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ORIGINAL ARTICLE

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

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

Key words bee decline; bumblebees; conservation; diploid males; premating recognition; reproductive traits

Introduction

About 90% of the world's plant species are pollinated by animals, and mainly bees (Ollerton *et al.*, 2011). This makes bees a critically important functional group for ecosystem services in wild environments as well as in crops (Free, 1993; Klein *et al.*, 2007). Among bees, females of the social genus *Bombus* (i.e., bumblebees) are among the most important pollinators of the northern temperate zone (Williams, 1985). However, some bumblebee species, as well as other wild bee species, are obviously experiencing a strong decline in their populations (Carvalheiro *et al.*, 2013; Rasmont *et al.*, 2015). Several factors were proposed to explain this decline: landscape modifications/fragmentations, intensive use of agrochemicals, pathogen infections, competition with alien species, climate change, and interactions between all of these factors (review in Winfree, 2010; Potts *et al.*, 2011). Ultimate consequences are a decrease of population size and an increase of population fragmentation that leads to a loss of genetic diversity through inbreeding and genetic drift (Zayed, 2009; Frankham *et al.*, 2010).

Loss of genetic diversity is particularly problematic in haplodiploid species such as bees (Zayed & Packer, 2005; Zayed, 2009). Like most Hymenoptera, bees have a single-locus complementary sex determiner (sl-CSD):

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

This vortex of extinction could be reduced if females are able to recognize and avoid mating with diploid males. In monandrous species, like most bumblebee species, the female choice requires premating indicators of male genetic equipment (Herzner *et al.*, 2006). Therefore, premating mechanisms of avoidance of diploid males could be selected in monandrous bumblebee queens in view of the deleterious consequences of mating with diploid males. First bioassays show that *Bombus* diploid males have the same mating success as haploid males in experimental conditions (Duchateau & Marien, 1995). However, such experiments could lead to unnatural mating because queens have no choice between different sexual partners. For example, sibling individuals mate in experimental condition even if *B. terrestris* queens seem to be less willing to mate with their siblings than with an unrelated partner when they have the choice (Whitehorn *et al.*, 2009). Therefore, we expect that premating cues produced by males could allow female premating discrimination to avoid diploid males in natural conditions. As diploid males show differences in body size and physiology (Duchateau & Marien, 1995; Gerloff *et al.*, 2003), we hypothesize that they could display original chemical reproductive traits.

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

Materials and methods

Collection and rearing of specimens

In order to produce diploid males (diploid male samples were also used in Gérard *et al.*, 2015), we obtained 3 colonies of *Bombus terrestris terrestris* (L.) from bee breeder Biobest N.V. We were reared these colonies at the laboratory of Zoology in Mons (UMons, Belgium) in a controlled climate room (see rearing protocol in Lhomme *et al.*, 2012, 2013; Gérard *et al.*, 2015): we obtained queens ($n = 11$) and males ($n = 21$) among the first generation from only one colony. We forced brother–sister mating by placing the newly emerged sibling queens and males in a flight cage exposed to natural light (see mating protocol in Lhomme *et al.*, 2013; Gérard *et al.*, 2015). We used these mated queens to produce a second generation of inbred colonies. These queens overwintered during 2 months prior to a activation by CO_2 narcosis (see protocol in Lhomme *et al.*, 2013). Then, we placed the queens in small boxes with 2 workers and a cocoon (i.e., to maximize the probability of colony initiation see protocol in Lhomme *et al.*, 2012, 2013). All 11 mated queens of the first generation initiated a colony, producing a total of 62 workers and 11 males of second generation (Table S1).

In order to produce haploid males, we reared 8 queenless microcolonies with 5 workers from 1 colony from bee breeder Biobest N.V. Each microcolony was maintained in a plastic box in a controlled climate room (same rearing conditions than for colonies). The microcolonies produced 41 haploid males (Table S1).

We increased the diversity in our dataset by sampling 38 wild males of *B. terrestris terrestris* in Belgium *in natura* with a net (Table S1). As wild males of *B. terrestris* could be confused with the closely related species *B. cryptarum*, the species identification was checked by CLGS analyses: the 2 species have species-specific CLGS composition used as diagnostic traits (e.g., Bertsch *et al*, 2005; see CLGS analyses protocol below).

All wild ($n = 38$) and reared ($n = 52$) males (n total $=$ 90) were killed by freezing at -20 °C. For the reared males, we used 5–10-d-old males when the *B. terrestris* males are the most attractive to females (Coppée *et al.*, 2011). For the wild males, we examined the wing condition (Tkalců, 1969) in order to avoid the sampling of old specimens that have a CLGS modification composition and are less attractive to females (Žáček *et al.*, 2009; Coppée *et al.*, 2011).

Determination of the degree of ploidy

We assessed the degree of ploidy by genotyped males with 13 microsatellite markers developed for *B. terrestris*: B11, B100, B121, B126, and B132 (Estoup *et al.*, 1993) and 0007_47n22, 0078_59o4, 0222_63d21, 0583_22I4, 0636_34m4, 0646_83e8, 0930_40o1, and 0939_33h17 (Stolle *et al.*, 2009). We extracted sample DNA following the method described by Maebe *et al.* (2013) with some small modifications: 200 μL of 5% Chelex (InstaGeneTM matrix, BioRad, Temse, Belgium) and 10 μL of proteinase K (20 mg/mL) were added to 1 middle leg and incubated at 56 °C for 2 h and 97 °C for 15 min. The microsatellite loci: B11, B100, B121, B126, and B132 were amplified in multiplex by PCR as described in Maebe *et al.* (2013). For allele detection of the primers originated from Stolle *et al.* (2011), we used a tailed-primer approach (Schuelke, 2000). We used the PCR protocol described in Maebe *et al.* (2015): simplex PCR reactions were performed, containing 1.5 μL template DNA, 1 μL of 10× PCR buffer (Qiagen, Antwerp, Belgium), 0.2 μL of 10 mmo/L dNTP's (Qiagen), 0.1 μL of 10 μmol/L forward primer, 0.4 μL of 10 μmol/L reverse primer, 0.4 μL of 10 μmol/L labeled M13-primer and 0.05 μL of 2.5 units/reaction Hotstar Taq DNA Polymerase (Qiagen). Initial denaturing at 95 °C for 15 min was followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 48, 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. Pooled samples were visualized on a ABI-3730xl sequencer (Applied Biosystems, Chesire, UK) and fragments were scored manually using Peak Scanner Software v 1.0 (Applied Biosystems).

Comparative chemical analyses of cephalic labial gland secretions

We extracted the CLGS of 90 males following the entire cut head protocol described in De Meulemeester *et al.*, 2011). Vials containing the solvent and sample were (i) kept for 24 h at room temperature (20 °C) to fulfill the extraction and (ii) stored at -40 °C for 2 months prior to the analyses. We (i) analyzed the CLGS samples and identified compounds using gas chromatography-mass spectrometry (GC/MS) and (ii) quantified them by gas chromatography with flame ionization detector (GC/FID) (see GC/MS and GC/FID specifications and temperature program in Lecocq *et al.*, 2015a). We identified the CLGS in Xcalibur$^{\mathrm{TM}}$ with their mass spectra compared to those in the National Institute of Standards and Technology Library (NIST, U.S.A) with NIST MS Search 2.0. We determined the double bond positions from (i) mass spectra of dimethyl disulphide adducts of unsaturated components (Francis, 1981) (reaction time: 4 h) and (ii) by chemical ionization with acetonitrile as a reaction gas (Oldham & Svatoš, 1999) (see protocol details in Lecocq *et al.*, 2013). We quantified the peak areas of CLGS compounds in GC-solution Postrun (Shimadzu Corporation) with automatic peak detection and noise measurement. We elaborated the data matrix (Table S2) with the relative amounts of each compound for each individual (the relative amounts are calculated by dividing the peak areas of compounds by the total area of compounds in each sample). We based our data matrix on the alignment of each compound between all samples performed with GCAligner 1.0 (Dellicour & Lecocq, 2013a,b).

We compared the CLGS between haploid and diploid males with nonmetric multidimensional scaling (nMDS) ordination using a Bray–Curtis similarity matrix, 3 dimensions and 50 runs (R-package ecodist, Goslee & Urban, 2007). We transformed [log (x + 1)] the data matrix to reduce the great difference of abundance between compounds in high and low concentration (De Meulemeester *et al.*, 2011). An hypothetic CLGS differentiation between haploids and diploids was assessed by performing permutation-based version of the multivariate analysis of variance (perMANOVA) using the Bray–Curtis similarity matrix and 10 000 permutations (R-package vegan, Oksanen *et al.*, 2011) (perMANOVA assumption; i.e., similar variance homogeneity between groups was checked by performing a distance-based test for multivariate homogeneity of group dispersions for a one-way ANOVA design; Anderson, 2006). We determined CLGS compounds that are potentially specific to and regular to diploid or haploid males with the indicator value (IndVal) method (Dufrêne & Legendre, 1997; see details in Lecocq *et al.*, 2015b). All statistical analyses were performed in R (R Development Core Team, 2013).

Results

Genetic analyses showed that reared queen-right colonies produced 9 diploid males (Table S1). One diploid male was also found in wild specimens (Table S1). Other males were unambiguously haploid (Table S1).

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

Discussion

Diagnostic features of diploid bumblebee males

In view of the dramatic consequences of diploid male production on population (Zayed & Packer, 2005), we expected that *B. terrestris* would display premating mechanisms avoiding virgin queen mating with diploid males as already observed in some Hymenoptera (Herzner *et al.*, 2006). Such premating mechanisms are most likely based on differences between reproductive traits used in premating recognition between diploid and haploid males. Therefore, we investigated 1 reproductive trait, the CLGS that acts as a key trait for the bumblebee premating recognition. However, our comparative statistical analyses based on CLGS composition did not detect obvious differences between diploid and haploid males in their CLGS, a main key trait for mate attraction (Fig. 1). The slight trend observed for the reared diploid males in the nMDS (i.e., these specimens are more or less grouped together at the edge of the nMDS; Fig. 1) could reflect a “family-effect” since all queens that produced these males are sister mated with their brothers as already observed in other study (Lecocq *et al.*, 2011). Nevertheless, our statistical analyses did not show significant differentiation between CLGS of diploid and haploid males (see perMANOVA results). Even if the components of the male CLGS that constitute the pheromones are not known (i.e., those components that release a female response or convey information for females), this does not invalidate the basic result that there is no significant differentiation in diploid male CLGS since there is no specific and regular chemical compounds to diploid or haploid males (see IndVal results in Table S2). Although we are aware of the limited power of our statistical analyses due to our small sample size (10 diploid males), our data clearly demonstrates that there is no strong difference in the CLGS composition between haploid and diploid males.

According to our results we speculate that the queens are not able to discriminate the diploid males through their CLGS. However, since the bumblebee male premating signal (Baer, 2003; Ayasse & Jarau, 2014; Streinzer & Spaethe, 2014) includes behavioral and other chemical features such as tendon glands (Jarau *et al.*, 2012) that are mainly poorly known, we cannot rule out the fact that the whole premating signal of diploid males are evaluated by females as being strictly similar to those of haploid males. Indeed other traits involved in the species communication (e.g., cuticular hydrocarbons) or in the reproduction could be different in diploid *B. terrestris* males such as observed for cuticular compounds and testis size of diploid drone in the close relative *Apis mellifera* L. (Herrmann *et al.*, 2005). Moreover, diploid *B. terrestris* males are distinct in other characters such as wing shape, body size, and physiology (Duchateau & Marien, 1995; Gerloff *et al.*, 2003; Gérard *et al.*, 2015). These features could allow the female to diagnose diploid males and/or decrease the fitness of diploid males (e.g., fitness of a bumblebee male is correlated with body size, Cueva del Castillo & Fairbairn, 2012). Further studies on other traits and ethological test allowing choices of virgin females could help to define whether the *B. terrestris* females are not able to recognize diploid males. Nevertheless, current evidence suggests that queens are not able to discriminate the

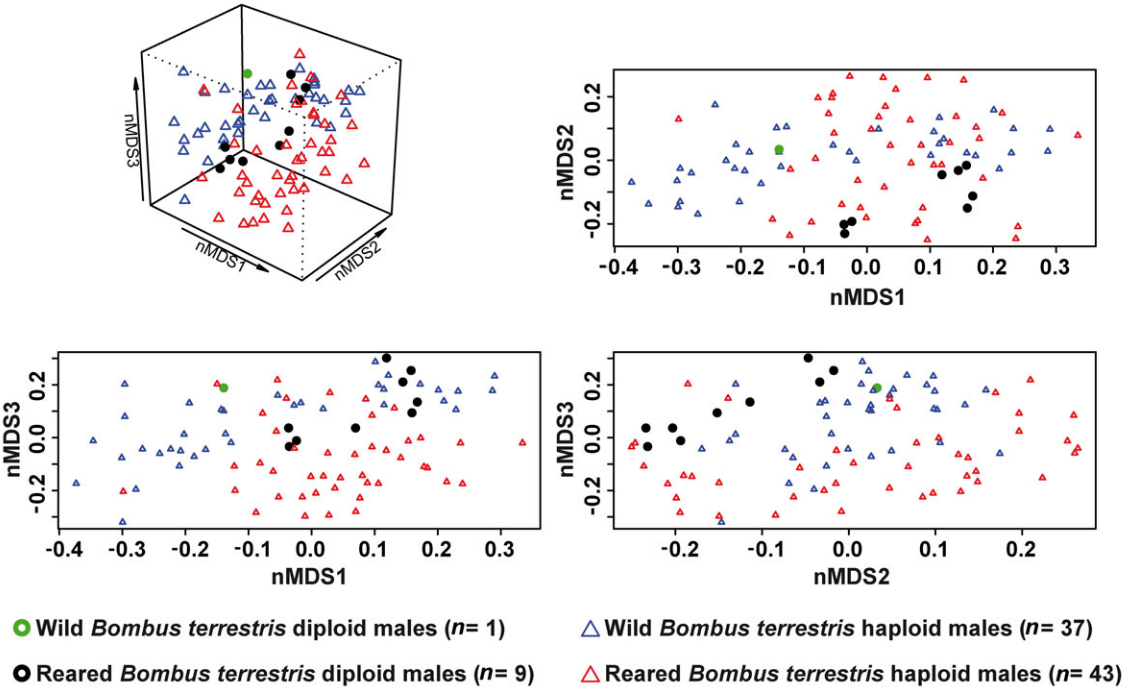

Fig. 1 Statistical analyses of cephalic labial gland secretions. Nonmetric multidimensional scaling (nMDS) ordination analysis of chemical reproductive trait of diploid and haploid *Bombus terrestris* males ($n = 90$). Three first axes of the nMDS ordination plot based on Bray–Curtis distances calculated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

diploid males during the premating recognition because (i) our results show that there are no diploid specific cephalic labial gland secretions that presumably contain the main long-distance signal of the bumblebee premating recognition (e.g., Ayasse & Jarau, 2014) and (ii) diploid males have the same mating success as haploid males in experimental conditions where short distance premating recognition signals should act (Duchateau & Marien, 1995).

Diploid males in the wild

Diploid males have been previously detected, mainly in bumblebee species with small and fragmented distribution like *B. florilegus* in Japan and *B. muscorum* in Great Britain, with a considerable proportion of such males of up to 50% (Darvill *et al.*, 2006; Takahashi *et al.*, 2008). However, diploid males were detected in populations of widespread species like *B. occidentalis*, *B. perplexus*, and *B. terricola* in about 5% frequency (Whidden & Owen 2011). Our results show a ratio (≈2%) of wild diploid males in *B. terrestris* similar to those found in other widespread bumblebee species. This low ratio was expected since *B. terrestris* is one of the most common and widespread bumblebee species in Europe (Rasmont *et al.*, 2008). Indeed, the large distribution and population density of this species (Rasmont *et al.*, 2008, 2015) make unlikely a limited genetic flow (i.e., genetic drift) between populations and therefore a high diploid ratio. However, our current sampling is limited to a small geographic region and to a small sample. Further studies on the diploid male ratio on large *B. terrestris* sampling and other European and non-European species are needed to validate this hypothesis.

Consequences for bumblebee conservation

If future studies confirm current evidence (see our results and Duchateau & Marien, 1995) that diploid males cannot be recognized through their reproductive traits by females, this rules out the potential role of premating recognition in curbing an ongoing vortex of extinction of diploid males in bumblebee species. Zayed & Packer (2005) and Hein *et al.* (2009) underline 4 crucial conditions that must be met to initialize the diploid male vortex: (i) fixed sex ratio, (ii) low reproductive rate, (iii) no mate choice (i.e., females cannot detect if potential mates are related mates), and (iv) extreme population fragmentation. Conservation plans should act on the 2 last factors since the 2 first conditions depend on intrinsic features of bumblebee species. The decrease of population fragmentation through the establishment of corridors (Holzschuh *et al.*, 2009) between populations could increase the genetic diversity of populations and thus prevent the initialization of diploid male vortex. Alternatively, individual translocations between populations (Arrendal *et al.*, 2004) could neutralize the fragmentation and enable females to choose exotic (unrelated) mates. Nevertheless, the beneficial effects of such translocations would appear to be restricted to areas in the immediate vicinity of the release sites (Arrendal *et al.*, 2004). Moreover, potential translocations should consider the inter-populational differentiations to maximize the genetic distinctiveness of translocated individuals while preserving the species diversity (i.e., avoid genetic homogenization through translocation; Lecocq *et al.*, 2015b).

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Disclosure

The authors declare that they have no conflict of interest.

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

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