateau *et al.*, 1994; Duchateau & Marien, 1995). These diploid males  
47 decrease the fitness of the local populations (Whitehorn *et al.*, 2009). First, the production of diploid males in  
48 place of workers implies that the number of the workers decreases up to 50% resulting in a small colony size and  
49 a low production of new queens (Duchateau *et al.*, 1994; Duchateau & Marien, 1995). Second, as diploid males  
50 produce diploid sperm (Ayabe *et al.*, 2004; Darvill *et al.*, 2012), the mating between monandrous virgin queens  
51 and diploid males produces 100% of triploid (i.e. sterile) offspring (Payne *et al.*, 2003; Takahashi *et al.*, 2008).  
52 Therefore, this process leads to a vortex of extinction: populations decreasing based on extrinsic factors (e.g.  
53 habitat fragmentation) show higher inbreeding, lower allelic diversity and production of diploid males, this  
54 production leading to an additional decrease of the population (Zayed & Packer, 2005). Consequently  
55 populations can be driven to quick extinction (Zayed & Packer, 2005) as currently observed in small and  
56 fragmented bumblebee populations (Darvill *et al.*, 2006; Takahashi *et al.*, 2008).

57 This vortex of extinction could be reduced if females are able to recognize and avoid mating with diploid males.  
58 In monandrous species, like most bumblebee species, the female choice requires pre-mating indicators of male

59 genetic equipment (Herzner *et al.*, 2006). Therefore, pre-mating mechanisms of avoidance of diploid males  
60 could be selected in monandrous bumblebee queens in view of the deleterious consequences of mating with  
61 diploid males. First bioassays show that *Bombus* diploid males have the same mating success as haploid males in  
62 experimental conditions (Duchateau & Marien, 1995). However, such experiments could lead to unnatural  
63 mating because queens have no choice between different sexual partners. For example, sibling individuals mate  
64 in experimental condition even if *B. terrestris* queens seem to be less willing to mate with their siblings than  
65 with an unrelated partner when they have the choice (Whitehorn *et al.*, 2009). Therefore, we expect that pre-  
66 mating cues produced by males could allow female pre-mating discrimination to avoid diploid males in natural  
67 conditions. As diploid males show differences in body size and physiology (Duchateau & Marien, 1995; Gerloff  
68 *et al.*, 2003), we hypothesize that they could display original chemical reproductive traits.

69 The present study investigates the cephalic labial gland secretions (CLGS) of haploid *versus* diploid males of *B.*  
70 *terrestris*. Courtship signals of bumblebees include both behavioral and chemical features (see Baer 2003 for a  
71 review). Among the features, the CLGS act as a key trait for the pre-mating recognition (Bergström *et al.*, 1981;  
72 Baer, 2003; Ayasse & Jarau, 2014): Most bumblebee males patrol along paths where they scent-mark objects  
73 with their CLGS that attract conspecific virgin females (see experimental demonstration that CLGS of  
74 bumblebee males attract virgin queens in Coppée *et al.*, 2011; Lecocq *et al.*, 2015b). This chemical reproductive  
75 trait consists of a species-specific complex mixture of (mainly aliphatic) compounds synthesized *de novo*  
76 (Calam, 1969; Lecocq *et al.*, 2011, 2013) with a interindividual variation (Coppée *et al.*, 2008; Žáček *et al.*,  
77 2009; Lecocq *et al.*, 2013) which leads to differential female preferences for different kinds of males (e.g. young  
78 *versus* old males, sibling *versus* unrelated males; (Coppée, 2010; Coppée *et al.*, 2011; Lecocq *et al.*, 2015c).  
79 Therefore variations in this trait could allow the virgin queen to detect diploids and avoid mating with them.

## 80 **Material and Methods**

### 81 *Collection and Rearing of Specimens*

82 In order to produce diploid males (diploid male samples were also used in Gérard *et al.*, 2015), we obtained three  
83 colonies of *Bombus terrestris terrestris* (L.) from bee breeder Biobest N.V. We were reared these colonies at the  
84 laboratory of Zoology in Mons (UMons, Belgium) in a controlled climate room (see rearing protocol in Lhomme  
85 *et al.*, 2012, 2013; Gérard *et al.*, 2015): we obtained queens (n=11) and males (n=21) among the first generation  
86 from only one colony. We forced brother-sister mating by placing the newly emerged sibling queens and males  
87 in a flight cage exposed to natural light (see mating protocol in Lhomme *et al.*, 2013; Gérard *et al.*, 2015). We

88 used these mated queens to produce a second generation of inbred colonies. These queens overwintered during  
89 two months prior to a activation by CO<sub>2</sub> narcosis (see protocol in Lhomme *et al.*, 2013). Then, we placed the  
90 queens in small boxes with two workers and a cocoon to (i.e. to maximize the probability of colony initiation see  
91 protocol in Lhomme *et al.*, 2012, 2013). All 11 mated queens of the first generation initiated a colony, producing  
92 a total of 62 workers and 11 males of second generation (Table S1).

93 In order to produce haploid males, we reared eight queenless micro-colonies with five workers from one colony  
94 from bee breeder Biobest N.V. Each micro-colony was maintained in a plastic box in a controlled climate room  
95 (same rearing conditions than for colonies). The micro-colonies produced 41 haploid males (Table S1).

96 We increased the diversity in our dataset by sampling 38 wild males of *B. terrestris terrestris* in Belgium *in*  
97 *natura* with a net (Table S1). As wild males of *B. terrestris* could be confused with the closely related species *B.*  
98 *cryptarum*, the species identification was checked by CLGS analyses: the two species have species-specific  
99 CLGS composition used as diagnostic traits (e.g. Bertsch et al 2005; see CLGS analyses protocol below).

100 All wild (n=38) and reared (n=52) males (n total=90) were killed by freezing at -20°C. For the reared males, we  
101 used five to ten days old males when the *B. terrestris* males are the most attractive to females (Coppée *et al.*,  
102 2011). For the wild males, we examined the wing condition (Tkalců, 1969) in order to avoid the sampling of old  
103 specimens that have a CLGS modification composition and are less attractive to females (Žáček *et al.*, 2009;  
104 Coppée *et al.*, 2011). *Determination of the Degree of Ploidy*

105 We assessed the degree of ploidy by genotyped males with 13 microsatellite markers developed for *B. terrestris*:  
106 B11, B100, B121, B126 and B132 (Estoup *et al.*, 1993) and 0007\_47n22, 0078\_59o4, 0222\_63d21, 0583\_22I4,  
107 0636\_34m4, 0646\_83e8, 0930\_40o1, 0939\_33h17 (Stolle *et al.*, 2009). We extracted sample DNA following the  
108 method described by Maebe *et al.* (2013) with some small modifications: 200 µl of 5% Chelex (InstaGene™  
109 matrix, BioRad) and 10 µl of proteinase K (20 mg/ml) were added to one middle leg and incubated at 56°C for  
110 2h and 97°C for 15 min. The microsatellite loci: B11, B100, B121, B126 and B132 were amplified in multiplex  
111 by PCR as described in Maebe *et al.* (2013). For allele detection of the primers originated from Stolle *et al.*  
112 (2011), we used a tailed-primer approach (Schuelke, 2000). We used the PCR protocol described in Maebe *et al.*  
113 (2015): simplex PCR reactions were performed, containing 1.5 µl template DNA, 1 µl of 10x PCR buffer  
114 (Qiagen), 0.2 µl of 10 mM dNTP's (Qiagen), 0.1 µl of 10 µM forward primer, 0.4 µl of 10 µM reverse primer,  
115 0.4 µl of 10 µM labeled M13-primer and 0.05 µl of 2.5 units/reaction Hotstar Taq DNA Polymerase (Qiagen).  
116 Initial denaturing at 95°C for 15 min was followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 48,

117 52 or 58°C for 30 s, and extension at 72°C for 30 s, and ended at 72°C for 10 min with a final extension step.  
118 Pooled samples were visualized on a ABI-3730xl sequencer (Applied Biosystems) and fragments were scored  
119 manually using Peak Scanner Software v 1.0 (Applied Biosystems).

#### 120 *Comparative Chemical Analyses of Cephalic Labial Gland Secretions*

121 We extracted the CLGS of 90 males following the entire cut head protocol described in De Meulemeester *et al.*,  
122 2011). Vials containing the solvent and sample were (i) kept for 24 h at room temperature (20°C) to fulfill the  
123 extraction and (ii) stored at -40°C for two months prior to the analyses. We (i) analyzed the CLGS samples and  
124 identified compounds using gas chromatography-mass spectrometry (GC/MS) and (ii) quantified them by gas  
125 chromatography with flame ionization detector (GC/FID) (see GC/MS and GC/FID specifications and  
126 temperature program in Lecocq *et al.*, 2015). We identified the CLGS in Xcalibur™ with their mass spectra  
127 compared to those in the National Institute of Standards and Technology Library (NIST, U.S.A) with NIST MS  
128 Search 2.0. We determined the double bond positions from (i) mass spectra of dimethyl disulphide adducts of  
129 unsaturated components (Francis, 1981) (reaction time: four hours) and (ii) by chemical ionization with  
130 acetonitrile as a reaction gas (Oldham & Svatoš, 1999) (see protocol details in Lecocq *et al.*, 2013). We  
131 quantified the peak areas of CLGS compounds in GCsolution Postrun (Shimadzu Corporation) with automatic  
132 peak detection and noise measurement. We elaborated the data matrix (Table S2) with the relative amounts of  
133 each compound for each individual (the relative amounts are calculated by dividing the peak areas of compounds  
134 by the total area of compounds in each sample). We based our data matrix on the alignment of each compound  
135 between all samples performed with GCAAligner 1.0 (Dellicour & Lecocq, 2013a,b).

136 We compared the CLGS between haploid and diploid males with non-metric multidimensional scaling (nMDS)  
137 ordination using a Bray-Curtis similarity matrix, three dimensions and 50 runs (R-package ecodist, Goslee &  
138 Urban, 2007). We transformed ( $\log(x+1)$ ) the data matrix to reduce the great difference of abundance between  
139 compounds in high and low concentration (De Meulemeester *et al.*, 2011). An hypothetic CLGS differentiation  
140 between haploids and diploids was assessed by performing permutation-based version of the multivariate  
141 analysis of variance (perMANOVA) using the Bray-Curtis similarity matrix and 10,000 permutations (R-  
142 package vegan, Oksanen *et al.*, 2011) (perMANOVA assumption; i.e. similar variance homogeneity between  
143 groups was checked by performing a distance-based test for multivariate homogeneity of group dispersions for a  
144 one-way ANOVA design; Anderson, 2006). We determine CLGS compounds that are potentially specific to and

145 regular to diploid or haploid males with the indicator value (IndVal) method (Dufrêne & Legendre, 1997; see  
146 details in Lecocq *et al.*, 2015b). All statistical analyses were performed in R (R Development Core Team, 2013).

## 147 **Results**

148 Genetic analyses showed that reared queen-right colonies produced nine diploid males (Table S1). One diploid  
149 male was also found in wild specimens (Table S1). Other males were unambiguously haploid (Table S1).

150 Fifty six compounds were detected in the CLGS of analyzed specimens (Table S2). The general pattern obtained  
151 for CLGS of diploid males corresponded to those of haploid males. No qualitative difference was detected  
152 between haploid and diploid males (including no differences in double bound positions). The main compounds  
153 (i.e. compounds that have the highest relative amount in one individual at least) were farnesol, 2,3-  
154 Dihydrofarnesol, geranyl citronellol, and 2,3-dihydrofarnesyl dodecanoate. The nMDS (stress value = 0.16;  
155 R<sup>2</sup>=0.83) did not show any obvious CLGS differentiations between haploid and diploid males except for a slight  
156 trend observed for the reared diploid males in the nMDS (i.e. these specimens are more or less grouped together  
157 at the edge of the nMDS) (Fig. 1). The perMANOVA (DF = 1, F = -9.17, P =0.79) analyses confirmed the lack  
158 of significant CLGS differentiation between the two groups. The IndVal method did not reveal indicator  
159 compounds with strong significance (IndVal>0.70) in haploid or diploid males (Table S2).

## 160 **Discussion**

### 161 *Diagnostic Features of Diploid Bumblebee Males*

162 In view of the dramatic consequences of diploid male production on population (Zayed & Packer, 2005), we  
163 expected that *B. terrestris* would display pre-mating mechanisms avoiding virgin queen mating with diploid  
164 males as already observed in some Hymenoptera (Herzner *et al.*, 2006). Such pre-mating mechanisms are most  
165 likely based on differences between reproductive traits used in pre-mating recognition between diploid and  
166 haploid males. Therefore, we investigated one reproductive trait, the CLGS that acts as a key trait for the  
167 bumblebee pre-mating recognition. However, our comparative statistical analyses based on CLGS composition  
168 did not detect obvious differences between diploid and haploid males in their CLGS, a main key trait for mate  
169 attraction (Fig. 1). The slight trend observed for the reared diploid males in the nMDS (i.e. these specimens are  
170 more or less grouped together at the edge of the nMDS; Fig. 1) could reflect a "family-effect" since all queens  
171 that produced these males are sister mated with their brothers as already observed in other study (Lecocq *et al.*,  
172 2011). Nevertheless, our statistical analyses did not show significant differentiation between CLGS of diploid

173 and haploid males (see perMANOVA results). Even if the components of the male CLGS that constitute the  
174 pheromones are not known (i.e. those components that release a female response or convey information for  
175 females), this does not invalidate the basic result that there is no significant differentiation in diploid male CLGS  
176 since there is no specific and regular chemical compounds to diploid or haploid males (see IndVal results in  
177 Table S2). Although we are aware of the limited power of our statistical analyses due to our small sample size  
178 (10 diploid males), our data clearly demonstrates that there is no strong difference in the CLGS composition  
179 between haploid and diploid males.

180 According to our results we speculate that the queens are not able to discriminate the diploid males through their  
181 CLGS. However, since the bumblebee male pre-mating signal (Baer, 2003; Ayasse & Jarau, 2014; Streinzer &  
182 Spaethe, 2014) includes behavioral and other chemical features such as tendon glands (Jarau *et al.*, 2012) that  
183 are mainly poorly known, we cannot rule out the fact that the whole pre-mating signal of diploid males are  
184 evaluated by females as being strictly similar to those of haploid males. Indeed other traits involved in the  
185 species communication (e.g. cuticular hydrocarbons) or in the reproduction could be different in diploid *B.*  
186 *terrestris* males such as observed for cuticular compounds and testis size of diploid drone in the close relative  
187 *Apis mellifera* L. (Herrmann *et al.*, 2005). Moreover, diploid *B. terrestris* males are distinct in other characters  
188 such as wing shape, body size, and physiology (Duchateau & Marien, 1995; Gerloff *et al.*, 2003; Gérard *et al.*,  
189 2015). These features could allow the female to diagnose diploid males and/or decrease the fitness of diploid  
190 males (e.g. fitness of a bumblebee male is correlated with body size, Cueva del Castillo & Fairbairn, 2012).  
191 Further studies on other traits and ethological test allowing choices of virgin females could help to define  
192 whether the *B. terrestris* females are not able to recognize diploid males. Nevertheless, current evidence suggests  
193 that queens are not able to discriminate the diploid males during the pre-mating recognition because (i) our  
194 results show that there are no diploid specific cephalic labial gland secretions that presumably contain the main  
195 long-distance signal of the bumblebee pre-mating recognition (e.g. Ayasse & Jarau, 2014), and (ii) diploid males  
196 have the same mating success as haploid males in experimental conditions where short distance pre-mating  
197 recognition signals should act (Duchateau & Marien, 1995).

#### 198 *Diploid Males in the Wild*

199 Diploid males have been previously detected, mainly in bumblebee species with small and fragmented  
200 distribution like *B. florilegus* in Japan and *B. muscorum* in Great Britain, with a considerable proportion of such  
201 males of up to 50% (Darvill *et al.*, 2006; Takahashi *et al.*, 2008). However, diploid males were detected in



202 populations of widespread species like *B. occidentalis*, *B. perplexus*, and *B. terricola* in about 5% frequency  
203 (Whidden & Owen 2011). Our results show a ratio ( $\approx 2\%$ ) of wild diploid males in *B. terrestris* similar to those  
204 found in other widespread bumblebee species. This low ratio was expected since *B. terrestris* is one of the most  
205 common and widespread bumblebee species in Europe (Rasmont *et al.*, 2008). Indeed, the large distribution and  
206 population density of this species (Rasmont *et al.*, 2008, 2015) make unlikely a limited genetic flow (i.e. genetic  
207 drift) between populations and therefore a high diploid ratio. However, our current sampling is limited to a small  
208 geographic region and to a small sample. Further studies on the diploid male ratio on large *B. terrestris* sampling  
209 and other European and non-European species are needed to validate this hypothesis.

### 210 *Consequences for Bumblebee Conservation*

211 If future studies confirm current evidence (see our results and Duchateau & Marien 1995) that diploid males  
212 cannot be recognized through their reproductive traits by females, this rules out the potential role of pre-mating  
213 recognition in curbing an ongoing vortex of extinction of diploid males in bumblebee species. Zayed & Packer  
214 (2005) and Hein *et al.* (2009) underline four crucial conditions that must be met to initialize the diploid male  
215 vortex: (i) fixed sex ratio, (ii) low reproductive rate, (iii) no mate choice (i.e. females cannot detect if potential  
216 mates are related mates), and (iv) extreme population fragmentation. Conservation plans should act on the two  
217 last factors since the two first conditions depend on intrinsic features of bumblebee species. The decrease of  
218 population fragmentation through the establishment of corridors (Holzschuh *et al.*, 2009) between populations  
219 could increase the genetic diversity of populations and thus prevent the initialization of diploid male vortex.  
220 Alternatively, individual translocations between populations (Arrendal *et al.*, 2004) could neutralize the  
221 fragmentation and enable females to choose exotic (unrelated) mates. Nevertheless, the beneficial effects of such  
222 translocations would appear to be restricted to areas in the immediate vicinity of the release sites (Arrendal *et al.*,  
223 2004). Moreover, potential translocations should consider the interpopulational differentiations to maximize the  
224 genetic distinctiveness of translocated individuals while preserving the species diversity (i.e. avoid genetic  
225 homogenization through translocation; Lecocq *et al.*, 2015b).

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231 **Disclosure**

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

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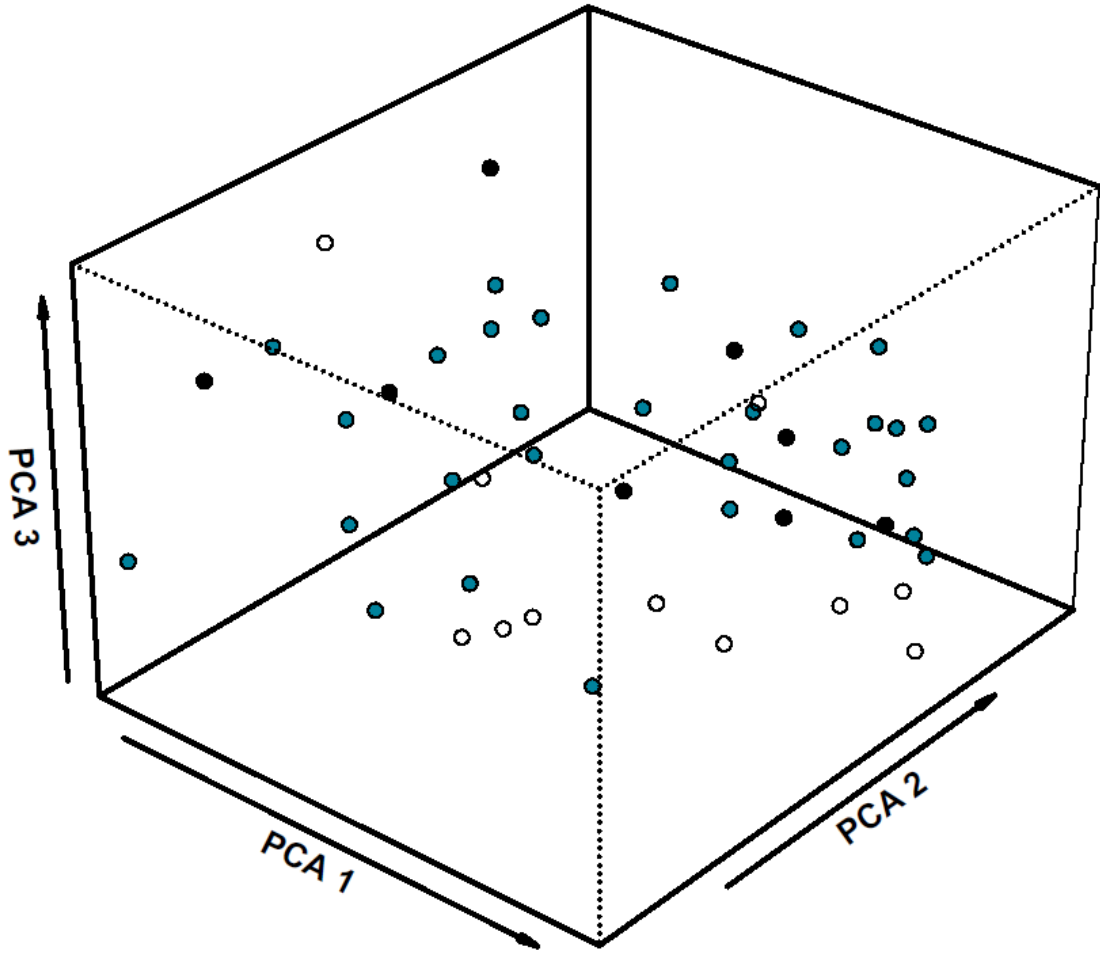
394 **Table**

395 **Table S1** Sampling table. The table gives the origin of samples and the ploidy determination by genotyping each  
396 specimen with 13 microsatellite loci with indication of the fluorescent dye used in the PCR amplification.

397 **Table S2** Data matrix of cephalic labial gland secretions (relative amounts of each compound) and list of the  
398 identified compounds in the sampling. MW is the molecular weight of the compound. IndVal results are  
399 indicator value obtained in IndVal method.

400 **Figure captions**

401 **Fig. 1** Statistical analyses of cephalic labial gland secretion. Non-metric multidimensional scaling (nMDS)  
402 ordination analysis of chemical reproductive trait of diploid and haploid *Bombus terrestris* males (n= 90). Three  
403 first axes of the nMDS ordination plot based on Bray-Curtis distances calculated on a male cephalic labial gland  
404 secretion matrix of *B terrestris*.



- Haploid males
- Diploid males (bred specimens)
- Diploid males (wild specimens)