UNIVERSITE DE MONS

FACULTE DES SCIENCES

LABORATOIRE DE ZOOLOGIE

Bombus terrestris (L. 1758) :

A complex species or a species complex ?

Intraspecific pheromonal and genetic variations of *Bombus terrestris* (L.)

-

Impacts on the speciation

Audrey Coppée

Thesis submitted in fulfilment of the degree of Doctor in Sciences

Mons – December 2010

Supervisor : Prof. P. Rasmont

Abstract

Bombus terrestris L. : A complex species or a species complex ?

Intraspecific pheromonal and genetic variations of *Bombus terrestris* (L.)

–

Impacts on the speciation

Bombus terrestris L. is a widespread European bumblebee species. Its distribution is centred on the Mediterranean Sea extending from the Canary Islands in the West to the Altai Mountains on the East, and from the Antiatlas Mountains of Morocco in the South to Southern Finland in the North. Nine subspecies are described showing morphological differentiations, particularly in isolated (e.g. insular) taxa. Males of this species have a patrolling premating behavior, i.e. they spread sexual pheromones along a circuit they patrol searching for a conspecific virgin female. These secretions, produced by the cephalic labial glands (CLG) are known to be species-specific. Some genetical studies have already shown distinctness of insular subspecies like *canariensis*, or *xanthopus*. Despite all the studies conducted on this biological model, specialists still do not agree on the taxonomic status several subspecies.

The goal of this study is to evaluate the chemical, the behavioural and the genetic variation inside the *B. terrestris* complex. Based on original results we propose new hypotheses about the species status of each previously described taxa.

We first demonstrated an age-dependent variation of the secretions of sexual pheromones, showing, in addition with behavioural test a preference of the virgin females for males aged of 10 days old.

A widespread sampling of the species was done, with special attention to the insular subspecies. The CLG secretions were analyzed using gas chromatography coupled with mass spectrometry. A Discriminant Linear Analysis was applied to the data matrix obtained (compounds x samples), showing great qualitative and/or quantitative differences in the secretions of *canariensis, sassaricus,* and *xanthopus.*

Using a simple behavioural test we also showed that the virgin queens preferred males belonging to their own subspecies. This choice can increase the divergences among subspecies by reinforcing the homogeneity within a given subspecies.

The genetic analyses, using mitochondrial DNA (COI and cytochrome b) revealed two well separated monophyletic groups: *xanthopus,* and *canariensis* and *africanus.*

Our results show that some taxa are poorly differentiated (*B. t. terrestris, B. t. dalmatinus)*, while others show no genetic differences but well characterized CLG secretions (*B. t. audax, B. t sassaricus*). Finally,two taxa show a conspicuous genetic divergence and/ or specific CLG secretions (*B. t. canariensis, B. t africanus, B. t. xanthopus)* raising questions about their taxonomic status. We suggest to consider four of the taxa as separate species: *canariensis, africanus, sassaricus* and *xanthopus*, becoming ***Bombus canariensis, Bombus africanus, Bombus sassaricus*** and ***Bombus xanthopus.***

Remerciements / Acknowledgements

Je tiens tout d'abord à remercier le Professeur Pierre Rasmont, initiateur de ce projet, pour m'avoir soutenu au long des 5 années passées dans le Laboratoire de Zoologie. Il m'a ouvert la voie au monde de la recherche et m'a appris à évoluer dans un nouveau cadre de réflexion, celui de la biologie. Je lui suis particulièrement reconnaissante de m'avoir fait confiance et de m'avoir laissé l'indépendance nécessaire à mon épanouissement.

I would also like to thank Irena Valterova (Prague, Czech Republic), my "mother" in the world of Chemical Ecology. She is a real inspiration, a model for me, someone who trusts me, I can always count on, even in last minute. I really hope we will continue to work the way we did.

Merci aussi à Denis Michez (aka PA), qui a su s'investir pour moi lorsque c'était nécessaire, m'a toujours soutenue et m'a remise sur le droit chemin quand je m'égarais. Il a fait preuve d'un courage sans égal pour supporter mes cris (de pleurs ou de joie), mes débordements d'humeurs en tout genre, ou encore mes crises existentielles. Les 5 années passées à partager un bureau avec Denis resteront gravées dans ma mémoire.

Je voudrais aussi remercier tous les membres, passés ou présents du Laboratoire de Zoologie, et aussi en profiter pour m'excuser pour les demandes urgentes (déjà trop tard), les coups de fils (de F.) et autres désagréments du quotidien. Ma thèse n'aurait jamais été la même si je n'avais pas été entourée et soutenue par mes collègues : Michaël Terzo, Stéphanie Iserbyt, Thibaut De Meulemeester, Patrick Lhomme, Thomas Lecocq, Maryse Vanderplanck, Dorothée Roelants, Laurent Crépin et tous les étudiants et mémorants avec qui j'ai beaucoup appris. Un merci particulier à Audrée Helderweirt dont j'ai un peu abusé ces derniers temps.

Je tiens également à exprimer toute ma gratitude au Professeur Jean-Yves Rasplus ainsi qu'à Gwenaëlle et Roula pour m'avoir accueillie avec leur bonne humeur et tout leur savoir faire au Centre de Biologie et de Gestion des Populations.

Un grand merci également au Docteur Pascal Gerbaux et à Michaël Boulvin pour m'avoir permis d'utiliser le matériel de chromatographie et m'avoir aidée pour la quantification de mes échantillons et toutes les autres analyses plus ou moins urgentes...

All my trips in Praha would have never been the same without the IOCB

team, which is just full of ready-to-help people, who taught me so much. I particularly want to thank Klara, Pavel and Petr for their help.

I also would like to thank the people who helped me for the sampling of bumblebees : Nigel Raine (London, UK), Javier Ortiz (Almeria, Spain), Andrew Hingston (Tasmania, Australia) and Nicolas Vereecken (Bruxelles, Belgium).

Finally, I thank my proofreaders who did a great job, Ramona Shleby, Véronique Brédas and Mike Wykes.

Sans mes proches, tout ceci n'aurait pas été possible. Ils ont été, sont et resteront, ma source d'inspiration, mon réconfort, mon soutien, ceux sans qui rien ne serait possible. Merci à toute ma famille, mes grand-mères, Maman, Séverine, Brice, Peggy Sue, Céline et Julien, mes trois FMMiennes préférées.

Pour terminer, un merci tout particulier à l'ange de patience qui me supporte au quotidien.

A mon Papa

“ How on earth do bees coming

separetely out of nest discover same place,

is it like dogs at corner-stone?”

Darwin, 1886

“The road to insect sociality was paved with pheromones”

Blum, 1974

Table of contents

Chapter 1

INTRODUCTION

Chapter 1. Introduction

1.1. The Species Concept: History and new insights

At the end of 1831, the Beagle was surveying and charting coasts and Charles Darwin (fig. 1) spent most of his time collecting living and fossil specimens and carefully noting observations and theoretical speculations. Twenty years later, he proposed the scientific theory that transformation of species resulted from a process that he called "Natural Selection" (Darwin, 1859).

Darwin's vision of species was as follows: "*…I look at the term species, as one arbitrarily given for the sake of convenience to a set of individuals closely resembling each other, and … it does not essentially differ from the term variety, which is given to less distinct and more fluctuating forms. The term variety, again, in comparison with mere individual differences, is also applied arbitrarily, and for mere convenience sake*". To summarise, as a paradox, Darwin's book "*The origin of Species*" (1859) denies any natural support of the species.

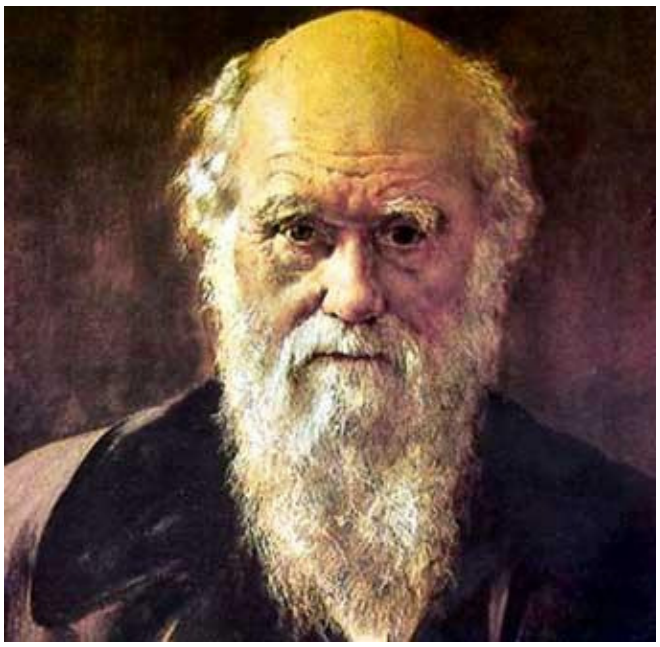


Figure 1. Charles Darwin (1809 – 1882)

It is only at the beginning of the 20th Century that the notion of species became part of the Evolutionist Biology again.

Oskar Vogt wrote the first paper trying to define and apply the species concept to a defined animal group (i.e. bumblebees) in his *Studien über das Artproblem* (1909, 1911). He first proposed that speciation results from subspecies differentiated in areas isolated by geological events.

Theodosius Dobzhansky (fig. 2) was one of the first to apply genetics to natural populations. He was the first to introduce the population as a keystone of the species concept: "*The evolutionary unit is not the individual, nor the species, nor even the subspecies, but the population (or deme)*". This is the local assemblage of specimens in panmictic conditions (that actually interbreed and among which they choose their mates) (Dobzhansky, 1937). Moreover, he introduced the genetic drift concept, which is "*The non-selective evolution resulting from the very low number of specimens in the smallest populations*" (Dobzhansky & Pavlovsky, 1957). For him, Evolution equated to a change in the frequency of an allele within a gene pool. He participated in the development of Modern Evolutionary Synthesis, along with many other major figures, including: R. A. Fisher, J.B.S. Haldane, Sewall Wright, E.B. Ford, Ernst Mayr, Bernhard Rensch, Sergei Chetverikov, George Gaylord Simpson, and G. Ledyard Stebbins (Reif et al., 2000).

Figure 2. Theodosius Dobzhansky (1900 -1975)

Mayr's (fig. 3) (1942, 1963) definition of species is nowadays the most commonly accepted concept. In his book *Systematics and the Origin of Species* (Mayr, 1942) he wrote that "*a species is not just a group of morphologically similar individuals, but a group that can breed only among themselves, excluding all others*". In 1963, he added an ecological dimension to his definition (Mayr, 1963). When populations of organisms become isolated, sub-populations will start to differ through genetic drift and Natural Selection over a period of

time, and thereby evolve into new species. Taking into account the genetic drift of Dobzhansky, he proposed that the most significant and rapid genetic reorganization occurs in extremely small populations that have been isolated (for instance on islands). Mayr's theory was based on interfecondity and isolation of the populations (later called the "Isolation Concept.")

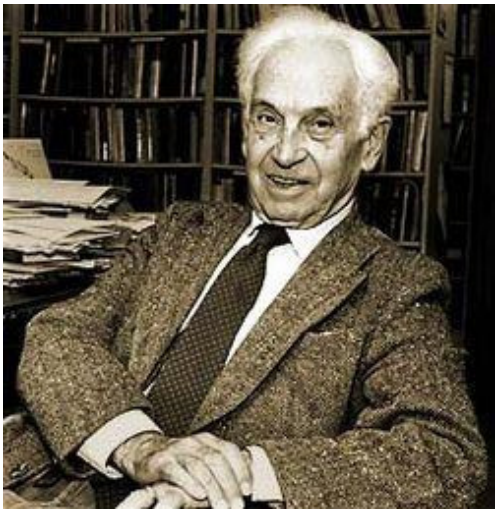


Figure 3. Ernst Mayr (1904-2005)

Other concepts were born during the 20th Century such as the morphological, genetic, ecological or phenetic concepts of species, which can all be helpful in understanding some special cases (Reif et al., 2000). But that will not be discussed here.

With the development of genetic tools, a new vision of the Species Concept emerged: Baum's Phylogenetical Concept of Species (PSC) (Baum, 1992). He actually distinguished two PSCs. In the first one (PSC1), a species is defined as the smallest group of organisms that share at least one diagnostic character. This character can be morphologic, biochemical, or molecular and is fixed in a cohesive unit of reproduction. This concept takes a lot of information into account: molecular aspects as well as mate recognition. The PSC2 considered a species as monophyletic and with one or more derivative characters. This concept tends to lead to "upgrade" most of the subspecies (as defined by e.g. Vogt, Mayr...) to the species level.

One of the most recent revolutions in the Species Concept definition occurred quite recently, when Paterson (Paterson, 1985, 1993) presented his Species Recognition Concept. The idea is that the pre-zygotic mechanisms presented and shared by mates to recognise each other define the species. Paterson calls them the "*Specific-Mate Recognition System*" (SMRS). The SMRS maximizes the specific recognition and generally prevents interspecific mating and hybridization as a result. According to Paterson, species isolation is a result or even a simple by-product of the evolution of SMRS, while for Mayr, the isolation is the keystone of speciation.

To summarize, for Mayr the individuals find their mate by avoiding other species, while for Paterson, they actively search for attractive mates, following their own species-specific criterion (SMRS).

Paterson's Concept of Species will be the paradigm used for the interpretation of the results in the present work. In order to test this concept, bumblebees are constituting a well adapted model since their SMRS is well known, particularly in *Bombus terrestris* (L.) which is used commercially, and consequently one of the most studied bumblebees.

Nowadays, it is more and more common to use a "Total evidence" approach in taxonomy (*sensu* Carnap, 1950). This implies using a maximum number of methods and tools to assess taxonomic status i.e.: morphology, phylogeny, SMRS …

1.2. *Bombus terrestris* (L. 1758)

1.2.1. General informations

Among the 250 species of bumblebees (Williams, 1998; Michener, 2007; Rasmont et al., 2008) described, *Bombus terrestris* is one of the species that has been the most studied. It is a very common bee in the West-Palaearctic region, with a distribution centered on the Mediterranean (fig. 4). It includes 9 subspecies [see Appendix I: (Rasmont et al., 2008)].

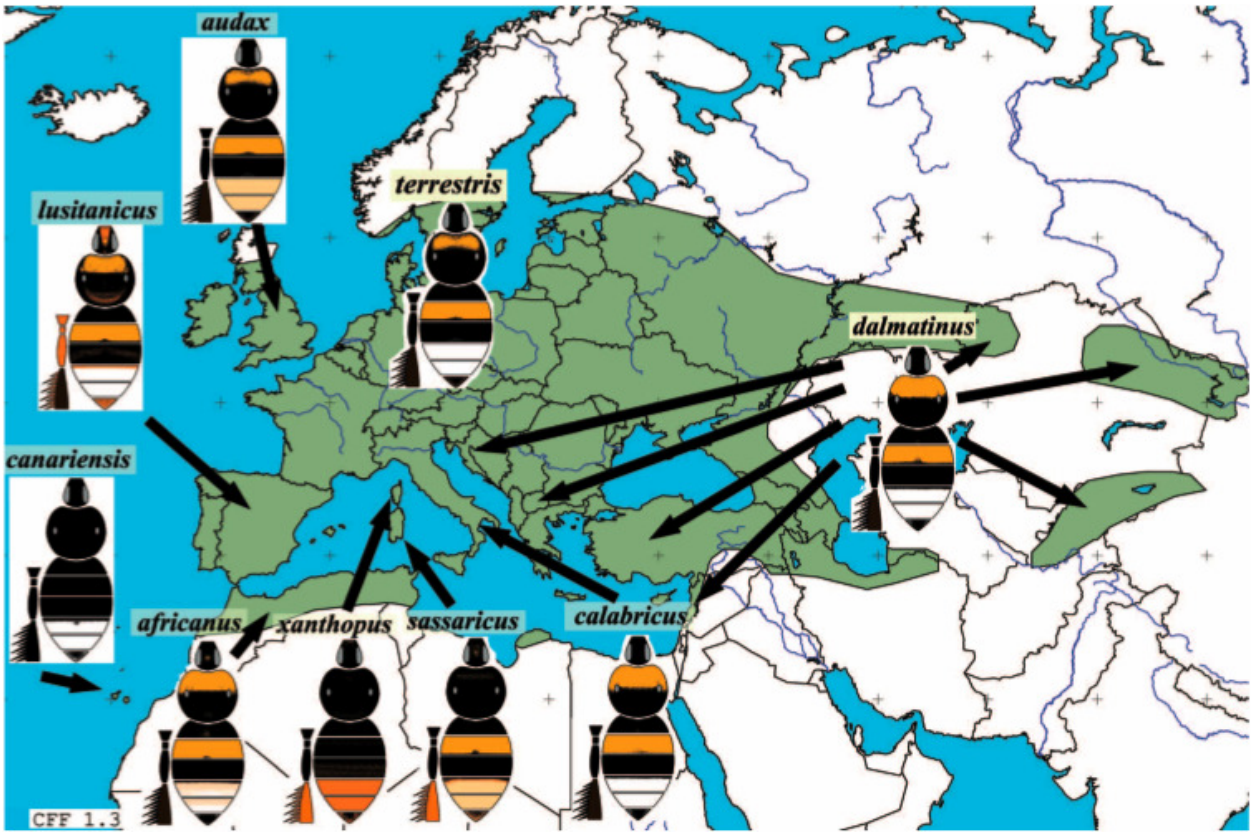


Figure 4. Geographic distribution and patterns of B. terrestris subspecies (from Rasmont et al. 2008).

In northern Europe, the phenology of *B. terrestris* extends from the early spring in March until August (Rasmont et al., 2008). This is not true in the Mediterranean regions, where the foundation of new colony occurs in autumn or winter, e.g. in the Maure Mts region (S.E. France) (Duhayon & Rasmont, 1993). In this case, the queens enter a diapause during the driest summer period and emerge in late-September with the first autumn rains. Except during these two-and-a-half-months diapause (June to mid-September), the

bumblebees are active all year long (Rasmont et al., 2008).

Bombus terrestris is a highly polylectic (or generalist) bumblebee. More than three hundred flower species have been listed as host plant for this species in France and Belgium (Rasmont, 1988). The autumnal flowering of some plants in the Mediterranean regions, like *Arbutus unedo* L., allows the development of winter colonies in these areas (Rasmont et al., 2005).

Bumblebees, like all Hymenoptera, are haplo-diploid (i.e. females are diploid and males that develop from unfertilized eggs are haploid). The sex determination was shown to be controlled by the system called "single locus – Complementary Sex Determination" (sl-CSD) (Cowan & Stahlhut, 2004; van Wilgenburg et al., 2006). In Hymenoptera, sex is determined by a single locus: heterozygotes are females and hemizygotes are males. With inbreeding, homozygous diploid and sterile males occur, which is a genetic burden for the population. Diploid males of bumblebees were shown to be smaller, more sensitive to diseases, and if not sterile, they may generate non-viable triploids (Duchateau & Marien, 1995).

1.2.2. The nine subspecies

1.2.2.1. Description

- ✓ **Ssp. *terrestris* (L.)**

This subspecies has a large distribution in Western and Central Europe (figs. 4 & 5). Its geographic distribution is overlapping with that of *lusitanicus* in South-western France. It also seems to overlap with *dalmatinus* in South-eastern France and Central Europe. It is a spring subspecies in the main part of its distribution.

- ✓ **Ssp. *africanus* Krüger 1956**

It is distributed in Northern Africa (N. of Morocco, N. of Algeria, and N. of Tunisia) (figs. 4 & 6). Males occur during the spring, which indicates, at least, a winter foundation (pers. obs.; Rasmont, com. pers.).

- ✓ **Ssp. *audax* (Harris 1780)**

The geographic distribution of *audax* is restricted to the British Islands (figs. 4 & 7), the queen of this subspecies emerge in spring, even if autumnal colonies have been found a few times (Stelzer et al., 2010).

- ✓ **Ssp. *calabricus* Krüger 1958**

This subspecies is found in Sicily and the Southern part of Italy (figs. 4 & 8), the queens and the workers occur in winter (pers. obs.).

- ✓ **Ssp. *canariensis* Pérez 1895**

It is the only bumblebee species that is found in the Canary Islands (figs. 4 & 9). It founds colonies in autumn and winter (Rasmont, com. pers.). The females are abundant in October, which seems to be the principal foundation period (Rasmont, com. pers.).

- ✓ **Ssp. *dalmatinus* Dalla Torre 1882**

Like the subspecies *terrestris*, *dalmatinus* as a wide distribution including the farthest reaches of South-eastern France, Northern Italy, the Balkan Peninsula and surrounding regions, Anatolia, Transcaucasia, Caucasus, Northern Iran, Southern Ural, Alaï, Altai (figs. 4 & 10) (Rasmont et al., 2008). This subspecies is found all year long, with a single spring or winter generation: in South-western Turkey, the foundation of the colony by the queen occurs in December (Yeninar et al., 2000; Gürel et al., 2008), while in Northern Turkey, it occurs in March (Rasmont, com. pers.).

- ✓ **Ssp. *lusitanicus* Krüger 1956**

The distribution of *lusitanicus* extends from the Iberian Peninsula to South-western France, where it overlaps the *terrestris* distribution. It also occurs in Madeira and the Balearic Islands (figs 4 & 11). This subspecies is found all year long, with spring, autumn or winter generations (Rasmont, 1988; pers. obs.).

- ✓ **Ssp. *sassaricus* Tournier 1890**

This subspecies only occurs in Sardinia (figs 4 & 12). It is found at least with a winter generation (Krausse, 1910).

- ✓ **Ssp. *xanthopus* Kriechbaumer 1870**

B. xanthopus is distributed over Corsica, Capraia Island and Elba Island (figs 4 & 13). This subspecies develops two generations per year: queens occur in spring and winter (Ferton, 1901; Rasmont & Adamski, 1995).

Besides the morphologic and phenologic aspects described, other characteristics were also compared between the 9 subspecies of *B. terrestris*. In addition to the different colour patterns, innate colour preferences, body size, learning abilities, flight speed, colony size, and phenology are also subject to appreciable significant variations between the subspecies (Chittka et al., 2004; Rasmont et al., 2008).

Figure 5. ***Bombus terrestris terrestris*****, male and female** ***in copula*****. Photo Pierre Rasmont, Belgium, 07. 01. 1991.**

Figure 6. ***Bombus terrestris africanus*****, worker. Photo Pierre Rasmont, Oukaimden (Morocco), 06.08.1998.**

Figure 7. ***Bombus terrestris audax*****, queen. Photo Pierre Rasmont, Bath (UK), 08.07.2009.**

Figure 8. ***Bombus terrestris calabricus*****, worker. Photo Laurent Crépin, Campofelice di Roccela (Sicily, Italy), 02.16.2009.**

Figure 9. ***Bombus terrestris canariensis*****, worker.Photo Pierre Rasmont, Teneriffe (Biobest), 03.07.2010.**

Figure 10. ***Bombus terrestris*** **cf.** ***dalmatinus*****, queen. Photo Pierre Rasmont, Gonfaron (S.E. France), 04.03.2008.**

Figure 11. ***Bombus terrestris lusitanicus*****, male. Photo Ferran Turmo Gort, Barcelona (Spain), 10.21.2009.**

Figure 12. ***Bombus terrestris sassaricus*****, worker. Photo Pierre Rasmont, Gonfaron (S.E. France), 04.16.1998.**

Figure 13. ***Bombus terrestris xanthopus*****, male. Photo Pierre Rasmont, Calvi (Corsica, France), 05.29.1985.**

Fig. 5

Fig. 6

Fig. 7

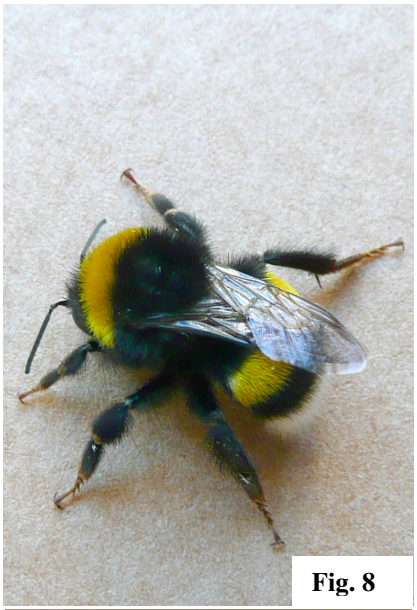


Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13

1.1.2.2. Hybridization

As far as we know now, at least some of these taxa are able to interbreed in experimental conditions (de Jonghe, 1986a; van den Eijnde & de Ruijter, 2000; Velthuis, 2002; Ings et al., 2005a). In natural conditions, hybrids rarely occur. In Corsica, introgressions of *sassaricus*, in the South of the island or *terrestris*, through the Toscan Archipelago, may occur and lead to hybridization with the endemic *xanthopus* (Rasmont & Adamski, 1995; Rasmont & Quaranta, 1997). In the Eastern Pyrenees (S. W. France), *lusitanicus* occurs side by side with *terrestris,* and several specimens that look like *lusitanicus* (f. *ferrugineus,* Schmiedeknecht) could be found as far North as Central Germany (Rasmont 1988).

1.1.2.3. Taxonomic status

Throughout the years, many authors have doubted the subspecific status of *canariensis* and to a lesser extent *maderensis*.

The questions about *canariensis* were brought about because of its high colour pattern differentiation and geographic isolation. First, it was described by Pérez (1985) as a variety of *B. terrestris*, then redescribed as a good species as *B. schmidti* (Pittioni, 1938). Afterwards, this taxon was generally considered as a subspecies of *B. terrestris* (Krüger 1951; Krüger, 1958; Rasmont et al. 1986; Rasmont 1988). However, Erlandsson (1979) defended a specific status for *canariensis*. Estoup et al. (1996) worked on the taxonomic status of *canariensis* using genetic analyses and confirmed the subspecific status.

Concerning *maderensis*, Erlandsson (1979) described it as a good species, even if there are no, or inconspicuous morphological differences with *lusitanicus*. Widmer et al. (1998) studied the population's genetic structure and colonization history of *B. terrestris*

from the Canary Islands and Madeira and concluded in the isolation of *canariensis*. These last authors considered *maderensis* from Madeira as a simple synonym of *lusitanicus*.

1.1.3. Life cycle

As eusocial animals, bumblebees present a social organisation with hierarchic levels. This social structure was defined by Wilson (1971) to include those organisms that have certain features: reproductive division of labour (with or without sterile castes), overlapping generations, cooperative care of offspring.

Queens spend the summer or winter (depending on the geographical origin) in diapause, in little cavities in the ground (fig. 14). After this period, they search for an abandoned rodent nest, in which they install their own nest. They make a first brood (fig.14, n°1) that they hatch, leaving the nest to collect nectar and pollen on host plants each day. The first emerged batch of workers forage instead of the queen and take care of the next broods (fig. 14, n°2). From that time the queen only lays eggs, hatches them and no longer forages outside of the nest. The queen seems to spread castrating pheromones, which inhibit egg-laying by the workers during the first weeks. After a few weeks, the queen begins to lay unfertilized eggs, developing into males. This is a called the *switch point*. Later, the number of workers is too great to be totally controlled by the queen and they start laying haploid eggs (fig.14, n°3), which also result in males. This is called the *competition point*. The production of new virgin queens (fig.14, n°3) is assured by the queen only, while the production of males is assured by the queen and the workers. New males leave the nest a few days after emergence, while new queens fly outside and return to the nest until they have mated. After mating, the queens dig a burrow for hibernation.

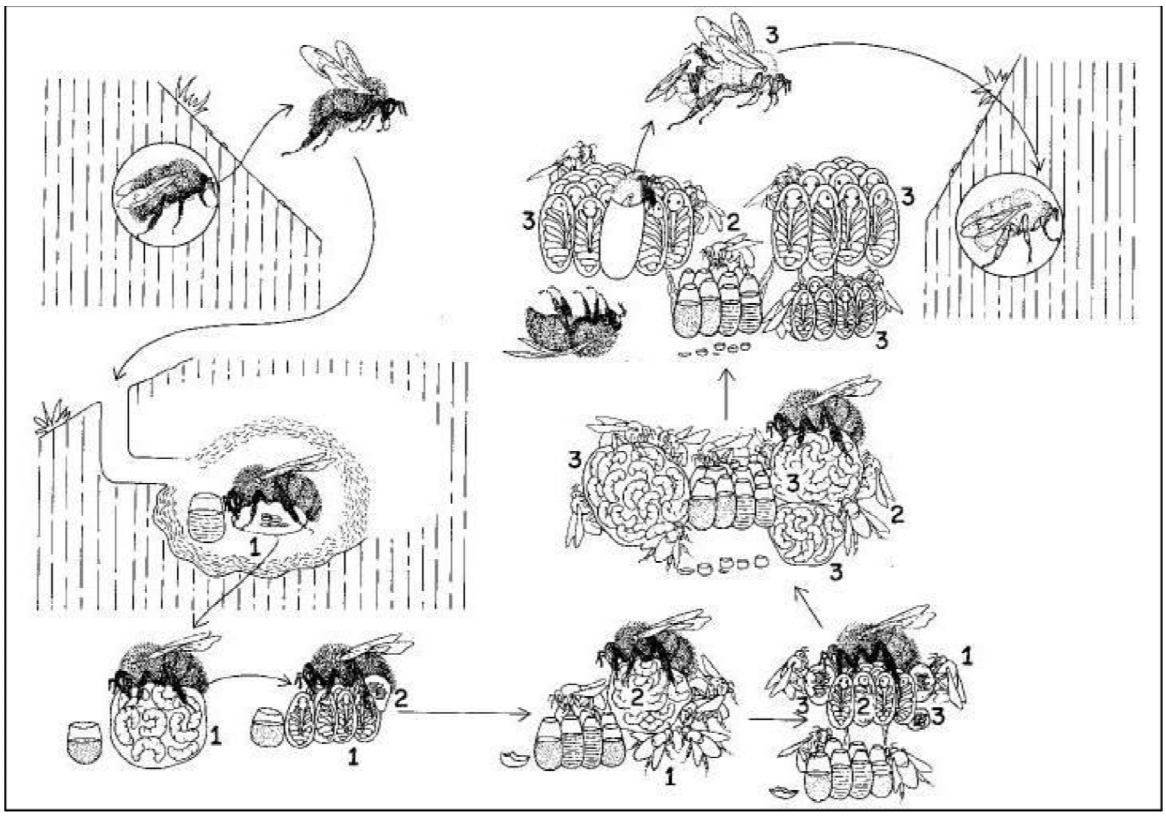


Figure 14. Cycle of a bumblebee colony (from Heinrich, 1979). The numbers (1, 2 and 3) represent the successive broods, "3" being the new generation constituted of virgin queens and males.

Bumblebees are considered as endotherms (i.e. maintaining a constant body temperature), while the eggs and developing larvae are ectotherms (internal temperature varies along with that of the ambient environmental temperature). The queen, for example, keeps a thoracic temperature of 35-38°C. This is due to their capacity to regulate their body temperature by using their thoracic muscles to heat the whole body (Heinrich, 1979).

1.1.4. Sexual pheromones and mating

To attract conspecific virgin females, males secrete sexual pheromones through large cephalic labial glands (CLG). Those pair glands (left and right) are situated in the head, behind the eyes, and they occupy most of the head volume. The CLG are acinous glands, and the secretions are excreted at the base of the mandibulae through a collecting duct (Ågren et al., 1979) (fig. 15).

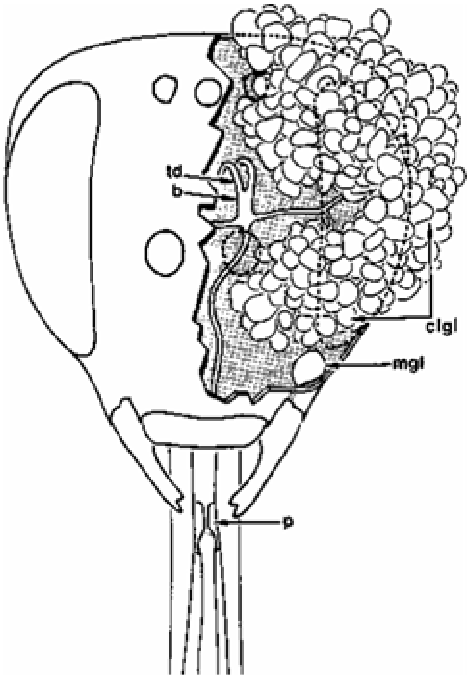


Figure 15. Semidiagrammatic drawing of a dissected *Bombus lapidarius* (L.) (from Ågren et al., 1979). td: thoracic duct; clgl : cephalic labial gland; b: bursae; mgl: mandibular gland, p: paraglossa.

These secretions are spread by the males of *B. terrestris* on twigs, leaves or trunks (fig. 16) all along a pathway they will patrol endlessly during the day to find a potential attracted conspecific virgin female.

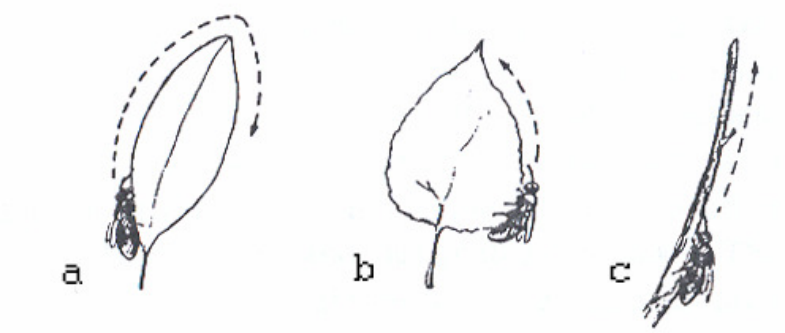


Figure 16. Marking behaviour of a male *Bombus lapponicus* (Fabricius): (a) on a *Salix glauca* L. leaf, (b) on a *Betula tortuosa* L. leaf (c) on a *Betula nana* L. twig. (Svensson, 1979).

This behaviour corresponds to the 'patrolling behaviour' described by Svensson (1979). This author summarized the previous observations of Haas (1949) and Krüger (1951) and made the distinction between three behaviours for male bumblebees searching for a mate: (1) the patrolling behaviour, (2) the perching behaviour, (3) the resting behaviour (near the nest containing the virgin queens).

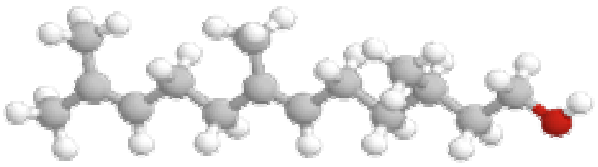


Figure 16. 3-dimensional representation of the 2,3-dihydro-6-transfarnesol.

The CLG secretions include two main types of chemical compounds: fatty acids and their derivatives, and terpenes and their derivatives. The functions described for these two types of compounds are: alcohols, aldehydes and esters. These two types of chemicals were shown to be synthesized *de novo* by males fed only with sugar water (Bergman, 1997).

Several authors have described the chemical composition of *Bombus terrestris* since the 1960s. Calam (1969) described the main compound of *B. terrestris*' CLG secretion as being a terpene with an alcohol function: the (*E*)-2,3-dihydrofarnesol (fig. 15). He called it "terrestrol", but this terminology is no longer used. The first analyses were made using entire head extracts, pooling 10 specimens to compensate for the low GC/MS sensitivity. The absolute configuration of (*E*)-2,3-dihydrofarnesol contained in the CLG secretions was determined (Stallberg-Stenhagen, 1970; Luxova et al., 2004), and it was shown that only one of the two isomers is synthesized by *B. terrestris*.

A few years later, with the improvement of gas chromatography coupled with mass spectrometry (GC/MS), and the discovery that CLG was the synthesis site of sexual

pheromones, the secretions of *B. terrestris* and other species were re-analysed (Svensson, 1977; Svensson, 1980). Despite these new contributions, those studies still focused on the main compounds and did not allow an exhaustive description of the CLG secretions due to technological limitations.

As GC/MS have become more and more accurate, CLG secretions have been re-analysed several times during the last 30 years (Bellés et al., 1987; Bergman, 1997; Bergström et al., 1981). Since 1996, new technologies have considerably increased the sensitivity of instruments and the analysis of individual CLGs extracted in hexane was applied frequently (Terzo et al., 2003). A recent development simplified the protocol allowing the analysis of a single specimen's head instead of dissected CLGs (See appendix 2, De Meulemeester et al., in press).

Recently, the compounds eliciting electroantennogram (EAG) responses of virgin queens were described for *B. t. terrestris* and *B. lucorum* (see appendix 3, Žaček et al., 2009). Six EAG-active compounds have been detected: ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecan-1-ol, octadeca-9,12,15-trienol, and geranylcitronellol. Moreover, Valterova et al. (2007) showed that only the (*E*)-2,3-dihydrofarnesol enantiomer elicited an EAG response, but not the (*Z*)-2,3-dihydrofarnesol.

This particular attention to CLG secretions has a taxonomical logic. The CLG secretions of bumblebees were shown to be very species-specific (Calam, 1969; Svensson, 1977; Svensson, 1980; Bergström et al., 1981; Bellés et al., 1987). Their taxonomical interest was particularly clear in highly complex taxonomic groups (polytypic taxa or cryptic sister species) (e.g. Svensson, 1980; Rasmont et al, 2005). Moreover, in the sl-CSD context explained before, behaviour adaptations to inhibit mating between kins include active dispersal from natal patches and mating preferences for non-relatives. The sexual

pheromonal cue may play a role in this context (Symonds & Elgar, 2008).

1.1.5. Genetics studies on *Bombus terrestris*

With the development of genetic tools, new studies have been conducted on *B. terrestris*. In their general overview, Cameron et al.(2007) reported the first nearly complete species phylogeny of bumblebees, including most of the 250 known species. In this analysis, *B. terrestris* is included in the monophyletic subgenerus *Bombus* Latreille *sensu stricto* (= *Terrestribombus* Vogt).

More specific studies have been conducted to clarify the taxonomical status of the insular and continental populations of *B. terrestris*, considered as subspecies. Estoup et al. (1996) studied the genetic differentiation of continental and insular populations of *B. terrestris*. Using ten microsatellite loci and a partial sequence of the COII mitochondrial gene, they discovered high levels of polymorphism in most populations. *B. t. canariensis* showed a significantly lower average calculated heterozygosity and presented allelic diversity as compared both to continental and island populations of *B. terrestris*. No significant differentiation was found among the continental populations. In contrast, island populations were all significantly -and most of them strongly- differentiated from continental populations. *B. terrestris* mitochondrial DNA was characterized by low nucleotide diversity: the only haplotype found in the Teneriffe population differed by a single nucleotide substitution from the most common continental haplotype of *B. terrestris*. Estoup *et al.* (1996) cast a doubt on the taxonomic status of *B. t. canariensis.* They explain that the great genetic distance between the Teneriffe and all other *B. terrestris* populations – estimated from microsatellite data –likely resulted from a severe bottleneck effect in the Canary Island population.

Later, Widmer et al. (1998) emphasized the genetic structure and colonization

history of *B. terrestris* in Madeira and the Canary Islands using, again, microsatellites and mitochondrial DNA (cytochrome b). They showed that the genetic differentiations among the islands, and between the islands and the continent, were extensive. They found that the distinctness of the Canary Islands population was strongly supported whereas the Madeira sample was genetically more similar to the continental populations of *B. terrestris* from Europe. Their results suggested that ancestral haplotypes occurred on the Canary Islands, whereas derivative haplotypes were found on the European continent. Moreover, they showed that bumblebees from the Canary Islands and Madeira do not share a common colonization history.

1.1.6. Commercial use of *Bombus terrestris*

Particular interest has been placed on *B. terrestris* over the years, because of its high value in crop pollination. Colonies of *B. terrestris* are used in glasshouses for the pollination of many fruits and vegetables: tomatoes, squashes, melons, strawberries, and others (Velthuis & Vandoorn, 2006) They are much more effective than honeybees thanks to their longer tongue and their quicker foraging speed (Chittka et al., 2004). Moreover, they are able to forage under rough conditions such as windy weather and low temperatures.

B. t. dalmatinus is nowadays the most commercialised subspecies in Western Europe because of its easy breeding and large colony size (Velthuis & van Doorn, 2006) and is nowadays exported all across Europe, except in Norway (for legal reasons) (De Jonghe, com. pers.). *B. t. audax* was implanted in New Zealand at the end of the 19th century (MacFarlane, 1995), while during the 1980's *B. t. sassaricus* and *B. t. dalmatinus* were domesticated and commercially spread. As is the case for many other reared animals, commercial uses are not without risk.

There is a possibility that commercial bumblebees may escape from glasshouses. If this happens, settlement or hybridization with local populations might occur. Recently, it has been shown that *B. terrestris* has invaded the peninsulas of Eastern Hokkaido, northern Japan (Inoue et al., 2009). *Bombus terrestris* could be more efficient in foraging native flowers than are the natural populations of bees. This results in a successful naturalization of *B. terrestris* that could negatively affect native bumblebees and wild bees.

Hingston (2002, 2005a, 2005b, 2006a, 2006b, 2007) showed that imported *B. terrestris* had negative effects on the natural plant populations in Tasmania. He showed that alien plants were preferred by *B. terrestris*, causing them to proliferate faster and eventually becoming invasive

Before the extensive use of *dalmatinus*, s*assaricus* had been imported and used for years. It has been established that this subspecies (originally from Sardinia) settled down in South-eastern France for a few years, but then was no longer found (Ings et al., 2010). It may have disappeared because of competition with native bumblebees, or because its gene pool was diluted by hybridization with native subspecies.

1.1.7. Taxonomic status of *Bombus terrestris* taxa

As explained earlier, the taxonomic status of *B. terrestris'* subspecies is still considered questionable by many authors. Most of the time, the geographic isolation of several taxa constituting *B. terrestris* is corresponding to a sexual isolation as well. The continental taxa (i.e. *terrestris, lusitanicus, dalmatinus, calabricus*) have overlapping geographic distributions, but the taxa that are geographically isolated in islands are generally separated by a greater distance than the dispersion distance known for virgin males and females. This means that *canariensis, africanus, xanthopus, sassaricus,* or *audax* have few chances to meet a potential mate belonging to another taxon in natural

conditions. Geographic isolation explains the small number of hybrids described in natural conditions.

The mating preferences of geographically isolated populations of *B. terrestris* that have unnaturally been brought together by the commercial trade of bumblebees have been tested. Crossings between *dalmatinus*, *sassaricus, terrestris* and *xanthopus* have been performed, resulting in a mating preference of *dalmatinus* for males from the same taxa (71%) (Ings et al. 2010). Nevertheless, it is important to highlight that hybrids of *xanthopus* x *terrestris* were obtained and produced viable F1 colonies with fertile offspring (de Jonghe, 1986a,b; Ings et al., 2010).

Since the sexual pheromones were shown to be species-specific (Calam, 1969), they might be a useful tool for a better understanding of the taxonomic situation inside the *B. terrestris* complex. The species concept applied to bumblebees could be improved using CLG secretions.

This is in line with Paterson's Species Recognition System (1985, 1993). The idea of this recent species concept is that the prezygotic mechanisms presented and shared by potential mates to recognise each other define the species. This can be stated in another way: "*members of a species share a common specific-mate recognition system*" (= SMRS) (Paterson, 1978). In 1978, Paterson explains that in sexually reproducing organisms, potential mates follow positive acts of recognition. The rise of new species occurs then, when all the members of a population of a species acquire a new specific-mate recognition system (SMRS).

This definition is opposed to the previous concept that defined a species in terms of "reproductive isolation", where post-zygotic incompatibility has the same importance as pre-zygotic recognition, as in Dobzhansky's *Genetics of the Evolutionary Process* (1937) or

Mayr's *Animal Species and Evolution* (1963).

The most important point of Paterson's Species Recognition Concept is that it finally solves the problem of the anthropocentric view of "what a species is". This later definition is thus no longer indirectly linked to the observer but directly linked to the mutual perception of the conspecific individuals themselves.

Paterson's Species Recognition System can only be applied to organisms for which the SMRS is already known. This is the case in bumblebees. They spread CLG secretions, which contain the sexual pheromones, along a patrolling circuit. This odorous cues will attract conspecific females looking for a potential mate. The first step in the Species Recognition System in bumblebees that exhibit a patrolling behaviour is then understanble by studying SMRS (CLG secretions), even if the next steps are still unknown (i. e. how does the female make the last step when she chooses the male she will copulate with?).

Applying Paterson's Species Recognition Concept will help to find the answer to the questions the "Reproductive Isolation" concept did not solve in particular organisms like bumblebees which present complexities like cryptic or sister species.

For all those reasons, the Paterson's Species Recognition System will be the keystone of the present work.

1.1.8. Unsolved problems

As developed in the previous paragraphs, there are still many unanswered questions about *Bombus terrestris*.

First of all, it would be interesting to determine if there are changes in the composition of the CLG secretions during the life of the male, our hypothesis being that most of the synthesis occurs when the male is out of the maternal nest, thus preventing any brother-sister copulations.

One of the major questions concerning the males' CLG secretions concerns the geographic variability. Since the subspecies differ by colour pattern, phenology, and many other aspects, does the chemical composition of CLG secretions also change with the geographic origin of males?

Logically, if there is a geographic variability of CLG secretions, we can wonder if the females perceive this variability, and of course, if they have any preference for consubspecific or heterosubspecific odour cues.

Finally, if there are variations in CLG secretions corresponding to the geographic origin of males, do they correspond to reproductive isolation, and/or to genetic drift, and to the definition of good species inside the *Bombus terrestris* complex?

Is the potential CLG secretion geographic variation meaning that a speciation is in progress or is it just a fortuitous differentiation?

1.2. Outline of the present study and implementation

The goal of this study is to evaluate the chemical, behavioural and genetic variations inside the *B. terrestris complex*. Based on original results, we propose new hypotheses about the species status of each previously described taxon. This is to answer the question: "Is *B. terrestris* a polytypic species made up of several subspecies or is it a complex of already differentiated species?". The Rasmont *et al.* (2008 – Appendix 1) review showed that some subspecies of *B. terrestris* are highly differentiated. Some of them have been fruitfully domesticated (*xanthopus, sassaricus, dalmatinus* and *canariensis*) while the others remain unused, or even unusable in crop pollination.

Hybridization between some of the subspecies seems to occur in the wild but its occurences appear to be rare events (Rasmont & Adamski, 1995). As the CLG secretions are known to be extremely species-specific, we will verify whether they are differentiated according to the geographic isolation of taxa (presently known as subspecies), and test whether the specimens are able to perceive those differences. These results will be studied in light of Paterson's Species Recognition Concept. We will also examine whether the phylogenetic differentiation follows the CLG differentiation.

We will first focus on age-dependent variation in CLG. This first analysis is of utmost importance, because it will influence the choice of the age of the males used in the next steps of the work. If there are any changes in function of the age of males, we will have to find out at which stage in their life they are the most attractive to females: subsequently we will study the CLG secretions of males in this age class only. To do so, we will test the behavioural responses of virgin females for different age classes of males (Chapter 2).

In order to highlight any variability in the composition of the CLG secretions with the geographic origin of the males, we will analyse the chemical composition of several subspecies and compare them. The CLG secretions of sympatric and allopatric subspecies will be analyzed using gas chromatography coupled with mass spectrometry. A multivariate analyses will be applied to the data matrix obtained (compounds x samples). This will be done to determine whether qualitative and/or quantitative differences occur in the secretions of some subspecies (Chapter 3) and to find out if sympatric heterosubspecific populations display a similar or different signal.

The preference of virgin queens for sexual pheromones from males of different geographic origins will be tested using a simple behavioural test. We will then demonstrate if the virgin queens prefer males belonging to their own subspecies or not. A better understanding of the existence of a prezygotic reproductive isolation by the means of the SMRS, will be possible in light of these results (Chapter 4).

A genetic review of the *B. terrestris* complex will be carried out, using mitochondrial DNA (COI and cytochrome b). This analysis is of utmost importance to measure the genetic distance between the subspecies (Chapter 5).

All the results obtained will be discussed in the last chapter (Chapter 6), taking into account previous and potential future works. Comparisons will be made between the pheromonal and genetic variability that exists between the nine *Bombus terrestris* subspecies described.

Chapter 2

SUBMITTED PUBLICATION

Coppée A., Mathy T., Cammerts M.-C., Verheggen F. J., Terzo, M., Iserbyt I ., Valterova I., Rasmont P.

Age-dependent attractivity of males' sexual pheromones in *Bombus terrestris* (L.) (Hymenoptera, Apidae). Submitted in *Chemoecology*. 15pp.

The first goal of the work was to determine if any variations occur with the age of males, in order to use appropriate age classes in further investigations. A commercially reared colony was used to control the age of males, and the chemical composition, and reactions of virgin queens were measured for various age classes.

AGE-DEPENDENT ATTRACTIVITY OF MALES'SEXUAL PHEROMONES IN BOMBUS TERRESTRIS (L.) [HYMENOPTERA, APIDAE]

Audrey Coppée [1*], Tifany Mathy, Marie-Claire Cammaerts, François J. Verheggen [2], Michael Terzo, Stéphanie Iserbyt [1], Irena Valterova [3] & Pierre Rasmont [1].**

*** audrey.coppee@umons.ac.be, +3265373436**

****valterova@uochb.cas.cz, +420220183298**

Publication submitted in Chemoecology

[1] Laboratoire de Zoologie, Université de Mons (UMons), Place du Parc 20, B-7000 Mons, Belgium

[2]Entomologie fonctionnelle et évolutive, Gembloux Agro-Bio Tech, Liège University, 2 Passage des Déportés , B-5030 Gembloux, Belgium

[3]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nam. 2, 16610 Prague, Czech Republic

This article is dedicated to the memory of Dr. Jan Tengö and his significant contribution to the field of chemical ecology of bees

Abstract -Males of Bombus terrestris (L.) adopt a patrolling behavior during their nuptial parade using cephalic labial gland (CLG) secretions containing sexual pheromones to attract conspecific virgin queens. The changes in chemical composition of their CLG secretions with age are quite well known. In this study, we investigate the evolution of CLG secretions with age in greater detail and compare behavioural reactions of conspecific virgin queens to the secretions.

We show that compounds of CLG secretions follow two profiles. Most of the compounds increase from the first day after emergence until the bees are 15 days old and then decrease. Others are less abundant in 1 to 15 day old males and then increase (e.g. tricosane, tricosene, henicosane, tetradecanoic acid, pentacosene, pentacosane, heptacosene, heptacosane, nonacosene, and gernaylcitronellyl tetradecanoate).

Differences in secretion composition lead to preferences of virgin queens for males according to the male's age. Virgin queens prefer the pheromonal gland secretions of bees of the following ages in decreasing order; 1 day = 3 days =30 days < 7 days < 15 days < 10 days. The virgin queens are much more attracted by secretions containing high amounts of 2,3-dihydrofarnesol, 2,3-dihydrofarnesal, ethyl dodecanoate, and hexadecanol. On the contrary, Geranylcitronellol is highly abundant in 30-day-old males.

Key Words- Bombus terrestris, Sexual Pheromones, Age-Dependent Variations, Behavioural Tests.

INTRODUCTION

It is well known that males of Bombus terrestris (L.) use cephalic labial gland (CLG) secretions containing sexual pheromones to attract conspecific virgin queens in order to mate (Calam 1969, Kullenberg et al. 1973). Male premating behavior consists of depositing small amounts of pheromones along a circuit and then patrolling it to find out if a virgin queen has been attracted by their scent marks (Svensson 1979).

The CLG secretions of bumblebees are used as a tool for taxonomical discrimination (Bellés et al. 1987; Coppee et al. 2008; Coppée et al. 2008; Rasmont et al. 2008). The chemical composition of B. terrestris' CLG secretions has been described by many authors since the improvement of the GC/MS technique (Bergman 1997; Bergström 1981; Calam, 1969; Coppée et al. 2008). Individual variations were highlighted in different species (Terzo et al. 2005) and are now well understood since histological (Ågren et al. 1979), physiological (Šobotník et al. 2008) and chemical (Žáček et al. 2009) studies were conducted. These authors showed that in B. terrestris ssp. terrestris, the CLG secretions evolve with the age of males. Just after males' emergence, the activity of secretory cells is high, but the level of synthesized secretions, as well as the electroantennographic-active (EAG-active) (on virgin queens) compounds concentrations are low (Žaček et al. 2009). At five days old, the secretory activity stops and the cells degenerate, but the level of secretions only decreases after 7 to 10 days old (Šobotník et al. 2008).

In spite of the numerous studies on the topic, a thorough list of compounds and an examination of the changes of all the coumpounds with age remains to be published. Moreover, no attention has yet been paid to behavioural reactions of virgin queens to chemical changes of the CLG secretions with age. This might lead to a better understanding of how bumblebees avoid inbreeding which might be hazardous for the population survival since the sex determination in bumblebees is under control of one single locus (Van Wilgenburg et al. 2006; Whitehorn et al. 2009). Copulations between brothers and sisters lead to a progeny made of 50% diploid males that are less adapted than the haploid ones, are smaller (Duchateau 1994, 1995) and have a weaker immune system (Gerloff et al. 2003).

In this paper, we describe the chemical composition of males'CLG secretions of B. terrestris ssp. dalmatinus between 0 to 40 days old and by means of a simple bioassay we measure preferences of virgin queens for secretions of males of various ages.

METHODS AND MATERIALS

Biological Material. Males of B. t. dalmatinus were provided by Biobest bvba (Westerlo, Belgium). The colony was raised in a dark room with the following breeding conditions: temperature (T) in the range: 25-32°C, relative humidity (RH) in the range: 25-55%. It was fed ad libitum with syrup (1kg water for 1kg sugar) and one stock of willow pollen (Salix sp.). Every day at a set time, newly emerged males were separated from the mother colony and labelled. They were gathered in small flight cages (13.5 x 12 x 8.5 cm) according to the age they were killed. These males had been maintained in the same breeding conditions as the mother colony for the first 3 days, and then placed in a room with the following conditions: 10,000 lux light for 12 hours and min. 20°C temp.

Males were killed by freezing at 0, 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30, and 40 days old. These different ages were chosen to see the evolution of secretions during the entire life of males while providing higher temporal resultion during the first days. Twenty males of each age were selected. Both cephalic parts of the labial glands of each specimen were dissected after dissection of eyes and placed in a glass vial for extraction in 200 µl of hexane. The vials were left for 24 hours at room temperature and then stored at -30°C until chemical analysis (Terzo et al. 2005).

Forty virgin queens of B. t. dalmatinus were provided by Biobest bvba (Westerlo, Belgium). These virgin queens were maintained, three by three, in wooden boxes. They were fed with pollen and sugar water ad libitum. The food was replaced every other day. The temperature ranged between 20-30°C and the relative humidity 45-55 %.

Chemical Analyses. Chemical analyses were performed using GC/MS. The mass spectrometer used was an ion trap instrument Finnigan GCQ. The capillary column specifications were as follows: a DB-5ms column (5% phenylmethylploysiloxane stationary phase of 0.25 µm thickness; 30 m column length; 0.25 mm inner diameter). The temperature of the injector was 220°C. The initial temperature of the column was held for 2 minutes at 70°C, then programmed to 320°C at 10°C/min and held for 3 min at 320°C. Helium was used as carrier gas at a constant velocity of 50 cm/sec. Mass spectra were obtained in electron ionisation mode, full scan (m/z 30-600). The extracted samples (1 µl) were injected in the GC in a random order.

Compounds were identified using their mass spectra by comparing to spectra in the National Institute of Standards Technology library (NIST, USA) using Nist MS Search 2.0.

For each chromatogram, the relative area (%) of peaks was integrated using Xcalibur software (v.1.3) according to the following parameters: baseline window = 100; area factor noise = 5; minimum peak width = 5; multiplet resolution = 10; area tail extension = 5; area scan window = 0.

Quantification of 2,3-dihydrofarnesol. This compound was chosen because of its high relative abundance and its EAG activity (Šobotník et al. 2008). Two males of each age class were used (in case one measurement would fail). Glandular extracts were quantified by gas chromatography coupled with flame ionisation detection (GC-FID) using a Thermo gas chromatograph model Focus. As internal standard, nonyl acetate (400 ng) was added to each sample. Aliquots of 1 µl were injected with a splitless injector held at 240°C. The column (15 m x 0.25 mm i.d.) was maintained at 40°C for 0.5 minute before being heated to 180°C at a constant rate of 20°C/min. The final temperature was maintained for 5 minutes. Quantification of compounds was performed by comparing their GC-peak areas with those of the internal standard using Chrom-Card software (v. 2.3.3.) (Interscience, Louvain-La-Neuve, Belgium).

Behavioural Experiments. Secretions of 1-, 3-, 7-, 10-, 15-, and 30-day-old males were tested. Those classes were representative of the main changes in CLG secretions (Tab.1). Males aged 0 and 40 days old were excluded from the behavioural tests; freshly emerged males do not scent-mark inside the colony and males do not survive until 40 days in natural conditions. The behavioural tests were performed in an olfactometer made of a glass tray (70 x 70 x 8 cm) covered with a polycarbonate plate with a central hole allowing the introduction of a Petri dish (9.2 cm diameter) containing a virgin queen. The arena was divided into 4 quarters (35 x 35 cm). A circular wire mesh (8 cm height) was set in the arena to avoid direct contact between the female and the scent marks. Males' secretions were put down in the corners of the arena. The different age secretions were tested 2 by 2. The four corners contained respectively and randomly: (1) a blank filter paper, (2) a filter paper with 2.5 µl of hexane, (3) a filter paper with 2.5 µl of secretions from a bee aged "X" days, (4) a filter paper with 2.5 µl of secretions from a bee aged "Y" days (fig. 2.). The different age pairs to be tested (33 times each) were randomly chosen. Forty-three control tests were performed following the same protocol. A digital camera (Philips SPC 900NC PC camera) connected to a PC was set and centred above the experimental arena, allowing the recording of the virgin female moves. The experimentation room was kept at 20-30°C temp., and 45-55% rH. Red light was used since bumblebees are unable to perceive this wavelength.

The following protocol was used to perform each test: The corner positions of blank, solvent, and males' extracts in the olfactometer were chosen randomly. Light was then switched to red. A virgin female was placed in a Petri dish (9.2 cm diameter) and set free 1 to 2 minutes later after she has calmed down. As soon as the

virgin female was free, the quarter in which she was located was recorded every 5 seconds, during 7 minutes (= 84 successive positions of the queen being tested). Each possible pair of ages was tested 33 times, each time with a different queen. After each test, the entire olfactometer was cleaned using acetone.

Virgin queens' reactions were quantified by the total number of approaches to each odor source (i.e. the presence of the female in each quarter of the arena). These positions were statistically compared with a χ^2 test, the null hypothesis being an identical number of approaches in the 4 zones. The approaches of solvent and an extract were also compared with a χ^2 test. The same test was applied to compare the two zones containing males' secretions.

Finally, we compared the attractivity of secretions (ranked from 1 to 4; 1 being the less attractive and 4 the most) with relative abundance (log+10) of EAG-active compounds determined in B. t. terrestris in Žáček et al.(2009) (fig.3).

RESULTS

Chemical Composition of Males' CLG Secretions.The chemical study is based on 112 interpretable chromatograms distributed among the 13 age classes (Table 1). Thirty-six compounds have been identified among the secretions: alkanes, alkenes, aliphatic alcohols, aldehydes, esters, and acyclic sesqui- and diterpenic alcohols and aldehydes. The 2,3-dihydrotransfarnesol (DHF) is the main component (in relative abundance) in males aged of 1 to 15 days old. In younger males (0 days old), it is henicosane that dominates while in older males (20-40 days old), tricosane is the most abundant component. There are two profiles of the relative concentration of the compounds with age. On the one hand, most of the compounds follow the same variation as DHF (i.e. high abundance in 1-15-day-old males, then decreasing abundance). On the other hand, tetradecanoic acid, tricosene, pentacosene, pentacosane, heptacosene, heptacosane, nonacosene, and geranylcitronellol tetradecanoate have the same profile as tricosane (i.e. low abundance in 1-15-day-old males, then increasing).

Quantification of the 2,3-dihydrotransfarnesol. The mean absolute quantity of DHF varies from 0.2 µg to 330 µg (fig. 1). Its variation follows the observed relative abundance (Table 1). It increases from 1 to 7 days of age and decreases from 10 to 20 days of age. Moreover, it is nearly totally absent in males of 0 and 25-40 days old.

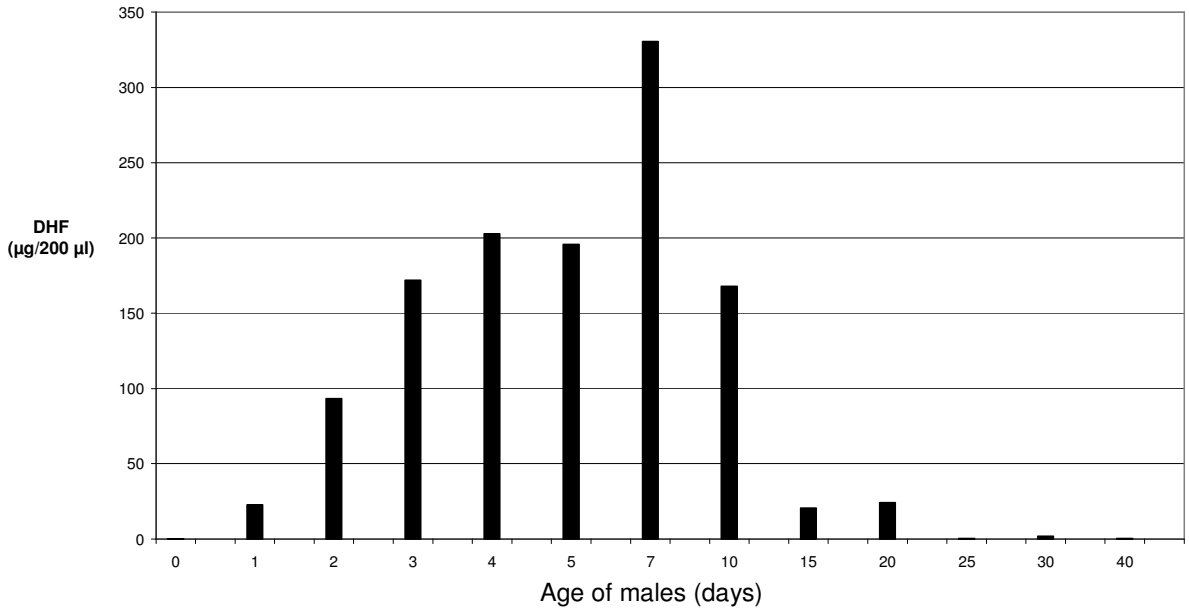


Figure 1. Quantification of the DHF in the different age classes (mean of two males), using nonyl acetate as internal standard.

Behavioural Experiments. Thirty-three control tests were performed previous to olfactory tests in order to check that no external stimuli could disturb the virgin queens. The χ^2 result of the control test has a non-significant p-value.

Results of the 2-by-2 tests are presented in figures 2a to 2f., The number of recorded approaches of virgin queens in each quarter is shown here as percentages. The virgin queen always shows a higly significant preference for one of the 4 olfactory stimuli presented (figs. 2a - 2f, "4 zones χ^2 "). The males' secretions, regardless of the males' age, are always prefered to pure solvent. An exception occurs in the 10- vs 15- day-old test (fig. 2f) in which the preference of the secretions of a 15-day-old male was not statistically different from the solvent.

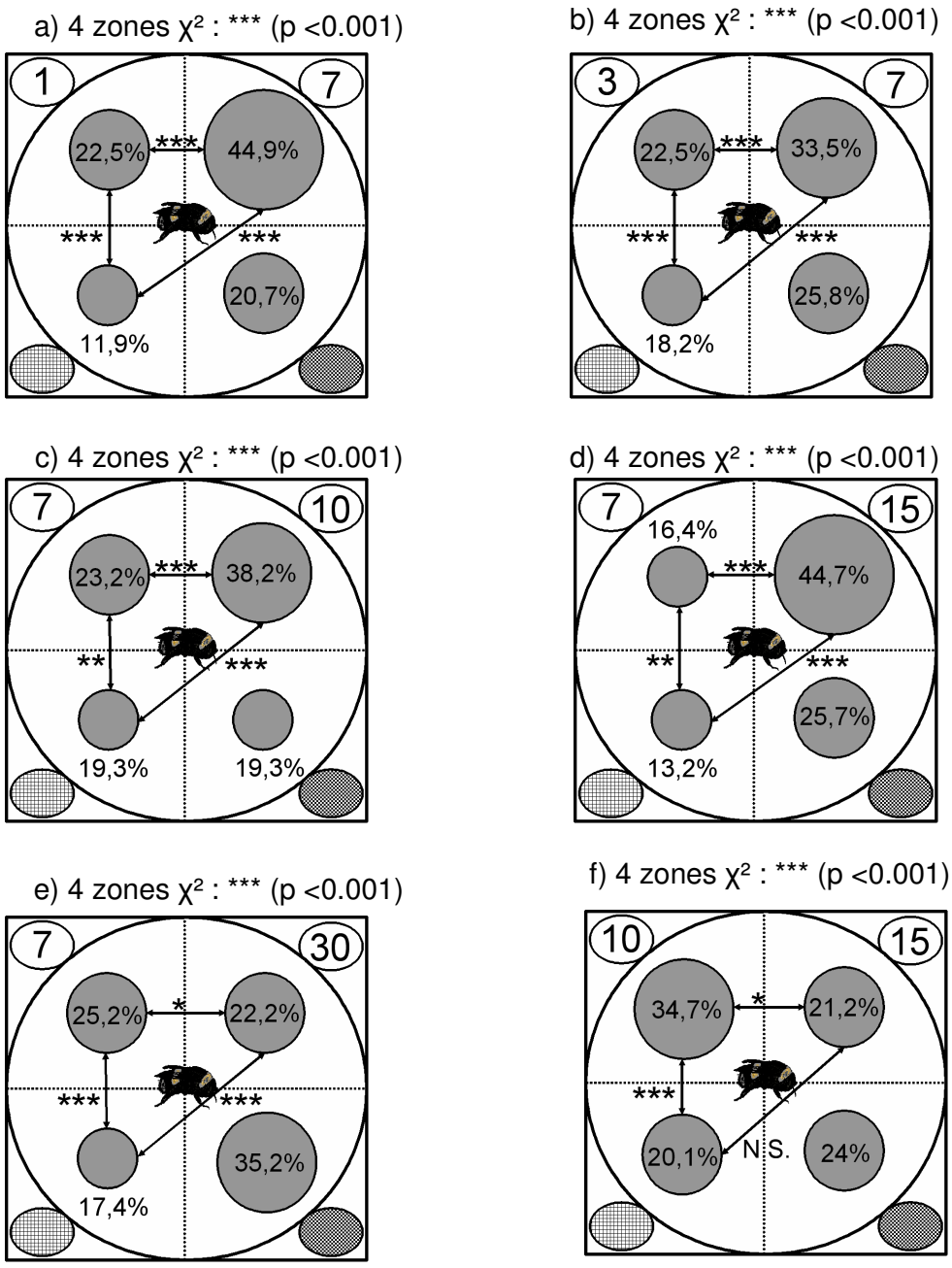


Figure 2. Approach (in %) of virgin queens in each quarter of the olfactometer, containing blank (the grey spot); pure hexane (the hatched spot) and 2 different ages secretions as follows: a) 1 Vs 7 days old; b) 3 Vs 7 days old; c) 7 Vs 10 days old; d) 7 Vs 15 days old; e) 7 Vs 30 days old; f) 10 Vs 15 days old. The χ^2 test result calculated on the approach in the 4 zones is given above the corresponding figure. The χ^2 test results of the secretions Vs secretions and secretions Vs solvent is given on the arrow linking the 2 corresponding zones. χ^2 results of secretions Vs blank are not shown, since the 4 zones χ^2 results were significant.

Table 1.- part 1

Compounds	0d (n=6)			1d (n=10)			2d (n=8)			3d (n=10)			4d (n=9)		
	Q1	M	Q3	Q1	M	Q3	Q1	M	Q3	Q1	M	Q3	Q1	M	Q3
Hexadecene	0,02	0,31	2,10	0,00	0,00	0,00	/	/	/	0,00	0,00	0,00	/	/	/
Ethyl dodecanoate	/	/	/	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,05	0,14	0,17	0,25	0,81
Tetradecanal	0,00	0,00	0,00	0,00	0,08	0,00	0,29	0,49	0,00	0,41	0,57	0,70	0,57	0,66	1,20
2,3-Dihydrofarnesal	0,07	0,13	1,04	1,47	3,51	1,47	1,97	3,15	0,11	1,82	2,01	2,48	1,81	1,88	2,06
Dodecyl acetate	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,05	0,54	0,06	0,08	0,12	0,08	0,10	0,13
2,3-Dihydrofarnesol	1,40	5,01	23,39	**22,11**	**72,45**	**22,11**	**33,60**	**74,85**	**3,43**	**35,81**	**71,27**	**84,94**	**37,63**	**81,37**	**86,75**
2,3-Dihydrofarnesyl acetate	0,00	/	0,00	0,00	0,00	0,00	0,00	0,00	0,07	0,02	0,08	0,10	0,01	0,12	0,24
Tetradecanoic	0,01	0,19	1,18	0,00	0,00	0,00	0,00	0,00	84,18	0,00	0,07	0,29	0,05	0,20	0,36
Hexadecenal	0,00	0,00	0,00	0,04	0,29	0,04	0,38	0,40	0,08	0,24	0,30	0,35	0,23	0,28	0,51
Hexadecanal	/	/	/	0,13	0,19	0,13	0,18	0,22	0,11	0,10	0,19	0,35	0,08	0,13	0,19
Hexadecan-1-ol	0,62	1,15	1,71	0,67	1,31	0,67	1,75	2,35	0,54	1,78	2,35	3,50	1,79	2,85	3,25
Octadecadienal	0,00	0,00	0,00	0,00	0,00	0,00	/	/	/	0,00	0,00	0,00	0,00	0,00	0,00
Octadecatrienal	0,00	0,00	0,05	0,00	0,00	0,00	/	/	/	0,00	0,00	0,04	0,00	0,06	0,07
Nonadecadienal	0,00	0,04	0,24	0,17	0,30	0,17	0,13	0,21	0,00	0,07	0,24	0,64	0,03	0,08	0,15
Hexadecyl acetate	0,00	0,00	0,11	0,23	0,33	0,23	0,20	0,51	2,88	0,12	0,39	0,83	0,09	0,12	0,35
Octadecadienol	/	/	/	0,42	0,66	0,42	0,51	1,50	0,00	1,39	1,99	3,41	0,74	2,53	3,56
Geranylcitronellal	/	/	/	0,00	0,00	0,00	0,00	0,81	0,00	0,21	1,35	2,58	0,56	1,10	2,95
Henicosane	**23,42**	**27,61**	**38,46**	5,14	8,04	5,14	2,78	4,27	0,00	2,22	4,24	5,38	0,99	1,97	3,90
Geranylcitronellol	/	/	/	0,64	1,21	0,64	1,35	1,98	0,00	1,05	1,87	7,61	1,16	1,80	9,09
Octadecadienyl acetat	0,54	0,95	1,61	0,00	0,00	0,00	0,00	0,00	0,40	0,00	0,00	0,12	0,00	0,07	0,35
Tricos-9-ene	4,40	6,34	8,52	0,48	0,61	0,48	0,29	0,66	0,65	0,36	0,82	1,77	0,10	0,19	2,45
Tricosane	8,68	16,22	23,29	0,98	1,59	0,98	0,90	1,57	0,03	0,71	1,34	13,22	0,42	0,55	12,11
Tetracosene	0,00	0,25	0,57	0,00	0,06	0,00	0,00	0,00	2,33	0,00	0,00	0,58	0,00	0,00	0,76
Pentacos-9-ene	1,25	4,53	9,00	0,14	0,19	0,14	0,04	0,23	1,22	0,05	0,28	1,37	0,00	0,05	1,56
Pentacosane	0,55	2,48	4,42	0,02	0,10	0,02	0,00	0,09	7,64	0,00	0,00	2,73	0,00	0,00	2,16
Hexacos-9-ene	0,00	0,00	0,20	0,00	0,00	0,00	0,00	0,00	5,84	0,00	0,00	0,34	0,00	0,00	0,32
Docos-15-enyl acetate	0,00	0,57	1,80	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,26	0,00	0,00	0,34
Heptacosene	0,84	3,73	7,22	0,33	0,70	0,33	0,18	0,30	2,54	0,13	0,47	3,48	0,09	0,12	3,51
Heptacosane	0,00	0,57	1,30	0,00	0,03	0,00	0,00	0,00	11,34	0,00	0,00	0,27	0,00	0,00	0,23
2,3-Dihydrofarnesyl dodecanoate	0,02	0,50	1,17	0,17	0,47	0,17	0,16	0,59	1,24	0,25	0,30	5,69	0,22	0,42	5,06
Nonacos-9)ene	1,96	5,55	9,83	0,16	0,39	0,16	0,08	0,30	2,34	0,02	0,35	0,56	0,00	0,06	0,54
2,3-Dihydrofarnesyl tetradecadienoate + 2,3-Dihydrofarnesyl tetradecenoate	0,41	0,54	0,70	0,18	0,25	0,18	0,12	0,36	1,69	0,14	0,26	2,04	0,12	0,30	1,63
2,3-dihydrofarnesyl hexadecanoate + Hexadecyl tetradecanoate + Tetradecyl hexadecanoate	0,30	0,45	0,55	0,00	0,06	0,00	0,00	0,00	0,01	0,00	0,03	0,08	0,00	0,00	0,11
Geranylcitronellyl dodecanoate	0,01	0,06	0,73	0,12	0,30	0,12	0,04	0,25	0,34	0,00	0,15	0,30	0,00	0,06	0,30
2,3-dihydrofarnesyl ooctadecenoate	0,00	0,00	0,05	0,00	0,00	0,00	0,00	0,00	0,80	0,00	0,00	0,05	0,00	0,00	0,06
Geranylcitronellyl tetradecanoate	1,36	1,71	1,98	0,19	0,59	0,19	0,10	0,33	3,77	0,04	0,20	0,48	0,02	0,19	0,39

Table 1. – part 2

Compounds	5d (n=10)			7d (n=10)			10d (n=10)			15d (n=9)			20d (n=8)		
	Q1	M	Q3	Q1	M	Q3	Q1	M	Q3	Q1	M	Q3	Q1	M	Q3
Hexadecene	/	/	/	/	/	/	/	/	/	/	/	/	0,00	0,00	0,00
Ethyl dodecanoate	0,18	0,46	1,77	0,08	0,20	0,30	0,14	0,31	0,48	0,20	0,47	2,59	0,00	0,23	3,54
Tetradecanal	0,85	0,91	1,11	0,92	1,04	1,43	1,18	1,39	1,60	0,99	1,24	2,00	0,00	0,06	0,81
2,3-Dihydrofarnesal	1,62	1,90	2,53	1,40	1,94	2,44	1,36	1,61	1,75	2,13	2,86	4,71	0,44	1,70	4,84
Dodecyl acetate	0,09	0,11	0,16	0,09	0,13	0,14	0,16	0,17	0,18	0,16	0,27	0,30	0,00	0,10	0,27
2,3-Dihydrofarnesol	**42,56**	**75,91**	**87,88**	**39,97**	**80,73**	**84,45**	**34,58**	**61,96**	**79,83**	**11,42**	**24,47**	**68,70**	3,05	8,58	37,44
2,3-Dihydrofarnesyl acetate	0,07	0,08	0,13	0,04	0,06	0,08	0,07	0,08	0,10	0,17	0,24	0,41	0,00	0,18	0,31
Tetradecanoic	0,19	0,23	0,37	0,13	0,20	0,30	0,17	0,22	0,30	1,02	1,18	2,07	0,33	1,34	1,63
Hexadecenal	0,14	0,23	0,28	0,11	0,18	0,25	0,14	0,18	0,26	0,21	0,30	0,38	0,00	0,71	1,17
Hexadecanal	0,07	0,14	0,19	0,10	0,12	0,15	0,11	0,15	0,18	0,15	0,26	0,64	0,00	0,36	0,45
Hexadecan-1-ol	2,21	2,76	3,09	2,63	3,12	3,43	2,98	3,27	5,05	2,27	4,77	5,34	0,00	0,08	1,19
Octadecadienal	0,00	0,05	0,07	0,00	0,06	0,07	0,00	0,00	0,06	0,00	0,00	0,00	0,00	0,00	0,02
Octadecatrienal	0,00	0,06	0,08	0,05	0,08	0,10	0,06	0,12	0,21	0,00	0,14	0,22	0,00	0,00	0,05
Nonadecadienal	0,01	0,06	0,08	0,06	0,07	0,13	0,01	0,06	0,14	0,10	0,17	0,29	0,10	0,30	0,42
Hexadecyl acetate	0,09	0,16	0,24	0,08	0,12	0,20	0,07	0,09	0,21	0,30	0,37	0,60	0,23	0,43	0,66
Octadecadienol	0,95	1,74	2,50	1,09	2,04	4,53	1,08	1,87	2,93	1,14	1,81	1,91	0,00	0,00	0,16
Geranylcitronellal	0,70	1,73	2,43	0,89	1,79	3,02	0,98	1,30	1,60	0,00	0,97	1,35	0,00	0,00	0,22
Henicosane	0,68	1,79	2,39	0,81	1,14	2,46	1,24	1,96	3,29	2,13	4,00	4,86	3,94	6,51	16,32
Geranylcitronellol	1,09	2,15	9,04	1,87	2,56	10,54	2,54	6,41	11,85	3,05	4,72	7,81	0,00	0,00	1,24
Octadecadienyl acetat	0,00	0,31	1,00	0,08	0,50	1,12	0,00	0,00	0,00	/	/	/	0,00	0,80	1,07
Tricos-9-ene	0,03	0,47	1,23	0,11	0,15	1,24	0,15	0,50	1,55	0,23	0,71	2,06	0,47	3,16	4,53
Tricosane	0,29	1,19	11,47	0,78	0,99	10,10	1,10	2,82	13,66	1,27	2,70	13,87	**1,74**	**17,55**	**21,93**
Tetracosene	0,00	0,00	0,56	0,00	0,00	0,00	0,00	0,00	0,39	0,00	0,00	0,00	0,00	0,00	0,76
Pentacos-9-ene	0,00	0,14	1,00	0,00	0,03	1,16	0,05	0,21	1,12	0,07	0,13	1,52	0,15	1,23	2,28
Pentacosane	0,01	0,08	2,15	0,05	0,07	1,74	0,07	0,31	2,38	0,17	0,27	2,79	0,15	1,95	4,05
Hexacos-9-ene	0,00	0,00	0,23	0,00	0,00	0,30	0,01	0,04	0,26	0,00	0,00	0,28	0,00	0,00	0,49
Docos-15-enyl acetate	0,00	0,00	0,20	0,00	0,00	0,11	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,39
Heptacosene	0,08	0,35	2,32	0,10	0,14	2,86	0,17	0,91	2,56	0,42	0,79	3,21	0,37	5,48	7,72
Heptacosane	0,00	0,00	0,16	0,00	0,00	0,20	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,00	1,32
2,3-Dihydrofarnesyl dodecanoate	0,21	1,21	4,93	0,29	0,51	6,05	0,41	2,06	4,85	0,62	2,22	10,54	1,81	3,18	17,92
Nonacos-9)ene	0,00	0,09	0,32	0,00	0,03	0,34	0,02	0,07	0,30	0,07	0,09	0,48	0,07	1,04	2,95
2,3-Dihydrofarnesyl tetradecadienoate + 2,3-Dihydrofarnesyl tetradecenoate	0,15	0,44	1,21	0,14	0,26	0,96	0,20	0,65	1,62	0,46	1,37	4,95	0,77	1,16	5,65
2,3-dihydrofarnesyl hexadecanoate + Hexadecyl tetradecanoate + Tetradecyl hexadecanoate	0,00	0,00	0,06	0,00	0,00	0,05	0,01	0,01	0,04	0,05	0,09	0,17	0,12	0,43	0,81
Geranylcitronellyl dodecanoate	0,00	0,07	0,16	0,00	0,00	0,11	0,02	0,05	0,15	0,10	0,26	0,42	0,27	0,50	1,52
2,3-dihydrofarnesyl ooctadecenoate	0,00	0,00	0,04	0,00	0,03	0,07	0,00	0,03	0,05	0,00	0,07	0,22	0,00	0,10	0,74
Geranylcitronellyl tetradecanoate	0,01	0,13	0,19	0,00	0,04	0,13	0,00	0,01	0,09	0,08	0,25	0,40	0,00	0,61	1,54

Table 1. – part 3

Compounds	25d (n=7)			30d (n=8)			40d (n=7)		
	Q1	M	Q3	Q1	M	Q3	Q1	M	Q3
Hexadecene	/	/	/	/	/	/	0,00	0,00	0,00
Ethyl dodecanoate	0,50	2,46	7,97	0,10	3,25	10,27	0,14	0,32	3,70
Tetradecanal	0,00	0,00	0,07	0,00	0,00	0,12	0,00	0,00	0,00
2,3-Dihydrofarnesal	0,40	0,90	5,00	0,16	0,52	2,07	0,00	0,10	0,15
Dodecyl acetate	0,00	0,00	0,11	/	/	/	0,00	0,00	0,00
2,3-Dihydrofarnesol	0,00	0,00	0,66	0,27	0,53	1,21	0,26	0,65	2,90
2,3-Dihydrofarnesyl acetate	0,00	0,00	1,04	0,00	0,80	1,73	0,00	0,00	0,27
Tetradecanoic	0,63	2,02	7,56	0,06	6,47	13,72	0,63	3,07	4,81
Hexadecenal	0,00	0,00	0,10	0,00	0,00	0,05	0,00	0,00	0,17
Hexadecanal	0,00	0,00	0,04	0,17	0,50	1,17	0,04	0,21	0,97
Hexadecan-1-ol	0,00	0,00	0,09	0,00	0,00	0,20	0,00	0,00	0,05
Octadecadienal	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Octadecatrienal	0,00	0,05	0,48	/	/	/	0,00	0,00	0,00
Nonadecadienal	0,00	0,00	0,10	0,00	0,17	0,59	0,00	0,09	0,14
Hexadecyl acetate	0,00	0,01	0,13	0,00	0,08	0,95	0,03	0,08	0,38
Octadecadienol	0,00	0,00	0,00	/	/	/	/	/	/
Geranylcitronellal	0,00	0,00	0,00	/	/	/	/	/	/
Henicosane	2,56	4,84	13,67	6,53	9,43	17,43	4,79	5,62	7,92
Geranylcitronellol	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Octadecadienyl acetat	0,00	0,05	0,40	0,00	0,74	1,86	0,56	0,73	0,87
Tricos-9-ene	0,42	2,07	3,59	2,15	2,40	3,52	1,53	2,82	3,10
Tricosane	**3,99**	**10,12**	**22,92**	**13,47**	**18,89**	**26,38**	**22,62**	**36,30**	**53,88**
Tetracosene	0,00	0,00	0,15	0,00	0,12	0,65	0,00	0,00	0,35
Pentacos-9-ene	0,06	0,44	2,82	1,18	1,83	2,31	0,69	2,35	3,04
Pentacosane	0,45	0,82	5,07	1,40	2,49	4,23	3,73	5,42	11,23
Hexacos-9-ene	0,00	0,08	0,57	0,00	0,18	0,46	0,00	0,00	0,39
Docos-15-enyl acetate	0,00	0,01	0,22	0,00	0,12	0,27	0,00	0,00	0,09
Heptacosene	1,31	3,80	11,79	2,98	6,37	9,36	3,29	7,13	7,51
Heptacosane	0,12	0,31	2,25	0,00	0,64	1,57	0,34	2,02	2,61
2,3-Dihydrofarnesyl dodecanoate	0,32	2,25	6,26	0,48	1,92	3,39	1,70	2,01	2,58
Nonacos-9)ene	0,00	0,26	5,10	0,45	1,33	3,57	0,41	0,96	1,64
2,3-Dihydrofarnesyl tetradecadienoate + 2,3-Dihydrofarnesyl tetradecenoate	0,04	0,20	1,49	0,77	1,35	2,83	1,14	1,49	1,67
2,3-dihydrofarnesyl hexadecanoate + Hexadecyl tetradecanoate + Tetradecyl hexadecanoate	0,01	0,24	0,65	0,00	0,10	0,46	0,00	0,00	0,26
Geranylcitronellyl dodecanoate	0,26	1,14	1,36	0,12	0,88	1,99	0,48	1,59	1,88
2,3-dihydrofarnesyl ooctadecenoate	0,00	0,00	0,26	0,00	0,06	0,30	0,00	0,00	0,22
Geranylcitronellyl tetradecanoate	0,61	2,28	3,29	1,12	3,65	6,87	0,00	0,00	0,66

Table 1.Median relative abundance (in %) of the 36 compounds identified, by age-class. Xd: age of the males; n: number of males analysed by age-class; "-": compound is absent in all specimens, M: median, Q1: first quartile; Q3: third quartile. The main compound of each age-class is given in bold on grey, and the EAG-active compounds are in bold. Compounds are listed in the retention order on a DB5-ms column.

The χ^2 results for 2-by-2 tests show that 7-day-old males were more attractive than 1 ($p < 0.001$), 3 ($p < 0.001$) and 30-day-old males ($p < 0.05$) but less attractive than 10- and 15-day-old males ($p < 0.001$). Ten-day-old males are more attractive than 15-day-old males ($p < 0.001$). To summarise and quantify the attractivity of the secretions of males of different ages, we can order them from the least to the most potent: 1 day = 3 days =30 days < 7 days < 15 days < 10 days.

Variation of the Response with Chemical Changes. Behavioural responses of the virgin queens to males' secretions follow the variation of chemicals as described before. The secretions become more and more attractive from 1 to 10 days and then the attractiveness decreases until 30 days of age. The virgin queens are much more attracted to males when a high amount of 2,3-dihydrofarnesol is present (fig. 3).

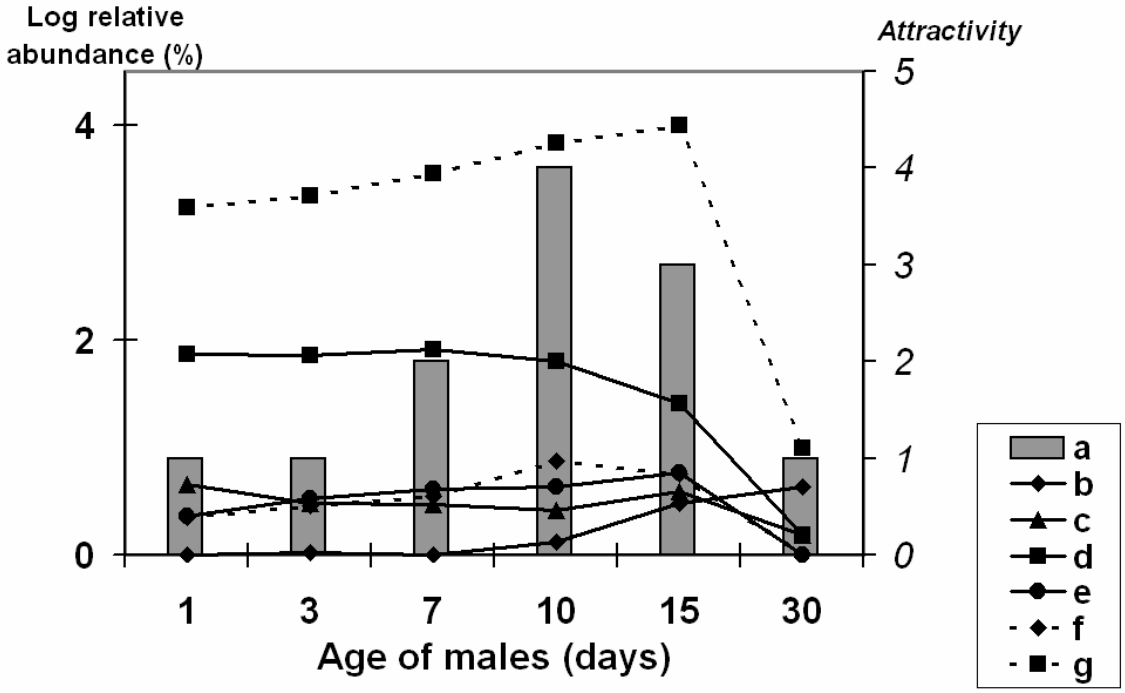


Figure 3. Graphic representation of the relative abundance of EAG-active compounds determined in Žáček et al. (2009) and attractivity of the males' secretions depending on the males'age. Relative abundance of EAG-active compounds is transformed (log10 + 1). Attractivity of males' secretions is ranked: 1 are the less attractive secretions and 5 the more attractive ones. a: rank of attractivity, b: ethyl dodecanoate, c: 2,3-dihydrofarnesol, d: 2,3-dihydrofarnesal, e: hexadecanol, f: geranylcitronellol, g: sum of all EAG-active compounds (from Žácek et al., 2009).

DISCUSSION

With a simple bioassay, we showed that chemical changes in aging males elicit different responses of virgin queens. Progressive changes in chemical composition of males' CLG secretions are related to preferences of virgin queens for a certain bouquet. The virgin queens are much more attracted to males with a high amount of 2,3-dihydrofarnesol as well as other EAG-active compounds (Žáček et al. 2009), i.e.: ethyl dodecanoate, 2,3-dihydrofarnesal, hexadecanol, and geranylcitronellol (fig. 3). Those males secrete a lower relative concentration of tricosane. Attractiveness of the bouquet seems to be due to a high level of EAG-active compounds (listed in Žácek et al. 2009) even if they are not at their highest level of relative abundance. In other words, attractivity seems to be due to the total ratio of EAG-active compounds.

Ten-day-old males are most attractive to queens and their secretions show a high fraction of EAG-active compounds. This includes a high DHF relative abundance (but not the highest), as well as 2,3-dihydrofarnesal, hexadecanol, and geranylcitronellol. Ethyl dodecanoate increases until the age of 30 days, which does not correspond to the decreasing trend of virgin queens' response. One could speculate that this compound may have a different function in the secretions (e.g. repellent instead of attractant). Males older than 30 days are rare in the wild and a female should avoid losing her energy mating with them for their possibly poor reproductive abilities (if they already mated, their sperm reserves may be low). It is also interesting to note that one EAG-active compound described in the subspecies B. t. terrestris (Žáček et al. 2009), i.e. octadecatrienol, was not detected in B. t. dalmatinus.

The profile of CLG secretions in B. t. ssp dalmatinus., with a peak of secretions at 7 days old, could be linked to having a maximal attraction potential to virgin queens. This might be an advantage in species that are monoandrous such as B. terrestris (Baer & Schmid-Hempel, 2001; Duvoisin et al., 1999). Having only few chances to mate, they will be favoured by being as attractive as possible. In multiple mating species on the other hand, males could be expected to be attractive during their entire life without age-dependent changes in their CLG secretions.

It is clear that the strategy of "age-dependent secretions composition" could play a role in inbreeding avoidance. As virgin queens are not attracted to males younger than 5 days old, they will not mate with their brothers in their parental nest (Ågren et al. 1979). Furthermore, as bumblebees sex determination is pledged to sl-CSD (single locus- Complementary Sex Determination) (Van Wilgenburg et al. 2006, Whitehorn et al. 2009), inbreeding is more than hazardous for the population survival by leeding to diploid and triploid progeny.

Acknowledgments- The authors acknowledge the "Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture", which awarded a grant to A.C, and the "Fonds de la Recherche Fondamentale et Collective (projet 2.4.564.06.F)". They are also grateful to Biobest for having provided the biological material. I.V. thanks the Ministry of Education of the Czech Republic (project No. 2B06007) and the Czech Science Foundation (project No. 203/09/1446) for financial support. A.C. is greatful to Mike Wikes for his kind proof reading.

References

ÅGREN L, CEDERBERG B ,and SVENSSON B G (1979). Changes with age in ultrastructure and pheromone content of male labial glands in some bumble bee species (Hymenoptera, Apidae). Zoon 7: 1-14

BAER B,SCHMID-HEMPEL P (2001). Unexpected consequences of polyandry for parasitism and fitness in the bumblebee, Bombus terrestris. Evolution 55(8): 1639-1643

BELLÉS X, GALOFRÉ A, and GINEBREDA A (1987). Taxonomic potential of the chemical constituents in the cephalic marking secretions of Bombus and Psithyrus species (Hymenoptera, Apidae): a numerical taxonomic study. Apidologie 18: 231-242

BERGSTRÖM G (1981). Chemical aspects of insect exocrine signals as a means for systematic and phylogenetic discussions in aculeate Hymenoptera. Entomologica scandinavica Suppl. 15: 173-184

CALAM D H (1969) Species and sex-specific compounds from the heads of male bumblebees (Bombus spp.). Nature 221: 856-857

COPPÉE A, TERZO M, VALTEROVA I, and RASMONT P (2008). Intraspecific variation of the cephalic labial gland secretions in Bombus terrestris (L.) (Hymenoptera: Apidae). Chemistry & Biodiversity 5: 2654-2661

DUCHATEAU M J, HOSHIBA H, and VELTHUIS H H W (1994). Diploid males in the bumble bee Bombus terrestris sex determination, sex alleles and viability. Entomologia Experimentalis Et Applicata 71: 263-269

DUCHATEAU M J, MARIEN J (1995). Sexual biology of haploid and diploid males in the bumble bee Bombus terrestris. Insectes Sociaux 42: 255-266

DUVOISIN N, BAER B, and SCHMID-HEMPEL P (1999). Sperm transfert and male competition in a bumblebee. Animal behaviour 58: 743-749

GERLOFF C U, SCHMID-HEMPEL P (2005). Inbreeding depression and family variation in a social insect, Bombus terrestris (Hymenoptera : Apidae). Oikos 111: 67-80

KULLENBERG B, BERGSTRÖM G, BRINGER B, CARLBERG B, and CEDERBERG B (1973). Observations on scent marking by Bombus Latr. and Psithyrus Lep. males (Hymenoptera, Apidae) and localization of site of production of the secretion. Zoon Suppl. 1: 23-30

RASMONT P, COPPEE A, MICHEZ D, and DE MEULEMEESTER T (2008). An overview of the Bombus terrestris (L.1758) subspecies (Hymenoptera: Apidae). Annales de la Société Entomologique de France 44: 243-250

ŠOBOTNÍK J, KALINOVA B, CAHLIKOVA L, WEYDA F, PTACEK V, and VALTEROVA I (2008). Age-dependent changes in structure and function of the male labial gland in Bombus terrestris. Journal of Insect Physiology 54: 204-214

SVENSSON B G (1979). Patrolling behaviour of bumble bee males (Hymenoptera, Apidae) in a subalpine/alpine area, Swedish Lapland. Zoon 7: 67-94

TERZO M, URBANOVÀ K, VALTEROVÀ I and RASMONT P (2005). Intra and interspecific variability of the cephalic labial glands' secretion in male bumblebees: the case of Bombus (Thoracobombus) ruderarius and B. (Thoracobombus) sylvarum [Hymenoptera, Apidae]. Apidologie 36: 85-96

VAN WILGENBURG E, DRIESSEN G, and BEUKEBOOM L W (2006). Single locus complementary sex determination in Hymenoptera: an "unintelligent" design? Frontiers in Zoology 3: 1

WHITEHORN P R, TINSLEY M C, BROWN M J F, DARVILL B, and GOULSON D (2009). Impacts of inbreeding on bumblebee colony fitness under field conditions. Bmc Evolutionary Biology 9:

ŽÁCEK P, KALINOVA B, ŠOBOTNÍK J, HOVORKA O, PTACEK V, COPPEE A, VERHEGGEN F,and VALTEROVA I. (2009). Comparison of age-dependent quantitative changes in the male labial secretion of Bombus terrestris and Bombus lucorum. Journal of chemical ecology 35: 698-705

Chapter 3

Coppée A., Terzo M., Valterova I., Rasmont P. 2008.

Intraspecific Variation of the Cephalic Labial Gland Secretions in *Bombus terrestris* (L.) (Hymenoptera: Apidae). *Chemistry & Biodiversity* **5**(12): 2654-2661.

Now that we have determined that particular attention should be paid to the age of males, and that 7- to 15-day-old males are the most appropriate, the geographic variability can be investigated. A chemical analysis of the CLG secretions composition is conducted on 4 populations of Bombus terrestris.

Intraspecific Variation of the Cephalic Labial Gland Secretions in *Bombus terrestris* (L.) (Hymenoptera: Apidae)

by **Audrey Coppée***[a]), **Michaël Terzo**[a]), **Irena Valterova***[b]), and **Pierre Rasmont**[a])

[a]) Laboratoire de Zoologie, Université de Mons-Hainaut, Place du Parc -20, B-7000 Mons (phone: +3265373436; e-mail: audrey.coppee@umh.ac.be)

[b]) Institute of Organic Chemistry and Biochemistry Academy of Sciences of the Czech Republic, Flemingovo nam. 2, CZ-166 10 Prague (e-mail: irena@uochb.cas.cz)

Variations of secretions of the cephalic part of the labial glands from four different subspecies of *Bombus terrestris*, *B. t. terrestris, B. t. lusitanicus, B. t. sassaricus*, and *B. t. dalmatinus,* were investigated. 95 compounds were detected in the whole data set: 54 in *B. t. terrestris*, 54 in *B. t. lusitanicus,* 48 in *B. t. sassaricus*, and 44 in *B. t. dalmatinus.* The (*E*)-2,3-dihydrofarnesol is the main compound in *B. t. dalmatinus* and *B. t. sassaricus*, while it is dihydrofarnesyl dodecanoate in *B. t. terrestris* and *B. t. lusitanicus.* A principal component analysis produced a pattern showing three well distinct groups corresponding to *dalmatinus, sassaricus*, and *terrestris* + *lusitanicus.*

Introduction. – The specific mate recognition system (SMRS *sensu Paterson* 1985 [1]) of bumblebees is largely based on the specificity of pheromonal secretions. Unmatted bumblebee queens (gynes) are attracted to species-specific pheromone blends synthesized in the cephalic part of the labial glands (CLG) of conspecific males [2–7]. These pheromones have, for a long time, been considered as species-specific with a low geographical variability.

Until recently, the few variations observed in male CLG secretions were thought to be related to seasons rather to place of collection [8]. However, individual variability reported in that paper was hidden by the necessity of pooling glands from several specimens to reach the sensitivity level of measuring instruments. Modern instruments are considerably more sensitive, allowing analysis of the secretions from the glands of a single individual [7]. Re-analysis of CLG secretions from previously studied bumblebee species (*i.e.*, before 1996) shows that the individual variability is important, and that the concentration of the major compounds can vary considerably [9].

Such individual variability in the pheromonal blend is already known for several insects including solitary bees (*Colletes cunicularius* (L.) Hymenoptera: Colletidae [10]), ants (Hymenoptera: Formicidae [11]), fruitflies (Diptera: Tephritidae [12–15]) and moths (Lepidoptera: Noctuidae [16–20]). Indeed, pheromonal variability in *Colletes cunicularius* is highly correlated with the distance between sampling localities [10], suggesting that specific recognition systems should be more effective with increasing distance between the geographical origins of potential mates.

Bombus terrestris (L.) is widely distributed in West-Palaearctic region. Its distribution is typically Mediterranean extending from the Canary Islands in the West

to the Altai to the East, and from the Antiatlas Mountains of Morocco in the South to Southern Finland in the North [21–23]. Within its wide distribution, there are important subspecific differences in morphological characters, *e.g.*, coat color [21][22][24–27], and behavior, *e.g.*, innate color preference [28][29] and learning performance [30] which underline the genetic differentiation among subspecies. Of these subspecies, the insular subspecies, *e.g.*, *Bombus terrestris sassaricus*, are the most genetically differentiated [23][30].

While the CLG secretions of *B. terrestris* have been previously studied [2][4][6][31–33], the extent to which pheromone blends vary among subspecies is unknown. In a more recent study, which deals with a population from the Netherlands, *Bergman* identified 23 compounds, including six isoprenoids (*Table 1*).

Hence, the aim of this study was to investigate the potential for variation in CLG secretions among *B. terrestris* males from four different subspecies.

Results and Discussion. – *Chemical Analysis.* 95 compounds were detected in the whole *B. terrestris* data set (*Table 1*): 54 from *B. t. terrestris*; 54 from *B. t. lusitanicus*; 48 from *B. t. sassaricus*, and 44 from *B. t. dalmatinus*. The four subspecies share only 14 compounds in common (*Table 1*), but the two continental subspecies (*B. t. terrestris* and *B. t. lusitanicus*) share 19 specific compounds. The (*E*)-2,3-dihydrofarnesol (DHF; *Fig. 1*), previously suggested to be the main pheromone component for the whole species [4], is not present in the same proportions across all studied subspecies. While it was the main compound found in the CLG extracts from *B.t. dalmatinus* and *B.t. sassaricus* (19–93%), it is the dihydrofarnesyl dodecanoate that was the most abundant compound (14–47%) for *B.t. terrestris* and *B.t. sassaricus*. The other differences between the four subspecies were principally due to the presence or absence of minor compounds.

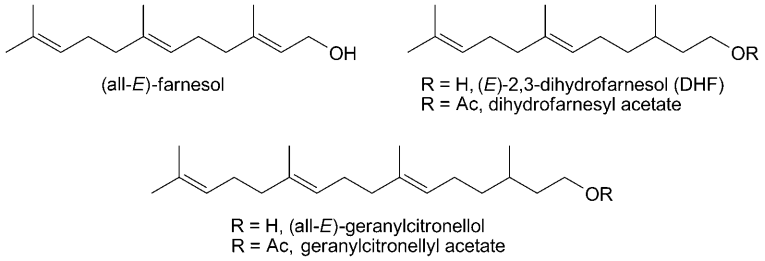


Fig. 1. *Chemical structures of the sesqui- and diterpenes together with their trivial names*

Statistical Analysis. Principal component analysis (PCA) of the data matrix, including data from all 95 compounds, leads a distinct pattern showing three distinct groups of specimens (*Fig. 2*): left to right *1*) *sassaricus, 2*) *dalmatinus,* and *3*) *terrestris* + *lusitanicus*. The first axis separates *terrestris* + *lusitanicus* from the two other subspecies groups, and the second axis separates clearly *dalmatinus* from both other groups.

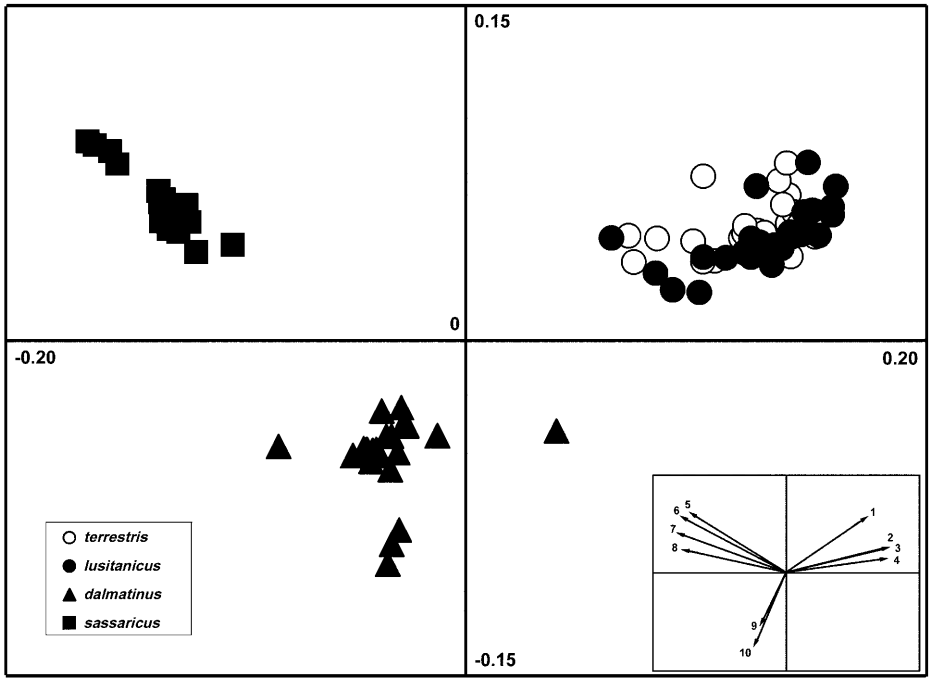


Fig. 2. *Two first axis projection of the PCA based on 95 compounds × 100 specimens.* Populations of *B. t. terrestris* ($n = 27$) and *B. t. lusitanicus* ($n = 27$) are widely overlapping (*top right*). Samples of *B. t. dalmatinus* ($n = 25$; *bottom*) and *B. t. sassaricus* ($n = 20$; *top left*) form two well-separated clouds. The ten most influential compounds in determinig the subspecies positions within the scatterplot are: *1*) farnesol; *2*) octadecatrienyl dodecanoate/octadecyl dodecanoate/hexadecyl tetradecanoate; *3*) hexadecyl dodecanoate; *4*) dihydrofarnesyl dodecanoate; *5*) tricosene 2; *6*) docosenol; *7*) octadecadienol; *8*) hexadecanol; *9*) tetradecanal; *10*) 2,3-dihydrofarnesal.

The *B. t. sassaricus* group is discriminated by the presence of tricosene 2, docosenol, octadecadienol, and hexadecanol. The *B. t. dalmatinus* group is discriminated by tetradecanal and 2,3-dihydrofarnesal. The *B. t. terrestris* + *sassaricus* group is discriminated by the amounts of farnesol, octadecatrienyl dodecanoate/octadecyl dodecanoate/hexadecyl tetradecanoate, hexadecyl dodecanoate, and dihydrofarnesyl dodecanoate.

The analysis of CLG secretions of four *B. terrestris* subspecies shows that, among 95 compounds identified, only 14 are common to the studied taxa. The geographical distributions of *B. t. terrestris* and *B. t. lusitanicus* overlap broadly in Southwest France. These two subspecies share 19 compounds among 54 in *B. t. terrestris* and in *B. t. lusitanicus*. The PCA shows three groups corresponding to: *B. t. dalmatinus*, *B. t. sassaricus* and *B. t. terrestris* + *B. t. lusitanicus*. Our study indicates that differences in CLG secretions exist among populations of *B. terrestris*. These differences affect the composition of secretions both qualitatively and quantitatively. Our results indicate that the divergence between the CLG secretions increases with the geographic distance. The subspecies (*B. t. terrestris* and *B. t. lusitanicus*) with the most similar CLG secretions overlap in their geographic range, while the other two subspecies, with distinct patterns of CLG secretions (*B. t. dalmatinus* and *B. t. sassaricus*), are also

Table 1. *List of the Identified Compounds.* Retention time (t_R [min]), median (M [%]), first and fourth quartile (Q1 and Q4, resp.) of the 95 identified compounds. The compounds we were not able to determine are indicated as U*x*. The 14 common compounds are shown on grey background. The main compounds are shown on black background. A: 2,3-dihydrofarnesyl tetradecadienoate + 2,3-dihydrofarnesyl tetradecenoate; B: 2,3-dihydrofarnesyl hexadecanoate + hexadecyl tetradecanoate + tetradecyl hexadecanoate; C: octadecatrienyl dodecanoate/octadecyl dodecanoate/hexadecyl tetradecanoate.

	t_R	*B. t. terrestris*			*B. t. lusitanicus*			*B. t. dalmatinus*			*B. t. sassaricus*		
		Q1	M	Q4	Q1	M	Q4	Q1	M	Q4	Q1	M	Q4
U1	11.30	0.08	0.14	1.82	0.07	0.11	0.58	–	–	–	–	–	–
Methyl dodecanoate	12.04	0.07	0.16	0.52	0.01	0.19	0.54	–	–	–	–	–	–
Dodecanoic acid	12.66	0.49	1.27	8.3	0.09	0.97	8.86	–	–	–	–	–	–
Ethyl dodecanoate	12.97	3.15	4.88	12.08	3.02	5.19	17.5	–	–	–	–	–	–
Dihydrofarnesene	13.06	–	–	–	–	–	–	0	0	0	–	–	–
Farnesol[a])	13.22	2.25	2.56	5.13	2.22	2.56	3.88	–	–	–	1.03	1.23	2.72
Hexadecene	13.34	–	–	–	–	–	–	0	0	3.37	–	–	–
Ethyl dodecanoate[b])	13.13	–	–	–	–	–	–	0	0	24.5	–	–	–
Ethyl dodecanoate 2	13.21	–	–	–	–	–	–	0.16	0.31	6.25	–	–	–
Tetradecanal	13.40	–	–	–	–	–	–	0.42	1.01	8.44	–	–	–
Isopropyl dodecanoate[b])	13.30	0.1	0.13	0.79	–	–	–	–	–	–	–	–	–
2.3-Dihydrofarnesal[b])	13.46	–	–	–	–	–	–	1.6	1.87	10.3	–	–	–
U2	13.96	0	0	0.59	–	–	–	–	–	–	–	–	–
Dodecyl acetate	14.16	–	–	–	–	–	–	0.09	0.13	1.18	–	–	–
DHF[a])[b])	14.12	4.77	8.35	17.61	4.24	8.41	19.01	26.45	45.02	93.01	19.24	20.67	26.43
2.3-Dihydrofarnesyl acetate[a])	15.15	–	–	–	–	–	–	0.06	0.09	2.21	–	–	–
7-Methylhexadecane	14.30	0.07	0.13	0.33	0.06	0.1	0.71	0.12	0.24	62.16	–	–	–
U3	14.72	–	–	–	0	0	0.07	–	–	–	–	–	–
Tetradecenoic acid	14.84	0.35	0.62	3.11	0.25	0.47	1.72	–	–	–	0.14	0.18	0.98
Tetradecanoic acid	14.92	0.27	0.48	4.78	0.36	0.56	2.57	–	–	–	–	–	–
U4	14.98	–	–	–	–	–	–	–	–	–	0.14	0.21	0.73
Ethyl tetradecenoate	15.09	0.24	0.44	1.91	0.22	0.39	4.7	–	–	–	–	–	–
Ethyl tetradecanoate	15.22	0.36	0.55	0.99	0.45	0.58	1.73	–	–	–	–	–	–
Dihydrofarnesyl acetate	15.34	1.24	1.77	10.52	1.75	2.43	10.99	–	–	–	0.05	0.1	0.34
U5	15.36	–	–	–	–	–	–	–	–	–	0.01	0.01	0.05
Hexadecenal	15.54	–	–	–	–	–	–	0.16	0.24	2.31	–	–	–
Hexadecanal	15.49	0.24	0.34	2.04	0.23	0.29	1.59	0.01	0.13	2.28	0.24	0.31	0.92
Pent-4-en-1-yl dodecanoate	16.11	–	–	–	–	–	–	0	0	0.13	–	–	–
Hexadecenol	16.24	–	–	–	–	–	–	0	0	0.43	0.21	0.27	0.54
Hexadecanol[b])	16.18	0.25	0.85	2.23	0.27	0.65	4.84	1.42	2.86	18.29	7.9	8.46	11.7
Heptadecane	16.50	0	0	0.23	–	–	–	–	–	–	–	–	–
Hexadecenoic acid	16.82	0.14	0.26	1.99	0.21	0.41	1.16	–	–	–	0.51	0.71	1.69
Hexadecenoic acid 2	16.90	0.19	0.31	3.26	0.21	0.3	1.84	–	–	–	0.14	0.18	0.84
Hexadecanoic acid	17.11	–	–	–	0.09	0.2	2.92	0	0	0.05	0.11	0.13	0.28
Octadecadienal	17.08	–	–	–	–	–	–	0	0	0.44	0.28	0.33	1.14
Octadecatrienal	17.34	0.28	0.38	0.89	–	–	–	0	0.06	1.78	0.49	0.6	1.75
Icosane	17.37	–	–	–	–	–	–	0	0	0.12	–	–	–
Nonadecadienal	17.43	–	–	–	–	–	–	0	0.08	3.09	0.02	0.03	0.08
Hexadecyl acetate	17.44	0.76	1.04	2.26	0.71	1.22	1.89	0.07	0.15	0.79	–	–	–

Table 1 (cont.)

	t_R	*B. t. terrestris*			*B. t. lusitanicus*			*B. t. dalmatinus*			*B. t. sassaricus*		
		Q1	M	Q4	Q1	M	Q4	Q1	M	Q4	Q1	M	Q4
U6	17.58	–	–	–	–	–	–	0	0	0.28	–	–	–
Octadecadienol[b])	18.04	–	–	–	–	–	–	0.30	0.81	6.41	9.76	11.21	15.31
Octadecatrienol[b])	18.09	–	–	–	–	–	–	–	–	–	3.67	4.39	13.62
Geranylcitronellal[b])	18.18	1.77	2.30	7.36	1.19	1.80	5.28	0.08	0.88	5.22	0.83	1.42	1.79
Henicosane[b])	18.36	1.34	1.86	9.49	1.09	1.34	2.47	1.19	2.19	23.63	2.08	2.37	2.99
3-Methylicosane	18.50	0.02	0.06	0.59	0.00	0.06	0.80	–	–	–	–	–	–
Geranylcitronellol[a])[b])	18.89	8.95	10.61	28.89	7.20	9.79	20.78	0.53	1.78	12.07	9.29	9.74	13.92
Ethyl octadecenoate	18.97	0.97	1.41	12.09	0.83	1.12	4.89	–	–	–	–	–	–
Octadecadienyl acetate	19.07	0.75	0.99	4.47	0.58	0.67	3.34	0.00	0.00	1.46	0.28	0.39	0.86
Octadecatrienyl acetate	19.13	0.81	1.08	2.13	0.83	1.08	6.46	–	–	–	0.20	0.29	0.63
Docosane	19.28	0.42	0.53	1.29	0.38	0.47	1.00	–	–	–	0.38	0.41	0.55
Octadecyl acetate	19.44	0.00	0.07	1.89	–	–	–	–	–	–	0.04	0.06	0.07
Icosadienol	19.43	–	–	–	–	–	–	–	–	–	0.07	0.10	0.25
Geranylcitronellyl acetate[a])	19.79	0.19	0.32	4.53	0.31	0.59	2.34	–	–	–	–	–	–
Tricosene[b])	19.18	0.75	1.01	2.30	0.62	0.75	1.80	0.09	0.54	6.55	2.57	2.88	3.88
Tricosene 2	19.97	0.40	0.90	2.83	0.00	0.35	0.70	–	–	–	9.10	10.19	15.09
U7	20.02	–	–	–	–	–	–	–	–	–	0.00	0.00	3.13
Tricosane[b])	20.17	5.74	6.36	8.87	4.55	5.54	8.21	0.55	4.72	34.09	5.48	6.08	7.83
U8	20.22	–	–	–	–	–	–	–	–	–	0.05	0.05	0.10
Tetracosene	20.81	–	–	–	–	–	–	0.00	0.00	5.75	–	–	–
Tetracosane	20.95	0.00	0.00	0.39	0.00	0.00	0.62	–	–	–	–	–	–
Icosenyl acetate	20.47	0.17	0.38	0.81	0.23	0.43	1.50	–	–	–	0.01	0.02	0.04
Icosenyl acetate 2	20.95	1.11	1.79	3.81	1.27	1.96	4.88	–	–	–	0.15	0.18	0.27
Icosyl acetate	21.05	0.00	0.18	0.92	0.00	0.23	0.85	–	–	–	–	–	–
Docosenol	21.10	–	–	–	–	–	–	–	–	–	7.55	8.50	12.01
Pentacosene[b])	21.75	0.97	1.22	2.64	0.69	1.16	4.14	0.00	0.08	3.17	–	–	–
Pentacosane[b])	21.67	2.20	2.37	3.70	1.72	2.22	3.48	0.04	0.43	5.46	1.14	1.34	2.20
Methylpentacosane	21.87	–	–	–	0.00	0.00	1.93	0.00	0.00	0.34	–	–	–
Hexacosene	22.15	0.11	0.13	0.45	0.11	0.14	0.96	0.00	0.00	0.60	0.19	0.27	0.38
Docosenyl acetate	22.46	0.72	1.49	4.04	1.39	1.73	5.37	–	–	–	0.02	0.04	0.08
Docosenyl acetate 2	22.56	–	–	–	–	–	–	0.00	0.00	0.48	–	–	–
Hexacosane	22.59	0.12	0.20	0.48	0.00	0.21	0.65	–	–	–	0.01	0.02	0.02
Docosyl acetate	22.65	0.00	0.07	0.45	0.01	0.12	0.46	–	–	–	–	–	–
Heptacosene[b])	22.72	1.89	2.33	3.18	1.60	2.04	10.32	0.10	1.22	12.03	2.77	3.27	4.23
Heptacosane	23.25	0.49	0.59	1.31	0.56	0.72	2.04	0.00	0.00	2.60	0.24	0.37	0.57
U9	23.41	–	–	–	–	–	–	–	–	–	0.40	0.51	0.80
Octacosene	23.40	–	–	–	–	–	–	–	–	–	0.06	0.07	0.12
Dihydrofarnesyl[b]) dodecanoate	23.95	14.32	18.39	31.46	16.72	21.32	47.49	0.30	0.64	17.62	–	–	–
Nonacosene	23.92	0.70	0.84	1.95	0.87	1.00	3.07	0.00	0.08	3.56	0.63	0.74	1.04
U10	24.71	–	–	–	–	–	–	–	–	–	0.17	0.25	0.40
Nonacosane	24.68	–	–	–	0.07	0.11	0.54	–	–	–	0.15	0.27	0.48
Dihydrofarnesyl tetradecanoate[b])	24.88	1.84	2.42	6.63	2.19	3.10	10.88	–	–	–	0.21	0.26	0.52
Hexadecyl dodecanoate	25.25	1.76	2.52	4.01	2.24	2.49	6.73	–	–	–	–	–	–
Hexacosenyl acetate	25.35	0.00	0.09	0.20	0.10	0.12	0.31	–	–	–	–	–	–
Hexacosenyl acetate 2	25.53	–	–	–	0.00	0.03	0.25	–	–	–	–	–	–

Table 1 (cont.)

	t_R	*B. t. terrestris*			*B. t. lusitanicus*			*B. t. dalmatinus*			*B. t. sassaricus*		
		Q1	M	Q4	Q1	M	Q4	Q1	M	Q4	Q1	M	Q4
A	25.62	–	–	–	–	–	–	0.13	0.51	11.83	–	–	–
Hentriacontene	26.04	–	–	–	–	–	–	–	–	–	0	0.02	0.04
B	26.21	–	–	–	–	–	–	0	0.04	2.32	0.04	0.05	0.12
Octadecadienyl dodecanoate	26.52	0.31	0.5	1.44	0.39	0.57	10.38	–	–	–	–	–	–
Dihydrofarnesyl hexadecenoate	26.53	–	–	–	–	–	–	0.02	0.14	3.39	–	–	–
Dihydrofarnesyl hexadecanoate	26.59	–	–	–	–	–	–	–	–	–	0.1	0.11	0.18
C	26.60	1.73	2.04	3.96	1.7	2.1	4.32	–	–	–	–	–	–
Geranylcitronellyl dodecanoate	26.94	0.95	1.51	2.56	1.2	1.86	4.89	–	–	–	–	–	–
Dihydrofarnesyl octadecenoate	27.40	–	–	–	–	–	–	0	0	1.34	–	–	–
Geranylcitronellyl tetradecanoate	27.63	–	–	–	–	–	–	0	0.08	9.41	–	–	–

[a]) Identified by *Bergmann* [6]. [b]) Chemical structures given in *Fig. 1.*

geographically isolated form one another and the other subspecies pair. Moreover, the main compound identified by *Bergman* [6] in *B. t. terrestris* from the Netherlands is the DHF (20.6%). These results suggest a North-South gradient within the *B. t. terrestris* subspecies, corresponding to the genetic gradient observed [23]. 17 of the 23 compounds identified by *Bergman* [6] are present in *Table 1*. On the one hand, all the isoprenoids listed by the latter author are detected here. On the other hand, seven fatty acid derivatives are missing: isopropyl dodecanoate, tetradecanol, tetradecanal, isopropyl tetradecenoate, icosanol, icosenol, and docosenol.

At present, we are not able to connect the phenetical characters of CLG secretions with the phylogeny of *B. terrestris* populations [23][30]. This analysis would be possible when the CLG secretions of most *B. terrestris* subspecies will be known.

The variations found in the CLG secretions of *B. terrestris* could reflect the geographical distance between subspecies, as formerly demonstrated in other taxa. In turnip moth *Agrotis segetum* DENIS and SCHIFFERMÜLLER (Lepidoptera Noctuidae), *e.g.*, various authors also found a geographic variation in the female sex pheromonal secretions and in the sensitive sensilla of males' antenna [18–20][34]. In the same way, in *Polistes dominulus* CHRIST (Hymenoptera Vespidae), *Dapporto et al.* [35] found significant differences in cuticular hydrocarbons from island and continental populations from the Tyrrhenian area. The same observations were made in *Colletes cunicularius* [10], in which different pheromonal 'dialects' are found in females originating from distant origins. However, in this latter case, *C. cunicularius* males are more attracted to the females of the most distinct populations. As we do not know if the *Bombus terrestris* virgin queens are more or less attracted to males from more or less distant origin, we cannot make further inference on the SMRS (Specific Mate

Recognition System) variation. We are currently conducting behavioral experiments to provide data about it.

The authors sincerely thank Prof. *Lars Chittka* and Dr. *Nigel E. Raine* (Queen Mary University of London, UK), and *Jan Vermeulen* (*Biobest bvba*, Westerlo, Belgium) for their help in collecting and providing the biological material. We are grateful to Dr. *D. Michez*, Dr. *S. Patiny*, and Dr. *N. E. Raine* for their kind proof-reading. *A. C.* was granted by the *Fonds pour la formation à la Recherche dans l'Industrie et l'Agronomie*. The authors thank the *Fonds de la Recherche Fondamentale et Collective* (2.4.564.06.F) for their financial support. A support by the *Ministry of Education of the Czech Republic* (I. V., project No. 2B06007) and by the *Academy of Sciences of the Czech Republic* (research project No. Z4055 0506) is also gratefully acknowledged.

Experimental Part

Biological Material. Four taxa were used for this study: *B. t. terrestris* (L.), *B. t. lusitanicus* KRÜGER, *B. t. sassaricus* TOURNIER, and *B. t. dalmatinus* DALLATORRE (*Table 2*). *B. t. terrestris* (27 males) and *B. t. lusitanicus* (27 males) of unknown age were collected in Southwestern France. Queens of *B. t. sassaricus* ($N=2$) from North Sardinia were reared in the laboratory of Prof. *L. Chittka* (Queen Mary University of London, UK) and ten males from each colony were used (age 4–27 d); *B. t. dalmatinus* (25 males, age 5–25 d) was obtained from one commercial bee breeders colony (*Biobest bvba*, Westerlo, Belgium).

Table 2. *Data on Collection of Biological Material.* Collecting sites and number (N) of samples collected for *Bombus terrestris terrestris, B. t. lusitanicus*, *B. t. dalmatinus*, and *B. t. sassaricus.* Coordinates are given with the reference to the WGS84.

Subspecies	Collecting sites	*N*
	France	
B. t. terrestris	Corbère, 42°39′N 2°40′E	4
	Millas, 42°42′N 2°42′E	13
	Ille-sur-Têt, 42°40′N 2°38′E	10
B. t. lusitanicus	Camélas, 42°39′N 2°42′E	1
	Corbère, 42°39′N 2°40′E	4
	Banyuls-dels-Aspres, 42°33′N 2°52′E	1
	Millas, 42°42′N 2°42′E	8
	Ille-sur-Têt, 42°40′N 2°38′E	13
	Greece	
B. t. dalmatinus	Rhodos (Biobest)	25
	Sardinia	
B. t. sassaricus	Luogo Santo, 41°01′N 09°12′E	20

CLG Extracts were prepared according to a protocol adapted from [9]. Secretions were extracted in hexane (200 µl).

Chemical Analysis. Gas chromatography/mass spectrometry (GC/MS) was used to identify chemical compounds (an 'ion trap' *Finigan GCQ*, with a *DB-5ms* non-polar cap. column (5% phenyl(methyl)polysiloxane stationary phase; 30-m column length; 0.25-mm inner diameter; 0.25-µm film thickness). Solns. (1 µl) were injected in splitless mode at the injector temp. set to 220°. The temp. of the column was initially held at 70° for 2 min, rising to 320° at 10°/min, and held at 320° for the last 5 min. The carrier gas was He at a constant velocity of 50 cm/s. Mass spectra were obtained in electron impact mode 'full scan (300–600)'. Compounds were identified using the retention times and mass spectra of each peak.

Statistical Analysis. The NTSYS [36] was used for statistics. The data set was analysed by Principal Component Analysis (PCA). The matrix consisted in records of molecules peaks (expressed as percentage relative abundance) by specimen in GC/MS.

REFERENCES

[1] H. E. H. Paterson, 'The recognition concept of species', in 'Species and speciation', Ed. E. S. Vrba, Transvaal Museum Monograph No. 4, Transvaal Museum, Pretoria, 1985, pp. 21–29.
[2] D. H. Calam, *Nature* **1969**, *221*, 856.
[3] B. G. Svensson, *Zoon* **1980**, *7*, 67.
[4] G. Bergström, B. Kullenberg, S. Ställberg-Stenhagen, E. Stenhagen, *Ark. Kemi* **1967**, *31*, 453.
[5] B. Cederberg, B. G. Svensson, G. Bergström, M. Appelgren, *Nova Acta Regiae Soc. Sci. Upsaliensis* **1984**, *3*, 161.
[6] P. Bergman, Ph. D. Thesis, Göteborg University, Göteborg, 1997.
[7] M. Terzo, I. Valterova, K. Urbanova, P. Rasmont, *Phytoprotection* **2003**, *84*, 39.
[8] B. G Svensson, G. Bergström, *Insectes sociaux* **1977**, *24*, 213.
[9] M. Terzo, K. Urbanova, I. Valterova, P. Rasmont, *Apidologie* **2005**, *36*, 85.
[10] N. J. Vereecken, J. Mant, F. P. Schiestl, *Behav. Ecol. Sociobiol.* **2007**, *61*, 811.
[11] M.-C. Cammaerts-Tricot, J.-C. Verhaeghe, *Insectes Sociaux* **1974**, *21*, 275.
[12] J.-M. Jallon, J. R. David, *Evolution* **1987**, *41*, 294.
[13] T. A. Markow, *Evolution* **1991**, *45*, 1525.
[14] M. D. Stennett, W. J. Etges, *J. Chem. Ecol.* **1997**, *23*, 2803.
[15] W. J. Etges, M. A. Ahrens, *Am. Naturalist* **2001**, *158*, 585.
[16] J. A. Klun, P. L. Anglade, F. Baca, O. L. Chapman, H. C. Chiang, D. M. Danielson, W. Faber, P. Fels, R. E. Hill, M. Hudon, C. S. Kania, A. J. Keaster, *Environ. Entomol.* **1975**, *4*, 891.
[17] J. R. Miller, W. L. Roelofs, *Environ. Entomol.* **1980**, *9*, 359.
[18] C. Löfstedt, J. Löfqvist, B. S. Lanne, J. N. C. Van Der Pers, B. S. Hansson, *Oikos* **1986**, *46*, 250.
[19] B. S. Hansson, M. Tóth, C. Löfstedt, G. Szöcs, M. Subchev, J. Löfqvist, *J. Chem. Ecol.* **1990**, *16*, 1611.
[20] M. Tóth, C. Löfstedt, B. W. Blair, T. Cabello, A. I. Farag, B. S. Hansson, B. G. Kovalev, S. Maini, E. A. Nesterov, I. Pajor, A. P. Sazanov, I. V. Shamshev, M. Subchev, G. Szöcs, *J. Chem. Ecol.* **1992**, *18*, 1337.
[21] P. Rasmont, *Notes faun. Gembloux* **1983**, *7*, 1.
[22] P. Rasmont, A. Coppée, D. Michez, T. De Meulemeester, *Ann. Soc. Entomol. Fr.* **2008**, *44*, 243.
[23] A. Estoup, M. Solignac, J.-M. Cornuet, J. Goudet, A. Scholls, *Mol. Ecol.* **1996**, *5*, 19.
[24] E. Krüger, *Tijdschr. Ent.* **1951**, *93*, 141.
[25] E. Krüger, *Tijdschr. Ent.* **1954**, *97*, 263.
[26] E. Krüger, *Tijdschr. Ent.* **1956**, *99*, 75.
[27] E. Krüger, *Tijdschr. Ent.* **1958**, *101*, 283.
[28] N. E. Raine, T. C. Ings, A. Dornhaus, N. Saleh, L. Chittka, *Adv. Study Behav.* **2006**, *36*, 305.
[29] L. Chittka, N. E. Raine, T. C. Ings, *Popul. Ecol.* **2004**, *46*, 243.
[30] A. Widmer, P. Schmid-Hempel, A. Estoup, A. Scholl, *Heredity* **1998**, *81*, 563.
[31] B. Kullenberg, G. Bergström, B. Bringer, B. Calberg, B. Cedreberg, *Zoon* **1973**, *Suppl. 1*, 23.
[32] L. Ågren, B. Cederberg, Bo G. Svensson, *Zoon* **1979**, *7*, 1.
[33] G. Bergström, Bo G. Svensson, M. Appelgren, I. Groth, 'Complexity of bumble bee marking pheromones: biochemical, ecological and systematical interpretations', in 'Systematics Association', Academic Press, New York, 1981, special volume 19, pp. 175–183.
[34] C. Löfsted, *Philos. Trans. R. Soc. London, Ser. B* **1993**, *340*, 167.
[35] L. Dapporto, E. Palagi, S. Turillazi, *J. Chem. Ecol.* **2004**, *30*, 2139.
[36] J. Rholf, NTSYS-pc Numerical Taxonomy and Multivariate Analysis System, version 1.80., Applied biostatistics Inc., New York, 1993, 241 + VII.

Received February 18, 2008

Chapter 4

SUBMITTED PUBLICATION

Coppée A., Helderweirt A., Mathy T., Cammaerts M.-C., Iserbyt S., Rasmont P.

Geographic-Dependant attractivity of males' sexual pheromones in *Bombus terrestris* (L.) (Hymenoptera, Apidae). Submitted in *Behavioral Ecology*. 18pp.

Since the geographic CLG secretion variability has been demonstrated in the previous chapter, the reactions of virgin queens toward sexual pheromones of various geographic origins have to be evaluated. This will determine if the virgin queens perceive the chemical variations observed.

GEOGRAPHIC-DEPENDENT ATTRACTIVITY OF MALES' SEXUAL PHEROMONES IN *Bombus terrestris* (L.) [HYMENOPTERA, APIDAE]

Short title : Geographic-dependent attractivity of males' sexual pheromones in *Bombus terrestris* (L.)

AUDREY COPPEE [a]), AUDREE HELDERWEIRT [a]), TIFANY MATHY [a]), MARIE-CLAIRE CAMMAERTS [b]), STEPHANIE ISERBYT [a]) & PIERRE RASMONT [a]).

[a]) Laboratoire de Zoologie, Université de Mons Place du Parc -20, B-7000 Mons
(phone: +3265373436; e-mail: audrey.coppee@umons.ac.be)
[b]) Evolution Biologique et Ecologique, Université Libre de Bruxelles, CP160/11, avenue F.D. Roosevelt 50, 1050 Bruxelles
(phone : +3226502411 ; e-mail : mtricot@ulb.ac.be)

Abstract: The sexual pheromones of *Bombus terrestris* are now well known (Calam 1969; Bergströrm 1981; Bergman 1997; Goulson *et al.*, 2000). Coppée *et al.* (2008) showed that the different subspecies present different CLG secretions but the queen's reactions and preferences towards those sexual pheromones are still poorly known. Here, we investigated virgin queens preferences for CLG secretions of males from different geographic origins, using a simple behavioural test. Avoiding inbreeding is very important for bumblebees, because of their sl-CSD system (Withehorn *et al.*, 2009), and the study of the species mating recognition system (SMRS, Paterson, 1993) may be a tool for understanding how females choose their mate.

The results highlighted a "two level" choice of the virgin queens for males CLG secretions. On one hand, virgin queens presented a significant preference for males' odour blend of their own subspecies. On the other hand, inside a single subspecies, virgin queens chose males from the furthest geographic origin. We showed that queens of *Bombus terrestris* prefer to mate with unrelated consubspecific individuals and are attracted to males with the most different CLG secretions within their taxon. It seems that sexual pheromones may contain important information used for inbreeding prevention.

Keywords: *Bombus terrestris*, CLG secretions, Behavioural tests, Geographic-dependent preferences.

INTRODUCTION

Bombus terrestris (L.) is one of the most widespread and abundant bumblebee species in the West Palearctic region (Rasmont *et al.,* 2008). It is used worldwide for its high effectiveness in crop pollination and for its easy breeding (Velthuis & Van Doorn, 2006), which explains why so many studies have been led on this biological model.

Nowadays, many studies have been conducted on this biological model and many differences have been highlighted between the different subspecies. These differences concern behaviour, flower colour choices (Chittka *et al.,* 2004); learning speed (Ings *et al.*, 2009; Molet *et al.*, 2009); morphology or geographical distribution (Rasmont *et al.*, 2008) and chemical communication (Coppée *et al.,* 2008).

Bombus terrestris includes nine morphological subspecies (Rasmont *et al.,* 2008): *Bombus terrestris africanus* Krüger (North Africa); *Bombus terrestris audax* Harris (British Isles); *Bombus terrestris calabricus Krüger* (S. Italia and Sicily); *Bombus terrestris canariensis* Pérez (Canary Islands); *Bombus terrestris dalmatinus* Dalla Torre (Southeastern France, Northern Italy, the Balkan Peninsula and surrounding regions, Ukraine, Anatolia, Transcaucasia, Caucasus, N. Iran, S. Ural, Alai, Altai); *Bombus terrestris lusitanicus* Krüger (Southwestern France, the Iberian Peninsula, Balearic Islands, Madeira); *Bombus terrestris terrestris* (L.) (Continental Europe); *Bombus terrestris sassaricus* Tournier (Sardinia); *Bombus terrestris xanthopus* Kriechbaumer (Corsica, Capraia Island, Elba Island).

Sexual pheromones allow specific recognition of potential mates (Calam, 1969). In bumblebees, sexual pheromones are secreted by the cephalic labial glands (CLG) (Ågren *et al.,* 1979). These CLG secretions are used by the males, during their nuptial behaviour, to attract the conspecific virgin queens in order to mate (Kullenberg *et al.,* 1973). The males of *B. terrestris* spread their CLG secretions on twigs, leaves and rocks all along a circuit. They regularly patrol this circuit waiting for a conspecific virgin queen to stop on a scent mark (Svensson, 1979). The composition of these CLG secretions is species-specific (Calam, 1969; Svensson, 1980).

The sexual pheromones of *Bombus terrestris* are now well known (Calam 1969; Bergströrm 1981; Bergman 1997; Goulson *et al.*, 2000). Coppée *et al.* (2008) showed that the different subspecies present different CLG secretions but the queen's reactions and preferences towards those sexual pheromones are still poorly known.

In Aculeates, the sex is determined by haplo-diploidy and the sex determination mechanism is the single-locus Complementary Sex Determination (sl-CSD) (Cook, 1993 Duchateau *et al.*, 1994; Paxton, 2005; Van Wilgenburg *et al.,* 2006; Whitehorn *et al.,* 2009). This mechanism dictates that in normal conditions females are diploids (heterozygotes) and

males are haploids (hemizygotes). Inbreeding can lead to the apparition of diploid males (homozygotes). Considering this mechanism, brother-sister and nephew-niece mating can lead to diploid male development (respectively 50 and 37.5%) and these diploids have reduced viability and fertility (Duchateau *et al.*, 1994; Duchateau *et al.*, 1995; Gerloff, 2003).

Avoiding inbreeding is then very important for bumblebees, and the study of the species mating recognition system (SMRS, Paterson, 1993) may be a tool for understanding how females choose their mate. Ings *et al.* (2005) tested the preferences of virgin commercial queens of *B. t. dalmatinus* for males of their own subspecies or males of *B. t. audax*. Most of the choices (71%) were made in favour of *B. t. dalmatinus* males. These results show a preference for consubspecific males, potentially leading to an increase of inbreeding.

The perception of male sexual pheromones by the queen has been demonstrated (Coppée *et al.,* in press), as well as geographic variabilty of the CLG secretions (Coppée *et al.*, 2008). The present study aims at answering the next questions: (i) Do the virgin queens perceive those chemical differences? (ii) if they do, do they prefer consubspecific males or not? (iii) and finally, within a subspecies, do the virgin queens prefer related or unrelated males (from the same geographic origin, or not?). Our hypothesis is that virgin queens prefer unrelated consubspecific males and geographically distant males within their own subspecies, to limit inbreeding risks.

To answer these questions we will carry out analyses on the reactions of virgin queens towards the male CLG secretions of various *Bombus terrestris* subspecies and towards the secretions of potential mates from a single subspecies that have various distant geographical origins.

MATERIAL AND METHODS

Biological material

Intraspecific tests.

Forty virgin queens of *B. t. dalmatinus* were provided by Biobest bvba (Westerlo, Belgium), and forty virgin queens of the Corsican *B. t. xanthopus* were raised and provided by the laboratory of Prof. L. Chittka (University of Queen Mary – London). Each queen was labeled with a coloured number. Females were maintained in a wood box and fed *ad libitum* with *Salix* pollen and syrup (1kg water for 1kg sugar). The rearing conditions were the following: temperature (T°) of 29°C and a relative humidity (RH) between 55% and 65%.

B. t. canariensis (Tenerife Island, Spain), *B. t. dalmatinus* and *B. ignitus* males were provided by Biobest bvba (Westerlo-Belgium). The *B. t. xanthopus* males and *B. t. sassaricus*

males were provided by the laboratory of Prof. L. Chittka (University of Queen Mary-London).

Virgin queens of *B. t. dalmatinus* and *B. t. xanthopus* were confronted with CLG secretions of *B. t. dalmatinus*, *B. t. xanthopus*, *B. t. canariensis*, *B. t. sassaricus* and *B. ignitus* Smith. This last species lives in China, Japan and Korea. It was chosen as the outgroup in order to verify the specificity of the sexual pheromones. Each possible pair was tested 10 times, randomly and with different queens.

Intrasubspecific tests.

Forty virgin queens of *B. t. terrestris* were provided by Biobest bvba (Westerlo, Belgium) and the rearing conditions were the same as above.

B. t. terrestris is a subspecies with a large geographical distribution: It spreads from Southern France to Scandinavian regions, crossing Central Europe. We obtained males from the southernmost to the northernmost locations of this subspecies. The males were collected from the field by P. Rasmont for most locations and by A. Coppée for the Pyrenees. The males were collected in Ille-sur-Têt (Pyrénées, Southwestern France), Gonfaron (SoutheasternFrance), Paris (Northern France), Münster (Germany), Stenved (Denmark), Veberöd (Sweden), Uppsala (Sweden) and Alvkalerby (Sweden). Belgian males of *B. t. terrestris* were provided by Biobest bvba (Westerlo - Belgium) (Fig. 1).

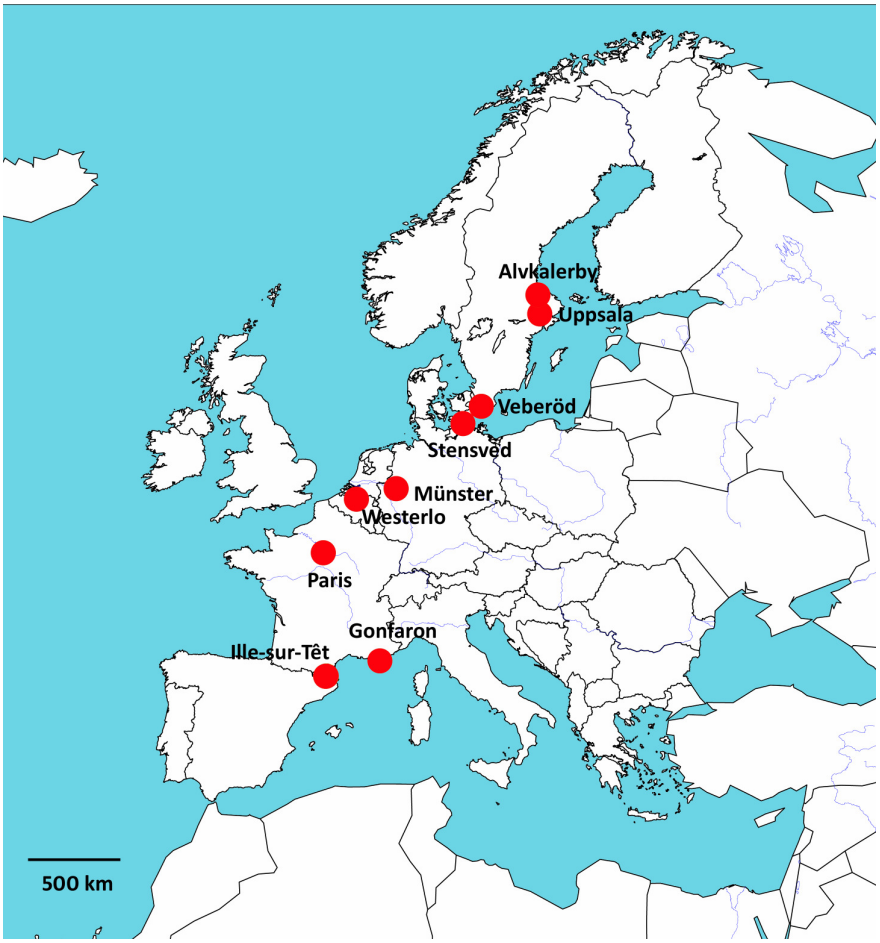


Figure 1. Map of the geographical origins of the different *B t .terrestris* males used for the

infrasubspecific tests.

Pheromonal extraction

Prior to dissection, males were killed by deep-freezing, the head was cut and placed in a glass vial containing 400 µl hexane. After 24 hours at room temperature, the samples were preserved in a deep freezer (-20°C) until the solutions were used.

CLG secretions of *B.t.terrestris* males were analysed using gas chromatography coupled with mass spectrometry (GC-MS). The mass spectrometer used was a Finnigan GCQ ion trap. The capillary column specifications were the following: a DB-5ms column (5% phenylmethylploysiloxane stationary phase of 0.25 µm thickness; 30 m column length; 0.25 mm inner diameter). The temperature of the injector was 220°C. The initial temperature of the column was maintained 2 minutes at 70°C, then programmed to 320°C at 10°C/min and held maintained 3 min at 320°C. Helium was used as the carrier gas at a constant velocity of 50 cm/sec. Mass spectra were obtained in full scan (m/z 30-600) electron ionisation mode. One µl of

each extract was injected in the GC.

A PCA (Principal Component Analysis) was applied to the data matrix (specimens x relative abundance in %) obtained from GC/MS results of *B. t. terrestris* males (the same samples as used for the intrasubspecific ethological tests). This PCA was based on the compounds found in the pheromonal blend (n=99) of the 30 males used for the behavioural tests.

A Mantel test (Mantel, 1967) was applied to the pheromonal matrix used for the PCA and to a distance matrix between populations (km) to detect the relation between pheromonal and geographic distance. This test (10,000 permutations) was based on the specimens x specimens' Euclidian matrix of pheromones versus the matrix of geographic distances. A Spearman test was applied to the pheromonal distance and to the geographical distance in order to evaluate the correlation between these two distances.

Olfactometer

The behavioural tests were performed in an olfactometer made of a glass tray (70 x 70 x 8 cm) covered with a polycarbonate plate. A circular wire mesh was placed at the centre to avoid direct contact between the virgin queens and the secretions (to avoid the arrestant effect). A Petri dish (9.2 cm diameter) allowed us to confine the queen until she calmed down.

The arena was divided into 4 square areas (35 x 35 cm) in which a 2cm x 2cm paper was laid down: 1) an empty one (indicator area), 2) with 2.5 µl hexane; 3) with 2.5 µl of CLG secretion of a male from the same origin and 4) with 2.5 µl of CLG secretions of a male from a different origin (Fig. 4-6).

Positions of the 4 paper filters were randomly chosen for each experiment. The queen was dropped in the olfactometer, her movements were recorded with a digital camera (Philips SPC 900 NC PC Camera) and her position was noted every 5 seconds for 5 minutes (60 positions/test). The positions in each area were summed up. All the tests were done in a room with a temperature of 29°C and a relative humidity of 55-65%. A red light was used for the tests to avoid any visual cue. Between each test, all the system was cleaned up with an odourless soap (Panama®) and ethanol. Each possible pair was tested 10 times, randomly and with a different queen.

Statistical tests

A non-parametric χ^2 test was applied to the sums of queens positions (Ho= no differences between the 4 zones), and to the "secretions-secretions", and "secretions-solvent" pairs (Ho = no differences between the 2 zones) (fig. 4 - 5).

Control tests

Before the tests, controls were performed to assure that nothing in the room would bias the attractivity effect of CLG secretions. For these tests, no secretions were used in the behavioural experiments and the queen's movements were recorded while she was in the arena. The χ^2 results were not significant: the controls showed that the queens moved randomly in the 4 areas.

RESULTS

Principal Component Analysis of *B .t. terrestris* CLG secretions

This analysis shows 3 distinct groups (fig. 2). A first group with CLG secretions of males from Southwestern France, a second group with CLG secretions of males from Sweden and a group with the CLG secretions of males from Belgium, Southeastern France, Northern Frnace, Germany and Denmark. It seems that the CLG secretions of the various populations present differences. A Spearman test was applied to the pheromonal distance and the geographical distance; the result is "R = 0.57", which means there is correlation between the pheromonal and geographical distance.

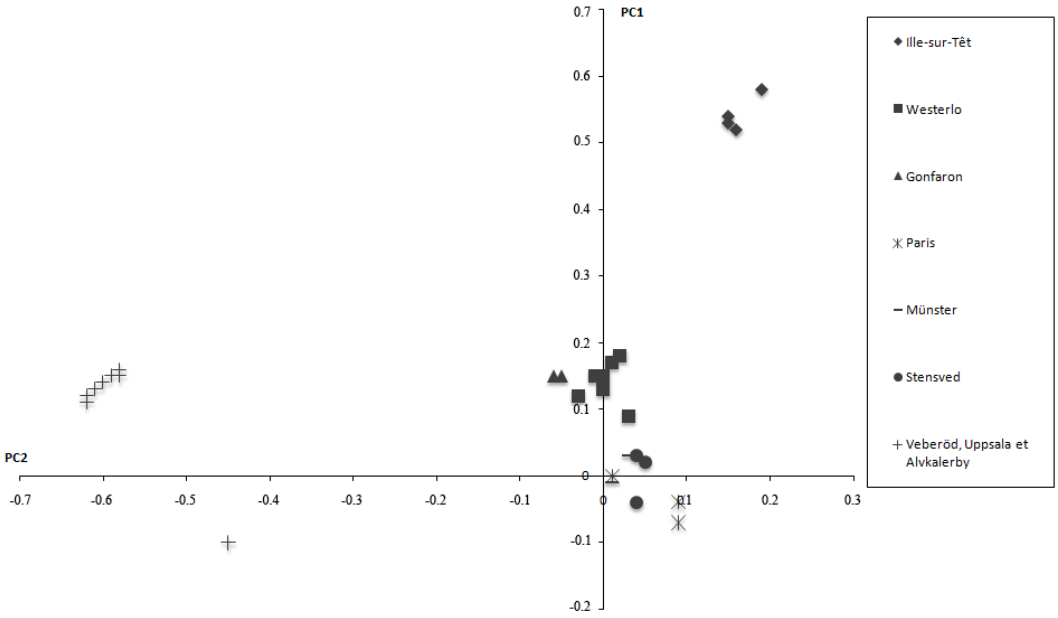


Figure 2. Two first axis of the PCA applied to CLG secretions of the males used for the infrasubspecific tests.

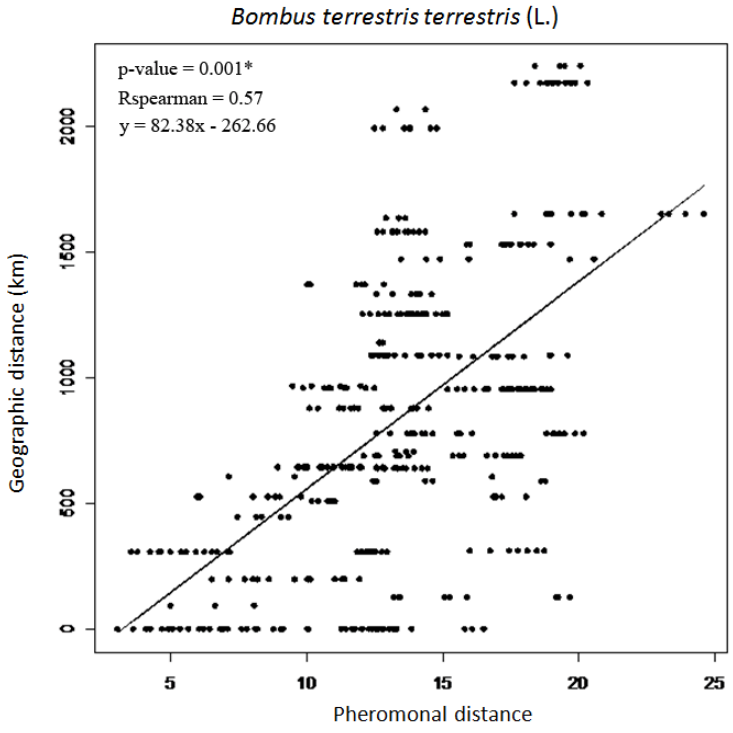


Figure 3. Mantel's correlogram and linear regression of Euclidian pheromonal distance and geographic distance of the specimens.

The null hypothesis of the Mantel test was: "no linear association between the pheromonal distance and the geographic distance". Our results (fig. 3) show a significant linear association between those two distances (p-value = 0.001). Moreover, the Spearman test resulted in R = 0.57, which also shows a correlation (even slight) between the pheromonal and geographical distances.

Infraspecific preferences.

Virgin queens of *Bombus terrestris dalmatinus*.

First of all, virgin queens of *B. t. dalmatinus* are obviously highly attracted to the consubspecific CLG secretions, no matter the other CLG secretions that are placed in the olfactometer (fig. 4a-e). The virgin queens of *B. t. dalmatinus* are more attracted by male secretions of different subspecies rather than by hexane, except when they have the choice between male secretions of *B. t. dalmatinus* and male secretions of *B. t. canariensis* (fig. 4b). In this last case, queens spent more time in the "solvent" zone. When the queen had to chose between 2 extracts of consubspecific males (fig. 4c), her presence did not statistically differ in one area or another. The χ^2 results of the secretions vs secretions and secretions vs solvent is represented by the arrows linking the 2 corresponding zones (fig 4a-e).

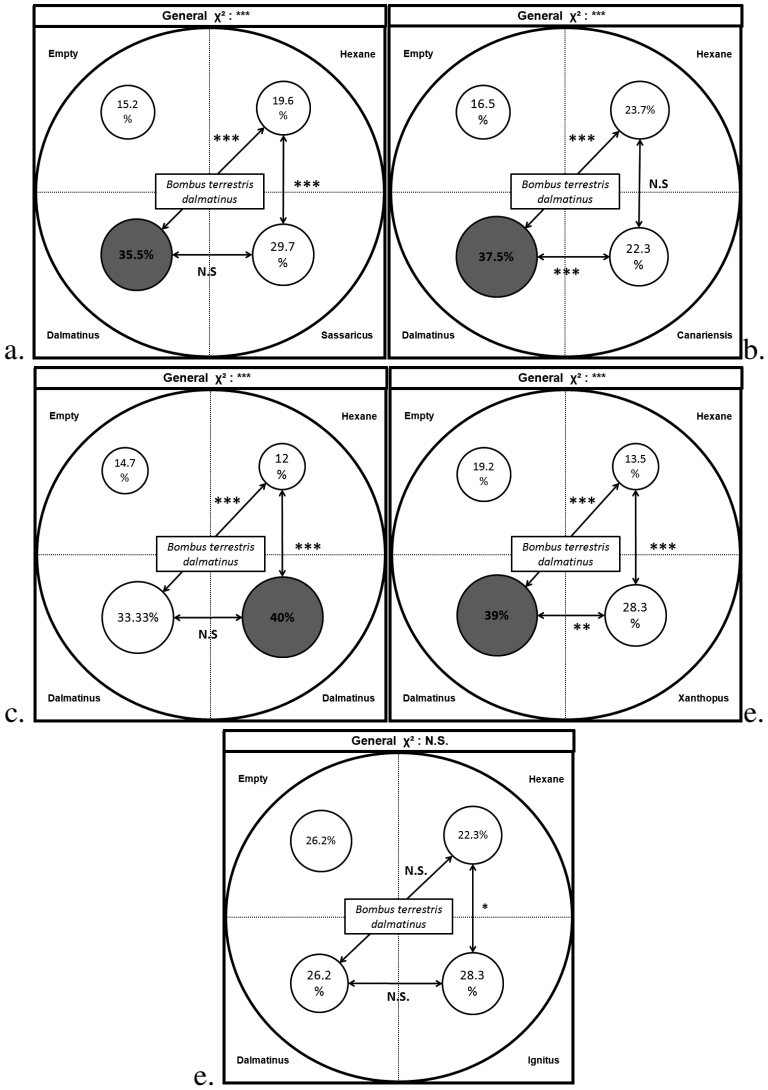


Figure 4. Presence (in %) of *B. t. dalmatinus* virgin queens in each area of the olfactometer, containing blank (Empty); pure hexane and different secretions. Down left is the consubspecific male secretion, and down left the non-consubspecific male secretions a: *dalmatinus* vs *sassaricus*; b: *dalmatinus* vs *canariensis*; c: *dalmatinus* vs *dalmatinus*; d: *dalmatinus* vs *xanthopus*; e: *dalmatinus* vs *ignitus* secretions.

Virgin queens of *Bombus terrestris xanthopus*

For this subspecies, the results obtained are similar to those for *B. t dalmatinus* (fig. 5a-e). In all cases, the queens spent most of the time in the consubspecific CLG secretions zone. When we tested two consubspecific extracts at the same time, the queen did not show a preference for either the extracts or the hexane (fig. 5a).

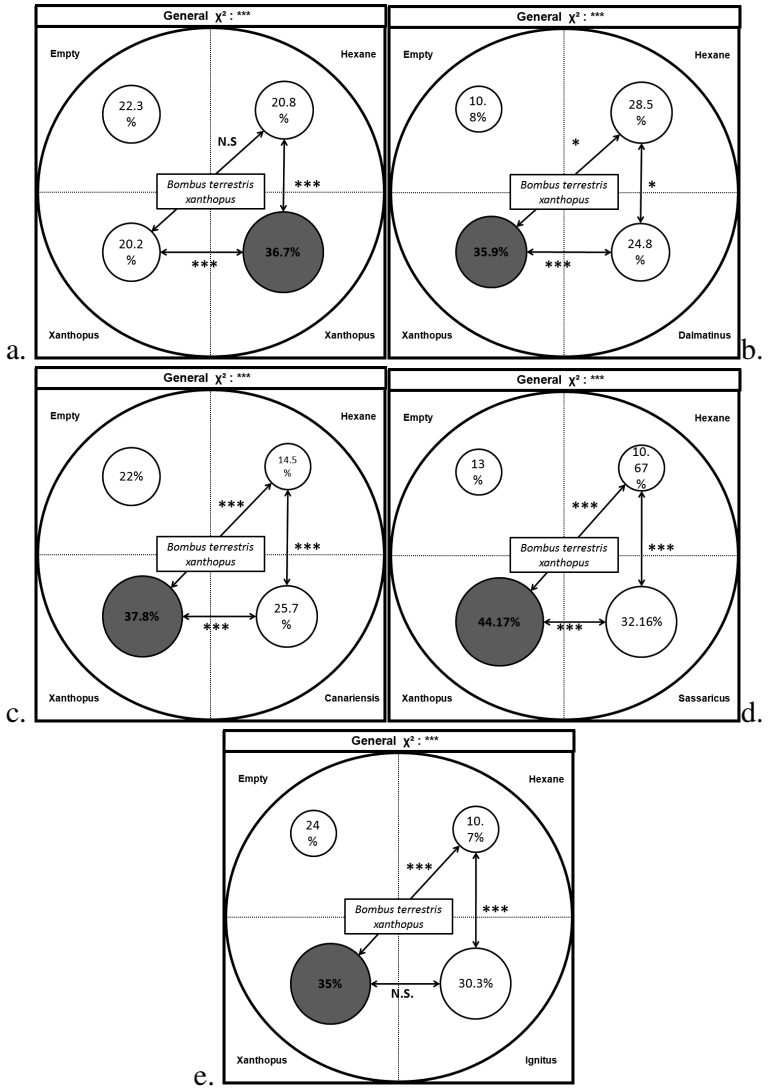


Figure 5. Presence (in %) of *B. t. xanthopus* virgin queens in each area of the olfactometer, containing blank (Empty); pure hexane and different secretions. Down left is the consubspecific male secretion, and down left the non-consubspecific male secretions a: *xanthopus* vs *xanthopus*; b: *xanthopus* vs *dalmatinus*; c: *xanthopus* vs *canariensis*; d: *xanthopus* vs *sassaricus*; e: *xanthopus* vs *ignitus*.

Confronted with *B. t. canariensis* male secretions, virgin queens of *B. t. xanthopus* and of *B. t. dalmatinus* had a strange behaviour (fig. 4b & fig. 5c): the queens moved all the time in the 4 areas and did not show any preference.

Infrasubspecific preferences

The queens prefer the CLG secretions of the more distant males. Moreover, in the PCA, those males have the most different CLG secretions (fig 2.).

Using the behavioural test, the queens from Belgium are highly attracted to the males from Sweden and by the males from Southwestern France (fig. 6a) in the same proportions. They do not seem to prefer the male secretions from Southeastern France or Germany: despite a greater presence in those zones, the result of the χ^2 test is not significant (fig. 6b). The queens from Belgium prefer hexane to male extracts from Paris (fig. 6c). Finally, comparing secretions of 2 related males from Belgium, the queen does not show any preference (fig. 6d).

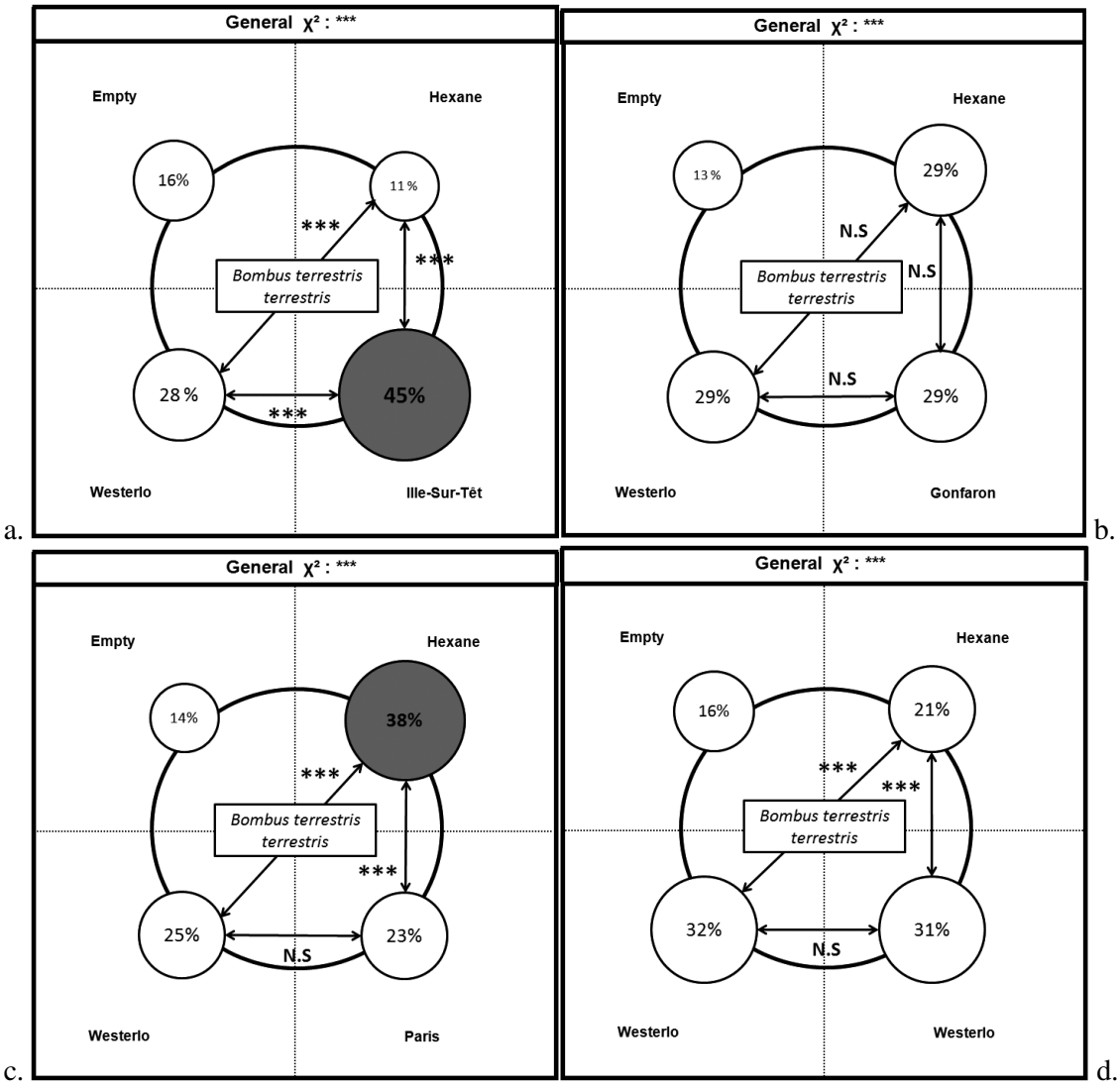


Figure 6. Presence (in %) of the virgin queen in each area of the olfactometer, containing as follows: a. secretion of males from Ille-sur-Têt (Southwestern France) vs secretions of males from Westerlo (Belgium); b. secretion of males from Gonfaron (Southeastern France) vs secretions of males from Westerlo (Belgium); c. secretion of males from Paris (Northern France) vs secretions of males from Westerlo (Belgium) ; d. secretion of males from Westerlo (Belgium) vs secretions of males from Westerlo (Belgium).

DISCUSSION

Infraspecific preference

The first aim of the present study was to compare the attractivity that virgin queens of *B. t. xanthopus* and *B. t. dalmatinus* exhibit towards CLG secretions of various *Bombus terrestris* subspecies.

The results obtained here are opposed to the sl-CSD theory. Virgin queens of *B. t. dalmatinus* or *B. t. xanthopus* showed an evident preference for males of their own subspecies, as described in Ings *et al.* (2005). This behaviour obviously presents inbreeding risks inside a population. Furthermore, this behaviour is contradictory with what has been described in other models like *Colletes cunicularius* among insects (Vereecken *et al.*, 2007) and fur seals (*Callorhinus ursinus*) among mammals (Hoffman *et al.*, 2007), in which the choice of the sexual partner is made in favour of unrelated or exotic conspecifics.

When *B. t. dalmatinus* virgin queens had to choose between *B. t. dalmatinus* and *B. t. sassaricus* male secretions, (fig. 4a) the results were non-significant. The CLG secretions of *B.t.sassaricus* were shown to be very different from those of other susbspecies (Coppée *et al.*, 2008; Coppée *et al.*, submit.), which could be an argument for to raise its taxonomic status to that of a good species, following the SMRS Concept of Paterson (1993). Ayasse *et al.* (2001) suggested that differences in sexual pheromones lead to reproductive isolation, which implies that populations no longer recognize sexual signals from distant populations.

B. t. canariensis CLG secretions cause strange behavioural responses in the virgin females (fig. 4b & 5c). There is also a possiblity that this taxon could be a species. Actually, the taxonomical story of *B. t. canariensis* is complicated. First, it was described by Pérez (1985) as a variety of *B. terrestris*, then redescribed as a good species (Pittioni, 1938b). Afterwards, this taxon was generally considered as a subspecies of *B. terrestris* (Krüger 1951; Krüger, 1958; Rasmont *et al.* 1986; Rasmont 1988; Hohmann *et al.*, 1993). However, Erlandsson (1979) defended a specific status for *canariensis*. Estoup *et al.* (1996) worked on the taxonomic status of *canariensis* using genetic analyses; they confirmed the subspecific status (Rasmont *et al.*, 2008). The present work clearly shows that *B. t. dalmatinus* virgin queens and *B. t. xanthopus* virgin queens recognize *B. t. canariensis* as non-conspecific.

Infrasubspecific preference

The second aim of the present study was to compare the attractivity of virgin queens of *B. t. terrestris* from Belgium towards CLG secretions of *B. t. terrestris* from different locations. Our hypothesis was that virgin queens were able to perceive the

chemical differences between the CLG secretions of consubspecific males from various locations.

B. t. terrestris virgin queens from Belgium are attracted to the CLG secretions of the most geographically distant consubspecific males. They prefer the CLG secretions of males from Southwestern France and Sweden to those of the ones from Belgium (Fig.6 a-d). They do not prefer secretions of males from the locations close to Belgium either (Paris and Germany). Instead, the virgin queens from Belgium only prefer the secretions of males from distant populations.

The PCA (fig.2) shows that males' CLG secretions from Southwestern France and from Sweden form 2 distinct groups. The other locations form a central homogenous group.

Many studies showed that individuals generally prefer to mate with proximate individuals (Boake, 2002; Wong *et al.,* 2004). Our results show that within the *Bombus terrestris terrestris* subspecies, virgin queens seem to prefer the CLG secretions of the more distant consubspecific males, which produce the most different CLG secretions.

Our results can be compared with those of Vereecken *et al.* (2006), considering the differences between *Colletes cunicularius* (L.) and *Bombus terrestris*. *C. cunicularius* is a monotypic solitary species, while *B. terrestris* is a polytypic eusocial species. Their results showing that *C. cunicularius* males are attracted by the sex pheromones of the distant (exotic) individuals have to be compared with the results we obtained in the infrasubspecific behavioural tests.

Our results also show that virgin queens prefer the distant consubspecific males. This is in line with the hypothesis expressed by Ayasse *et al.* (2001), and the sl-CSD theory (Cook, 1993).

B. terrestris is monandrous (Estoup *et al.*, 1995) and a male has to be the first to copulate with any of the new virgin queens to ensure his reproductive success. Prefering the most different, unrelated individuals can be an additional mechanism to prevent inbreeding. Females are the limiting sex. Their choice is very important since they invest more than males in the offspring. Their picking the fittest male is thus essential (Baer *et al.*, 2001).

Sl-CSD is very important because inbreeding increases the risk of homozygote production, which results in diploid male offspring instead of workers. Then, diploid males can lead to triploid offsprings, which are not viable. Inbreeding and its negative effects must be avoided. It has been shown that *Bombus terrestris* queens are able to recognize related individuals (Whitehorn *et al.*, 2009). It seems that other mechanisms also exist to prevent this phenomenon from occurring.

In conclusion, we showed that queens of *Bombus terrestris* prefer to mate with unrelated consubspecific individuals and are attracted to males with the most different CLG secretions within their taxon. It seems that sexual pheromones may contain important information used for inbreeding prevention.

Aknowledgements. We would like to thank the Fonds de la Recherche Fondamentale et Collective" (Project 2.4.564.06 F) and the Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture, which awarded a grant to Audrey Coppée. We are also grateful to Biobest bvba for having provided the biological material. We thank Dr. R. De Jonghe, Jan Vermeulen, Dr. N. E. Raine and Prof. Lars Chittka for their help in providing biological material.

References

Ayasse M, Paxton RJ, Tengö J. 2001. Mating behavior and chemical communication in the order hymenoptera. Annual Review of Entomology. 46:31-78.

Boake CRB. 2002. Sexual signaling and speciation, a microevolutionary perspective. Genetica. 116:205-214.

Bergman P. 1997. Chemical communication in bumblebee premating behaviour. In:

Department of Chemical Ecology. Göteborg University: Göteborg; 26p.

Bergström G. 1981. Chemical aspects of insect exocrine signals as a means for systematic and phylogenetic discussions in aculeate Hymenoptera. Entomologica scandinavica. Suppl. 15:173-184.

Calam DH. 1969. Species and sex-specific compounds from the heads of male bumblebees (Bombus spp.). Nature. 221:856-857.

Coppée A, Terzo M, Valterova I, Rasmont P. 2008. Intraspecific variation of the cephalic labial gland secretions in Bombus terrestris (L.) (Hymenoptera: Apidae). Chemistry & Biodiversity. 5:2654-2661.

Dapporto, L. E. Palagi and Turillazzi S. 2004. i S (2004) Cuticular hydrocarbons of Polistes dominulus as a biogeographic tool: a study of populations from the Tuscan Archipelago and surrounding areas. J Chem Ecol 30:2139–2151.

Dettner K, Liepert C (1994) ChemicaDuchateau M J, Hoshiba H, Velthuis HHW. 1994. Diploid Males in the Bumble-Bee Bombus-terrestris Sex Determination, Sex Alleles and Viability. Entomologia Experimentalis Et Applicata. 71(3):263-269.

Erlandsson A. 1979. Bombus canariensis Pérez, 1895 n. stat and Bombus maderensis n. sp. from the Macaronesian Islands. Entomologica scandinavica. 10:187-192.

Estoup A, Scholl A, Pouvreau A, Solignac M. 1995. Monoandry and polyandry in bumble bees (Hymenoptera - Bombinae) as evidenced by highly variable microsatellites. Molecular Ecology. 4(1):89-93.

Estoup A, Solignac M, Cornuet JM, Scholl A. 1996. "Genetic differentiation of continental and island populations of Bombus terrestris (Hymenoptera: Apidae) in Europe." Molecular Ecology. 5(1):19-31.

Hohmann H, La Roche F, Ortega G, Barquin J. 1993. Bienen, Wespen und Ameisen der Kanarischen inseln (Insecta : Hymenoptera : Aculeata). Übersee-Museum, Bremen, 894p.

Krüger E. 1951. Phänoanalytische Studien an einigen Arten der Untergattung Terrestribombus O. Vogt (Hymen. Bomb.). I. Teil. 93:141-197 (1950).

Krüger E. 1958. Phänoanalytische Studien an einigen Arten der Untergattung Terrestribombus O. Vogt (Hymen. Bomb.). III. Teil. 101:283-344.

Kullenberg B, Bergström G, Bringer B, Carlberg B, Cederberg B. 1973. Observations on scent marking by Bombus Latr. and Psithyrus Lep. males (Hym., Apidae) and localization of site of production of the secretion. Zoon Suppl. 1:23-30.

Pérez J. 1895. Voyage de M Ch. Alluaud aux Iles Canaries (Novembre 1889 – Juin 1890), 4e mém Hyménoptères. Annls Soc. Ent. Fr. 64:191-204.

Pittioni B. 1938. Die Hummelnu nd Schmarotzerhummedlne rB alkan-Halbinsel mit besondere Brerucksichtigung der Fauna Bulgariens.l : Allgemeiner Teil. Mitteilungen aus den Kôninglichen Nantrwissenschaftlichen Instituten in SoJia.

11:12-69.

Rasmont P, Scholl A, De Jonghe R, Obrecht E, Adamski A. 1986. Identité et variabilité des mâles de bourdons du genre Bombus Latreille sensu stricto en Europe Occidentale et Centrale (Hymenoptera, Apidae, Bombinae). Revue Suisse Zoology. 93:661-682.

Rasmont P. 1988. Monographie écologique et biogéographique des Bourdons de France et de Belgique (Hymenoptera, Apidae, Bombinae). Thèse de doctorat, Faculté des sciences agronomiques de l'Etat : Gembloux. 309p.

Rasmont P, Adamski A. 1995. Les bourdons de la Corse (Hymenoptera, Apidae, Bombinae). Notes fauniques de Gembloux. 31:3-87.

Rasmont, P, Coppee A, Michez D, De Meulemeester T. 2008. An overview of the Bombus terrestris (L. 1758) subspecies (Hymenoptera : Apidae). Annales De La Societe Entomologique De France. 44(2):243-250.

Svensson BG. 1979. Patrolling behaviour of bumble bee males (Hymenoptera, Apidae) in a subalpine/alpine area, Swedish Lapland. Zoon. 7:67-94.

Velthuis HHW, Van Doorn A. 2006. A century of advances in bumble bee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie. 37:421-451.

Vereecken N, Mant J, Schiestl F. 2007. Population differentiation in female sex pheromone and male preference in a solitary bee. Behavior Ecology Sociobiology. 61:811-821.

Whitehorn PR, Tinsley MC, Brown MJF, Darvill B, Goulson D. 2009. Impacts of inbreeding on bumblebee colony fitness under field conditions. Bmc Evolutionary Biology. 9.

Widmer, A, Schmid-Hempel P, Estoup A, Scholl A. 1998. Population genetic structure and colonization history of Bombus terrestris s.l. (Hymenoptera : Apidae) from the Canary Islands and Madeira. Heredity. 81:563-572.

Wong BBM, Keogh JS, Jennions MD. 2004. Mate recognition in a freshwater fish : geographical distance, genetic differentiation and variation in female preference for local over foreign males. J Evol Biol. 17:701-708.

Chapter 5

PUBLICATION IN PREPARATION

Coppée A., Lecocq T., Rasplus J.-Y., Michez D., Rasmont P.

Phylogenetic relationships among lineages of the *Bombus terrestris* (L.) [Hymenoptera, Apidae] complex using mitochondrial DNA sequences. In preparation.

Since the CLG secretions vary with the geographic origin of males and constitute a pre-zygotic isolation between subspecies, we wondered if there were any corresponding genetic differences within the model studied.

Phylogenetic relationships among lineages of the *Bombus terrestris* (L.) [Hymenoptera, Apidae] complex using mitochondrial DNA sequences

Audrey Coppée [1], Thomas Lecocq[1], Jean-Yves Rasplus[2], Denis Michez[1] & Pierre Rasmont[1]

[1]Laboratoire de Zoologie, Université de Mons- 20 Place du Parc B-7000 Belgium. audrey.coppee@umons.ac.be

[2]Centre de Biologie et de Gestion des Populations – Montferrier-sur-Lez, France. rasplus@supagro.inra.fr

Abstract – Bombus terrestris (L.) is a biological model of high interest among pollinators. It is not only a common species, easy to rear in laboratory conditions, but it also present a high commercial value, due to its use in glasshouse. Nine subspecies are described inside the *B.* terrestris species, but many authors do not agree on their subspecific status. Moreover, the analysis of the Specific-Mate Recognition System (SMRS) revealed intraspecific variations that raised questions about the taxonomic status of the taxon constituting *B. terrestris.* Several genetic studies have already been conducted on *B. terrestris* using mitochondrial DNA coupled with microsatellites. Those studies revealed a high polymorphism of several taxa, and a strong differentiation of the insular taxa compared to the continental taxa. The goal of this study was a better understanding of the phylogenetic relationships among the *B. terrestris* complex, using mitochondrial DNA only. We found two well supported (in MP, BM and ML) monophyletic groups constituted of the taxa *xanthopus* and *canariensis* + *africanus*. These results suggest a revision of the subspecific status of these 3 taxa using a comparison with the SMRS variations as well.

Keywords- Bumblebees, *Bombus terrestris*, Cytochrome Oxydase I, Cytochrome b.

Introduction

Since the 60s, *Bombus terrestris* has been the topic of many studies. That bumblebee species is a biological model of high interest: it is a very common eusocial Hymenoptera, with a large distribution across the West Paleartic region (fig. 1), and it is widely used in glass houses for crop pollination (Velthuis & van Doorn, 2006). *Bombus terrestris* includes 9 currently recognized subspecies (Rasmont et al., 2008), of which 5 are geographically isolated (fig. 1): *Bombus terrestris africanus* (Krüger) (North Africa), *Bombus terrestris audax* (Harris) (British Islands), *Bombus terrestris canariensis* Pérez (Canary Islands), *Bombus terrestris sassaricus* Tournier (Sardinia), *Bombus terrestris xanthopus* Kriechbaumer (Corsica, Capraia Island, and Elba Island). The subspecies *canariensis* has been considered as a good species by Erlandsson (1979). The last 4 subspecies live in sympatric conditions (fig. 1): *Bombus terrestris calabricus* Krüger (South Italia and Sicily), *Bombus terrestris dalmatinus* Dalla Torre (South-East France, North Italia, Balkanic Peninsula, Ukraine, Anatolia, Transcaucasia, Caucasus, Northern Iran, Southern Ural, Alaï, Altaï), *Bombus terrestris lusitanicus* Krüger (South-Western France, the Iberian Peninsula, the Balearic Islands, and Madeira), *Bombus terrestris terrestris* (L.) (Continental Europe). According to Erlandsson (1979), a supplementary subspecies, *Bombus terrestris maderensis* (Madeira), could be considered as living in parapatric conditions. However, *Bombus terrestris maderensis* is considered as a simple synonym of *Bombus terrestris lusitanicus* by Rasmont et al. (2008).

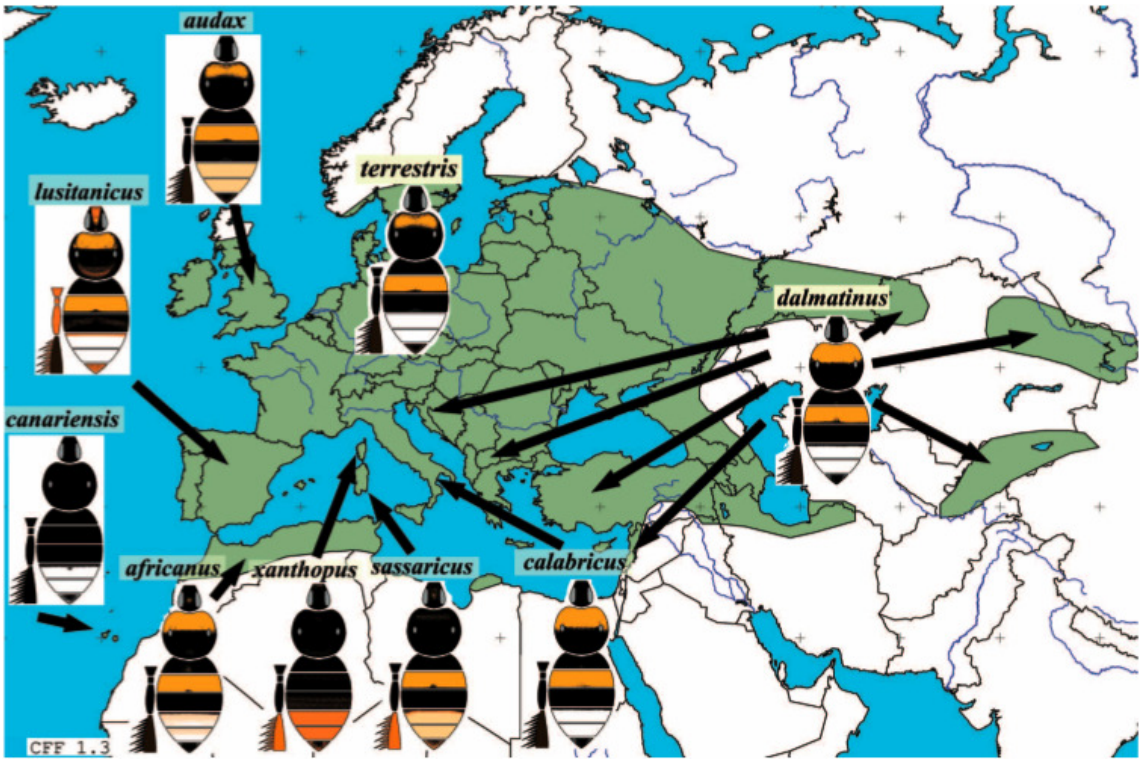


Figure 1. Geographic distribution of *Bombus terrestris* and colour patterns of the 9 described subspecies (from Rasmont et al., 2008).

Coppée et al. (2008, submitted) highlighted important variations among the sexual pheromonal blend displayed by males to attract virgin females, and particularly in *B. t. canariensis*, *B. t. sassaricus* and *B. t. xanthopus*. These pheromones involved in the Specific-Mate Recognition System (Paterson, 1985), are species-specific and suggest that the variations observed could correspond to specific differences rather than subspecific ones. Considering those variations through Paterson's Concept of Species Recognition (Paterson, 1985, 1993), there is a possibility for this variable Mate Recognition System to have impacts on the reproductive isolation of the different subspecies.

Unfortunately, the studies conducted on *Bombus terrestris* until now did not clarify the situation.

Several genetic studies have been conducted on *B. terrestris*, using mitochondrial DNA and microsatellites. Estoup et al. (1996) studied the genetic differentiation of

continental and insular populations of *B. terrestris*. Using ten microsatellites loci and a partial sequence of the COII mitochondrial gene, they discovered high levels of polymorphism in most populations. *B. t. canariensis* showed a significantly lower average calculated heterozygosity and observed allelic diversity as compared both to continental and island populations of *B. terrestris*. No significant differentiation was found among the continental populations. In contrast, island populations were strongly differentiated from continental populations. *B. terrestris* mitochondrial DNA (COII) was characterized by a low nucleotide diversity: the only haplotype found in the Tenerife population differed by a single nucleotide substitution from the most common continental haplotype of *B. terrestris*. Estoup *et al.* (1996) cast a doubt on the taxonomic status of *B. t. canariensis.* They explain that the genetic distance between the Tenerife and all other *B. terrestris* populations – estimated from microsatellite data – resulted likely from a severe bottleneck effect in the Canary Island population.

Later, Widmer et al. (1998) emphasized the genetic structure and colonization history of *B. terrestris* in Madeira and the Canary Islands using miscrosatellites and mitochondrial DNA (cytochrome b). They showed that the genetic differentiation among the islands, and between the islands and the continent as well, were extensive. They found that the distinctness of the Canary Islands population was strongly supported whereas the Madeira population was genetically closer to the continental populations. Their results suggested that ancestral haplotypes occurred on the Canary Islands, whereas derivative haplotypes were found on the European continent. Moreover, they showed that bumblebees from the Canary Islands and Madeira do not share a common colonization history.

In 2004, Beton compared 4 microstellites loci of *Bombus terrestris* populations from North Turkey (Ankara) and North Cyprus, which exhibited significant genetic distances.

The goal of this work is to review the taxonomical status of the nine *B. terrestris* subspecies thanks to phylogenetic analyses using mitochondrial DNA sequences.The results obtained could help to resolve the taxonomic uncertainties remaining.

Materials and Methods

Taxa examined

Due to its wide European distribution (fig. 1), we sampled all of the nine described subspecies of *B. terrestris* on the mainland and across different islands (tab. 2). A total of 94 specimens were collected (see tab. 2). Subspecies with a large-range distribution were sampled in a higher number of locations (fig. 2 – tab. 2). Sampled individuals were morphologically determined based on the susbspecies description available in Rasmont et al. (2008). The East Asian well distinct species *Bombus ignitus* was selected as outgroup for its phylogenetic affinities with the studied species (Kawakita et al. 2003, 2004; Shao et al., 2004; Cameron et al., 2007).

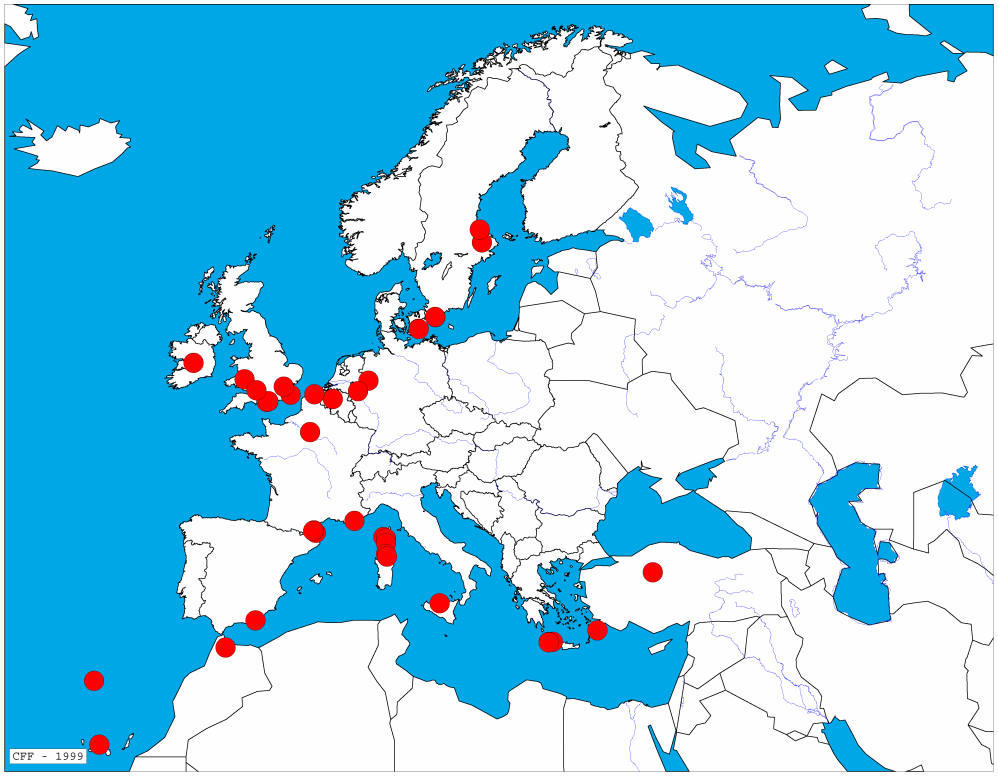


Figure 2. Geographic representation of the sampling.

Four techniques were used to collect males: 1) Capture of males in the field; 2) Capture of post-diapause queens and rearing in laboratory conditions until the end of the colony cycle and sexual production; 3) Capture of workers and rearing of orphan nests in laboratory condition until male production; 4) Rearing of commercial colonies (provided by Biobest bvba, Belgium) until male production.

Males were killed by deep freeze prior to dissection; their head was cut off and placed in a glass vial containing hexane (for the analysis of sexual pheromones) (De Meulemeester et al., in press) and their bodies were conserved in ethanol 99% for the molecular analysis.

Molecular Analysis

Genomic DNA was extracted from ethanol-preserved thoracic muscles or dried legs of each bee thanks to the QIAGEN Dneasy ® Animal Tissues kit following the manufacturers' protocols. The legs were crushed in liquid nitrogen before DNA extraction.

Genes Selection

As the taxonomic relationships between the sampled taxa have to be clarified, we selected mitochondrial genes known to be variable and useful to assess relationships at species and subspecies levels (Hines, 2008): two fragments of the Cytochrome oxydase I (COI) and one fragment of the Cytochrome b (Cyt b) (Gibbs, 2009; Pedersen, 2002; Shao et al., 2004).

For each specimen, the first section of the COI gene (COIa, ~658bp), the second part of COI gene (COIb, ~758bp) and the Cytb gene (~433bp) were amplified by PCR (see tab.1) using the primers LCO1490 and HCO2198 (Folmer et al., 1994; Hebert et al., 2003), C1-J-2183 and TL2-N-3014 (Simon et al., 1994), and CB1 and CB2 (Jerminn and Crozier,1994),

respectively. PCR conditions included an initial denaturation step of 3 min at 94°C and a final extension of 10 min at 72°C as shown in Table 1. Amplification products were purified and both strands of each PCR product were sequenced (using ABI 3730 sequencers) at the *Genoscope* (*Centre National de Séquençage*; Evry, France).

	COIa	COIb	Cyt b
Number of cycles	40	37	40
Denaturation	45sec/94°C	30sec/94°C	1min/94°C
Annealing	1min15/48°C	1min/48°C	1min30/47°C
Extension	1min30/72°C	2min/72°C	1min30/72°C
PCR mix (in µl) for 50 samples (total volume = 25µl/vial, including 2µl extracted DNA)	Taq (Qiagen): 6.25 MgCl2 (Qiagen): 62.5 Buffer 10x (Quiagen): 125 dNTP (MP biomedical): 25 Primer F (Eurogentec): 8.75 Primer R (Eurogentec): 8.75	Taq (Qiagen): 6.25 MgCl2 (Qiagen): 50 Buffer 10x (Quiagen): 125 dNTP (MP biomedical): 25 Primer F (Eurogentec): 8.75 Primer R (Eurogentec): 8.75	Taq (Qiagen): 6.25 MgCl2 (Qiagen): 50 Buffer 10x (Quiagen): 125 dNTP (MP biomedical): 25 Primer F (Eurogentec): 8.75 Primer R (Eurogentec): 8.75

Table 1. PCR Conditions and reagents used for the PCR amplification.

DNA Sequences

Sequences were edited with CodonCode Aligner 3.0.1 (Dedham, MA) and their bumblebee origin was checked with BLAST 2.2.20 (Zhang et al., 2000). After confirming the identity of the sequences from specimens of each sampled location, only one randomly-selected sequence for each subspecies/location was used in the following analyses (tab. 2). The alignement was performed by MAFFT ver.6. (default parameters) (Katoh et al., 2002). The translation in proteins (using the drosophila mtDNA genetic code) for verification of the data matrix was performed in Mesquite 2.6 (Maddison & Maddison, 2007). New sequences were deposited in GenBank under accession numbers xxxx to xxxx (tab. 2) and inferred trees can be downloaded from Treebase (Sanderson et al., 1993) (tab. 2 & 3).

Vouchers and PCR products are conserved at the CBGP (Monteferrier-sur-Lez, France).

A test of saturation (performed in Paup* version 40b10 Swofford, 2010) was applied to each fragment. The absolute number of transitions and transversions for all pairwise comparisons of individuals were plotted against pairwise genetic distance to determine if either had reached saturation

Sample n°	Taxon (n = number of analysed sample)	Males origin (rec)	Origin (WGS 84)	Genbank accession numbers		
				COIa	COIb	Cytb
AC154	*B. ignitus* n= 5	CC (AC)	Sapporo, Japan	XXXX	XXXX	XXXX
AC113	*canariensis* n= 4	CC (AC)	Teneriffe, Canaries Islands	XXXX	XXXX	XXXX
Sas026 (consensus)	*sassaricus* n= 7	QR (NR)	Sardinia 40°47'27"N 08°03'12"E	XXXX	XXXX	XXXX
AC215	*terrestris* n= 4	W (AC)	S.E. France 43°25'32"N 06°25'23"E	XXXX	XXXX	XXXX
PR019 (consensus)	*terretris* n= 4	W (PR)	N. France 48°50'37"N 02°21'35"E	XXXX	XXXX	XXXX
TD039	*terrestris* n= 4	W (TD)	S.W. France 42°28'38"N 01°55'04"E	XXXX	XXXX	XXXX
AC219	*terrestris* n= 4	W (AC)	Belgium 50°50'22"N 04°23'44"E	XXXX	XXXX	XXXX
PR026	*terrestris* n= 3	W (PR)	Germany 51°56'27"N 07°33'03"E	XXXX	XXXX	XXXX
PR032	*terrestris* n= 3	W (PR)	Danemark 54°59'01"N 12°00'32"E	XXXX	XXXX	XXXX
PR060	*terrestris* n= 5	W (PR)	Sweden 59°51'43"N 17°38'00"E	XXXX	XXXX	XXXX
AC257	*xanthopus* n= 5	W (AC)	France 42°17'25"N 08°52'40"E	XXXX	XXXX	XXXX
AC413	*dalmatinus* n= 5	W (AC)	Crete 35°23'28"N 23°34'30"E	XXXX	XXXX	XXXX
HU003	*dalmatinus* n= 4	W (MA)	Turkey 39°55'54"N 32°51'48"E	XXXX	XXXX	XXXX
PR008	*dalmatinus* n= 4	W (PR)	S.E. France 43°25'32"N 06°25'23"E	XXXX	XXXX	XXXX
AC435	*lusitanicus* n= 3	W (AC)	Madeira 32°48'01"N 16°50'43"W	XXXX	XXXX	XXXX
TD037	*lusitanicus* n= 3	W (TD)	S.W. France 42°28'51"N 01°55'25"E	XXXX	XXXX	XXXX
DM044	*lusitanicus* n= 5	W (DM)	Spain 36°42'59"N 04°25'03"W	XXXX	XXXX	XXXX
AC459	*africanus* n= 5	W (AC)	Morocco 35°03'40"N	XXXX	XXXX	XXXX

			05°09'60"W			
AC476	*calabricus* n= 5	WR (AC)	Sicily 35°59'35"N 13°52'49"E	XXXX	XXXX	XXXX
DT008	*audax* n= 5	W (TD)	Ireland 52°57'03,3"N 9°04'41,8"W	XXXX	XXXX	XXXX
PR222	*audax* n= 3	W (PR)	N.UK 51°05'43,0"N 0°38'33,2"E	XXXX	XXXX	XXXX
RG016	*audax* n= 5	W (RG)	S.W. UK 50°21'33"N 03°50'15"W	XXXX	XXXX	XXXX

Table 2. Voucher number and origin of the sequences used in the alignments.

Consensus means that a consensus was built from several samples to obtain a complete dataset for each taxa included in the analyses. Genbank accession numbers are given to each gene fragment obtained by PCR. Origin of males: CC= commercial colony; QR= queen rearing; WR= worker rearing; W= wild males. Rec.= recorder; AC= Audrey Coppée; PR= Pierre Rasmont; TD= Thibaut De Meulemeester; NR= Nigel E. Raine ; DM = Denis michez ; MA = Murat Aytekin.

Phylogenetic Analyses

We analyzed each gene (COIa, COIb, Cytb) separately and in combination for a total of 22 taxa using Maximum Parsimony (MP), Maximal Likelihood (ML) and Bayesian methods (BM). All trees were rooted with sister-species *B. ignitus* (tab. 3).

MP analyses were performed with PAUP*version 40b10 (Swofford 2001) using a heuristic search (1000 random additions and TBR branch swapping, keeping the best trees only). Clades support values were estimated using non-parametric bootstrapping (Felsenstein, 1985) in Paup* (10000 replicates, 1000 random additions, 500 trees saved per replicate).

For ML and BM analyses, the Akaïke information criterion (Akaïke, 1974) in Jmodeltest (Posada, 2008) was used to determine the model of evolution that best fitting our data. Selected models for each dataset are presented in table 3.

Dataset	Number of base pair	Informative characters	N° Treebase	Model 1st position	Model 2nd position	Model 3rd position
COIa	~658	26	MP: XXX ML: XXX BM: XXX	GTR	F81	GTR
COIb	~758	36	MP: XXX	GTR	F81	HKY+G

			ML: XXX BM: XXX			
Cytb	~433	3	MP: XXX ML: XXX BM: XXX	GTR	F81	GTR+G
Combined dataset	~1849	65	MP: XXX ML: XXX BM: XXX	See above	See above	See above

Table 3. Number of parsimony-informative characters, Treebase accesion numbers and models of evolution used for Parsimony, Bayesian and likelihood analyses.

ML analyses were implemented in GARLI (Zwickl, 2006). A random starting tree and the automated stopping criterion were used (stop when the ln score remains constant for 20,000 consecutive generations). Ten independent runs were carried out for each gene and for combined data. The topology and log-likelihood were identical among replicates. The best likelihood of those runs was kept. Statistical confidence in nodes was evaluated using 100 non-parametric bootstrap replicates (Felsenstein, 1985) using the automated stopping criterion set at 10,000 generations. Branches present in ≥ 70% of the bootstrap trees were considered well supported following Hillis & Bull (1993).

BM analyses were implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Four independent analyses were carried out for each gene and for the combined data set (5,000,000 generations; 4 chains with mixed-models; default priors; saving trees every 100 generations). The analyses were stopped after checking to log-likelihood with Tracer 1.2 (Rambaut & Drummond, 2003). The first million generations were discarded as burn-in. The phylogeny and posterior probabilities were then estimated from the remaining 4,000,000 trees and resumed in a consensus tree (MJ50). Topology with ≥95% posterior probabilities were considered as well supported following (Wilcox et al., 2002).

Results

Molecular analysis

A total of 658bp, 758bp and 433bp were obtained for the COIa, COIb and Cytb, respectively. The results of independent analyses (MP, BM and ML) realized on 4 matrices (each gene separately and in combination) (a total of 12 trees, see tab. 3) showed highly resolved and well-supported phylogenies. Whatever the data matrix considered, *xanthopus* and *africanus* + *canariensis* formed two distinct basal monophyletic groups strongly supported (see fig. 3), while the other populations form a single monophyletic group. No common geographic differentiation patterns have been highlighted among the continental populations.

Nevertheless, in the BM analysis (using the combined dataset) the S.W. France and the UK + Sardinia samples form two monophyletic well supported groups, separated from the continental samples. The North France sample is separated from the undifferentiated continental samples in MP. No monophyletic separation inside the continental samples was observed in ML.

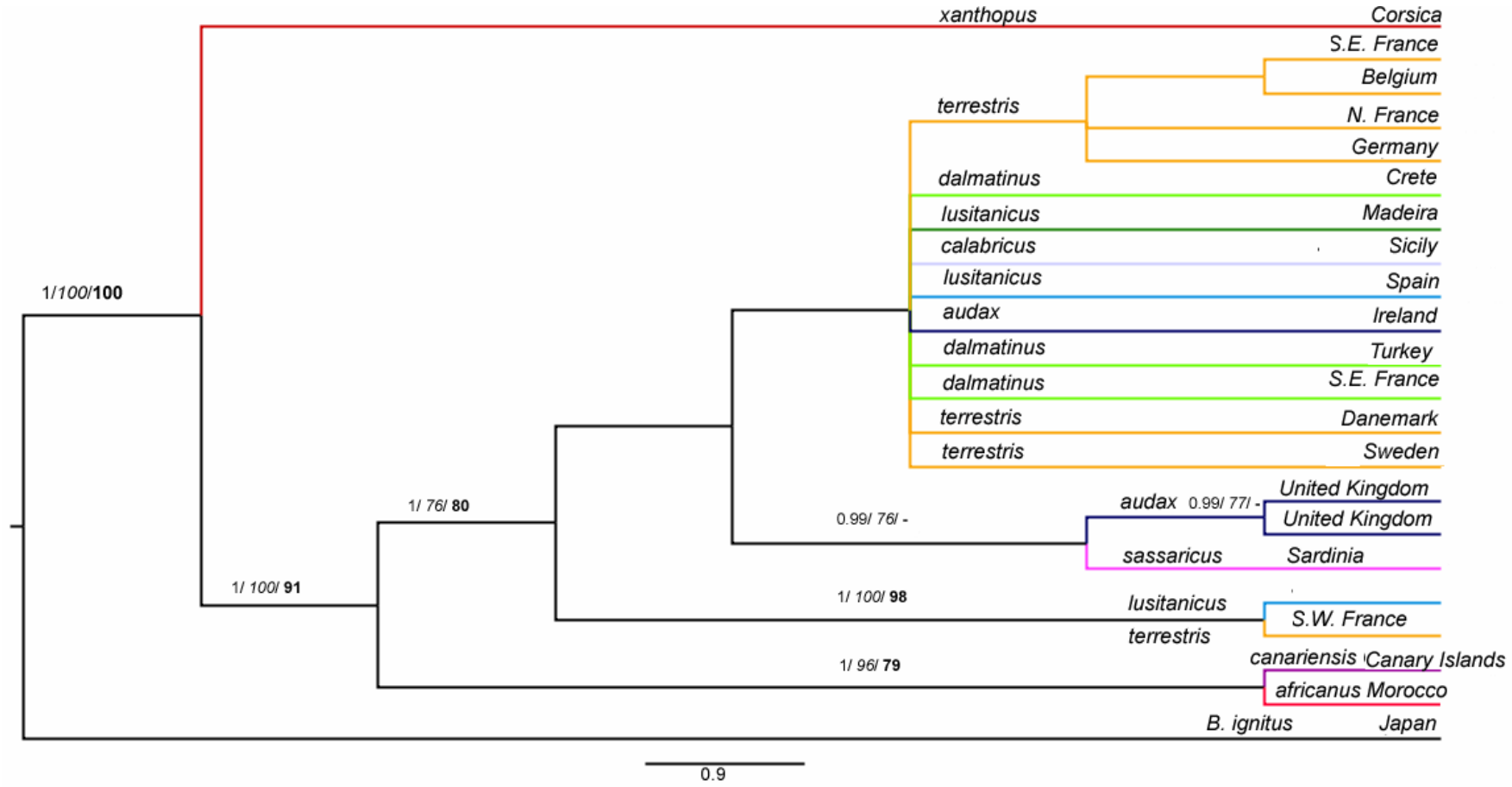


Figure 3. Maximum Parsimony tree consensus of 48 trees obtained by 50% majority rule; Tree lengh: 223bp; 65 parsimony informative characters; CI=0,8655, HI= 0,1345, RI= 0,7391; RC= 0,6397), rooted with *B. ignitus.* MB posterior priorities are given in normal font, MP bootstrap values are given in italic font and ML bootstrap values are given in bold font. Tree lengh: 223bp; 65 parsimony informative characters; CI=0,8655, HI= 0,1345, RI= 0,7391; RC= 0,6397.

Discussion

Our genetic results match those that were shown previously, but we took more populations into account. Estoup et al. (1996) showed a high polymorphism between insular populations using mitochondrial DNA and microsatellites. The insular populations were characterized by a higher polymorphism than the one observed between the continental ones. For Estoup et al (1996) the most differentiated subspecies was *B. t. canariensis*. Using the same techniques, Widmer et al.(1998) highlighted the genetic distinctness of *B. canariensis* and the similarity of *B. maderensis* with *B. terrestris* continental population. In the present study, we confirmed the genetical distinctness of *canariensis* + *africanus.* Furthermore, we showed that it is a sister group of all the other *terrestris*. We also proved the genetic divergence of *xanthopus* situated at the more basal position, instead of *canariensis* as previously described (Estoup et al. 1996).

The basal phylogenetic position of the subspecies from North-Africa, Canary Islands and Tyrrhenian Islands can be explained by post Ice-Ages continental colonization events or by the island rule (review in Blondel 1995; Rasmont & Adamski, 1995; Rasmont & Quaranta, 1997). On one hand, the island would have been colonized during the last Ice-Ages by plesiomorphic populations. Thereafter, these populations should have been extincted and replaced by other apomorphic populations. On the other hand, Millien & Damuth (2004) show the evolution of insular taxa to have decelerated due to the island effect. This observation can explain the phylogenetic topology of our study too. Further studies on the consequence of isolation on bumblebees evolution would probably allow to explain these observations.

The more differentiated taxa in our phylogeny are the same as those having the most different Mate Recognition System. The taxa x*anthopus, canariensis* and *africanus* show a genetic differentiation that raise question about their taxonomic status. Since the genetic tools avalaible at this time are not sufficient to solve the taxonomic questions we suggest to use

other tools as sexual pheromones for a better understanding of the situation.The recent studies about the sexual pheromones of *Bombus terrestris* highlighted variations of the specific recognition cues among the different subspecies described. Following Paterson's Recognition Concept of Species, these variations could have impact on the reproductive isolation of the different taxa. If these variations are congruent with the phylogeny described here, a revision of the taxonomic status of some of the *B. terrestris* subspecies will be necessary.

Acknowledgements

AC & TL thank the Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture (FRIA). The authors would like to thank Drs. Denis Michez, Murat Aytekin, Thibaut De Meulemeester for their kind help in collecting bees; and Gwenaëlle Genson for her kind help

References

Akaike, H. 1974. A New Look at the Statistical Model Identification. IEEE Trans. Autom. Control Volume AC-19:716-723.

Beton, D. 2004. Morphometric and genetic differentiation between Anatolia and Cyprus *Bombus (Bombus) terrestris* (L. 1758) populations*in* Graduate school of natural and applied sciences.

Blondel, J. 1995. Biogéographie : Approche écologique et évolutive, Paris.

Cameron, S. A., H. M. Hines, and P. H. Williams. 2007. A comprehensive Phylogeny of the Bumble Bees (*Bombus* Latreille). Biological Journal of the Linnean Society 91:161-188.

Coppée, A., M. Terzo, I. Valterova, and P. Rasmont. 2008. Intraspecific Variation of the Cephalic Labial Gland Secretions in Bombus terrestris (L.) (Hymenoptera: Apidae). Chemistry & Biodiversity 5:2654-2661.

Coppée A., Helderweirt A., Mathy T., Cammaerts M.-C., Iserbyt S., Rasmont P. Geographic-Dependant attractivity of males‘ sexual pheromones in *Bombus terrestris* (L.) (Hymenoptera, Apidae). Submitted.18pp.

De Meulemeester T., Gerbaux P., Boulvin M., Coppée A., Rasmont P. In press. A simplified protocol for bumble bees species identification by cephalic secretion analysis. Insectes Sociaux, 21 pp.

Erlandsson S. 1979. Bombus canariensis Pérez, 1895 n. stat. And B. maderensis n. sp. From the Macaronesian Islands.Entomologica Scandinavica 10: 187-192

Estoup, A., M. Solignac, J.-M. Cornuet, J. Goudet, and A. Scholl. 1996. Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. Molecular Ecology 5:19-31.

Felsenstein, J. 1985. Phylogenies and the Comparative Method. The American Naturalist 125:1.

Folmer O., M. Black, W. Hoeh, R. Lutz, R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxydase subunit I form diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 3:294-299.

Gibbs, J. 2009. Integrative taxonomy identifies new (and old) species in the Lasioglossum (Dialictus) tegulare (Robertson) species group (Hymenoptera, Halictidae). Zootaxa:1-38.

Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. DeWaard. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London Series B-Biological Sciences 270:313-321.

Hillis, D. M. & J. J. Bull. 1993. An Empirical-Test of Bootstrapping as a Method for Assessing Confidence in Phylogenetic Analysis. Systematic Biology 42:182-192.

Hines, H. 2008. Historical Biogeography, Divergence Times, and Diversification Patterns of Bumble Bees (Hymenoptera: Apidae: Bombus). Systematic Biology 57:58-75.

Jerminn & Crozier .1994. The cytochrome b region in the mitochondrial DNA of the ant *Tetraponera rufoniger*: sequence divergence in hymenoptera may be associated with nucleotide content. Journal of Molecular Evolution. 38:282-294.

Katoh, K., K. Misawa, K. Kuma, and T. Miyata. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30:3059-3066.

Kawakita, A., T. Sota, J. S. Ascher, M. Ito, H. Tanaka, and M. Kato. 2003. Evolution and Phylogenetic Utility of Alignement Gaps Within Intron Sequences of Three Nuclear Genes in Bumble Bees (*Bombus*). Molecular Biology and Evolution 20(1):87-92.

Kawakita, A., T. Sota, M. Ito, J. Ascher, H. Tanaka, M. Kato, and D. W. Roubik. 2004. Phylogeny, historical biogeography, and character evolution in bumble bees (*Bombus*: Apidae) based on simultaneous analysis of three nuclear gene sequences. Molecular Phylogenetics and Evolution 31:799-804.

Maddison, W., & D. Maddison. 2007. Mesquite : a modular system for evolutionnary analysis.

Millien, V. & J. Damuth. 2004. Climate change and size evolution in an island rodent species: New perspectives on the island rule. Evolution 58:1353-1360.

Paterson, H. E. H. 1985. The Recognition Concept of Species. Transvaal Museum Monograph, Pretoria.

Paterson, H. E. H. 1993. Evolution and the recognition concept of species. The Johns Hopkins University Press.

Pedersen, B. V. 2002. European bumblebees (Hymenoptera: Bombini) - phylogenetic relationships inferred from DNA sequences. Insect Systematics and Evolution 33:1-23.

Posada, D. 2008. jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution 25:1253-1256.

Rambaut, A. & A. Drummond. 2003. Tracer.

Rasmont, P. & A. Adamski. 1995. Les Bourdons de la Corse (Hymenoptera, Apoidea, Bombinae). Notes Fauniques de Gembloux, 31:3-87.

Rasmont, P. & M. Quaranta. 1997. I Bombi dell'Arcipelago Toscano (Hymenoptera Apidae). Boll. Soc. entom. ital. 129 (1):31-38.

Rasmont, P., A. Coppée, D. Michez, and T. De Meulemeester. 2008. An overview of the *Bombus terrestris* (L.1758) subspecies (Hymenoptera: Apidae). Annales de la Société Entomologique de France 44:243-250.

Ronquist, F. & J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.

Sanderson, M. J., B. G. Baldwin, G. Bharathan, C. S. Campbell, C. Vondohlen, D. Ferguson, J. M. Porter, M. F. Wojciechowski, and M. J. Donoghue. 1993. The Growth of Phylogenetic Information and the Need for a Phylogenetic Data-Base. Systematic Biology 42:562-568.

Shao, Z.-Y., H.-X. Mao, W.-J. Fu, M. Ono, D.-S. Wang, M. Bonizzoni, and Y.-P. Zhang. 2004. Genetic structure of asian populations of *Bombus ignitus* (Hymenoptera: Apidae). Journal of Heredity 95:46-52.

Simon 1994. Evolution, Weighting and phylogenetic utility of mitochonfrial gene sequences and a compilation of conserved polymerase chain reaction primers . Annals of the Entomological Society of America. 87 (6).

Swofford, D. L. 2001. PAUP 4.0b10, version 4.0b10. Sinauer Associates, Sunderland.

Velthuis, H. H. W. & A. van Doorn. 2006. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie 37:421-451.

Widmer, A., P. Schmid-Hempel, A. Estoup, and A. Scholl. 1998. Population genetic structure and colonization history of *Bombus terrestris* s.l. (Hymenoptera: Apidae) from the Canary Islands and Madeira. Heredity 81:563-572.

Wilcox, T. P., D. J. Zwickl, T. A. Heath, and D. M. Hillis. 2002. Phylogenetic relationships of the dwarf boas and a comparison of Bayesian and bootstrap measures of phylogenetic support. Molecular Phylogenetics and Evolution 25:361-371.

Zhang, Z., S. Schwartz, L. Wagner, and W. Miller. 2000. A greedy algorithm for aligning DNA sequences. Journal of Computational Biology 7:203-214.

Zwickl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion.The University of Texas at Austin, Austin.

Chapter 6

DISCUSSION

Chapter 6: Discussion

6.1. Pheromonal variations

6.1.1. Age-dependent variations

As described in chapter 2, it is now clear that sexual pheromones evolve with age quantitatively as well as qualitatively. The CLG secretion dynamics that we observed in *B. terrestris* closely recalls the description of Ågren et al. (1979) for *Bombus lapidarius* and *B. hypnorum*. Those authors explained this evolution pattern considering the fact that males do not leave the nest during the first two days following emergence. At this time, they are unable to fly and their production of pheromones is not fully operational. They may stay in the nest for 2-7 days depending on weather conditions (Hoffer, 1882; Free & Buttler, 1959). During this time, males develop both physically and sexually so that the sexual pheromone production reaches a maximum after a few days, by the time the males leave the nest. At the same time, females should save and store as much energy as possible for the subsequent period of hibernation. They should therefore minimize their energy expenditure while they are searching for a mate. Thus, it is the male that expends the most energy in maintaining the chemical signals and the premating activities in this system.

In our study, we showed that the CLG secretions evolve according to the histological pattern previously described by Ågren et al. (1979) and Šobotník et al. (2008) with slight differences in the age at which changes occur. We considered many more age classes than was previously done, thanks to improvements in rearing techniques. Nevertheless, we observed a low quantity of secretions in 0-day-old males. This quantity increased gradually until the age of 15 days, and then decreased until the age of 40 days.

Besides the quantitative description, we also conducted an exhaustive qualitative study of the CLG secretions, their age-dependent variations, and their attractivity to virgin females. We showed that the compounds of CLG secretions follow two profiles. Most of the compounds increase (in relative abundance) from the first day after emergence until males are 15 days old and then decrease. The others are less abundant in 1- to 15-day-old males and then increase (i.e. tricosane, tricosene, henicosane, tetradecenoic acid,

pentacosene, heptacosene, heptacosane, nonacosene, and geranylcitronellyl tetradecanoate). Differences in secretion composition lead to preferences of virgin queens for males according to the males' age. Virgin queens prefer the pheromonal gland secretions of bees of the following ages in increasing order: 1, 3 and 30 days < 7 days < 15 days < 10 days. The virgin queens are much more attracted by secretions containing high amounts of 2,3-dihydrofarnesol, 2,3-dihydrofarnesal, ethyl dodecanoate and hexadecanol. On the other hand, they are not attracted to 30-day-old males, likely senescent, which have a highly abundant level of geranylcitronellol. These observations were in agreement with the electroantenographic (EAG) study conducted by Žácek et al. (2009) (Appendix 3). They observed significant changes in the EAG-active compounds over the lifetime of males, with a maximal concentration at 7 days after emergence.

Age-dependent variations have already been described in other animals. In *Myrmica rubra* (Hymenoptera, Formicidae) the trail pheromone is not produced in young workers, but its secretion increases with age and worker function, reaching a maximum in old, forager workers (Cammaerts & Verhaeghe, 1974). The same is true for the sexual pheromones of the Sorghum plant bug *Stenottus rubrovittatus* (Oku & Yasuda, 2010).

In bumblebees this "age-dependent" strategy might play a role in the avoidance of inbreeding. *Bombus terrestris* has an sl-CSD (single-locus Complementary Sex Determination) system of sex determination. As a consequence, inbreeding increases the number of diploid males, which are less fit than haploid ones. The males spend the first day of their life inside the nest for a final physiological and physical maturation (Ågren et al., 1979). During this time, they should not be attractive to their sister virgin females, to avoid inbreeding risks. This is what is observed here: males are attractive for virgin females only at the time they leave the nest.

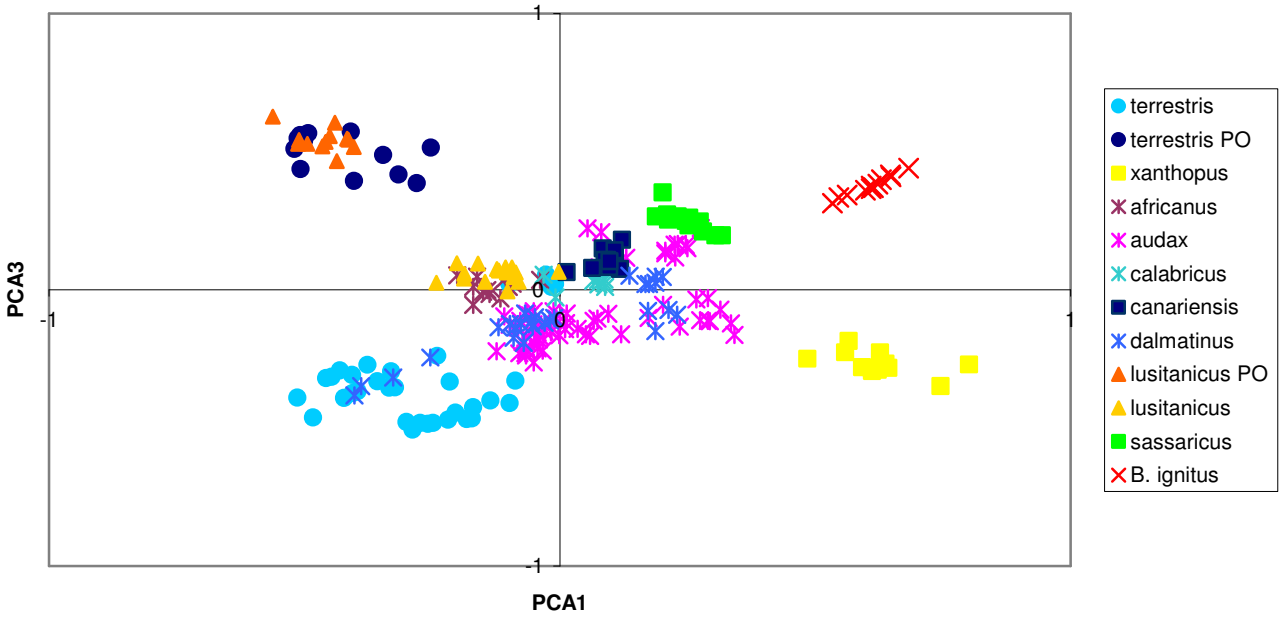
Anyway, the composition of CLG secretions changes during the lifetime of male *Bombus terrestris* and this has to be taken into account in further studies. When workers or emerging queens are reared in laboratory conditions, the age of studied males can easily be controlled. This not the case when males are caught in the wild. Nevertheless, it is impossible to catch males that are too young since they have not left the nest yet. Very old males are easily recognizable by the loss of thoracic hairs, the damaged wing margin and the faded coat colour (Tkalců, 1969). Analytically, older males have a lesser relative

amount of CLG secretions, which can be observed thanks to the Total Ion Current (TIC) of the GC. In males from 7 to 15 days old, it is at least 10^7. In the very young or very old specimens, the TIC is always below 10^7. In our different studies, males with a relative secretion concentration presenting a TIC lower than 10^7 where excluded from the analyses. As a further development, it would be interesting to have a statistical tool determining whether a male is too old or not, depending on the TIC.

6.1.2. Geographic-dependent variations

Cephalic Labial Gland secretions not only vary during the males' lifetime, but also depend on their geographic origin. In chapter 3, we showed that there were significant differences between the CLG secretion compositions of 4 subspecies of *Bombus terrestris*: *terrestris*, *lusitanicus*, *sassaricus* and *dalmatinus*, in allopatry or sympatry. All the CLG secretions of the taxa were analysed using GC/MS (unpublished results, see Appendix 4), compared using a Principal Component Analysis (PCA) (fig. 17 a & b) and a Linear Discriminant Analysis (LDA) (fig. 18 a & b) The method of extraction and analysis of the results were the same as used in Chapter 3 and Appendix 2. The relative areas were normalised (to 100%/sample), transformed (log1) and standardised (-mean/standard deviation).

a. Axis 1 & 3 of the PCA



b. Axis 2 & 3 of the PCA

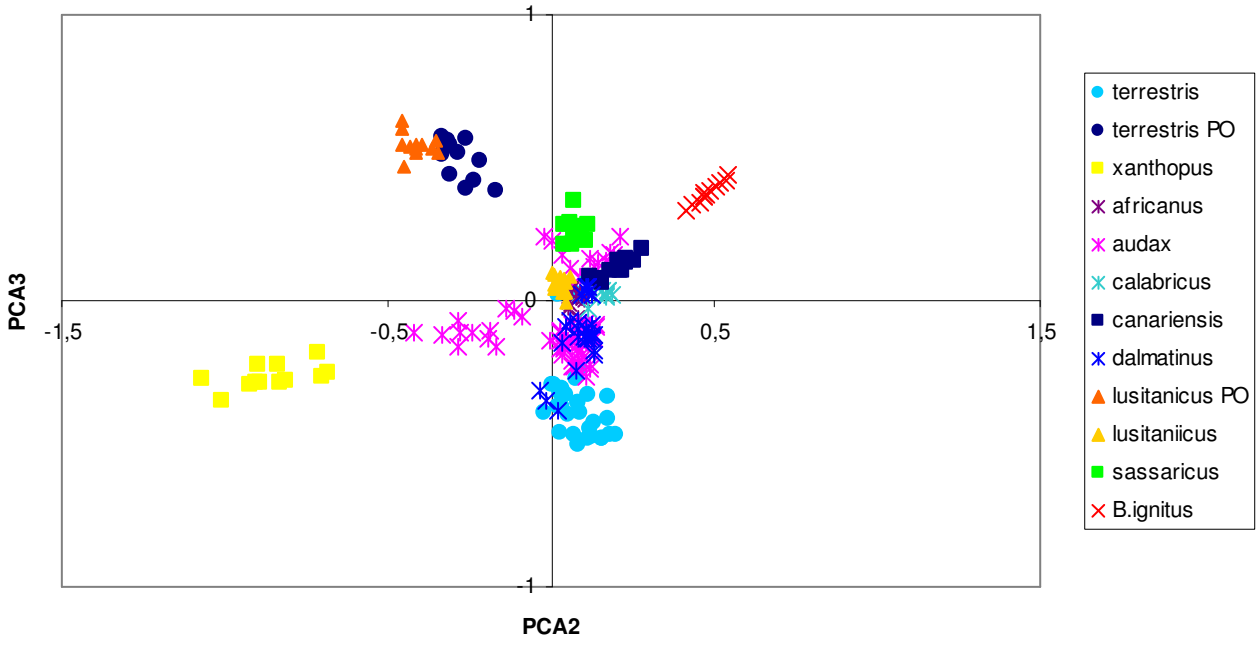


Figure 17. a. Principal Component Analysis of the compounds x specimens'matrix, projection of axis 1 & 3; b. Principal Component Analysis of the compounds x specimens'matrix, projection of axis 2 & 3. PO is for "Pyrénées-Orientales" populations sampled in S.W. France.

The PCA shows obvious differences among the population studied. Each taxon presents a well defined group. The larger geographic samplings of *terrestris*, *audax*, and *dalmatinus* lead to the apparition of larger groups corresponding to a geographic gradient inside their distribution. The first axis (fig. 17 a) separates *B. ignitus* and *xanthopus* from the *terrestris* and South-western France specimens. The taxa *xanthopus*, and the population from South-western France are as separated from the other taxa as *B. ignitus*, the outgroup. Those last populations are the most differentiated, comparing them to all the other (fig. 17 a & b). The observations are the same on the figure 17 b. In a 3-dimensional view, the separation of *canariensis* and *sassaricus* is also observable, but it can not be represented in a 2-dimensional view.

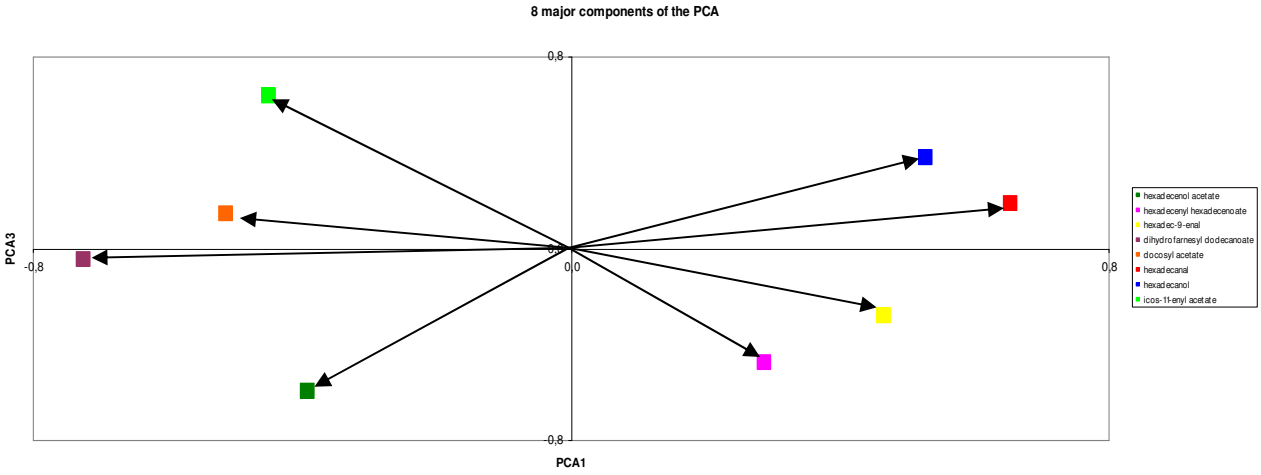


Figure 18. Eight major vectors defining the positions of specimens on the first and third axis of the PCA. Dark green : hexadecenol acetate – pink: hexadecenyl hexadecenoate – yellow : hexadec-9-enol – purple: dihydrofarnesyl dodecanoate – orange: docosyl acetate – red: hexadecanal – blue: hexadecanol – light green : icos-11-enyl acetate.

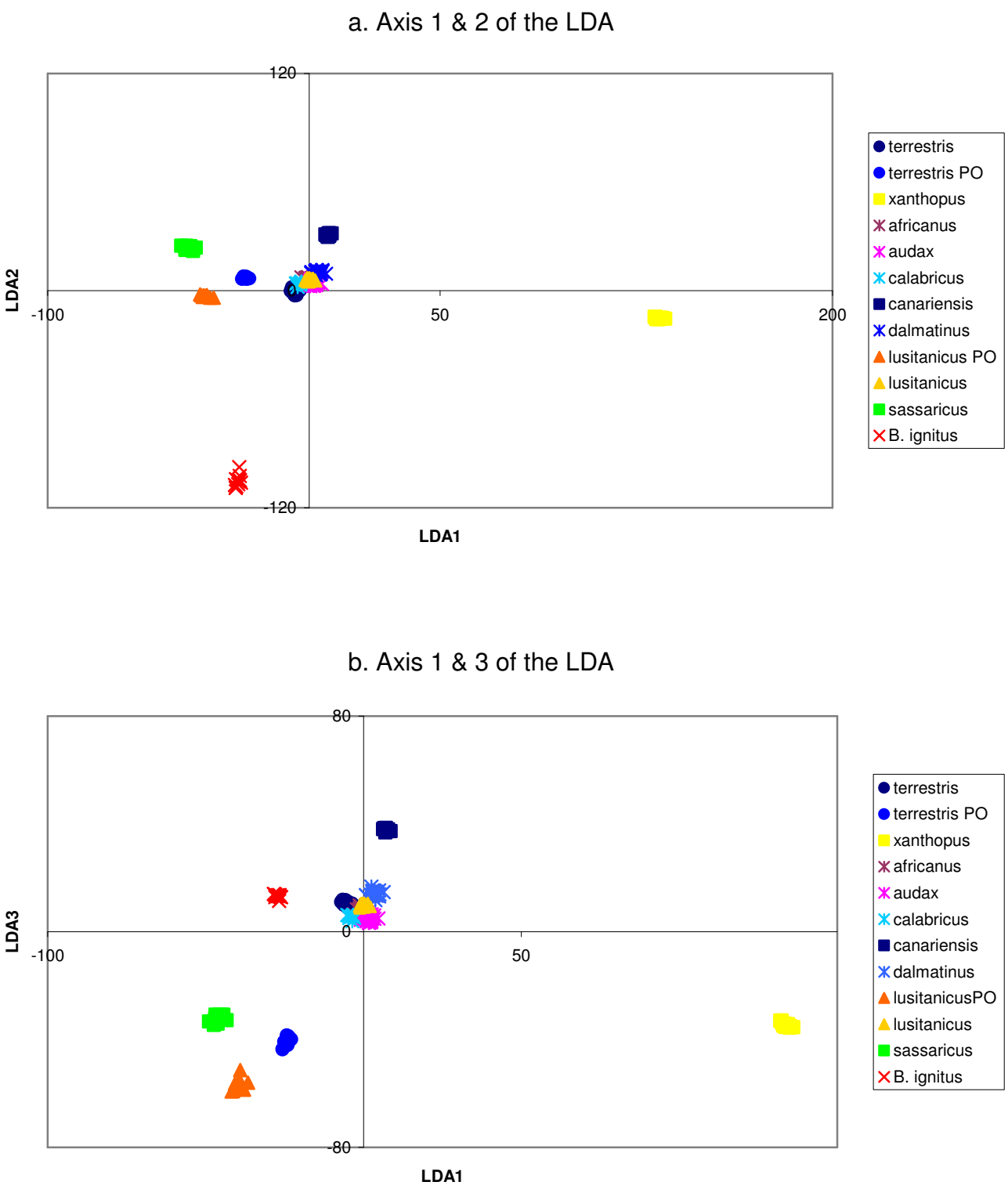


Figure 19. a. Discriminant Linear Analysis of the compounds x specimens matrix, projection of axis 1 & 2; b. Discriminant Linear Analysis of the compounds x specimens matrix, projection of axis 1 & 3. PO is for "Pyrénées-Orientales" populations sampled in S.W. France.

The Linear Discriminant Analysis results in the same topology as the PCA. A central group is constituted of poorly differentiated taxa: *terrestris, audax, dalmatinus, calabricus* and *africanus*, while *canariensis*, *sassaricus,* the S.W. France sampling and *xanthopus* are well separeted groups. The first axis of the LDA is clearly separating *xanthopus* from all the other populations and the second axis separates *B. ignitus* from the *terrestris* complex (fig. 19a). On the figure 19b, the third axis separates clearly *sassaricus*, *xanthopus*, and the S.W. France populations from all the other taxa. As this statistical analysis is group-discriminant, the groups obtain are more tight are more separated than when applying a PCA, but the conclusions are the same.

It is important to pay a particular attention to the EAG-active compounds described in *B. t. terrestris* (see Appendix 3): ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecan-1-ol, octadeca-9,12,15-trien-1-ol and geranylcitronellol. Those EAG-active compounds have been described for *terrestris* only and they might be different for the other subspecies.

The chemical characteristics of each taxon are the next (see Appendix 4):

- ***xanthopus:*** it is the most differentiated taxa using the PCA and the LDA. Its main compound is the tricosane. Three of the EAG-active compounds described in *B. t. terrestris* are absent from its CLG secretions: dodecanoic acid ethylester, 9,12,15-octadecatrien-1-ol and geranylcitronellol. Its secretions also show a high relative quantity of geranylcitronellal and hexadecenyl hexadecenoate.

- ***sassaricus:*** the CLG secretions of this taxon are particular and lead to a great separation of this taxa using PCA or LDA. The main compound of the secretions is the dihydrofarnesol, but the high relative quantities of 9,12-octadecadienol and tricos-11-ene are also characteristic. The dodecanoic acid ethylester (EAG-active in *B. t. terrestris)* is absent from the secretions.

- ***canariensis***: the 2,3-dihydrofarnesol is in high relative abundance in this taxon. This is the only taxon in which the 2,3-dihydrofarnesal is totally absent, all the others EAG-active compounds of *B. t. terrestris* are present.

The tricosane, hexadecanol and dihydrofarnesyl dodecanoate are in high relative abundance.

- ✓ ***africanus:*** this taxon presents dodecanoic acid ethylester and 2,3-dihydrofarnesol, but contrary to the other taxa the ratio of their relative abundance is inverted. The geranylcitronellol is in high relative abundance, but the other EGA-active compounds of *B. t. terrestris* are in lower concentration. Tricosane and dihydrofarnesyl are relatively abundant.

All the other taxa show high relative concentration of 2,3-dihydrofarnesol.

The remaining taxa present a positive [2,3-dihydrofarnesol:dodecanoic acid ethylester] ratio and low relative concentrations in 2,3-dihydrofarnesal, hexadecanol and 9,12,15-octadecatrien-1-ol. The *dalmatinus* strains have a low geranylcitronellol relative concentration while *terrestris, audax* and *calabricus* have a higher relative concentration of this last compound.

The Eastern Pyrenees populations of *terrestris* and *lusitanicus* and South-eastern France *dalmatinus* have a similar [2,3-dihydrofarnesol:dodecanoic acid ethylester] ratio but their main compound is the dihydrofarnesyl dodecanoate. The *lusitanicus* samples from this region do not seem to produce 9,12,15-octadecatrien-1-ol (EAG-active in *B. t. terrestris*). Rasmont(1988) and Ings et al. (2010) described these Southern France populations as tri-hybrids in a introgression zone between *lusitanicus*, *terrestris* and *dalmatinus*.

The taxon *audax* presents a high relative abundance in 2,3-dihydrofarnesol, low relative abundances in dodecanoic acid ethylester, 2,3-dihydrofarnesal and geranylcitronellol. Hexadecanol and 9,12,15-octadecatrien-1-ol are absent. This taxon is similar to *terrestris* and *lusitanicus* regarding to the EAG-active compounds described for *terrestris*.

The italian taxon *calabricus* has a CLG secretion composition close to that of *dalmatinus* but presents a higher relative abundance of geranylcitronellol. The next compounds also differ in relative concentration from those of *dalmatinus*: docos-17-enol, dihydrofarnesyl dodecanoate. The EAG-active in *B. t. terrestris* compound, 9,12,15-octadecatrien-1-ol is absent from its secretions.

Generally, the insular taxa present a higher relative abundance of 2,3-dihydrofarnesol than the continental ones, except for *xanthopus*. The highest relative concentration in this compound is found in *dalmatinus* Biobest (from Sporades), *canariensis* Biobest (from Teneriffe) and *calabricus* (from Sicily). The cretan *dalmatinus* are the only one with a lower relative abundance.

There are many other differences that can be observed when comparing the chemical compositions of the diverse taxa, but they do not allow any interpretation.

Summarising the differences in the CLG secretions compostition between the taxa analysed, we observed that *xanthopus* and *sassaricus* are the most differentiated taxa, due to the absence of dodecanoic acid ethylester, and the low relative concentration in 2,3-dihydrofarnesol in *xanthopus*. The taxon *africanus* present an inverted [2,3-dihydrofarnesol:dodecanoic acid ethylester] ratio. Despite its high relative abundance in 2,3-dihydrofarnesol, *canariensis* is remarkable because of a lot of particular chemical differences.

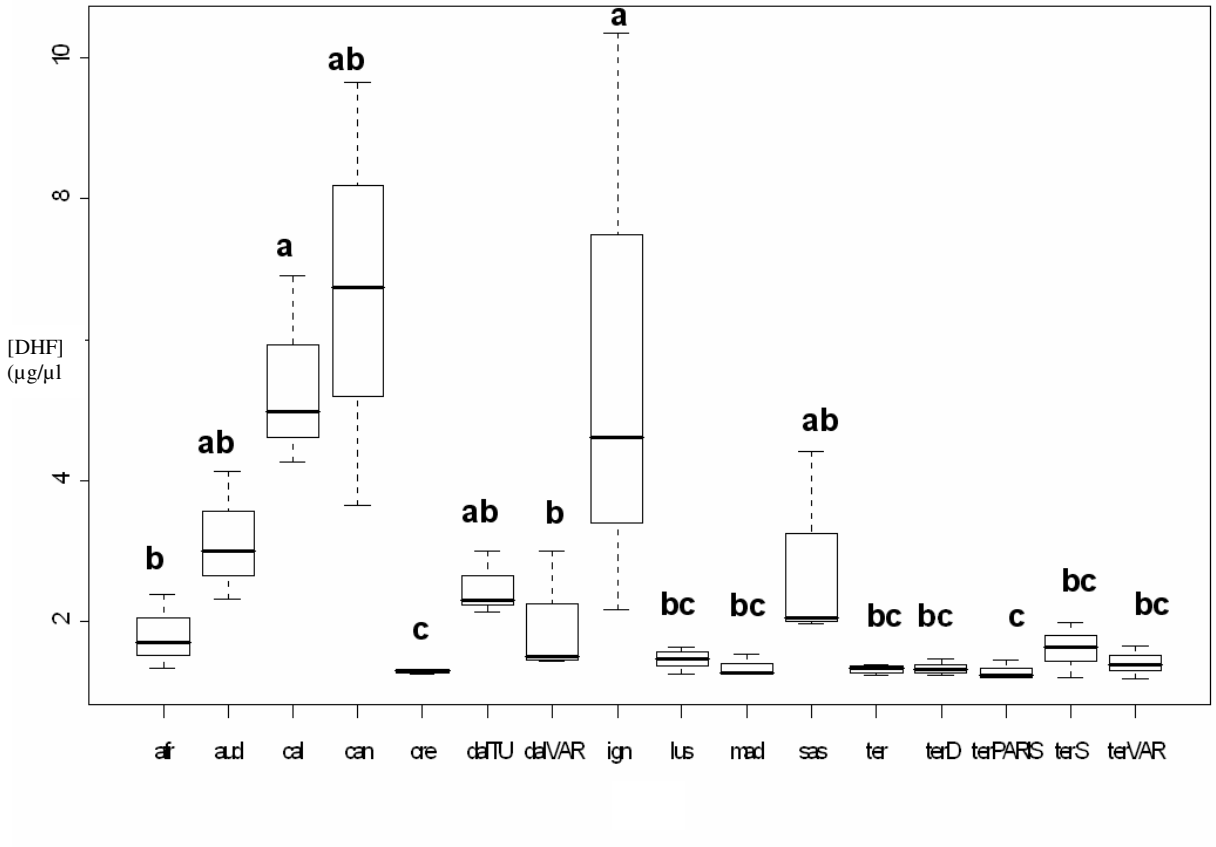


Figure 20. Quantification of the 2,3-dihydrofarnesol, using citronellol as IS. [DHF] in µg/µl. Afr: B. t. africanus; aud: *B. t. audax*; cal: *B. t. calabricus*; can: *B. t. canariensis*; cre: *B. t. dalmatinus* (Crete); dalTU: *B. t. dalmatinus* (Turkey); dalVAR: *B. t. dalmatinus* (South eastern France); ign: *B. ignitus*; lus:*B. t. lusitanicus* (Spain); mad: *B. t. lusitanicus* (Madeira); sas: *B. t. Sassaricus* (Sardinia); ter: *B. t. terrestris* (Belgium); terD: *B. t. terrestris* (Germany); terPARIS: *B. t. terrestris* (Northern France); terS: *B. t. terrestris* (Sweden); terVAR: *B. t. terrestris* (Southeastern France).

We also quantified the (*E*)-2,3-dihydrofarnesol in 17 populations and discovered huge differences in the absolute concentration of this compound between the populations. The subspecies *canariensis*, and *calabricus* synthesized 10 times more (*E*)-2,3-dihydrofarnesol than *terrestris* or *lusitanicus* (fig. 20). This information is of utmost importance since it is known that this compound is EAG-active, at least for *terrestris* virgin queens

The differences highlighted were then not only quantitative, but also qualitative. First of all, the (*E*)-2,3-dihydrofarnesol was not detected as the major compound in all the subspecies. Secondly, the main differences in qualitative composition concern medium

and/or minor compounds while the relative quantitative variations occur in all the compounds. Finally, it is important to note that many unsaturated compounds present several double bound positions, BUT only one double position exists in every taxon studied. In Lepidoptera, variations of the double bounds position frequently occur, and two isomers of the same unsaturated compound may be synthesized in the same sexual pheromonal blend. Several authors considered these variations in regard to the potential role of pheromones in the speciation process. The population divergence in SMRS and associated mate preferences can potentially lead to the establishment and evolution of pre-zygotic isolating barriers among segregated populations (Paterson, 1993). In the European small ermine moth (*Yponomeuta*), 6 species displayed different ratios of (Z)- to (E)-11-tetradecenyl acetate (Löfsted et al., 1991). It was suggested that the ancestral ermine moth pheromone was a mixture of (Z)- 11- and (E)-11-tetradecenyl acetate and the corresponding alcohols. The sexual pheromones of present-day species might have evolved through *reproductive character displacement* upon secondary contact between populations that had already diverged genetically in allopatry. *Bombus terrestris* does not seem to present the same kind of character displacement; the possible isomers are never found in the same blend, but are specific to one taxon. This might increase the reproductive isolation between taxa expressing conspicuously different cues. The unsolved questions remain "How does the female perceive these variations?" or "How did the receiver evolve in comparison with the variations?".

Geographic-dependent variability has been highlighted in numerous organisms. In the turnip moth *Agrotis segetum* Denis & Schiffermüller (Lepidoptera, Noctuidae), the variations in female sex pheromones correspond to those of the males' sensitive sensilla on the antennae (Löfsted et al., 1986; Hansson et al., 1990; Tóth et al., 1992). In *Polistes dominulus* Christ (Hymenoptera, Vespidae) differences between cuticular hydrocarbons (CHCs) of insular and continental populations have been highlighted (Dapporto et al., 2004). These differences suggest that the similarities in proportions of CHCs might reflect the closer genetic relatedness among individuals in island populations vs. those in mainland populations. In *Colletes cunicularius* (L.) (Hymenoptera, Apidae) (Vereecken et al., 2006), intraspecific sexual pheromone variations were found in distant populations, suggesting the existence of "dialects" expressed by females to attract males.

With this biological model (i.e. *C. cunicularius*), behavioural tests were conducted to determine the preference of males for allopatric or sympatric female sexual pheromones (Vereecken et al., 2006). The results showed that males were more sensitive to "exotic" odour blends, secreted by the females from more distant populations. The same situation occurs in many other animals e.g.: moths (Yponomeutidae) (Löfsted et al., 1991), fur seals (*Callorhinus ursinus)* (Hoffman et al., 2007), bark beetles (Dendroctonus & Ips) (Symonds & Eldgar, 2004), *drosophila* (Boake, 2002). But many other studies on sexual selection have underlined that individuals often recognise and prefer local (or proximal) populations (e.g.: Boake, 2002; Wong et al., 2004).

In chapter 4, the results of behavioural tests brought out a "two-level choice" preference of virgin females for male sexual pheromones. On one hand, virgin females displayed a clear attraction for males of their own subspecies. On the other hand, they prefer the more distant males within their subspecies.

Several authors considered these variations in regard to the potential role of pheromones in the speciation process. The population divergence in SMRS and associated mate preferences can potentially lead to the establishment and evolution of pre-zygotic isolating barriers among segregated populations (Paterson, 1993). Evolutionary simulations using the model showed that sexual selection of males causes an indirect stabilizing selection on the pheromone blends produced by females. The strength of the selection was analysed, and it was suggested that this indirect stabilizing selection becomes particularly important in situations where geographically close populations have evolved different pheromonal blends (Bengtsson & Löfsted, 2007). In the present study, the opposite situation occurs: females might cause a stabilizing selection on the pheromone blends produced by males of their own subspecies. It should be noted that this hypothesis was also suggested by Mayr (1963), Ayala (1978), and it is the keystone of Paterson's theory (1985).

Nowadays, most authors agree that sexual selection can be a very powerful force in driving speciation (e.g. Lande, 1981), with the persistence of hybrid zones in some cases e.g.: birds (Brelsford & Irwin, 2009), lizards (Böhme et al., 2007), reef fishes (Crow et al., 2007). In these cases, the authors underlined that the species maintain their integrity thanks to sexual selection against hybrids. This follows the most common speciation

process which was advocated by Mayr (1963). It has to be noted that for Paterson (1993), the selection against hybrids makes the allelic frequency equilibrium metastable, thus very efficiently eliminating these hybrids.

For years, it has been assumed that sexual pheromone blends undergo a very high degree of stabilizing selection that would strongly resist any shifting from the species' norm (Cardé & Baker, 1984; Baker, 1989). Recently, this view has been questioned and evidence has accumulated that an asymmetry exists between the strength of selection pressure on senders and receivers (Löfsted, 1993). This asymmetry, with lower pressure exerted on senders, should allow for much higher variance in the senders' blend. The corresponding higher selection pressure on receivers' response systems should lead some subset of receiver populations to follow major variations in the sender's blend. Through asymmetric tracking, their olfactory response spectrum will be adjusted over generations. This phenomenon is known as "saltational shifts". On the other hand, the results in *Drosophila* species (Symonds & Wertheim, 2005) strongly suggest that aggregation pheromones exhibit a gradual, and not saltational, mode of evolution. These findings reflect the function of aggregation pheromones, which do not hinge on species specificity. To summarize, species-specific blends could evolve following a saltational mode of evolution, while non-species-specific ones evolve gradually. The same conclusions can be drawn from our model, keeping in mind that in bumblebees the sender (the male) is not only subject to the pressure of the receiver's (the female) choice, but also to general sexual selection.

What we observed in the odour choice of *B. terrestris* virgin females, and in males' CLG secretion variation, is congruent with these theories. On one hand, major saltational shifts might have occurred in sexual pheromones among the subspecies of *B. terrestris*. The corresponding high selective pressure on virgin females led their response to follow those major variations. On the other hand, within a single subspecies (e.g. *B. t. terrestris*) minor changes could occur, and they are subject to females' preferences, resulting in the preference for unrelated males.

Inbreeding in Hymenoptera is hazardous for the species perennity. In inbreeding mating, their single-locus Complementary Sex Determination leads to the development of diploid males (50% of the progeny in brother-sister mating, in *B. terrestris*), which are less

adapted. Hybridization may still occur (de Jonghe, 1986a; Rasmont & Adamski, 1995, Rasmont & Quaranta, 1997; Ings et al., 2005, Kanbe et al., 2008) and allow an admixture of gene pools. No proved hybrid was found in the wild. In the present analysis, based on the CLG secretions study (Coppée, unpublished results, Appendix 4), except in the case of *lusitanicus* and *terrestris* populations that display similar CLG composition in sympatry.

The questions about a potential geographic variability are now partly resolved. We highlighted chemical changes in the composition of the males' CLG secretions depending on the geographic origin; they can be qualitative as well as quantitative. We also proved that females are sensitive to those chemical changes and show a preference for males of their own subspecies. It is important to keep in mind that the sexual pheromones of the males constitute the first step in the courtship. The way the female finally accepts the copulation with the male remains unknown.

Using Paterson's Species Recognition Concept to interpret our results helped us to better understand the implications of the chemical changes in the sexual pheromones. The pre-zygotic isolation system seems to be very efficient in bumblebees in light of the behavioural answers of virgin females towards non-conspecific males' sexual pheromones. In this case, the "Reproductive Isolation" concept of species, as described by Dobzhansky (1937) and Mayr (1963) is no longer applicable since the only important cue is the pre-zygotic recognition, and no longer the post-zygotic differences, which are considered as by-products. The Specific-Mate Recognition System determines the choice of the female before the copulation itself, and this system seems to be very powerful in bumblebees.

The SMRS is not considered as a means to reduce the risk of mismating or hybridization, but as a chance to maximise specific mate recognition.

6.2. Genetic differences

Our results (chapter 5) show well supported differentiation of some isolated subspecies of the *B. terrestris* complex (*africanus, canariensis,* and *xanthopus*). Estoup et al. (1996) and Widmer et al. (1998) previously observed genetic differences of an insular taxon, *canariensis*, but not of any other one, even if they observed that island populations were all significantly strongly differentiated from continental populations. Beton (2004)

also described genetic and morphometric differences between *B. terrestris* from Anatolia and Cyprus. Despite those previous studies, a complete analysis of an exhaustive sampling needed to be done.

Several situations are observed in the *Bombus terrestris* complex:

Most of the taxa (*terrestris, dalmatinus, lusitanicus, sassaricus, calabricus* and *audax*) are genetically undifferentiated. No geographic isolation occurs in their distribution, and hybrids have only been found from time to time (Rasmont & Adamski, 1995; Rasmont & Quaranta, 1997). Except for the taxa *sassaricus* their CLG secretions are not differentiated.

The "isolated" insular subspecies (*xanthopus, canariensis* and *africanus*) present significant differences from the continental ones. They have CLG secretions that are much more differentiated from those of the "continental" taxa except for *africanus*. Moreover, they are genetically different. These taxa seem to have undergone genetic drift since their isolation, with no possibility of hybridization. The CLG secretions also show saltational changes, driven by sexual selection or genetic drift.

Nevertheless, more specific, or well-adapted genes could be studied in order to have a better resolution of the group phylogeny, particularly the genes involved in the metabolic pathways involved in the sexual pheromone synthesis. More information could probably be obtained from other genes e.g. desaturases involved in the double position of unsaturated compounds.

6.3. Overview

The CLG secretions analyses along with the behavioural tests conducted on the studied taxa showed a high differentiation of all 9 previously described subspecies. The variability can be easily observed using simple statistical and behavioural tests. Comparing these results with the phylogeny obtained, the genetic and pheromoneal results are congruent for 2 taxa: *xanthopus* and *canariensis*, while *africanus* is well isolated in the phylogeny, and *sassaricus* displays very different CLG secretions.

These evidences strongly advocated in favour of the revision of the taxonomic status of these taxa. The chemical composition of CLG secretion being the major argument

following Paterson's Specific-Mate Recognition System

- ✓ **Ssp. *terrestris*:** this subspecies is not genetically differentiated in regard of our analysis, but they have a particular chemical composition of the CLG secretions which allows the recognition by consubspecific females. There are slight changes, in this wide distributed taxa, which reflects the origin of each studied populations. In the Pyrénées-Orientales (S.W. France), the composition of the CLG secretions is similar to that of the local *lusitanicus.* It seems that free hybridization occurs between the two subspecies in this region. Their subspecific status is conserved.

- ✓ **Ssp. *audax*:** the specimens present particular CLG secretions allowing the recognition by consubspecific females, but no genetic differentiation. The subspecific status is conserved.

- ✓ **Ssp. *africanus*:** this taxa displays differentiated CLG secretions, and is combined with *canariensis* in the phylogeny, in a isolated group. These observations lead to consider *africanus* as a good species: ***Bombus africanus* Krüger 1956 comb. nov.**

- ✓ **Ssp. *calabricus*:** the males of *calabricus* display subspecific pheromones but are not genetically differentiated from other continental subspecies. The subspecific status is conserved.

- ✓ **Ssp. *canariensis*:** the CLG secretions of *canariensis* are highly differentiated from those of other *B. terrestris* subspecies. During behavioural tests, non-consubspecific virgin queens showed "strange" reactions to the *canariensis* sexual pheromones, as if they were in panic. Moreover, this taxa is isolated in the phylogeny, together with *africanus.* This suggests us to consider this taxon as a good species: ***Bombus canariensis* Pérez 1895 resurrected species status.**

- ✓ **Ssp. *dalmatinus*:** males of this subspecies display their own chemical blend, like all the other subspecies, but are not differentiated in the phylogeny obtained here. There are minor changes in the CLG secretion composition, depending on the geographic origin of the population studied, but it did not interfere in the subspecific recognition system. The problem of hybridization in overlapping zones (with *terrestris*) is not resolved at that time, a sampling all along this zone may help to understand if hybridization naturally occurs or not. The subspecific status is conserved.

- ✓ **Ssp. *lusitanicus*:** the pheromonal blend displayed by males of *lusitanicus* form Spain or Madeira are not different from those of other subspecies, except from the Pyrénées-orientales (S.W. France) population which are overlapping the *terrestris* of the same geographic origin. This argues in favour of natural hybridization in this zone. Nevertheless no genetic differentiation was highlighted in this study. The subspecific status is conserved, the Madeira population being included in this subspecies.

- ✓ **Ssp. *sassaricus*:** this taxa has a well differentiated pheromonal blend. The phylogenetic results raised questions about the genetic marker used. Nevertheless following Paterson's definition of species, we can consider *sassaricus* as a good species: ***Bombus sassaricus* Tournier 1890 resurrected species status.**

- ✓ **Ssp. *xanthopus*:** the CLG secretions of this taxa are particular and allow the recognition by *xanthopus* virgin queens. Moreover, the taxon is isolated in the phylogeny, suggesting it to be a good species and not a subspecies of *B. terrestris*. The taxon is raised at the species level, becoming ***Bombus xanthopus* Kriechbaumer 1870 resurrected species status.**

In 2008, Rasmont *et al.* published the following classification:

One species, *Bombus terrestris* including 9 subspecies: *B. t. africanus*, *B. t. audax, B. t. calabricus, B. t. canariensis, B. t. dalmatinus, B. t. lusitanicus, B. t. sassaricus, B. t. terrestris, B. t. xanthopus.*

In light of our results, we propose this new classification :

Five species;

- *Bombus terrestris* including 5 subspecies: *B. t. audax, B. t. calabricus, B. t. dalmatinus, B. t. lusitanicus, B. t. terrestris*.
- *Bombus xanthopus* (resurrected species status)
- *Bombus canariensis* (resurrected species status)
- *Bombus africanus* (comb. nov.)
- *Bombus sassaricus* (resurected species status).

This new taxonomical description of the *Bombus terrestris* complex could have impacts on the commercialisation of reared bumblebees for crop pollination in glass houses, since taxa that were considered as conspecific no longer belong to the same species.

Chapter 7

CONCLUSION

7. Conclusion & perspectives

7.1. General conclusions

Bombus terrestris L. is a widespread European bumblebee species. Its distribution is centered on the Mediterranean Sea extending from the Canary Islands in the West to the Altaï Mountains on the East, and from the Antiatlas Mountains of Morocco in the South to Southern Finland in the North (Rasmont et al., 2008). It was described as including 9 subspecies showing morphological differentiations, particularly in isolated (e.g. insular) taxa. Despite all the studies conducted on this biological model, specialists still do not agree on the taxonomic status of several subspecies. We used modern tools to review the taxonomy of this biological model.

We first demonstrated an age-dependent variation in the secretions of sexual pheromones. Additionally, the behavioural tests we carried out showed that virgin females have a preference for 10-day-old males. Taking these results into account, we strongly suggest using males between 7 and 15 days old in any further study of CLG secretions. This is easy when males are obtained by breeding queens or workers. In the wild, the age of males can not be determined, but attention has to be paid to the appearance of mature males: they should not have hairless parts on their body, their wings should not be damaged, and they should have bright colours. Another possibility is to carefully check the TIC (which would be greater than 10^6) when analysing the CLG secretions using GC/MS.

A widespread sampling of the species was done, with special attention to the insular subspecies. The CLG secretions were analyzed using gas chromatography coupled with mass spectrometry. A Discriminant Linear Analysis, and Principal Component Analysis were applied to the data matrix obtained (compounds x samples), showing great qualitative and/or quantitative differences in the secretions of *canariensis, sassaricus,* and *xanthopus.* Our sampling methods were various: wild males, breeding of queens or workers and commercial colonies, but our results do not show any bias linked to the sampling method and are all congruent. Quite curiously, males from a single colony present the same variability as males caught in a natural population. Nevertheless, it is

important to sample as many populations as possible within taxa distributed over a large geographic area, since slight variations seem to occur between distant locations.

Using a simple behavioural test we also showed that the virgin queens preferred males belonging to their own subspecies. This choice can increase divergences among subspecies and reproductive isolation by reinforcing homogeneity within a given subspecies.

The genetic analysis, using mitochondrial DNA (COI and cytochrome b), revealed two well separated monophyletic groups: *xanthopus,* and *canariensis* and *africanus.* Further analysis with other genetic markers might be helpful for a better understanding of the phylogenetic relations existing between the *Bombus terrestris* subspecies. Our results show that some taxa are poorly differentiated (*B. t. terrestris, B. t. dalmatinus)*, while other taxa present no genetic differences but do have well characterized CLG secretions (*sassaricus*). Finally, two taxa show a conspicuous genetic divergence as well as specific CLG secretions (*B. t. canariensis* and *B. t. xanthopus)*, raising questions about their taxonomic status. We suggest to consider four former subspecific taxa as good species: *canariensis, sassaricus, africanus,* and *xanthopus* becoming *Bombus canariensis, Bombus sassaricus, Bombus africanus* and *Bombus xanthopus*.

Our principal argument for considering the 4 taxa as species different from terrestris sensu stricto is not only based on the phylogeny, but mainly on CLG secretion variability. Considering the virgin queens' preferences, this indicates a pre-zygotic specific-mate recognition system. Paterson's definition was of utmost importance to understand the taxonomic relations between the studied taxa (Paterson, 1985, 1993).

7.2. Perspectives

Many questions remain unsolved, and many new questions appeared during this work.

✓ It would be interesting to have an idea of the composition of CLG secretions of hybrids of numerous combinations, and test how the virgin queens perceive them. The same analyses that were used in this work could be applied.

✓ The EAG-active compounds should be determined, as well as the absolute quantification of those compounds, in every single taxon for a better understanding of the system. In all the chemical changes observed, which are the important ones? Could it be possible to recreate a synthetic sexual pheromone?

✓ We solved the problem of specific recognition in the first step of the courtship of *Bombus terrestris*, but what happens next? The subsequent steps in recognising potential mates have to be studied. The female might also produce some cues, or be more or less attracted by the cuticular hydrocarbons of males. Those points might be decisive for the success of the mating.

✓ The phylogenetic relations between the undifferentiated taxa could be clarified by a character mapping between the CLG secretion composition and the sequences obtained in this work. The genes involved in desaturase production could be very helpful for a better resolved phylogeny of the *Bombus terrestris* subspecies.

- ✓ The commercial use of *Bombus terrestris dalmatinus* has to be carefully studied. It might no longer be considered as "indigenous" or conspecific with taxa from the Canary Islands, Morocco, Algeria, Tunisia, Corsica and Sardinia. That could raise new legal questions that have to be solved urgently.