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Exploring Plant-Pollinator interactions: critical studies for the safeguard of wild Apoidea and spontaneous plant populations

Presentata da: **Gherardo Bogo**

Coordinatore Dottorato

Prof.ssa Barbara Mantovani

Relatore

Prof.ssa Marta Galloni

Co-relatore

Dott.ssa Laura Bortolotti

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"According to the laws of aerodynamics, the bumblebee can't fly,
but bumblebee doesn't know anything about the laws of aerodynamics,
so it goes ahead and flies anyway"

Mary Kay Ash

To my wonderful parents

CONTENTS

CHAPTER 1

General introduction

1.1. Plant-pollinators relationship

Plant-pollinators interactions are considered essential in the evolutionary process of Angiosperms and several mutual relations developed due to this connection (Biernaskie et al., 2005; Brunet, 2005). This co-evolution led entomophilous plant species to evolve lure and reward mechanisms that guarantee visits and fidelity of pollinator insects. Pollinators, in their turn, developed specific adaptations to interact with different types of flowers, ensuring plant pollen transfer from one individual to another, favouring allogamy and the fitness of the plant population (Richards, 1997). Even in some cases in which pollination is triggered by other vectors, pollinators can facilitate it and make it more effective. This relationship sometimes reached an extreme specialization level, in which the plant totally depends from a specific animal taxon for its reproduction.

Plant-pollinator interactions can be very complex, since pollinators behaviour is influenced by many factors, such as flowers' morphology, their arrangement, the plant density within the population, the plant population size, in addition to other biotic and abiotic factors (Kunin, 1993; Routley et al., 1999; Mitchell et al., 2004; Brunet, 2005).

Pollination is the first step in sexual reproduction: pollen carriage is essential to preserve biodiversity of terrestrial ecosystems (Kevan, 1999). In case of zoophilous plants, especially if rare, threatened or particularly sensitive to environmental changes, the interaction network with pollinators plays a primary role. Pollinators are therefore extremely important, both from ecological and economical point of view.

The delicate balance between plants and pollinators is constantly jeopardized by a whole series of anthropogenic factors, including fragmentation of habitat, land-use changes, modern agricultural practices, use of chemicals such as pesticides and herbicides and invasions of non-native plants and animals (Kearns et al., 1998). The need for active conservation of pollinators, with the aim of preserving plant-pollination interactions, is being appreciated only in the last few years.

1.2. Global decline of bees

In the whole spectrum of animal pollinators, bees (both managed and wild) are among the best ones. There are many potential threats that affect bee biodiversity in general and their abundance and diversity in particular (National Research Council, 2007). It is important to consider that these drivers are not independent factors (Brook et al., 2008), but they can interact with each other (Didham et al., 2007). Decline factors (Tab. 1) are identified in: land-use change, with the consequent loss and fragmentation of habitats (Steffan-Dewenter et al., 2002; Hendrickx et al., 2007; Goulson et al., 2008), increase of pesticide application and environmental pollution (Potts et al., 2010; Goulson et al., 2015), decrease of resource diversity (Biesmeijer et al., 2006), introduction of alien species (Thomson, 2006; Stout & Morales, 2009), the spread of pathogens (Cox-Foster et al., 2007; Neumann & Carreck, 2010) and climate change (Williams et al., 2007; Dormann et al., 2008).

Table 1. Causes of bee decline. The geographic and taxonomic impact of various factors (Brown & Paxton, 2009). ×: strong effect; ?: suspected effect.

	Solitary bees				Bumble bees	Honey bees		
	Australia	Central and South America	Europe, Mediterranean and North Africa	Sub-Saharan Africa and Madagascar	Worldwide	Asia	Europe	Africa
Habitat loss, fragmentation degradation	×	×	×	×	×	×	×	×
Invasive species[a]	?	×			×	×	×[d]	
Parasites and disease[b]			?		×	×	×	×
Exploitation						×		×
Extinction cascades[c]	?	?	?	?	?			
Climate change	?	?	?		?	?	?	?

The habitat loss (Fig. 1) seems to be the major causal factor in the decline of bees, as it is for the decline of biodiversity in general (Foley et al., 2005). Habitat fragmentation, a direct result of habitat loss, impacts on surviving populations, either through genetic isolation and subsequent inbreeding (Zayed, 2009) or simply due to the inability of small habitat islands to support viable bee populations (Ellis et al., 2006).

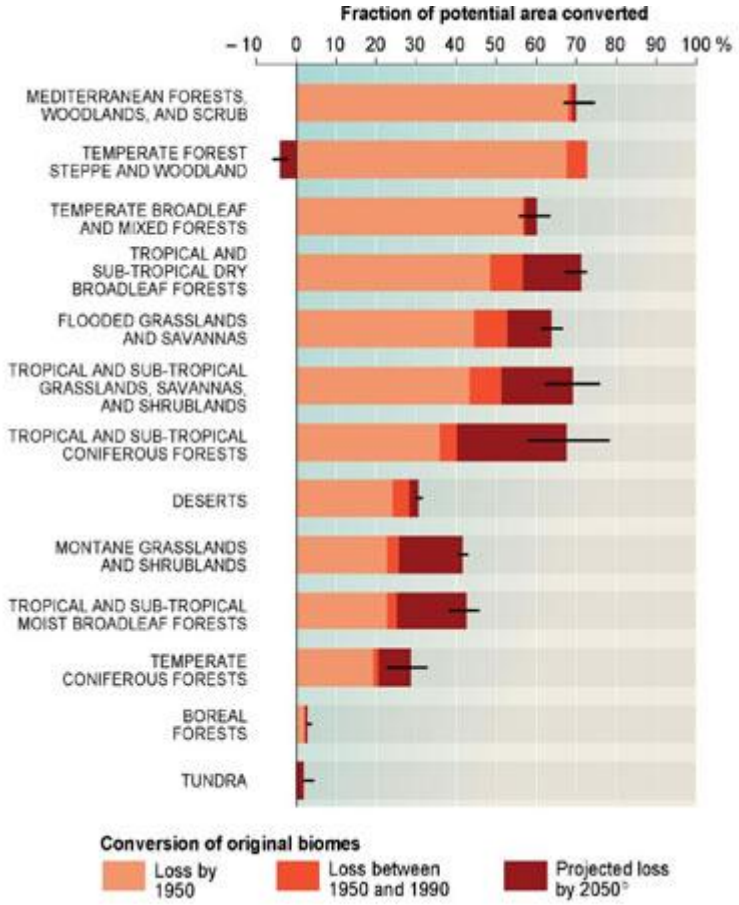

Figure 1. Terrestrial habitat transformation (Assesment M. E. 2005).

Insecticides can cause mortality by direct intoxication that might result in local shifts in wild bee diversity and abundance (Brittain et al., 2010), whereas herbicides and fertilisers can affect pollinators indirectly by decreasing floral resource availability (Gabriel & Tscharntke, 2007). Sub-lethal effects of pesticides have been demonstrated (Morandin et al., 2005) with implications for the longer term survival of populations. In addition, the effects of agrochemicals might not be restricted to agricultural lands themselves because agrochemicals can drift into semi-natural habitats where pollinators nest and forage.

Alien plants with showy floral displays and large rewards decrease the the visitation rate of native plants by native bees (Stout & Morales, 2009), the introduction of managed pollinators for crop pollination and honey production can have a negative impact on native pollinators (Thomson, 2006) through competition for resources or direct interaction. In addition, invasive alien bees can act as vectors of novel pathogens that can infect native con-specifics and other closely related species.

Finally, climate change-induced mismatches in temporal and spatial co-occurrence (Hegland et al., 2009), and morphological and physiological interdependencies of plants and pollinators can potentially disrupt their interactions (Memmott et al., 2007).

1.3. Eusocial Apidae: bumble bees and honey bees

Bumble bees (genus *Bombus* Latreille, 1802) and honey bees (*Apis mellifera* L., 1758) are social bees belonging to family Apidae, order Hymenoptera. Their social structure is defined "eusociality" because of three main characteristics: the coexistence of reproductive and sterile individuals, the overlap of various generations and the cooperative brood care (including brood care of offspring from other individuals) (Wilson, 1971). Bumble bees are defined as "primitive eusocial" because their life cycle includes both a solitary and a social phase.

Bees are very important pollinators of both spontaneous plants and crops. It is estimated that almost all the fruit trees and at least 85% of entomophile angiosperms are dependent by honey bees (Tautz, 2009). Bumblebees are effective pollinators of many wild plants and exclusive pollinators of many crops of economic interest such as Solanaceae.

Foraging honey bees are able to cover large distances in flight, up to 3 km (Ricciardelli D'Albore & Intoppa, 2000), while bumble bees are able to maintain a good physical efficiency even at low temperatures and consequently they can offer a pollination service even in adverse conditions (Corbet et al., 1993). Due to their considerable physical strength, bumble bees are able to carry out the "buzzpollination", in which the insect shakes the flower with rapid muscle movements to release a greater amount of pollen. Moreover, bumble bees are the best pollinators in greenhouses (Dag & Kammer, 2001).

Due to their feeding on nectar and pollen, honey bees and bumble bees show morphological traits relating to the collection and transport of those rewards. One of the most relevant adaptations regards the modification of hind legs for the collection of pollen: in *Bombus* spp. female, tibiae are flattened and hairless on the external surface, with a fringe of long hairs curved at the sides to form the corbicula (Thorp, 1979). In

Apis mellifera workers, in addition the first segment of the tarsus is wide and equipped on the inner side of numerous bristles which are called "pollen comb", while the tibia is externally covered with hair which delimit a smooth and concave surface call "pollen basket" (Hodges, 1952; von Frisch & Giavarini, 1984). In both cases, pollen stored in the corbicula is packed into small cohesive masses becoming no longer available for pollination (Faegri & Van der Pijl, 1979; Thorpe, 2000). Another adaptation concerns the mouthparts for nectar collection. In bumble bees and honey bees mouthparts are composed by two portions with different roles: proboscis and mandibles. Proboscis is not a permanent organ, but it becomes functional when the insect combines maxilla, labial palpi and glossa modelling a duct to suck liquids. Mandibles are mainly used to open anthers to extract pollen, to manipulate wax or grab enemies (Winston, 1991).

1.3.1. Caste differentiation

Honey bee and bumble bee colonies are composed by three kinds of adult individuals: a single fertile female or queen, diverse sterile females (several thousands in honey bee colonies and up to 300 in bumble bee ones) or workers and few hundreds of male present only in late summer and in early autumn (D'Ambrosio & Zappi Recordati, 1990).

In honey bees and bumble bees sex determination is haplodipoid: male emerge from unfertilized haploid eggs, while the two female castes (queens and workers) emerge from fertilized diploid eggs. Queen and worker eggs are genetically indistinguishable, and the fate of the hatched larvae toward a queen or a workers is called "caste determination".

In *A. mellifera* caste determination is triggered by the different diet by which are fed the female larvae during their development. Young workers (called "nurse bees") produce, secrete, and feed a substance called royal jelly to developing larvae. In the initial three days of development, all larvae are fed with royal jelly but thereafter only larvae designated to become queens will receive it. In its place, a mixture of honey, pollen, and water is fed to larvae selected to become workers (Drapeau et al., 2006). In bumble bees instead, queens are about three times bigger than workers but this difference is not due

to different feeding rates (Riberio et al., 1999) or food composition (Pereboom, 2000) but it seems to be linked to the food quantity and to the feeding period. In fact, the total feeding period of queen larvae is 3 days longer, which permit queens to become bigger (Cnaani et al., 2000). The different developmental path (caste differentiation) between queen and worker is driven in both species by the queen mandibular pheromone, which leads a differential genetic expression in the larvae of two castes (Grozinger, 2003; Pereboom et al., 2005). Accordingly, Harrison et al. (2015) found that bumble bee reproductive workers resembles very closely to queens regarding their gene expression.

1.3.2. Life cycle

The colony life cycle of honey bees and bumble bees shows several differences. A brief description follows here.

Cycle duration: bumble bee colonies are annual, starting in early spring and lasting until autumn, when the queen and the workers die and only newborn queens (gynes) survive. Honey bee colonies instead can last potentially for many years because the family survive the winter and new queens are generate when the old one is not efficient any more.

Overwintering: bumble bee gynes survive to winter in a state of diapause, exploiting the fat accumulated in autumn as metabolic reserve (Horber, 1961). Regarding honey bees, in temperate regions the queen and workers survive the winter in a cluster inside the nest, producing heat from the flight muscle, thanks to food supplies stored during the good season (Tautz et al., 2004).

Solitary phase: Only bumble bee colonies pass through a solitary phase, that starts in late summer, when gynes mate, and continues during winter up to early spring, when gynes exit the diapause. Gynes survived to diapause restore the energy reserves lost in winter by foraging on flowers, then find a nesting site, where they lay the first egg-cells (Alford, 1978). When the first worker emerges the solitary phase ends up and the social phase begin, whith the gyne becoming the queen of the colony.

Queen role: bumble bee queen found the colony by herself. After laying the first eggs, she takes care of the brood, maintaining the temperature around 30-32°C (Heinrich, 1979), feeding the larvae, increasing the nest, providing pollen and nectar and protecting the colony (Prys- Jones & Corbet, 1991). On the contrary, the honey bee queen is highly specialized and her only role is to lay eggs, while workers take charge of all the nest duty, like care of offspring and nest building (Contessi, 2004).

Worker division of labour: in honey bee workers an age polyethism occurr in which they carry out different tasks depending on the age and on physiological changes as the maturation of different glands. Newly emerged bees perform tasks within the colony, such as cleaning the comb and feeding the old larvae. Subsequently, hypopharyngeal glands develop and they become able to produce royal jelly by which they feed the queen and the young and queen larvae. From the tenth day, the hypopharyngeal glands wastes in favour of wax glands and workers become comb builders. At 20 days workers become guard bees and, from the third week until the end of their life, foragers. However, this age-related division of labour is flexible according to colony needs, which can accelerate, delay, or reverse worker behavioral development (Huang & Robinson, 1996). In bumblebee colonies only a weak age-related division of labour occurs (Cameron, 1989), but several studies have revealed a correlation between a worker's size and the probability of performing a certain task. Large workers were found to have a higher probability of foraging for nectar and pollen, whereas small workers tended to stay inside the nest and attend to nest duties (Cumber, 1949; Free, 1955; Spaethe & Weidenmüller, 2002).

Male production: both bumble bees and honey bee colonies produce few hundreds of males, but in bumble bees male production occurr only in summer, when the queen switch the deposition of diploid eggs to that of haploid eggs, and this event is called "switch point" (Duchateau et al., 1988).,Contrarily honey bee queen produce haploid and diploid eggs at the same time following colony needs (Winston, 1991).

Gyne production and mating: in bumble bees, gynes derive from the latest fertilized eggs laid by the founder queen before the switch point (Prys- Jones & Corbet, 1991). The founder queen can never be replaced, all the gynes leave the colony to mate and never return. Bumble bee gynes can mate one (monandry) or more times (polyandry) depending on the species (Foster, 1992; Brown et al., 2002; Baer et al., 2003). In honey

bees, queen larvae are reared in three cases: when the founder queen dies, when an old queen has to be replaced, or when the colony reach an adequate size to swarm. In all these cases several queen cells are produced, and the first emerged one kills the others before they exit the cell. In case of swarming, the old queen leaves the colony before the emergence of the new one, together with about an half of the workers, to found a new colony (Bortolotti & Costa, 2014). The newly emerged queen leaves the hive after few days to mate with about fifteen males, then come back to the hive and she will never leave again, except for a swarming in the following years (Contessi, 2004).

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CHAPTER 2

General purposes

Wild bees (Hymenoptera, Apoidea) are a large and various group of insects, very meaningful for the preservation of all the ecosystems. They provide for pollen transfer, thus undertaking the production of fruits, seeds, fodder, and the conservation of natural habitats (Corbet et al., 1991; Goulson, 2003; Morandin & Winston, 2006; Klein et al., 2007; Garibaldi et al., 2013).

Currently, reared and wild Apoidea are threatened by human activities and they are encountering a severe decline, both in the abundance and in the number of species, with high risk of local extinction (Goulson et al., 2008; Potts et al., 2010; Winfree, 2010). Several causes have been identified: climatic changes, soil use changes, habitat fragmentation, use of pesticides in agriculture and environmental pollution in general; but the most common and widespread reasons for the decline of a pollinator species are the scarcity of floral resources and the scarcity of nesting sites (Kremen et al., 2007; Lebuhn et al., 2013). Many of the above mentioned factors may affect the populations of these insects by increasing their reproductive isolation and consequently reducing their offspring fitness due to inbreeding (Keller & Waller, 2002).

The fates of plants and bee pollinators are strictly connected: if bees decline, the plants they pollinate set less seeds and consequently produce less food for the bees in the following year; this in turn leads to a higher decline in bee populations (Meffe, 1998; Biesmeijer et al., 2006). This situation, known as “extinction vortex”, in which mutually dependent species drive each other to extinction, has been recently described for an increasing number of plant-pollinator relationships (Buchmann & Ascher, 2005; Ghazoul, 2005).

This research was finalized to improve knowledge on plant-pollinator interactions, in ecological context with conservation needs. I approached this subject from three different points of view: 1) ecosystemic, where I considered the relationships between a rare plant and the community of its flower visitors and pollinators; 2) on nectar-pollinator interface, exploring the role of this leading floral rewards, and especially of

its non-protein amino acids component, on pollinators' preference and behaviour; 3) on the genus *Bombus*, investigating different aspects of this model organism for pollinator studies, such as artificial rearing and inbreeding related problems.
I carried out field work on natural populations of two locally protected perennial herbs, *Dictamnus albus* and *Gentiana lutea*, and their wild Apoidea pollinator community and I performed laboratory experiments on specific critical aspects (wild bees artificial rearing, role of different nectar components, inbreeding in bumble bees) using *Bombus terrestris* adults and colonies from laboratory reared populations.

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Chapter 3

Evaluation and enhancement of wild Apoidea populations: pollinator community of *Dictamnus albus*

3.1. LIFE+ PP-ICON project

LIFE+ (2007-2013) is the EU's financial instrument supporting environmental and nature conservation projects throughout the EU. It follows previous EU's *LIFE* programme. It includes three components: *LIFE+ Nature and Biodiversity*, *LIFE+ Environment Policy and Governance*, *and LIFE+ Information and Communication*. *LIFE+ Nature and Biodiversity* supports projects that contribute to the implementation of the EU's "Birds" (79/409/CEE) and "Habitats" (92/43/CEE) Directives, the "Natura 2000" network of protected areas, and that contribute to the EU's goal of halting the loss of biodiversity. Inside this last components, it is located the PP-ICON (Plant Pollinator Integrated CONservation approach: a demonstrative proposal) project.

About 90% of angiosperm species profits from animal pollination for their reproduction. The mutualistic pollination interactions are beneficial to both plants and animals but also favour humanity, directly through crop productivity and indirectly through ecosystem health. Pollination systems are under increasing threat from anthropogenic sources, including fragmentation of habitat, changes in land use, modern agricultural practices, use of pesticides and herbicides (Stokstad, 2008; Biesmeijer et al., 2006). Rare plants are commonly more sensitive to habitat loss and fragmentation and often occur in small populations, which may decrease the attractiveness to pollinators and reduce pollinator service.

The PP-ICON project will focus on the conservation of a locally rare plant (*Dictamnus albus* L.) and the community of its natural pollinators. At present, European natural populations of *Dictamnus albus* are declining because of scarcity of pollination service;

in addition to this, suitable habitats (woodland fringes and clearings) are becoming rare due to land-use changes as result of the abandonment of traditional agro-sylvo-pastoral activities. Wild pollinators are facing a widespread decline, mainly due to climatic changes, soil use changes, habitat fragmentation and pollution, which cause a scarcity of floral resources and nesting sites.

The main objectives of this project was to ensure the persistence of an isolated population of *Dictamnus albus*, located in a protected area (Parco Regionale dei Gessi Bolognesi e Calanchi dell'Abbadessa) included in the Natura2000 network (pSCI IT4050001), and to restore the community of its natural pollinators. This was a pilot demonstration project: the integrated techniques proposed could be easily applied for the management of other European populations of *Dictamnus albus*, as well as for the conservation of several plant species and respective pollinators that are facing the same risks in Europe.

Actions focused on habitat management, safeguard of insect natural pollinators, awareness and dissemination. The total duration of the project was 4 years. At the beginning of the project the habitat was managed in order to establish the best environmental condition for the future persistence of the target plant population. At the same time the effective pollinators of the target plant was identified and collected, in order to artificially rear and introduce them in the target area during the following three years. The maintenance of the introduced pollinators was then assured by the growing of autochthonous nectariferous plant species, which were planted in the target area. For all the duration of the project the fitness of the plant and the presence of its effective pollinators was monitored; pollinator insects in the target area were also monitored, to evaluate the impact of the intervention on the pollinating fauna.

3.2. *Dictamnus albus*

Dictamnus albus L. (Rutaceae) (Fig. 2) is a long-lived perennial herb, characterized by thick storage roots. The potential lifespan of an individual is estimate to be at least 30 years (Jäger et al., 1997). Each individual produces one (rarely two or three) stem that bears many pentamerous and slightly zygomorphic white-purple flowers, on a long and

loose raceme (Fisogni et al., 2011). Stamens are arranged in two whorls; the nectary is placed at the base of the gynophore, below the ovary (Weryszko-Chmielewska et al., 2001). Flowering begins after 5-7 years and occurs between the end of April and June. Fruits are capsules composed of 5 carpels with a locular opening; the black pear-shaped seeds are dispersed by autochory, due to increasing turgor pressure, with a maximum dispersal distance of approximately 4 m (Frey, 2000). Like most species of the Rutaceae, plants of *D. albus* are characterized by oils, which are found both in leaves and in oil glands disposed throughout the stem and flowers. Secondary chemistry of the genus is unique within Rutaceae: *Dictamnus* has limonoids instead of coumarins, and special quinolones (Da Silva et al., 1988).

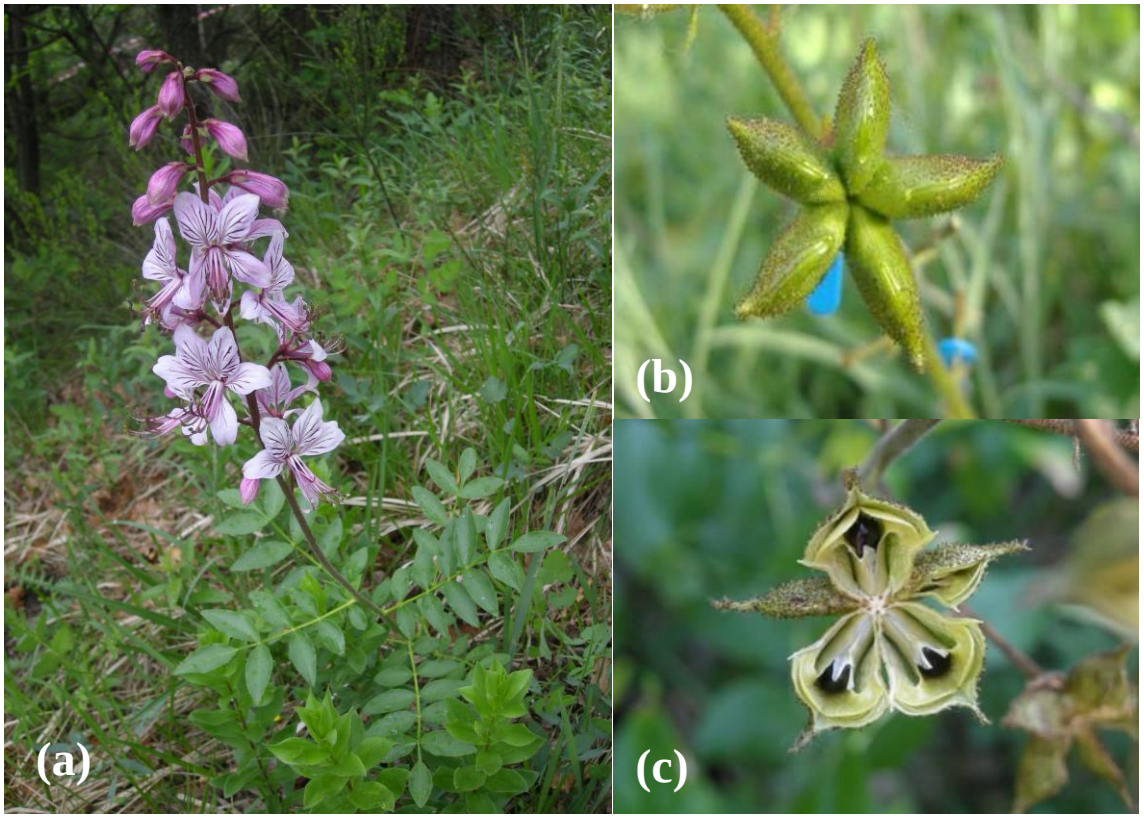

Figure 2. (a) Flowering stem of *D. albus*; (b) star-shaped fruit and (c) ripened fruit with seeds (Fisogni 2011).

D. albus is found at the fringes between xerothermic woodlands and (semi)natural grasslands, or within open oak forests, in the southern warm-temperate regions of Europe and Central and Eastern Asia (Hensen & Oberprieler 2005, Fisogni et al., 2011). The species has been designated as "vulnerable" in several European countries

(Schnittler & Gunther, 1999), and is locally protected across Europe. In Italy it is protected at regional level (Fisogni, 2011).

3.3. Aim of the study

This part of my thesis is included the PP-ICON project (Plant-Pollinator Integrated CONservation approach: a demonstrative proposal – LIFE09/NAT/IT000212), started in 2011 and aimed at protecting the *Dictamnus albus* population in a small area of Parco dei Gessi Bolognesi e Calanchi dell'Abbadessa (Bologna, Italy), by acting on habitat management and pollinators' enhancement.

Pollination limitation is one of the main risk factor for the target population of dittany: previous studies have shown that seed production suffer from a reduced pollen supply, indicating a deficit in the pollination service (Fisogni, 2011; Fisogni et al., 2015). The great majority of insect visitors and pollinators of dittany in the area are bees (Hymenoptera, Apoidea), including both social (honey bees and bumble bees) and solitary (Megachilidae, Andrenidae, Anthophorinae, Halictidae) bees.

To support the local bee fauna and consequently favour the pollination of dittany, we pursued three actions, aiming at enhancing the number of native bee plants, providing artificial nesting sites for solitary and social bees, and reinforcing the bumble bee population through the rearing and releasing of wild colonies. Moreover, under the current global warming scenario (Memmott et al., 2007; Hegland et al., 2009), the study of the abiotic factors affecting the dynamics of pollinator community of *D. albus* is particularly relevant, in order to identify potential mismatches in this plant-pollinator system and to develop specific conservation programs.

Considering the increasing pollinators' decline and the undisputed importance of pollinators in the ecosystem, the aim of this study is to investigate *D. albus* most efficient pollinators and to develop different conservation strategies, to be applied not only within the LIFE project, for the safeguard of *D. albus* pollinator community, but useful and reproducible to protect natural populations in many different contexts.

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3.5. Integrated conservation of bee pollinators of a rare plant in a protected area near Bologna (Italy)

Submitted to *Conservation Evidence*

Laura Bortolotti[1]*, Gherardo Bogo[1,2], Natasha de Manincor[2,3], Alessandro Fisogni[2], Marta Galloni[2]

[1]Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA) – Unità di ricerca di apicoltura e bachicoltura, Via di Saliceto 80, 40128, Bologna, Italy.
[2]Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Università di Bologna, Via Irnerio 42, 40126, Bologna, Italy.
[3]Univ. Lille, CNRS, UMR 8198 - Evo-Eco-Paleo, F-59000 Lille, France.
* Corresponding author: Laura Bortolotti

SUMMARY

An integrated approach was proposed for the conservation of the bee pollinators of the locally rare plant dittany *Dictamnus albus*. Based on previous studies that revealed the most efficient pollinators, we performed three strictly connected actions to improve their presence in the area: (i) we placed artificial nests for bumble bees and solitary bees; (ii) we added bee plants to support local populations of pollinators throughout their life cycle, and (iii) we reared and released bumble bee colonies from wild queens collected in the area. Artificial nests were occupied at high rates by cavity nesting species such as mason bees, leafcutter bees and carpenter bees, while we did not observe any ground nesting bee. Artificial nests for bumble bees did not attract any wild queen. All the bee plants settled at different rates: transplanted adult individuals survived better than seeds directly sown in the site. In three consecutive years, we reared and released several colonies of buff-tailed bumble bees, which survived through the flowering season but did not develop new gynes.

BACKGROUND

In the last decades large relevance has been given to honey bee colony collapse, but also wild bees are declining worldwide, both in abundance and specie richness (Burkle *et al.* 2013, Ollerton *et al.* 2014). The main drivers of the decline are habitat fragmentation, land-use change, pollution and climatic changes, which may affect pollinators and reduce floral resources and nesting sites (Vanbergen *et al.* 2013, Goulson *et al.* 2015). Although honey bee has been generally considered the most important insect pollinator, recent studies have demonstrated a functional complementarity of wild bees in the pollination of several crops (Garibaldi *et al.* 2013, Mallinger & Gratton 2014), wild plants (Ollerton *et al.* 2011) and floral resources in urban landscapes (Lowenstein *et al.* 2015). The provision of artificial nesting sites is among the most common methods used to support local populations of pollinators, and appropriate food resources are needed to ensure their sustenance throughout the colony development.

From 2011 to 2015 a LIFE+ Biodiversity demonstrative project (www.pp-icon.eu) has been carried out focusing on the conservation of an isolated population of dittany *Dictamnus albus* and its wild pollinators, within the Natural Park "Gessi Bolognesi e Calanchi dell'Abbadessa", located nearby Bologna (Emilia Romagna, Italy). In this region, dittany is protected under Regional Law (L.R. 2/1977), and the studied area is included within a Natura 2000 site (SCI-SPA IT4050001).

The study site is mainly composed of abandoned coppice and abandoned pastures, interspersed with rural buildings and private land. The natural vegetation is dominated by downy oak *Quercus humilis* and ash *Fraxinus ornus*, and by mesophilous shrubberies of blackthorn *Prunus spinosa*, dog-rose *Rosa canina* and common dogwood *Cornus sanguinea*. Residual post-cultural grasslands are dominated by orchard grass *Dactylis glomerata*, common meadow-grass *Poa pratensis* and couch grass *Agropyron repens*. The vertebrate fauna of the Natural Park is well known; by contrast, no inventory of arthropods of this area is currently available.

In recent studies Fisogni and colleagues (2011, 2015) reported that the great majority of insect visitors and pollinators of dittany in the area are bees (Hymenoptera, Apoidea), including both social (honey bees *Apis mellifera* and bumble bees *Bombus* spp.) and

solitary (e.g. mason bees *Osmia* spp., carpenter bees *Xylocopa* spp., mining bees Andrenidae, digger bees Anthophorinae, sweat bees Halictidae) bees.

One of the main risk factor for the target population of dittany is pollination limitation: seed production may suffer from a reduced pollen supply, indicating a deficit in the pollination service (Fisogni 2010, Fisogni *et al.* 2015).

To support the local bee fauna and consequently favour the pollination of dittany, we pursued three actions, aiming at enhancing the number of native bee plants, providing artificial nesting sites for solitary and social bees, and reinforcing the bumble bee population through the rearing and releasing of wild colonies.

ACTION

Providing nests for pollinators

We built artificial nests for bumble bees using an upside-down terracotta flower pot, with a diameter of about 20 cm, filled with straw and bedding for caged hamsters (which is known to be attractive for bumble bee queens). Nest entrance was provided by a 25-30 cm long piece of garden hose (internal diameter 18 mm). The base of the pot and the hose were buried and covered with soil and leaves, leaving only the outer end of the hole free (Figure 1a). An accurate description of bumble bee nest construction can be found in Bortolotti *et al.* (2015). Nest material cost €5 each. Ten bumble bee nests were placed in the area in spring 2011, preferably in sheltered places such as tree bases. Nests were rearranged and supplied with new litter every year in early spring to increase their attractiveness (Figure 1b), and the occupancy was checked during the season, by periodical observations of the nest entrance, and at the end of the season, by opening the nest to search for signs of bumble bee presence.

Figure 1. Artificial bumble bee nest placed in the area (a) and its periodic upkeep (b).

Artificial nests for solitary bees were re-adapted throughout the project to respond to the indications arisen from the project results (i.e. identification of the best pollinators of dittany), and to avoid the problems emerged during the first year (see below). In 2011, we assembled 15 nests for cavity-nesting solitary bees. Each nest contained 28 holes of seven different sizes, from 0.2 to 1.4 cm. Nests were placed on trees at a minimum height of one meter and a half to avoid mammal predation (Figure 2a). Since 10 nests out of 15 were completely hollowed out by acrobat ants *Crematogaster scutellaris*, in April 2012 we put six different nests on a pole fixed in the ground and spread with ant glue (Glu arboricole Pelton 2) (Figure 2b). Each nest contained eight wooden cubes presenting cavities of different size, from 0.6 to 1.4 cm. Smallest holes were excluded because they hosted small sized bees that act as pollen and nectar robbers in dittany. Nest material cost €80 each. Nest occupancy was followed by visual inspection until October 2013.

In spring 2014 we installed two "bee hotels" (40 × 70 × 150 cm) in the area: each one contained the above described wooden cubes and canes of various length and diameter. Canes of at least 60 cm length and 1.2 cm diameter were added for the mating and nesting of carpenter bees (Vicedomini 2009). In addition, to favour digger bees, we added perforated clay bricks filled with mud, and cleared the ground of the bee hotels (about 80 × 160 cm) from wild plants, turned it over, covered it with soil and sand and surrounded it with tuff bricks (Figure 2c). Bee hotel legs were fixed in the ground and

covered by ant glue. The material and manufacturing costs amounted to €600 for each bee hotel. Nest occupation was not recorded in 2014-2015, but nest holes were periodically inspected to check for problems or new achievements.

Figure 2. Progression in solitary bee nest shape during project duration: (a) 2011 nests; (b) 2012-2013 nests; (c) 2014-2015 bee hotels.

Planting of bee plants

In order to assure and increase food resources to pollinators throughout their life cycle, we planted several native bee plants (i.e. plants that provide nectar and pollen for bees) with scalar flowering (Table 1). Selection of species was based on their attractiveness to bees, flowering period and environmental suitability (Mossetti 2015).

We limited as much as possible the use of species that flower in May, in order to reduce the possibility of competition with dittany for pollination services. The propagation strategy comprised seeds and/or adult plants collection (depending on species lifespan), seed germination in greenhouse, seed propagation, and juveniles and/or adults transplantation in the abandoned pastures or at the wood fringes in the study site. Seeds and adult individuals have been collected from local (regional) wild populations, except for deadnettle *Lamium* spp. and lungwort *Pulmonaria vallarsae* that were taken from the Bologna Botanic Garden, since they were of regional provenance.

Table 1. Details of the bee plants transplanted and established in the area. Year = sowing or transplantation of individuals; Adult = vegetative adult plants; Wild = local populations; BG = Botanic Garden. Flowering period refers to the study area.

Species	Flowering period	Sampling	Provenance	Year	No. total seeds and planted individuals	No. established plants
Helleborus viridis	Feb, Mar	Adult	Wild	2011-2012	30 plants	30
Pulmonaria vallarsae	Mar, Apr	Adult	BG, Wild	2012	>25 plants	8
Lamium purpureum	Mar, Apr	Adult	BG	2012	>25 plants	> 20
Lamium maculatum	Mar, Apr	Adult	BG	2012	>25 plants	> 20
Vicia sativa	May-Jul	Seeds	Wild	2012	≈ 70 seeds, 5 plants	5
Lathyrus latifolius	May-Aug	Seeds	Wild	2012-2013	≈ 60 seeds, 5 plants	5
Securigera varia	May-Aug	Seeds, adult	Wild	2012-2013	> 300 seeds	> 10
Hedysarum coronarium	Jun, Jul	Seeds, adult	Wild	2011	15 plants	2
Trifolium pratense	Jun, Jul	Adult	BG, Wild	2012	≈ 250 seeds, >25 plants	> 25
Scorpiurus muricatus	Jun, Jul	Seeds	Wild	2012-2013	> 60 seeds, 5 plants	5
Trifolium repens	Jun-Aug	Seeds	Wild	2012-2013	> 300 seeds	> 25
Prunella laciniata	Jun-Aug	Seeds	Wild	2012	> 60 seeds, 5 plants	> 25
Melilotus officinalis	Jun-Sep	Adult	Wild	2012-2013	> 200 seeds, 5 plants	5
Veronica spicata subsp. barrelieri	Jun-Sept	Seeds, adult	Wild	2011	15 plants	15
Vicia cracca	Jun-Sept	Seeds, adult	Wild	2013	> 60 seeds, 10 plants	> 10
Cephalaria transsylvanica	Jul-Sept	Seeds	Wild	2012-2013	10 plants, ≈ 30 seeds	> 10
Clinopodium nepeta	Jul-Sept	Adult	Wild	2012	5 plants	> 5

In November 2011 we directly dispersed in the target area some diaspores collected during summer and we planted adult individuals of long-lived perennials; in early spring 2012 we transplanted adults and plantlets germinated at the Botanic Garden (Table 1). During late spring and summer 2012, we collected mature seeds of the selected plants following "ENSCONET Seed Collecting Manual for Wild Species" recommendations (http://www.bgci.org/resources/news/0632). A technical data form was compiled for each sourcepopulation. From July 2012, the collected seeds were both

potted at the Botanic Garden and directly dispersed in the target area, in order to increase the possibilities of propagation. To reinforce populations introduced the previous years, during autumn 2012 we repeated transplantations of green hellebore *Helleborus viridis*, deadnettle and lungwort, and in April 2013 we also transplanted a few individuals from newly germinated juveniles at the Botanic Garden.

Rearing and releasing of bumble bee species

Queens of the most common bumble bee species were collected every year in the surroundings of the target area and reared in controlled conditions; the resulting colonies were released in the area before the beginning of the flowering season. The protocol applied for bumble bee rearing and releasing is described in Bogo & Bortolotti (2015). In September 2011, 15 queens of the buff-tailed bumble bee *Bombus terrestris* and eight queens of the common carder bee *B. pascuorum* were collected and hibernated in the laboratory, but failed to survive. Therefore, we decided to collect in the field only post-diapausing bumble bee queens. Ten queens were collected at the end of March 2012, but the colonies obtained did not develop adequately, probably due to an early spring and consequently to a delay in our collecting campaign. As a consequence, in 2012 we purchased three commercial colonies of buff-tailed bumble bees by a local supplier (Bioplanet Soc. Coop. A.R.L., Cesena, Italy), to implement the pollination service on dittany. Each commercial colony cost €50. Colonies were removed before the emergence of males and queens, to avoid genetic contamination of the native populations. Contrarily, in 2013 spring was late and rainy, so we could collect only eight post-diapause buff-tailed bumble bee queens and two post-diapause common carder bee queens at the end of March (Figure 3). Queens of the common carder bee did not develop a colony due to breeding difficulties, therefore we proceeded in the following years only with buff-tailed bumble bees, whose artificial rearing is easier to obtain. Colonies were periodically inspected both outside, to verify the presence of flying bumble bees, and inside, to check the survival and the eventual presence of nuisances (Figure 4).

Figure 3. Bumble bee queens collection in the field (a) and their management in the laboratory: buff-tailed bumble bee *B. terrestris* (b), common carder bee *B. pascuorum* (c).

Figure 4. An example of artificially reared bumble bee colony (a), its releasing in the area (b) and its periodical check for colony development and presence of pests (c).

CONSEQUENCES

Providing nests for pollinators

Bumble bee artificial nests were not occupied by queens for all the project duration. Total occupation of solitary bee nests increased through years, particularly in medium- and large-sized cavities (Table 2).

Canes with 0.6 cm diameter were the most occupied in both 2011 and 2012, while there was a slight reduction in 2013. By contrast, there was a drastic increase in the occupation rate of larger canes, especially in the last year of monitoring. The placement of bee hotels considerably increased the number and shape of cavities compared with previous types of nest. In 2014 we observed for the first time occupation of larger canes by carpenter bees, both during the early mating season and at later stages (Figure 5).

Table 2. Nest occupation during the first three years of the project. * = percentage calculated excluding the smallest (0.2 and 0.4 cm) cavities.

Cavity size (cm)	Occupied cavities 2011		Occupied cavities 2012		Occupied cavities 2013	
	No./tot	%	No./tot	%	No./tot	%
0.2	31/60	52	---	---	---	---
0.4	25/60	42	---	---	---	---
0.6	13/60	21.7	117/294	39.8	83/294	28.2
0.8	3/60	5	7/216	3.2	60/216	27.8
1	1/60	1.7	10/150	6.7	31/150	20.7
1.2	0/60	0	3/48	6.2	16/48	33.3
1.4	0/60	0	1/48	2.1	2/48	4.2
TOT	73/420	17.4 (5.7)*	145/756	19.2	192/756	25.4

Figure 5. Solitary bee species nesting in artificial nests; adults of mason bees *Osmia cornuta* (a) and *Osmia* sp. (b), and carpenter bee *Xylocopa violacea* (c).

During spring 2014, a random sample of individuals nesting in canes of different size were collected soon after emergence for identification. We found mason bees, leafcutter bees *Megachile* spp. and wool carder bees *Anthidium manicatum*. We did not observe any ground nesting bees at the base of the bee hotels nor digger bees in the mud-filled bricks. Solitary bee nests were partly colonised by nest competitors, parasites and predators in different amounts (Figure 6). In the first group, we found grass-carrying wasps *Isodontia mexicana*, who feeds its larvae on paralysed crickets stored in the nest cavities; pedotrophic nests of different spider catching wasps (Sphecidae, Eumenidae

and Pompilidae) were also found inside holes. Among parasites, we mainly observed the bee-fly *Anthrax anthrax*, which lays eggs in the open cells of solitary bees. During the first year of the study 67 % of nests of solitary bees were predated by acrobat ants.

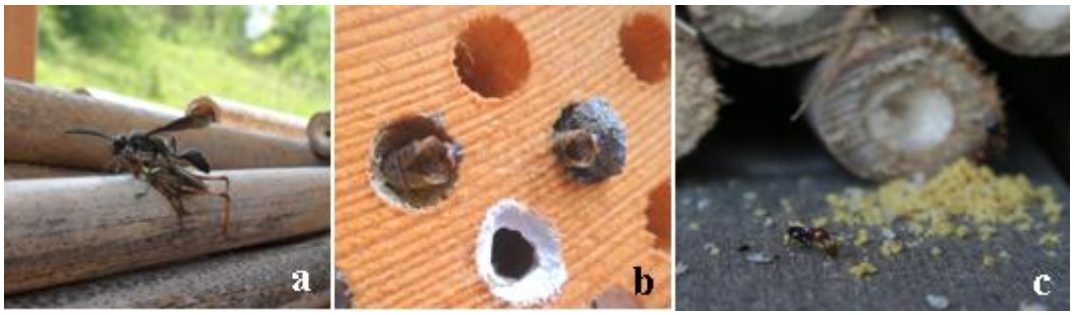

Figure 6. Nest intruders, parasites and predators of solitary bee nests: (a) adult of the grass-carrying wasp *Isodontia mexicana* nesting in bee hotel, (b) exuviae of the bee-fly *Anthrax anthrax* emerged from nest cavities and acrobat ant *Crematogaster scutellaris* feeding on bee stored pollen (c).

Planting of bee plants

Regarding the long-lived adult individuals directly transplanted in the site, green hellebore and spiked speedwell *Veronica spicata* subsp. *barrelieri* had the highest and full success, with 30 and 15 established individuals, respectively. The plantation of French honeysuckle *Hedysarum coronarium* gave full establishment of two out of five individuals (Table 1). In 2012, the majority of individuals belonging to deadnettle and lungwort, despite flowering during spring, did not overcome the summer due to the conjunction of an extremely dry season and presumable eradication by boars. In 2013 there was a recovery of deadnettle plants after spontaneous germination by seed, and by the end of the project several individuals were well established in the area. By contrast, only three plants of lungwort were observed in 2014 and 2015, likely due to a prominent eradication activity by boars.

Several individuals planted in 2012 from seeds germinated at the Botanic Garden were observed in the following seasons (2013-2015). All the species transplanted in April 2013 survived to some extent (Table 1), and during surveys performed in 2014 and 2015 several individuals were in bloom and actively visited by bees (e.g. mason bees, carpenter bees and bumble bees).

Bumble bee rearing

The rearing process and release of bumble bee colonies in the project area improved from 2013 to 2015 (Table 3). In 2013 three out of 10 buff-tailed bumble bee queens produced a medium-size colony (10-20 workers and large brood) that were released at the beginning of May. Two other queens with a reduced brood (only few larvae and no workers) were placed in two of the artificial bumble bee nests which were not previously occupied, but they did not produce a colony. The first three colonies survived during the flowering of dittany, and one of them developed until gynes production. At the beginning of May 2014 we released nine colonies. After one month, six of them were still in good condition, but during summer they were severely attacked by parasites and predators, and failed in producing new gynes. In 2015, all the seven released colonies survived throughout the season, but again they suffered the attack by parasites and predators and they did not develop until gynes production.

Table 3. Number of wild queens collected in the field and corresponding number of colonies released and survived in the field throughout the project duration.

Bumble bee rearing progress	**2012**	**2013**	**2014**	**2015**
Collected queens	9	10	32	26
Egg laying queens	5	7	18	15
Colonies released in the target area	0	5	9	7
Colonies survived in the field	0	1	6	7

Among parasites we observed larvae and puparia of the fly *Brachycoma devia* and the lesser house fly *Fannia canicularis*, and larvae of the bee moth *Aphomia sociella*. Among predators we mainly observed the hornet *Vespa crabro*.

DISCUSSION

The introduction of artificial nests and bee plants to enhance and support wild pollinators is a quite widespread practice in conservation programs, as well as in bee-friendly gardening. In this project we proposed an integrated approach that combines

habitat management, such as the plantation of bee plants, and conservation actions towards pollinators (Bogo *et al.* 2015).

The pollinating species targeted by our actions are among the most common and efficient pollinators in the study area and of dittany in particular. Among solitary bees, we observed a good artificial-nest occupation by mason bees and leafcutter bees, and a sporadic but promising presence of carpenter bees at our artificial shelters. On the contrary, we did not observe the presence of digger bees in the artificial clay bricks or ground nesting bees in the turned soil below the bee hotel; other surveys also indicate a low effectiveness of artificial nests for these bees (Gibbs 2004).

The most common cavity nesting species are usually easily attracted by bee hotels and artificial nests, and several studies report encouraging results (e.g. Wilkaniec & Giejdasz 2003, Gaston *et al.* 2005). Nevertheless it is complicated to calculate the benefits of these measures on local bee populations, and additional investigation to understand the pitfalls and benefits of bee hotels on bee biodiversity and pollination is needed (MacIvor & Packer 2015).

The high occupation rate of our artificial nests by the medium-sized mason bees and leafcutter bees and by the large carpenter bee suggests an increased availability of efficient pollinators for dittany, and consequently for the other flowering plants in the site. Accordingly, the survey on dittany pollinators during the four years of the project (Fisogni *et al.* 2015) showed a significant increase of megachilids in 2014 with respect to the previous years. By contrast, no increase in bumble bee visits to dittany have been recorded, despite the strong efforts in colony release. The reason can be that the number of released colonies was not adequate, or that bumble bees were attracted by other co-flowering plants. Other variations in dittany pollinators were probably independent of our actions: for example, the two abundance peaks observed in 2013 and 2014 for the digger bee *Habropoda tarsata*, a species which was not attracted by our artificial nests, and the high inter-annual fluctuation of honey bees, most likely related to human activity.

The presence of parasites and predators in artificial nests apparently did not severely affect the nest occupancy by solitary bees, which increased through years. The most aggressive predators in solitary bee nests were acrobat ants, which destroyed two-thirds of the nests in 2011. In the following years ant predation was easily prevented by

placing nests on a pedestal covered by ant glue. Other methods of protection from ants are described in the literature (Zammit *et al*. 2008).

The artificial nests and bee hotels were left in the area to increase nesting places for solitary bees after the end of the project.

Wild bumble bees did not use the provided artificial nests and these results support other works that highlight the low occupation success of artificial shelters by wild social species (Gaston *et al*. 2005, Lye 2009). However, a significant number of colonies of the buff-tailed bumble bee were released after rearing wild queens in controlled conditions. Artificial rearing is more expensive and time consuming compared to the placing of artificial nests, specially due to the maintaining of a climate room and the continue upkeep of the colonies, but despite costs it can be regarded as a good conservation measure for bumble bees (Goulson *et al.* 2002). Nevertheless, our released colonies struggled to survive throughout the season, likely due to the presence of parasites and predators that caused a developmental arrest before the emergence of queens and males. Therefore, although we enhanced the bumble bee population during the flowering of dittany, we did not succeed in establishing a new bumble bee generation for the forthcoming years.

All the bee plants transplanted as plantlets or adults settled in the area, while directly sown seeds showed a lower germination success. The plants that flowered attracted to some extent bees of several species throughout the year. In particular, early flowering species such as the green hellebore could represent an important food resource for bumble bee queens and early pollinators like mason bees, carpenter bees and other solitary bees. Considering the results obtained after three years from the actions, these plants are expected to maintain themselves autonomously and eventually increase in abundance.

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3.6. Seasonal and annual variations in the pollination efficiency of a pollinator community of *Dictamnus albus* L.

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Alessandro Fisogni [1*], Martina Rossi [2], Fabio Sgolastra [3], Laura Bortolotti [4], Gherardo Bogo [1,4], Natasha de Manincor [1], Marino Quaranta [5], Marta Galloni [1]

[1] Università di Bologna, Dipartimento di Scienze Biologiche, Geologiche e Ambientali. Via Irnerio, 42 - 40126 Bologna, Italy.

[2] Istituto di Bioscienze e Biorisorse, Consiglio Nazionale delle Ricerche, Via Università 133 - 80055 Portici (NA), Italy

[3] Università di Bologna, Dipartimento di Scienze Agrarie. Viale G. Fanin 42 – 40127 Bologna, Italy.

[4] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria - Unità di Ricerca di Apicoltura e Bachicoltura (CRA-API). Via di saliceto, 80 – 40128 Bologna, Italy.

[5] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria - Centro di Ricerca per l'Agrobiologia e la Pedologia (CRA-ABP) Via di Lanciola, 12/A – 50125 Firenze, Italy.

[*] Corresponding author: Alessandro Fisogni

SUMMARY

The interplay between insect and plant traits outlines the patterns of pollen transfer and the subsequent plant reproductive fitness. We studied the factors that affect the pollination efficiency of a pollinator community of *Dictamnus albus* L. by evaluating insect behaviour and morphological characteristics in relation to flowering phenology. In order to extrapolate the pollinator importance of single taxa and of the whole pollinator guild, we calculated an index distinguishing between potential (PPI) and

realized (RPI) pollinator importance. Although the pollinator species spectrum appeared rather constant, we found high intra- and inter-annual variability of pollinator frequency and importance within the insect community. Flower visitation rate strictly depended on insects abundance and on the overlap between their flying period and flower blooming. All the pollinators visited flowers from the bottom to the top of the racemes, excluding intra-plant geitonogamous pollination, and most of them showed high pollen fidelity. Only medium-large sized bees could contact the upward bending stiles while feeding on nectar, highlighting a specialization of the plant towards bigger pollinators. Moreover, we found evidence of functional specialization, since all pollinators were restricted to a single taxonomic group (order: Hymenoptera; superfamily: Apoidea). Both the PPI and RPI indices indicate Habropoda tarsata as the most important pollinator of *D. albus*. Following hand-cross pollination experiments we revealed the presence of pollination limitation in one of the three years of field study. We discuss this result in relation to flowering abundance and to possible mismatches of phenological periods between plants and insects.

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RESEARCH PAPER

Seasonal and annual variations in the pollination efficiency of a pollinator community of *Dictamnus albus* L.

A. Fisogni[1], M. Rossi[2], F. Sgolastra[3], L. Bortolotti[4], G. Bogo[1,4], N. de Manincor[1], M. Quaranta[5] & M. Galloni[1]

1 Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Università di Bologna, Bologna, Italy
2 Istituto di Bioscienze e Biorisorse, Consiglio Nazionale delle Ricerche, Portici (NA), Italy
3 Dipartimento di Scienze Agrarie, Università di Bologna, Bologna, Italy
4 Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria – Unità di Ricerca di Apicoltura e Bachicoltura (CRA-API), Bologna, Italy
5 Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria – Centro di Ricerca per l'Agrobiologia e la Pedologia (CRA-ABP), Florence, Italy

Keywords
Dictamnus albus; phenology; plant–pollinator interaction; pollination limitation; pollinator behaviour; pollinator importance; wild bees.

Correspondence
Alessandro Fisogni, Università di Bologna, Dipartimento di Scienze Biologiche, Geologiche e Ambientali. Via Irnerio, 42 – 40126 Bologna, Italy.
E-mail: alessandro.fisogni2@unibo.it

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ABSTRACT

The interplay between insect and plant traits outlines the patterns of pollen transfer and the subsequent plant reproductive fitness. We studied the factors that affect the pollination efficiency of a pollinator community of *Dictamnus albus* L. by evaluating insect behaviour and morphological characteristics in relation to flowering phenology. In order to extrapolate the pollinator importance of single taxa and of the whole pollinator guild, we calculated an index distinguishing between potential (PPI) and realized (RPI) pollinator importance. Although the pollinator species spectrum appeared rather constant, we found high intra- and inter-annual variability of pollinator frequency and importance within the insect community. Flower visitation rate strictly depended on insect abundance and on the overlap between their flying period and flower blooming. All the pollinators visited flowers from the bottom to the top of the racemes, excluding intra-plant geitonogamous pollination, and most of them showed high pollen fidelity. Only medium large-sized bees could contact the upward bending stiles while feeding on nectar, highlighting a specialisation of the plant towards bigger pollinators. Moreover, we found evidence of functional specialisation, since all pollinators were restricted to a single taxonomic group (order: Hymenoptera; superfamily: Apoidea). Both the PPI and RPI indices indicate *Habropoda tarsata* as the most important pollinator of *D. albus*. Following hand cross-pollination experiments we revealed the presence of pollination limitation in 1 of the 3 years of field study. We discuss this result in relation to flowering abundance and to possible mismatches of phenological periods between plants and insects.

INTRODUCTION

How pollinator behaviour, plant traits and their variability affect pollination in natural communities is among the key issues for pollination ecologists in order to improve knowledge on plant–pollinator interactions (Mayer *et al.* 2011). Typically, only a fraction of the insect community includes efficient pollinators for a given plant species, and the identification of these key pollinators could be extremely important in the framework of the conservation programmes (Potts *et al.* 2011). Flower visitors become pollinators only when they transport compatible viable pollen from a flower to the receptive stigma of a conspecific flower. A plant species may be defined as 'specialist' or 'generalist', depending on the number of visitor taxa that effectively transfer pollen among flowers, which may be regarded as 'effective pollinators' (Johnson & Steiner 2000; Ollerton *et al.* 2007).

Pollinator performance basically depends on the frequency of visits and effectiveness in pollen transfer, which in turn are influenced by behavioural, morphological and phenological traits. Temporal variability in the activity and abundance of pollinators may be considerable both within and among years (Ivey *et al.* 2003; Castro *et al.* 2013). Frequency of visits may vary in relation to plant population size and density: increased abundance or density of flowering at the population level may enhance insect visits as a direct consequence of increased attraction (Dauber *et al.* 2010). Visitation rates may also vary depending on the natural fluctuations of insect population abundance, or as a consequence of local mismatches between the phenological periods of both plants and insects (Olesen *et al.* 2008). Flowering phenology especially plays an important role in defining the interaction with pollinators. Timing of pollen presentation and stigma receptivity at the individual and population level may strongly force mate availability, and species with long flowering periods may avoid pollination limitation and reproductive deficit as a consequence of increased overlap with insect activity (Aronne *et al.* 2014). However, variations in the visitation rates among pollinating taxa and the local disruption of plant–pollinator interactions may lead to pollination limitation and to consequent seed reproductive failure in the population (Fernández *et al.* 2012; Forrest 2015).

Flower arrangement and reward availability within plants can drive pollinator foraging strategies by altering behaviour and directionality during flower probing, thus shaping mating success (Goverde *et al.* 2002; Iwata *et al.* 2012). Pollen transfer within and among plants has a striking relevance in natural systems since it defines the genetic structure of a population (García *et al.* 2007). Self-pollen deposition within inflorescence may be affected by floral display size and by the quality and quantity of insect pollen load, depending on visitation sequence in consecutive flowers (Karron *et al.* 2009; Howard & Barrows 2014). Visitation rate and foraging behaviour within plants influence the amount of geitonogamous self-pollen transferred and may eventually lead to pollen limitation in self-incompatible species (Sigrist & Sazima 2015). However, geitonogamy may also mitigate the presence of pollen limitation in the case of reduced mate availability in self-compatible species (Delmas *et al.* 2015).

The main objective of this study is to identify the pollinator community of a rare and threatened plant, *Dictamnus albus*, and the factors that affect the pollination efficiency within and among years. We aim to clarify the different contributions of each pollinating taxon throughout the different stages of plant blooming. A previous study found that gender-biased nectar production in *D. albus* flowers induces in honeybees and bumblebees a foraging behaviour that reduces intra-plant pollination (Fisogni *et al.* 2011): we want to evaluate if the entire pollinator guild of *D. albus* would present a comparable foraging directionality within racemes. Moreover, insect size in relation with flower structure can influence the effectiveness of pollen deposition (Willmer & Finlayson 2014; Sigrist & Sazima 2015): we want to determine to what extent the interplay between floral structure and pollinator behaviour influences the pollination effectiveness of each insect taxon. Given the spatial separation between nectaries and the stigmatic surface in *D. albus* flowers, we hypothesise that larger insects that forage for nectar would show higher effectiveness than smaller or pollen-gathering insects. In particular, we address the following questions: (i) which climatic parameters influence the extent of flowering in the studied population and what is the phenological development of *D. albus* racemes throughout anthesis; (ii) to what extent does the visitor community of *D. albus* vary within and among years in relation to flower phenology; (iii) which are the most important pollinators of *D. albus* and which parameters determine their efficiency; and (iv) how does the temporal variability of flowering abundance and of the pollinator community influence pollination limitation in the population?

MATERIAL AND METHODS

Species and study site

Dictamnus albus L. (Rutaceae) is a long-lived perennial herb with thick storage roots. Fertile plants produce flowering racemes that bear numerous pentamerous and slightly zygomorphic hermaphroditic flowers. Flowering within the raceme proceeds from the bottom to the top and single flowers show herkogamy and protandry; therefore, at full blooming, older flowers at the bottom of the raceme are in female phase while upper younger flowers are in male phase (Fisogni *et al.* 2011). Fruits are star-shaped capsules that autonomously disperse black pear-shaped seeds at ripening. We studied the relationships between *D. albus* and its visitor community in a natural population in a Natura 2000 site (SCI-SPA IT4050001) situated within the Regional Park Parco dei Gessi Bolognesi e Calanchi dell'Abbadessa, on the hills nearby the city of Bologna, Italy (168 m a.s.l., 44°25′11″ N, 11°23′54″ E). Typical habitats of *D. albus* are fringes between xerothermic woodlands and (semi)natural grasslands and clearings in open oak forests in the southern, warm-temperate regions of Europe and Central-Eastern Asia (Hensen & Oberprieler 2005). In the study site, *Dictamnus* plants are found at the edge of a wood mainly composed of downy oak (*Quercus pubescens*) and ash (*Fraxinus ornus*). In Italy, *D. albus* reaches its southern distribution limit and is protected at regional level. The species is locally protected across Europe and has been designated as 'vulnerable' in several European countries (Schnittler & Günther 1999).

Long-period flowering observations

In order to characterise the magnitude of flowering in the population through time in relationship to environmental factors, we counted the number of flowering stems in eight consecutive years (2007–2014) in a permanent plot of 500 m^2 at the edge of the studied site. For each year (from October to April) we considered the following variables: temperature (minimum and mean $month^{-1}$, °C), rainfall (mean $month^{-1}$, mm), snow cover (mean and total $month^{-1}$, snow water equivalent). Data were downloaded by the Regional Agency for the Environment Protection (ARPA Emilia-Romagna; permanent weather station of Settefonti, Bologna, 44°23′ N, 11°27′ E).

Insect observations

We studied the behaviour of insect visitors while foraging on *D. albus* flowers, performing field observations in a fixed patch. We randomly chose a subset of plants at the wood edge that were followed throughout their flowering period: eight plants in 2011, 2012 and 2013; five plants in 2014. We made a total of 6 days of observations per year: 2 days at the beginning of flowering, 2 days during the full blooming and 2 days towards the end of the flowering period. For each day, we repeated observations at four intervals (09:00 and 12:00 h, 15:00 and 18:00 h), each consisting in two 15-min periods, separated by 10 min of rest. After the two morning and afternoon intervals, we performed a 30-min period of net sampling throughout the area, collecting insects that alighted on flowers of *D. albus*. Collected individuals were put in separate vials with ethyl acetate and brought to the laboratory for taxonomic identification and pollen analysis. Observations and samplings of flower visitors were performed under favourable weather conditions. During observations, for each visitor we recorded the phenological stage of visited flowers, their position within the raceme, the reward sought (pollen, nectar) and the contact with receptive stigma.

Flowering phenology

We investigated flower anthesis during insect observations, recording the phenological stage of all the flowers within the fixed patch. Flower development was followed for 10–15 days

between April and May. In particular, for each flower we recorded whether it was functionally male or female by observing anther dehiscence and the upward bending of the style as an indicator of stigma receptivity (Fisogni *et al.* 2011). Dates of observations and flower availability changed among years depending on weather conditions and the development of the flowering.

Pollinator importance

To evaluate the potential contribution of flower visitors to pollination, we considered four parameters based on field observations: (i) pollinator effectiveness; (ii) visitation rate; (iii) foraging directionality; (iv) pollinator fidelity. Since it was not always possible to distinguish insect species during observations, and since different species belonging to the same genus or family had similar behaviours, for some taxa we considered the genus or family level.

We considered contacts with receptive stigmas by insects as a proxy for pollen deposition (hereafter: pollinator effectiveness, *sensu* Ne'eman *et al.* 2010). Individuals that contacted stigmas were considered as potential pollinators and the proportion of touches to receptive stigmas on the visited female phase flowers was then calculated.

For each group of potential pollinators we calculated the flower visitation rate per 15-min observation period using the following formula:

$$\frac{\text{number of visited flowers/plant/observation period}}{\text{number of open flowers/observation period}} \times \frac{\text{number of visited plants/observation period}}{\text{total observed plants}}$$

This allowed us to consider both flower and plant availability at the time of observations.

Thereafter, for each potential pollinator we assessed the direction of visit to flowers within the racemes. We regressed the number and the position of flowers visited on a given plant, and calculated the mean angular coefficient (b). Values equal or higher than 1 indicate upward movements, while values close to 0 indicate random visits, and negative values result from downward movements. We then calculated a modified Student's *t*-test and the related *P*-value to evaluate the consistency of the upward movement (Fisogni *et al.* 2011).

Insects caught during sampling periods were brought to the laboratory and pollen was removed from their bodies using a needle under a dissecting microscope. Since pollen actively gathered in the collecting structures of honeybees and bumblebees has been assessed as unavailable for pollination, we only considered sparse pollen on insect bodies, with the exception of megachilids, where pollen collected in ventral scopae remains highly vital and available for pollination (Pinzauti *et al.* 2002). Pollen grains were mounted on microscope slides with glycerine and observed under an optical microscope at 400× magnification. We counted 100 pollen grains per slide or all the grains if <100, and calculated the pollinator fidelity as the proportion of conspecific pollen to the total pollen load. Pollen grains sampled from anthers of *D. albus* were used as reference. Only insects with a total of five or more grains were considered for further analyses.

Taxa that showed both effectiveness and fidelity higher than 0.10 were considered as the main pollinator groups. For these groups we calculated an index of Potential Pollinator Importance (PPI) as effectiveness × visitation rate × directionality × *fidelity*, considering mean values over the 4 years of study. To integrate this index within the observed natural context, we calculated the index of Realized Pollinator Importance (RPI) for the three periods of flowering of *D. albus* (early, full and end) as PPI × %female phase flower × insect abundance.

Pollination limitation

To assess the presence of pollination limitation (*i.e.* pollen and/or pollinator limitation), we performed pollen augmentation experiments on randomly chosen plants throughout the population, for three consecutive years. We selected 40 individuals in 2012 and 2013, 32 plants in 2014; each year we assigned half of the plants to the pollination treatments which were performed as follows. We hand cross-pollinated all or the majority of flowers on the selected plants with pollen from at least two different pollen donors on two consecutive days. Dehiscent anthers were collected from flowers at least 25 m distant, to avoid genetic similarities, and brushed directly on receptive stigmas. Manipulated flowers were then tagged individually and assigned to the 'supplementation' (S) treatment (2012: 140, 2013: 173, 2014: 118 flowers, respectively). Non-manipulated flowers were chosen on the remaining plants, marked with different tags and left open to natural pollination ('control', C; 2012: 144, 2013: 171, 2014: 117 flowers). At the end of the flowering season we counted the number of developed fruits, the number of vital seeds produced and the number of unfertilised or aborted ovules. We then calculated fruit set (fruit:flower ratio) and seed set (seed:ovule in developed fruits) of control and pollen-augmented flowers.

Data analysis

Normality was assessed using Shapiro-Wilk test. Appropriate transformations were applied to improve normality and, when it could not be achieved, non-parametric tests were used. Relationships between the number of flowering stems and meteorological variables were calculated by means of Spearman's rank correlation. Variation in the visitor abundance within and among years was analysed using chi-square tests. We performed multiple correlations between the frequency of visits of *D. albus* insect community and the following variables: open/total flowers, female phase/open flowers, total number of open flowers, date of observation. For each pollinator group, differences in visitation rate among years and among flowering periods were tested using several-sample tests as Kruskall–Wallis and one-way ANOVA (followed by Mann–Whitney and Tukey's *post-hoc* pair-wise comparisons, respectively). Differences in fruit set between supplemented flowers and open-pollinated controls were evaluated using chi-square tests, while differences in seed set were evaluated with non-parametric Mann–Whitney *U*-tests. A 95% confidence interval was considered. Means ± SE are given. All statistical analyses were performed with R version 3.1.2 (R Core Team 2014).

RESULTS

Flowering abundance

We found high variability in the number of flowering stems among years. In the permanent plot of 500 m^2 we recorded <50 flowering stems in 2007, 2009 and 2014 (43, 29 and 27, respectively), up to 10-fold reproductive stems in 2008, 2010, 2011 and 2012 (380, 475, 287, 160, respectively), and a peak of 1114 flowering plants in 2013. We found a significant negative correlation between the number of flowering stems and mean winter temperature ($rs = -0.810$, $P = 0.022$) that ranged between 7.02 °C (2009/2010) and 10.64 °C (2006/2007) over the 8 years of study. Other meteorological variables did not correlate significantly.

Insect observations

We performed a total of 48 h of observations and 24 h of insect sampling from 2011 to 2014. The spectrum of insects that visited plants of *D. albus* was relatively constant through years and was composed by 12 main taxonomic groups (Fig. 1), 10 of which were bees (family: Hymenoptera, superfamily: Apoidea). For bumblebees we recorded *Bombus terrestris* (Linnaeus 1758), *B. pascuorum* (Scopoli 1763), *B. lapidarius* (Linnaeus 1758) and *B. pratorum* (Linnaeus 1761); megachilids included *Osmia aurulenta* (Panzer 1799), *O. cornuta* (Latreille 1805) and *O. bicornis* (Linnaeus 1758), *Megachile circumcinta* (Kirby 1802) and *Rhodanthidium septemdentatum* (Latreille 1809); among halictids we found *Halictus* gr. *simplex*, *Lasioglossum albipes* (Fabricius 1871), *L. pygmaeum* (Schenck 1853), *L. glabriusculum* (Morawitz 1872), *L. politum* (Schenck 1853) and *L. interruptum* (Panzer 1799). We also found the long-horned bees *Eucera longicornis* (Linnaeus 1758) and *E. nigrescens* (Pérez 1879), and the carpenter bees *Xylocopa violacea* (Linnaeus 1758) and *X. iris* (Christ 1791). Individuals of *Cerinatina* (Latreille) spp. were only found during sampling periods, so they were included in the analyses of pollinator fidelity. On average, *Habropoda tarsata* (Spinola 1838), halictids, bumblebees and megachilids were the most frequent visitors. Chi-square tests showed significant variations in the relative abundances of insect groups among years. The highest fluctuations were found for *Apis mellifera* (Linnaeus 1758), which was completely absent in 2012 while it was the second most abundant species in 2014 ($\chi^2 = 638.66$, $df = 3$, $P < 0.001$); for *H. tarsata* that showed two peaks in 2013 and 2014, when it was the most abundant species ($\chi^2 = 822.75$, $df = 3$, $P < 0.001$); and for megachilids that significantly increased in 2014 with respect to the previous years ($\chi^2 = 260.79$, $df = 3$, $P < 0.001$). Regarding floral rewards, all insect groups fed on nectar in more than 80% of the flower visits, with the exception of halictids (Fig. 2f) and hoverflies, which alighted directly on dehiscent anthers and were observed gathering or eating pollen in 34% and 46% of the visits, respectively.

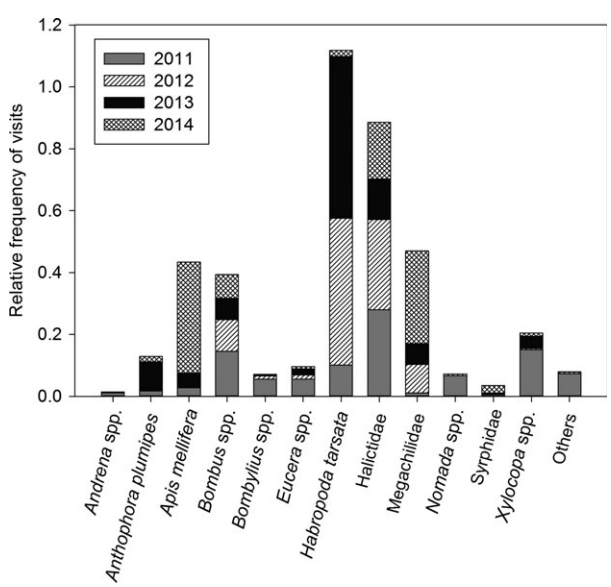

Fig. 1. Relative frequency of visits of the insect community of *Dictamnus albus* in the 4 years of study. Taxonomic groups are defined at the species, genus or family level. The group *Others* includes unidentified and sporadic insects.

Flowering phenology

In the observation patch we counted a total of 103, 123, 153 and 104 flowers in 2011, 2012, 2013 and 2014, respectively. In general, at the beginning of the observations we estimated that approximately 20–30% of the flowers were open; after 2–5 days we observed a peak of flowering with up to 90% of open flowers, followed by a decrease due to the progressive withering of older flowers at the base of the raceme (Fig. 3). The proportion of female phase flowers increased significantly with blooming ($rs = 0.869$, $P < 0.001$), following the protandrous development of flowers. In 2013 there was a marked delay of the beginning of blooming, therefore we started our observations 1 week later compared to other years. The frequency of visits of *Eucera* spp. and *Habropoda tarsata* negatively correlated with both the percentage of female phase flowers available ($rs = -0.520$, $P < 0.01$ and $rs = -0.480$ $P < 0.05$, respectively) and the flowering date, *i.e.* the later in the season the fewer the visits ($rs = -0.278$, $P < 0.01$ and $rs = -0.564$, $P < 0.01$, respectively). Frequency of visits also negatively correlated with the total number of open flowers for *Bombus* spp. and megachilids ($rs = -0.424$, $P < 0.05$ and $rs = -0.444$, $P < 0.05$, respectively).

Pollinator importance

Only a portion of flower visitors touched the receptive stigma, depending on insect dimensions and behaviour: these taxa were therefore counted as potential pollinators (Fig. 2a–e). Visiting taxa that showed the highest effectiveness were *Xylocopa* spp. (1.00), *H. tarsata* (0.92), bumblebees (0.86), *Anthophora plumipes* (Pallas 1772) (0.83) and *A. mellifera* (0.70) (Fig. 4). Megachilids and *Eucera* spp. touched the receptive stigma in about half of the visits to female phase flowers (0.54 and 0.41, respectively); other taxa showed an effectiveness below 0.10 (*Nomada* (Scopoli) spp. and *Bombylius* (Linnaeus) spp.) or close to zero (halictids, hoverflies and *Andrena* spp.). Halictids were sporadically observed (<1% of visits) alighting directly on receptive stigmas, likely to eat pollen previously deposed by other insects.

Mean visitation rates varied markedly among years: *A. mellifera* ranged from 0.002 to 0.51 ($H = 9.50$, $P < 0.01$), *H. tarsata* from 0.009 to 0.91 ($H = 8.75$, $P = 0.03$), megachilids from

Fig. 2. Visitor and pollinator insects foraging on flowers of *Dictamnus albus*. Large bees touching the receptive stigma while seeking nectar (a–d): *Habropoda tarsata* (a) *Anthophora plumipes* (b), *Bombus terrestris* (c), *Xylocopa violacea* (d). Medium-sized *Osmia* sp. (e): feeding on nectar without touching the receptive stigma. Small *Lasioglossum* sp. (f): eating or collecting pollen directly from dehisced anthers. Photographs by Francesca Rovetti.

0.001 to 0.46 ($H = 17.45$, $P < 0.001$) and halictids from 0.002 to 0.27 ($F = 5.43$, $P < 0.01$). We also found significant differences in visitation rates among flowering phases within years: *Habropoda tarsata* showed highest rates at the beginning of blooming, while it decreased substantially towards the end of anthesis ($H = 7.67$, $P = 0.02$), and a similar trend was found for *Eucera* spp. ($H = 7.47$, $P = 0.02$). Our records did not highlight any clear trend of visits for other taxa.

On average, all the observed insect taxa performed upward visits among flowers on the racemes of *D. albus*. Mean angular coefficients (b) were significantly higher than 1 for bumblebees, *H. tarsata*, megachilids and *A. plumipes*, indicating visits to non-consecutive flowers (Table 1); the other taxa consistently visited consecutive flowers from the bottom to the top of stems, excluding the possibility of geitonogamous pollination.

All the insect groups carried a certain amount of pollen grains of *D. albus* on their bodies. Nine taxa out of 13 showed levels of fidelity higher than 0.60 (Fig. 4). Bumblebees presented a frequency of conspecific pollen of 0.36, while only *Ceratina* spp. (0.28), syrphid flies (0.25) and *Bombylius* spp. (0.07) had values below 0.30.

The indices of PPI showed the higher potential contribute of *H. tarsata*, megachilids, *A. mellifera* and bumblebees, with lower values found for *Xylocopa* spp., *Eucera* spp. and *A. plumipes* (Table 2). The index of RPI varied greatly within flowering periods and among years for each pollinator taxon,

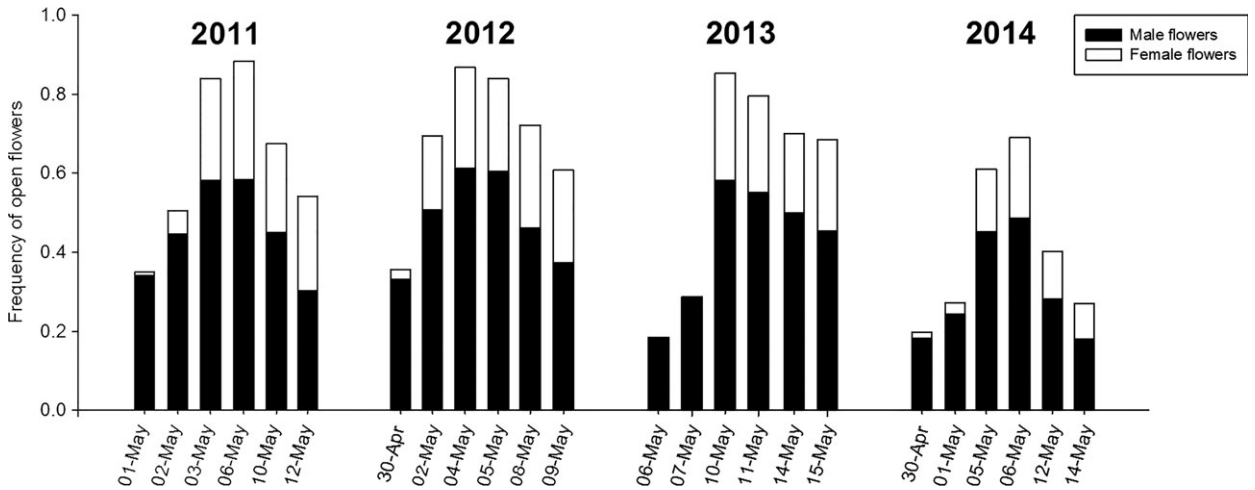

Fig. 3. Flower anthesis and availability of *Dictamnus albus* in the observation patch in the 4 years of study.

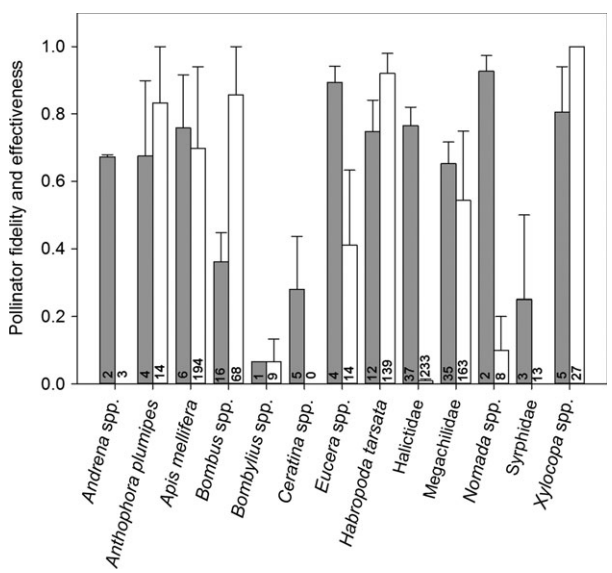

Fig. 4. Mean (±SE) fidelity (filled bars) and effectiveness (empty bars) of the visitor community of *Dictamnus albus* over the 4 years of study (see text for details on definitions). Sample size inside bars indicates sampled and observed insects, respectively. Taxa with fidelity or effectiveness values <0.10 were excluded from calculations of pollinator importance indices.

mainly as a consequence of the number of approaches to the patch (Table 3). Community values of RPI for each year showed higher contributions in 2012 (10.358) and 2014 (7.709), while very low values were found in 2011 (1.110) and 2013 (1.818).

Pollination limitation

We found statistically significant differences in both fruit and seed set between open-pollinated controls and hand-crossed flowers in 1 year of manipulation experiments (Fig. 5). Chi-square tests revealed a significant increase in the mean fruit/flower ratio in supplemented flowers compared to controls in 2013 (S: 0.60 ± 0.04, C: 0.41 ± 0.04; $\chi^2 = 11.175$, df = 1, $P < 0.001$). Similarly, Mann–Whitney tests showed a higher seed/ovule ratio (U = 1747, $P < 0.001$) in developed fruits after pollen augmentations than in controls (S: 0.69 ± 0.02, C: 0.57 ± 0.02). No significant differences were observed in fruit or seed production in 2012 or in 2014.

Table 1. Direction of visits among flowers within racemes of *D. albus* during foraging bouts for the main pollinator taxa, expressed as the mean angular coefficient of the linear regression between the flowers visited and sequence of visits (b ≥ 1 indicates upward movements).

Pollinator taxa	n	b	SE	Student's *t*	*P*(*t*)
Anthophora plumipes	16	1.69	0.38	1.83	0.04
Bombus spp.	61	1.61	0.23	2.62	0.01
Eucera spp.	9	1.49	0.57	0.86	0.20
Megachilidae	122	1.43	0.22	1.95	0.03
Habropoda tarsata	187	1.24	0.11	2.23	0.02
Xylocopa spp.	14	1.20	0.49	0.41	0.34
Apis mellifera	88	1.01	0.24	0.04	0.49

n = number of insects that visit two or more flowers within a raceme; *P* (*t*) = probability associated with the modified Student's *t*-test.

Table 2. Potential pollinator importance (PPI) for the main taxonomic groups of pollinators of *D. albus*, calculated by multiplying effectiveness × %visitation rate × directionality × fidelity (see text for details on the single components).

Pollinator taxa	Effectiveness	Visitation rate	Directionality	Fidelity	PPI
Habropoda tarsata	0.92	0.09	1.24	0.75	0.077
Megachilidae	0.54	0.06	1.43	0.65	0.030
Apis mellifera	0.70	0.05	1.01	0.76	0.027
Bombus spp.	0.86	0.03	1.61	0.36	0.015
Anthophora plumipes	0.83	0.01	1.69	0.68	0.010
Xylocopa spp.	1.00	0.01	1.20	0.81	0.009
Eucera spp.	0.41	0.01	1.49	0.89	0.005

Table 3. Realized Pollinator Importance (RPI) index of the main pollinator taxa calculated by multiplying Potential Pollinator Importance index(PPI) × %female phase flowers × number of approches to the observed patch, in three flowering periods (I: early, II: full, III: end) in the 4 years of study. Community RPI/year is obtained as the sum of all the RPIs per year.

Year	Flowering period	% Female phase flowers	*Anthophora plumipes* (PPI = 0.010)		*Apis mellifera* (PPI = 0.027)		*Bombus* spp. (PPI = 0.015)		*Eucera* spp. (PPI = 0.005)		*Habropoda tarsata* (PPI = 0.077)		Megachilidae (PPI = 0.030)		*Xylocopa* spp. (PPI = 0.009)		Community RPI/year
			Approaches	RPI	Approaches	RPI	Approaches	RPI	Approaches	RPI	Approaches	RPI	Approaches	RPI	Approaches	RPI	
2011	I	07.9	0	0	0	0	4	0.048	2	0.008	8	0.490	1	0.024	0	0	1.110
	II	32.4	0	0	0	0	0	0	6	0.010	10	0.249	1	0.010	27	0.079	
	III	38.1	3	0.011	5	0.051	22	0.126	2	0.004	0	0	0	0	0	0	
2012	I	20.5	0	0	0	0	51	0.157	12	0.012	455	7.182	56	0.344	0	0	10.358
	II	28.8	3	0.009	0	0	43	0.186	2	0.003	49	1.087	19	0.164	4	0.010	
	III	37.2	0	0	0	0	20	0.112	0	0	28	0.802	26	0.290	0	0	
2013	I	0.0	19	0	0	0	28	0	4	0	152	0	14	0	0	0	1.818
	II	31.3	16	0.050	16	0.135	0	0	2	0.034	58	1.398	12	0.113	2	0.006	
	III	31.2	2	0.006	3	0.025	0	0	2	0.003	0	0	1	0.009	14	0.039	
2014	I	09.2	14	0.013	4	0.010	2	0.003	8	0.004	14	0.099	55	0.152	0	0	7.709
	II	27.9	8	0.022	269	2.026	12	0.050	2	0.003	8	0.172	154	1.289	9	0.023	
	III	31.2	0	0	194	1.634	85	0.398	0	0	4	0.096	182	1.704	4	0.011	

Fig. 5. Mean (±SE) fruit set (fruit:flower ratio) and seed set (seed:ovule ratio) in open controls (empty bars) and hand-pollinated (filled bars) flowers in three consecutive years. ***$P < 0.001$.

DISCUSSION

Flowers of *D. albus* have abundant and well exposed rewards that are potentially easily exploitable by a wide array of pollinator species: nectar could be reached by both short- and long-tongued bees, the relatively long and patent stamens could work as a landing platform for butterflies, and petals present showy nectar guides. Based on these traits we can define flowers as morphologically and phenotypically generalised (*e.g.* Fenster *et al.* 2004; Ollerton *et al.* 2007). However, the realized generalisation or specialisation depends on the relationships that occur between the plant and its pollinator community. The way in which pollinators interact with flowers determines their potential efficiency as pollen vectors. In the studied site, *D. albus* is only efficiently pollinated by insects belonging to two families, Apidae and Megachilidae, and is therefore specialised on bees (Hymenoptera: Apoidea). Moreover, the floral structure functions as a selective factor for effective pollination. As hypothesised, the spatial separation between reproductive structures and the nectary located at the base of the ovary requires medium-large bees to effectively touch the receptive stigma and transfer pollen grains while they feed on nectar. In fact, carpenter bees (*Xylocopa* spp.) show the highest effectiveness, bumblebees (*Bombus* spp.) and large solitary bees (*H. tarsata* and *A. plumipes*) show slightly lower values, and the smaller mason bees (*Osmia* spp.) and leafcutter bees (*Megachile* spp.) have the lowest effectiveness. Conversely,

small halictids (*Halictus* and *Lasioglossum* spp.) act as nectar or pollen robbers and may have potential negative effects on both female and male plant fitness, since the pollen collected is not further available for pollination (do Carmo & Franceschinelli 2004; Castro *et al.* 2008; Hargreaves *et al.* 2010; Hanna *et al.* 2014). However, the consequence of robbing on plant reproductive success should be specifically investigated since positive effects might also be observed (Fumero-Cabán & Meléndez-Ackerman 2013; Mayer *et al.* 2014; Singh *et al.* 2014).

Our estimates of pollinator fidelity highlight how *D. albus* is a favourite resource of floral rewards for its pollinator community, suggesting that pollen uptake is not a limiting factor for plant reproductive success. Foraging pollinator behaviour defines pollen transfer within and among plants and contributes in shaping the population genetic structure. Preferential direction of visits has been observed in several species and is due to a combination of innate behaviour and learning abilities connected to foraging experience (Iwata *et al.* 2012; Valtueña *et al.* 2013; Morawetz *et al.* 2014). *Dictamnus albus* racemes display a bottom-to-top nectar gradient due to gender-biased nectar production towards the older female stage flowers (Fisogni *et al.* 2011). Our analysis of insect directionality while foraging on *D. albus* shows that pollinators visit plants from lower to upper flowers, confirming previous findings on honeybees and bumblebees (Fisogni *et al.* 2011) and extending analogous pattern of movements to all the main pollinating taxa. This implies the absence of intra-plant pollination and of the following potential negative effects on reproductive fitness related to geitonogamy, such as pollen discounting, stigma clogging (de Jong *et al.* 1993; Harder & Barrett 1995; Gross 2005; Howard & Barrows 2014) and inbreeding depression, since *D. albus* is partially self-compatible and self-fertilisation results in reduced seed germination (Fisogni *et al.* 2011).

The proportion of female phase flowers increases as flowering progresses in the population, with a consequent rise in nectar availability, reaching a climax before the decline towards the end of blooming. However, we did not observe an increase of pollinator abundance or of flower visitation rate. On the contrary, visits decreased over time. *Habropoda tarsata* is the most important pollinator of *D. albus*, according to both the index of potential pollinator importance (PPI = 0.077) and the index of realized pollinator importance (maximum RPI = 7.182). This solitary bee is one of the earliest bees to emerge in the study site (personal observation) and is therefore more abundant during the beginning of flowering, while its presence decreases naturally with time due to the end of its nesting period, independently of resource availability. In contrast, the pollen requirement for bumblebee and honeybee brood nutrition increases during the season, as the colony becomes established or increases in size, respectively (Jeffree & Allen 1956; Duchateau & Velthuis 1988; Tasei & Aupinel 2008). The increased proportion of female phase flowers following blooming progress also implies that the amount of available pollen decreases in the population. Social bees and late-spring flying solitary bees are therefore more likely to seek other plants in the surroundings as pollen donors, in order to supplement their diet. Moreover, as spring advances the number of available flowering species increases in the study area (personal observation), with expected consequences on dietary choices, flower constancy and increased potential for competition among plants for pollinator services (Fontaine *et al.* 2008; Mitchell *et al.* 2009; Flanagan *et al.* 2011; Somme *et al.* 2015).

High values of cumulative pollinator importance at the community level should reflect strong positive plant–pollinator interactions that would lead to increased plant fitness in the population. We observed an opposite trend between community pollinator importance and pollination limitation in 3 years. Highest values of importance were associated with the lack of pollination limitation, while we found evidence of pollination limitation for both fruit and seed production in conjunction with the lowest value of importance. We hypothesise that the main cause of limitation was the exceptionally high blooming that occurred during the spring of 2013. Increased floral resource availability may lead to an allee effect and enhance pollinator abundance (Groom 1998; Meyer *et al.* 2007), although plants may face decreased visitation rates due to competition for pollinator services (Goulson *et al.* 1998; Steven *et al.* 2003; Ghazoul 2006; Dauber *et al.* 2010). This particular year, flower abundance in the population increased one order of magnitude compared to the other years. The number of flowers available might have exceeded a threshold through which the benefits from insect attraction and pollination service are balanced, leading to a 'dilution' in plant–animal interactions with reduced efficiency of the local pollinator community. This may have caused a shortage of pollen transferred between flowers (*i.e.* quantitative pollen limitation), but also reduced quality of the pollen delivered to stigmas. In fact, bees frequently move between nearest neighbour plants, depositing higher pollen amounts at short distance (Morris 1993; Cresswell *et al.* 1995). Since *D. albus* fruits autonomously release seeds within a short distance of the mother plant (Frey 2000; personal observations), we assume that the increased number of neighbour flowering plants might have augmented the probability of pollen transfer between genetically related individuals, thus reducing their reproductive fitness. Finally, climatic conditions may have altered the overlap between flowering and the period of pollinator activity. Monthly temperatures that precede flowering can influence plant phenology; warmer temperatures frequently corresponding to advanced blooming in spring-flowering species (Fitter & Fitter 2002; Menzel *et al.* 2006). Winter temperatures of 2012–2013 were lower than in the other years, and corresponded to more abundant but delayed blooming in the observed *D. albus* population during the spring of 2013. Winter temperatures can also determine the emergence time of bees, affecting the diapause termination process (Yocum *et al.* 2006; Gosterit & Gurel 2009; Sgolastra *et al.* 2010). Thus synchronisation between the insect emergence and the flowering period could be altered by extreme climate conditions due to the different thermal physiological adaptations between the insect populations and the plant phenology (Forrest & Thomson 2011). This occasional mismatch can explain the pollination limitation observed in spring 2013, where the number of active early pollinators was not sufficient to guarantee an adequate pollination service.

In conclusion, this study shows that for *Dictamnus albus* the importance of single pollinators is mainly defined by their body size, frequency of visits, foraging behaviour and by the phenological phase of the visited flowers. Bigger bees may be more effective than smaller insects as potentially depositing larger

amounts of pollen, but smaller bees may have higher visitation rates, thus increasing their importance as pollen vectors. At the community level, the interplay between early and late flying pollinators throughout the flowering span of *D. albus* can guarantee an efficient pollination service. However, under increased abundance of flower availability and influence of climate variables there might be a disruption in the plant–pollinator interaction, with negative consequences for plant reproductive fitness.

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3.7. Temporal activity patterns in a pollination community of *Dictamnus albus* L. in relation to some biotic and abiotic factors

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Fabio Sgolastra[1*], Alessandro Fisogni[2], Marino Quaranta[3], Gherardo Bogo[2,4], Laura Bortolotti[4], Marta Galloni[2]

[1] Dipartimento di Scienze Agrarie, Università di Bologna, Italy

[2] Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Università di Bologna, Italy

[3] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria - Centro di Ricerca per l'Agrobiologia e la Pedologia (CREA-ABP), Firenze, Italy

[4] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria - Unità di Ricerca di Apicoltura e Bachicoltura (CREA-API), Bologna, Italy

[*] Corresponding author: Fabio Sgolastra

SUMMARY

Pollinator behaviour depends on several biotic (e.g. interspecific interactions, floral reward availability) and abiotic (e.g. weather and climate variables) factors that determine the variation in seasonal and daily patterns of activity among species. In turn, these modifications will influence the composition and abundance of a pollinator community and ultimately the extent of their pollination service.

The aim of this four-year study was to assess the effect of some abiotic and biotic factors (ambient temperature, relative humidity, flower availability and pollinator competition) on the abundance and activity pattern of flower-visiting insect groups throughout the blooming of a locally rare plant species, *Dictamnus albus L.* (Rutaceae). Moreover, we assessed whether the climate conditions during wintering can explain the annual differences observed in the abundances of each flower-visiting insect group.

We found a large inter-annual variation in the abundance and activity of pollinators, with up to a six-fold increase in the total number of individual insects observed. Moreover, a similar fluctuation among years was also highlighted by biodiversity

indices and by the changes in the relative frequency of each flower-visiting group. Annual and daily variations were explained by a certain level of "response diversity" between flower-visiting groups in relation to different environmental conditions (e.g. ambient temperature, relative humidity and flowering availability) during *D. albus* blooming. In fact, the pollinator activity was related to ambient temperature in two flower-visiting groups (*Anthidium* spp. and *Bombylius* spp.), to relative humidity in five groups (positive relation in *A. plumipes* and *H. tarsata*; negative relation in *Megachile* spp., *Xylocopa* spp. and Syrphidae) and to both factors in two groups (Halictidae and Vespidae). On the contrary, the climate conditions during the winter did not affect the phenology of flower-visiting groups, except for honey bees, *Megachile* spp. and *Eucera* spp.

In conclusion, our results show that the pollinator community of *D. albus* is quite variable among years and during the days of blooming, but the different spectrum of activity related to different environmental responses might guarantee a stable pollination service of this plant species also in years with extreme environmental conditions.

Key words: *Dictamnus albus* L., Hymenoptera Apoidea, bees, plant-pollinator interactions, climate change, weather conditions

INTRODUCTION

Pollinator behaviour depends on several biotic (e.g. predation and competition interactions, pollen and nectar availability) and abiotic (e.g. ambient temperature, solar radiation, relative humidity and wind speed) factors that determine the variation in seasonal and daily patterns of activity among species. Therefore, the presence of different pollinator species visiting the flowers of a given plant species in the course of a day and during the flowering period depends on the interspecific differences in temporal activity. Moreover, the climatic conditions during the winter period and early spring can affect the timing of pollinator emergence in diapausing insects and thus the synchronization with the flowering period (Schweiger *et al.*, 2008; Forrest, 2011). In

fact, some studies on bumblebees and solitary bees have shown that the temperature during overwintering and the accumulation of degree-day in early spring can affect their diapause termination (Gosterit and Gurel, 2009; White *et al.*, 2009; Jiang *et al.*, 2010; Sgolastra *et al.*, 2010; Bartomeus *et al.*, 2011).

The daily activity of pollinators can vary considerably among taxa. For example, Herrera (1990) observed a variation in the timing of foraging on *Lavandula latifolia* Medicus, both among major groups (hymenopterans, dipterans and lepidopterans) and among species within groups. In these cases, weather conditions is often one of the main abiotic factors affecting pollinator activity. Tuell and Isaacs (2010) showed an effect of the ambient temperature, wind speed, humidity and solar radiation on the foraging community composition on highbush blueberry, *Vaccinium corymbosum* L. In the aforementioned study, honey bees resulted more abundant during good weather conditions (high temperature and solar radiation, low wind speed and humidity), whereas bumble bees dominated during poor weather. Similar results were also observed in an apple orchard where *Apis mellifera* L. showed a peak of activity in late morning and early afternoon, when the ambient temperatures were higher; in contrast, *Osmia cornuta* (Latr.) was equally abundant throughout the day (Vicens and Bosch, 2000).

Because the weather conditions can vary significantly between years, the abundance of pollinators during the flowering season of a given plant can show annual variation with possible effects on the pollination success and the percentage of fruit-set (Ivey *et al.*, 2003). Although the pollinator seasonal activity is usually associated to the flowering period of pollinated plants, in particular in oligolectic species, the recent climate changes could potentially lead to phenological mismatches in plant-pollinator interactions (Bartomeus *et al.*, 2011). Over the period 1880 to 2012 , the global surface temperature calculated as linear trend shows a warming of 0.85 [0.65 to 1.06] °C and some ecological responses to climate change are already evident (Walther *et al.*, 2002; IPCC, 2013). The animal and plant phenology (i.e. the timing of seasonal activities) is probably the simplest process in which the climate changes can affect the ecology of animal and plant species observable in relatively few years (IPCC, 2007; Bartomeus *et al.*, 2011). Walther *et al.* (2002) reported that spring activities, including early blooming, have occurred progressively earlier since the 1960s both in Europe and in

North America. Because not all taxonomic groups respond similarly to the temperature variations, differences in the magnitude of phenological responses may affect food-web interactions with important ecological consequences (Winder and Schindler, 2004). Shifts in seasonal events due to climate change have been observed also in several plant-pollinator systems (Kudo and Ida, 2013; Robbirt *et al.*, 2014; Forrest, 2015).

Among the biotic factors, flower availability in terms of quantity or density may affect the abundance of flower-visiting insects as a direct consequence of increased attraction (Dauber *et al.*, 2010). At the same time, the possible competition for pollen and nectar sources between different pollinator species can affect their relative abundance (Paini, 2004). In this case, we should expect a stronger competition among generalist than specialist pollinators (Blüthgen and Klein, 2011).

The identification of the abiotic and biotic factors that affect the plant-pollinator interaction is essential to understand and mitigate the decline of threatened plant species. In Italy, *Dictamnus albus* L. (Rutaceae) reaches its southern distribution limit and is protected at regional level. The species is locally protected across Europe and has been designated as 'vulnerable' in several European countries (Schnittler and Gunther, 1999). Previous studies reported an occasional pollination deficit that, in isolated population, might lead to fitness reduction (Fisogni, 2011; Fisogni *et al.*, 2016). Under the current global warming scenario, the study of the abiotic factors affecting the pollinator community activity of *D. albus* is particularly important in order to identify potential mismatches in this plant-pollinator system and to develop specific conservation programs.

The aim of this study was to assess the daily, the seasonal and the annual activity of a pollinator community of *D. albus* in Northern Italy in relationship to some abiotic and biotic factors. The study was performed in the framework of the PP-ICON project (Plant Pollinator Integrated CONservation approach: a demonstrative proposal), a Life+ European Project, with the aim to ensure the persistence of an isolated population of *D. albus* located in a protected area near Bologna (North Italy) and to restore the community of its natural pollinators. Here, in particular, we addressed the following questions: 1) do weather conditions (ambient temperature and relative humidity) during the flowering period affect the abundance of the visitor community of *D. albus*? 2) what is the daily activity pattern of each flower-visiting group? 3) are there associations of

flower-visiting insect occurrences during the same units of observation? 4) do the climatic conditions (mean temperature and degree-day) during wintering affect the annual abundance of each flower-visiting group?

MATERIALS AND METHODS

Study system

Pollinator surveys were performed on *Dictamnus albus* L., a perennial entomophilous herb that produces flowering racemes bearing numerous white-purple slightly zygomorphic flowers. Single flowers show marked herkogamy and protandry that strongly reduce the possibility of autonomous self-pollination; flowering develops from the bottom to the top of the raceme (Fisogni *et al.*, 2011). Observations were carried out in a natural population in the SCI-SPA IT4050001 - Natura 2000 site, situated within the Regional Park "Parco dei Gessi Bolognesi e Calanchi dell'Abbadessa", on the hills nearby the city of Bologna, Italy (168 m a.s.l.; 44°25'11'' N, 11°23'54'' E). The study population is found on the fringe of a xerothermic wood mainly composed of downy oak (*Quercus pubescens* Willd.) and ash (*Fraxinus ornus* L.).

Insect observations

We performed insect observations in a randomly chosen patch of *D. albus* plants in four consecutive years (2011-2014). From 2011 to 2013 the patch consisted of eight plants, while in 2014 it included five plants due to the scarcity of flowering in the area. All the plants in the patch were followed throughout the flowering season. We carried out six days of observation per year: two at the beginning of the flowering, two in full blooming and two at the end of the flowering season. Discrepancies in flowering times among plants in the patch were negligible. Each day we performed 4 observation units, two in the morning (9:00 and 12:00 h) and two in the afternoon (15:00 and 18:00 h). Each observation unit consisted of two 15-minute surveys, separated by an interval of

10 minutes, for a cumulative observation time of 2 hours per day and a total of 12 hours per year. Observations of flower visitors were performed in favourable weather conditions (i.e., low cloud cover, dry weather and low wind speed). During each survey we recorded the number of approaches to the patch and the number of flowers probed for nectar and/or pollen by each visiting insect. Insect visitors were identified at the lower taxonomic level allowed by visual recognition, i.e. family, genus or species. We did not capture insects for the purpose of this study. In order to correlate the number of flowers available to visitor abundance, prior to each observation unit we counted the number of open flowers in each plant of the observed patch.

Climatic factors

Each year of the study we performed two types of climatic data collection. During daily surveys, at the beginning and at the end of each observation unit we recorded air temperature above ground (°C) and relative humidity (HR %), by means of a LCD thermo-hygrometer placed adjacent to the observed patch. Mean values for every unit were further considered for data analysis.

In addition, in order to take into account the effect of overwinter climatic conditions on the abundance of pollinators during the flowering period of *D. albus*, for each year we calculated the degree-hour (DH) accumulated during wintering and the hourly mean temperature recorded from January 1st to the observation dates. The DH was calculated with the equation:

$$\sum_{1}^{n}(T - Tt)$$

where *T* was the recorded hour temperature and *Tt* was the baseline temperature. We used the 7 °C threshold considering the different response to increasing winter temperature conditions among taxa (Fründ *et al.*, 2013) and the temperatures required to emerge in early spring-flying bees (White *et al.*, 2009; Sgolastra *et al.*, 2010; Ahn *et al.*, 2014). The accumulation of the DH started on January 1st of each year because, in most species, this is considered the beginning of the post-diapause period (Hodek, 1996). Data were downloaded from the Regional Agency for the Environment Protection

(ARPA Emilia-Romagna, www.arpa.emr.it; permanent weather station of Settefonti, Ozzano nell'Emilia (Bologna) - 44°24'09'' N, 11°27'42'' E).

Data analysis

For each year, we counted the total number of insects and the abundance of each flower-visiting group, and we calculated the Shannon's diversity (expH'), Simpson's diversity (1/D) for a finite community and Berger-Parker (1/d) biodiversity indices (Magurran, 2004). Moreover, the abundance of each visitor taxon on *D. albus* was related with the weather conditions (hourly temperature and relative humidity) and flower availability during each observation unit by means of Spearman's rank correlations.

In order to assess the activity of each flower-visiting group during the day, the mean abundance values (individuals year^{-1}) were obtained for each observation unit, and expressed as percentages of the mean abundance value at its daily peak of activity (i.e. period with maximum abundance).

The associations between two flower-visiting insects in the same unit of observation were measured with a phi coefficient ($\varphi 2$) for dichotomous nominal-scale data, and its significance was tested with a contingency table with a Bonferroni correction for multiple tests (Zar, 1999).

Separate Pearson correlation analyses have been performed to assess significant relationships between the annual climatic conditions during wintering (DH and hourly mean temperatures) and the annual abundance of each flower-visiting group. Only groups with at least 20 individuals recorded during the four-year study have been considered in the analysis. Normality was confirmed using Shapiro-Wilk test.

Analyses were performed using STATISTICA version 7.1 software (Statsoft, 2005).

RESULTS

We observed 15 taxonomic groups that visited flowers of *D. albus* in the study period (table 1). Some species or genera were considered together at higher taxonomic levels

due to similar behaviour while foraging on flowers and due to difficulties in visual discrimination (e.g. similarity in morphology and very small body sizes). In total, we counted 71, 426, 132 and 404 individual insects in 2011, 2012, 2013 and 2014 respectively. The Shannon and Simpson indices showed similar trends among years, with the maximum value recorded in 2011 and the minimum value in 2012, while the Berger-Parker index showed the minimum value in 2012 but the maximum value in 2014 (table 1). Halictidae were the most abundant flower-visiting group in three out of the four study years (39.4, 49.5 and 34.9% in 2011, 2012, 2014, respectively). *Habropoda tarsata* Spinola was the second most abundant visitor but with strong fluctuations among years: the number of individuals counted per year and their relative frequency ranged from 6 to 133 insects/year and from 2 to 36%, respectively.

The relative daily abundance of each flower-visiting group showed a large variation among observation units and taxonomic groups (figure 1). Several taxonomic groups displayed a unimodal activity pattern, with maximum abundance occurring in the morning (Halictidae, *Osmia* spp., *Megachile* spp., *Anthidium* spp., *Nomada* spp., Syrphidae, *Bombylius* spp.), or in the afternoon (Vespidae and *Xylocopa* spp.), or in the middle of the day (*A. mellifera*). A bimodal activity pattern was observed only in *Bombus* spp. and *Anthophora plumipes* Pallas. *Habropoda tarsata* and *Eucera* spp. showed a homogeneous activity for most of the day (figure 1).

Table 1. Biodiversity indices, total insects and abundance of individual insects counted in each group during the four-year study. Relative frequencies of each group in bracket.

Flower-visitor group	**2011**	**2012**	**2013**	**2014**
Apis mellifera	3 (4.2)	0 (0.0)	8 (6.1)	78 (19.3)
Andrena spp.	2 (2.8)	0 (0.0)	0 (0.0)	2 (0.5)
Anthidium spp.	0 (0.0)	2 (0.5)	2 (1.5)	3 (0.7)
Anthophora plumipes	1 (1.4)	1 (0.2)	11 (8.3)	4 (1.0)
Bombus spp.	7 (9.9)	34 (8.0)	7 (5.3)	17 (4.2)
Eucera spp.	7 (9.9)	7 (1.6)	7 (5.3)	5 (1.2)
Habropoda tarsata	6 (8.5)	133 (31.2)	48 (36.4)	8 (2.0)
Megachile spp.	0 (0.0)	7 (1.6)	2 (1.5)	88 (21.8)
Osmia spp.	2 (2.8)	21 (4.9)	6 (4.5)	35 (8.7)
Xylocopa spp.	4 (5.6)	1 (0.2)	4 (3.0)	3 (0.7)

Halictidae	28 (39.4)	211 (49.5)	35 (26.5)	141 (34.9)
Nomada spp.	0 (0.0)	4 (0.9)	0 (0.0)	0 (0.0)
Syrphidae	1 (1.4)	0 (0.0)	1 (0.8)	20 (5.0)
Vespidae	5 (7.0)	0 (0.0)	0 (0.0)	0 (0.0)
Bombylius spp.	5 (7.0)	5 (1.2)	1 (0.8)	0 (0.0)
Total insects	71	426	132	404
Shannon Index (expH')	7.56	3.35	5.64	4.14
Simpson Index (1/D)	5.33	2.85	4.62	4.60
Berger-Parker Index (1/d)	2.54	2.02	2.75	2.87

The mean temperatures recorded during the observation units showed a similar trend between years with inverted U-shape observed in 2012, 2013 and 2014 (figure 2). On the other hand, the higher temperatures in 2011 were recorded in the second half of the day. The mean relative humidity showed a U-shape trend in 2012 and 2014 with the minimum values recorded in the middle of the day. In 2011 and 2013 the relative humidity decreased slightly from the morning to the afternoon (figure 2).

The abundances of *Anthidium* spp., Halictidae, Vespidae and *Bombylius* spp. were positively correlated with the ambient temperatures measured during the observation units on *D. albus*. The abundances of *A. plumipes* and *H. tarsata* were positively correlated with the relative humidity, whereas the abundance of *Megachile* spp., *Xylocopa* spp., Halictidae, Syrphidae, and Vespidae decreased with increasing relative humidity. The flower availability was only correlated negatively with Syrphidae (table 2).

The test for association between flower-visiting insect groups showed a positive correlation between the presence of *A. mellifera* and the presence of *Andrena* spp., *Megachile* spp. and hoverflies. The presence of *Megachile* spp. was positively correlated with Halictidae and Syrphidae occurrences, whereas the presence of *Bombylius* spp. was positively associated with wasps. We found only a negative association between *H. tarsata* and hoverflies (table 3).

We found a significant positive correlation between the annual abundance of *A. mellifera* (r_s = 0.993, df = 4, *P* = 0.006) and *Megachile* spp. (r_s = 0.967, df = 4, *P* = 0.03) with the hourly mean temperature recorded during wintering. The abundance of

Eucera spp. was negatively correlated with the temperature during wintering (r_s = -0.98, df = 4, P = 0.02). No other significant correlations were found.

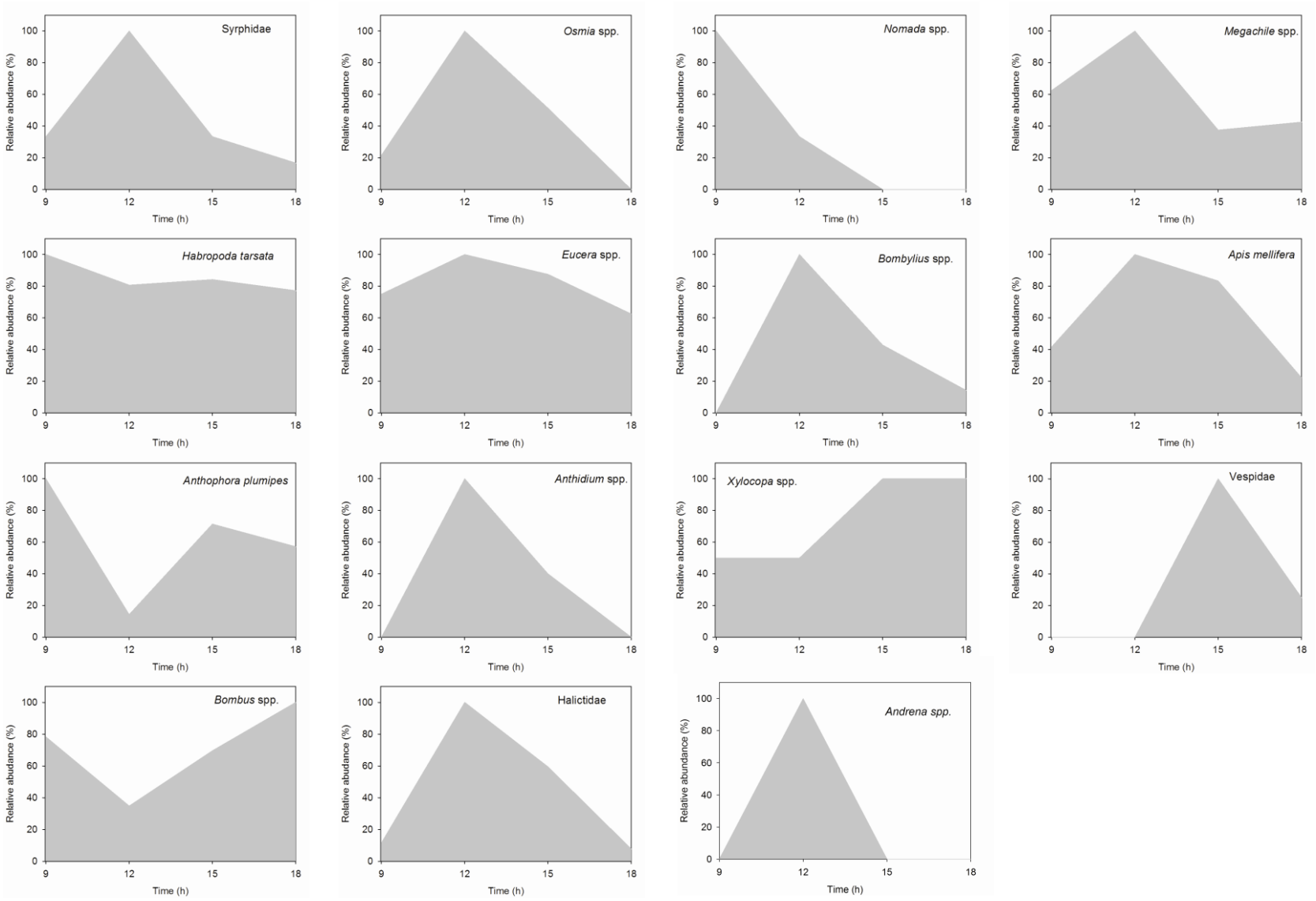

Figure 1. Daily variation in the abundance (mean of the four years) of 15 flower-visiting groups on *Dictamus albus*. For each group, abundance at a given hourly period is expressed as a percentage of its abundance at the daily peak.

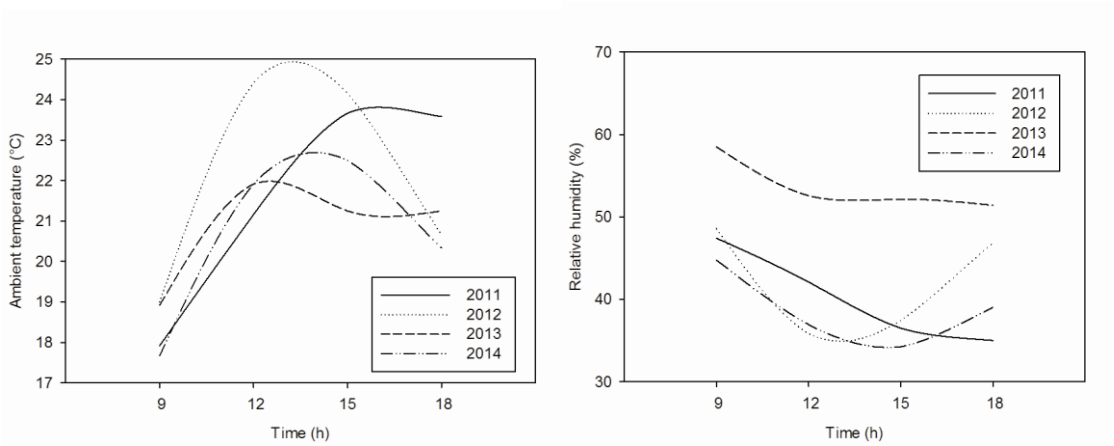

Figure 2. Annual mean temperatures and relative humidity recorded in the observation units.

Table 2. Spearman correlation values (r_s) between the abundance of each flower-visitor group and the ambient temperature (°C), the relative humidity and the flower availability during the flowering period of *Dictamus albus*. * indicates a statistically significant correlation (P<0.05).

Flower-visitor group	**Temperature**	**RH**	***Flower availability***
Apis mellifera	0.19	-0.19	*-0.02*
Andrena spp.	0.14	-0.01	*-0.15*
Anthidium spp.	0.28*	-0.18	*0.10*
Anthophora plumipes	-0.14	0.23*	*0.10*
Bombus spp.	-0.03	-0.06	*-0.15*
Eucera spp.	-0.08	0.11	*0.06*
Habropoda tarsata	-0.20	0.33*	*0.03*
Megachile spp.	0.13	-0.35*	*-0.06*
Osmia spp.	0.11	0.07	*-0.12*
Xylocopa spp.	0.12	-0.24*	*0.18*
Halictidae	0.63*	-0.47*	*0.13*
Nomada spp.	-0.05	-0.01	*0.07*
Syrphidae	0.12	-0.32*	*-0.23**
Vespidae	0.26*	-0.27*	*-0.04*
Bombylius spp.	0.22*	-0.19	*0.04*

Table 3. The associations between two flower-visiting insects in the same intervals of observation. * indicates a statistically significant correlation using Bonferroni correction (P<0.003).

	Apis mellifera	*Andrena* spp.	*Anthidium* spp.	*Anthophora plumipes*	*Bombus* spp.	*Eucera* spp.	*Habropoda tarsata*	*Megachile* spp.	*Osmia* spp.	*Xylocopa* spp.	Halictidae	*Nomada* spp.	Syrphidae	Vespidae	*Bombylius* spp.
Apis mellifera															
Andrena spp.	* phi=0.3144 *P*=0.002														
Anthidium spp.	phi=0.2897 *P*=0.004	phi=-0.0409 *P*=0.692													
Anthophora plumipes	phi=0.0901 *P*=0.382	phi=-0.0525 *P*=0.612	phi=-0.1009 *P*=0.328												
Bombus spp.	phi=-0.1361 *P*=0.186	phi=0.0590 *P*=0.568	phi=-0.1026 *P*=0.320	phi=-0.1014 *P*=0.325											
Eucera spp.	phi=-0.1231 *P*=0.232	phi=0.0940 *P*=0.362	phi=-0.0576 *P*=0.577	phi=0.0373 *P*=0.718	phi=-0.1537 *P*=0.135										
Habropoda tarsata	phi=-0.2902 *P*=0.004	phi=-0.1259 *P*=0.221	phi=0.0008 *P*=0.993	phi=0.0200 *P*=0.847	phi=0.0994 *P*=0.335	phi=0.0804 *P*=0.436									
Megachile spp.	* phi=0.3314 *P*=0.001	phi=-0.0795 *P*=0.441	phi=0.2284 *P*=0.025	phi=-0.0405 *P*=0.695	phi=0.0602 *P*=0.560	phi=-0.0614 *P*=0.552	phi=-0.0198 *P*=0.848								
Osmia spp.	phi=0.0308 *P*=0.766	phi=-0.0748 *P*=0.469	phi=-0.0452 *P*=0.662	phi=-0.0235 *P*=0.820	phi=0.0415 *P*=0.688	phi=0.1475 *P*=0.152	phi=0.0756 *P*=0.464	phi=0.565 *P*=0.584							
Xylocopa spp.	phi=0.1991 *P*=0.052	phi=-0.0497 *P*=0.630	phi=0.0355 *P*=0.731	phi=0.1985 *P*=0.053	phi=-0.0828 *P*=0.423	phi=-0.1048 *P*=0.310	phi=-0.2255 *P*=0.027	phi=0.1386 *P*=0.178	phi=-0.0070 *P*=0.946						

Halictidae	phi=0.2077 *P*=0.042	phi=0.1342 *P*=0.192	phi=0.2580 *P*=0.011	phi=-0.1285 *P*=0.212	phi=-0.0564 *P*=0.585	phi=0.1534 *P*=0.136	phi=-0.0511 *P*=0.621	* phi=0.3026 *P*=0.003	phi=0.1115 *P*=0.279	phi=0.1084 *P*=0.293					
Nomada spp.	phi=-0.0677 *P*=0.512	phi=-0.0213 *P*=0.837	phi=0.2396 *P*=0.019	phi=-0.0525 *P*=0.612	phi=0.0590 *P*=0.568	phi=-0.0795 *P*=0.441	phi=0.1689 *P*=0.100	phi=0.0940 *P*=0.362	phi=-0.0748 *P*=0.469	phi=-0.0497 *P*=0.630	phi=0.1342 *P*=0.192				
Syrphidae	* phi=0.4328 *P*<0.001	phi=0.1765 *P*=0.085	phi=0.1507 *P*=0.143	phi=0.0759 *P*=0.462	phi=0.1102 *P*=0.285	phi=-0.1183 *P*=0.251	* phi=-0.3106 *P*=0.002	* phi=0.4263 *P*<0.001	phi=-0.0235 *P*=0.820	phi=0.0914 *P*=0.376	phi=0.1340 *P*=0.193	phi=-0.0525 *P*=0.612			
Vespidae	phi=0.2303 *P*=0.024	phi=-0.0262 *P*=0.800	phi=-0.0504 *P*=0.626	phi=0.1233 *P*=0.231	phi=0.0081 *P*=0.938	phi=-0.0979 *P*=0.343	phi=-0.1551 *P*=0.131	phi=-0.0979 *P*=0.343	phi=-0.0921 *P*=0.372	phi=-0.0612 *P*=0.553	phi=0.1652 *P*=0.108	phi=-0.0262 *P*=0.800	phi=0.1233 *P*=0.231		
Bombylius spp.	phi=0.0798 *P*=0.440	phi=-0.0409 *P*=0.692	phi=0.2295 *P*=0.024	phi=0.0249 *P*=0.810	phi=-0.1026 *P*=0.320	phi=0.0377 *P*=0.715	phi=-0.0802 *P*=0.438	phi=-0.1529 *P*=0.137	phi=-0.0452 *P* =0.662	phi=-0.0956 *P*=0.354	phi=0.1776 *P*=0.083	phi=0.2396 *P*=0.019	phi=0.0249 *P*=0.810	* phi=0.4102 *P*<0.001	

DISCUSSION AND CONCLUSIONS

In the present study we assessed the effect of some abiotic and biotic factors on the abundance of flower-visiting insect groups throughout the blooming of *D. albus*. Moreover, we assessed whether the climate conditions during wintering can explain the annual differences observed in the abundances of each flower-visiting insect group.

Inter-annual variation in the abundance and activity of pollinator guilds is a common trend in natural communities (Petanidou *et al.*, 2008; Couvillon *et al.*, 2015). In our study, the insect visitor spectrum did not change severely among years with 7 out of 15 flower-visiting groups constantly present every year. However, we found marked fluctuations in the insect abundance within group among years. The total number of individual insects observed in the 4-year study showed a six-fold increase from 71 in 2011 to 426 in 2012. The lowest insect abundance observed in 2011 corresponded to the highest diversity values found in the same year for both Shannon's and Simpson's indices. On the contrary, the highest insect abundance in 2012 corresponded to the lowest diversity values for the Shannon's, Simpson's and Berger-Parker indices. These patterns are mostly due to the large fluctuations of insect abundance within taxonomic group; in 2012 we observed a striking dominance of both *H. tarsata* and Halictidae, while in the other years the abundance distribution were more equally distributed among groups. This was also reflected by comparable values of the Berger-Parker dominance index in the remaining years, indicating a lower contribution of a dominant single species to the total diversity in the area. In a previous study Fisogni *et al.* (2016) showed that different taxa have different importance in the pollination efficiency of *D. albus*, and that the cumulative effect of the pollinator community may or may not guarantee an adequate pollen flow in the studied population (i.e., the authors found evidence of pollination limitation in 2013). The diversity values found in the present study indicate that the pollination service in the population is not strictly related to insect diversity and confirms that it is more connected to the frequency of the most important pollinator species, such as *H. tarsata*, *Megachile* spp. and *Osmia* spp., and to a lesser extent bumble bees and honey bees. Moreover, our data suggest the importance to have a certain level of complementary among different flower-visiting groups in

order to buffer possible mismatches between the blooming period of *D. albus* and the foraging activity of the main pollinator groups.

Habropoda tarsata was the most important pollinator of *D. albus* (Fisogni *et al.*, 2016), and the most abundant species in 2013 (36.4%, present study). However, the highest contribution in terms of total insect abundance was offered by Halictidae (frequency range: 26.5-49.5%), which showed a correlation with the ambient temperature and the relative humidity during the flowering period. In fact, the number of Halictidae observed in the patch increased with increasing ambient temperatures and decreasing relative humidity. This response was also in accordance with the daily pattern of activity of this group with a peak in the middle of the day. Despite the higher frequency of Halictidae, their presence was not positively connected to the pollination of *D. albus* due to behavioural and morphological constraints that exclude pollen deposition on receptive stigmas (Fisogni *et al.*, 2011; 2016). On the contrary, they may act as pollen and/or nectar robbers and have therefore potential negative effects on plant male and female fitness. On the one hand, a reduction of flower nectar might reduce the attractiveness towards the main pollinators; on the other hand, the pollen collected by Halictidae is no longer available for pollination.

Variation in responses to environmental conditions among pollinator groups represents an "environmental complementarity" which is important in the insurance hypothesis (Yachi and Loreau, 1999). This hypothesis implies that two pollinator species can show long-term complementarity in the pollination service when they present different response to stress and environmental changes. Our data showed a certain level of "response diversity" between flower-visiting groups in relation to different environmental conditions (e.g. ambient temperature, relative humidity and flowering availability) during *D. albus* blooming. In fact, the activity of flower-visiting groups was related to ambient temperature in two cases (*Anthidium* spp. and *Bombylius* spp.), to relative humidity in 5 cases (positive relation in *A. plumipes* and *H. tarsata*; negative relation in *Megachile* spp., *Xylocopa* spp. and Syrphidae) and to both factors in two cases (Halictidae and Vespidae). In addition, the mutual interactions between hourly

activity of the different groups might guarantee an efficient pollination service also in years with extreme weather conditions during the flowering of *D. albus*.

The differences in the hourly pattern activity observed in our study can be therefore explained by the different specific temperature and humidity responses. Pollinator daily activity, however, can depend not only on their temperature and humidity tolerance, but also on the caloric reward offered by their host plants; in fact, it has been showed that some pollinators can adjust their collection activities to the rhythms of nectar production (Abrol, 2012). Sugar concentration in nectar varies daily and seasonally depending on the activity of nectaries (secretion or re-absorption), on the equilibrium with the humidity of the air (evaporation and condensation) and on removal of nectar by flower visitors (Abrol, 2012). These variations may affect the spectrum of flower visitors. In our study we found mainly significant negative effects of the relative humidity on the daily pollinator activity. High humidity can increase the nectar availability while decreasing its sugar concentration, thus negatively affecting the visits of those insects with a preference for high-sugar concentration. Honey bees, for example, stopped to visit almond flowers following intermittent rains because the nectar concentration dropped to 9-15% (Abrol, 2012).

The abundance of flower availability and the competition among pollinators during blooming of *D. albus* seems to play a marginal role in shaping the pollinator community composition of this plant species. Only one group (i.e., Syrphidae) showed a negative correlation between their abundance and the flower abundance in the patch, while no significant correlations were observed in the other groups. These results confirm that high flower density is not necessarily related with pollinator abundance (Filella *et al.*, 2013). Moreover, Syrphidae abundance resulted negatively related with the presence of *H. tarsata* and positively related with the presence of *A. mellifera* and *Megachile* spp. Although some studies have demonstrated that honey bees may compete for food resources with wild pollinators and reduce their abundance or flower visits (Shavit *et al.*, 2009; Hudewenz and Klein, 2013; 2015), other studies showed that interspecific competition did not negatively affect the abundance and species richness of wild bees (Steffan-Dewenter and Tscharnke, 2000; Garibaldi *et al.*, 2013). Our data show that

honey bees, when present, did not have negative effects on other foraging insects. On the contrary, we found some positive relations between honey bee abundance and that of some pollinators. This finding suggests the absence of competition between domestic and wild bees in the observed environmental conditions. It is possible that the abundance of *A. mellifera* in the site is not detrimental for wild bees, due to the absence of intensive beekeeping activities in the surroundings (A.F., personal observation). The absence of significant negative associations between the main pollinators of *D. albus* also suggests the overlap in the diet breadth of wild bees when feeding on this early blooming species, likely as a consequence of the abundant display of nectar and pollen throughout its flowering period. However, further studies on pollen loads found on insect bodies would help to clarify the actual feeding choices and possible niche overlap between taxa, and possible competition among plants for pollinator services.

Climate conditions during the winter can also affect the phenology of flower-visiting groups, thus causing possible mismatches between the presence and/or abundance of different pollinator groups and the flowering period of *D. albus*. Only the annual abundance of honey bees and *Megachile* spp. was positively correlated with the mean temperature during wintering, while a negative correlation was observed for *Eucera* spp. This result suggests that we should expect a higher number of honey bees and Megachilidae on *D. albus* under a scenario of global warming. The annual abundance of other flower-visiting groups were affected neither by mean temperature nor by degree-hour accumulated during the winter. This indicates that the pollinator community composition of *D. albus* is likely more affected by weather during blooming period than by the climatic conditions experienced during the previous winter.

In conclusion, our results show that the pollinator community of *D. albus* is quite variable among years and during the days of blooming. This variation could be explained by different environmental responses among flower-visiting groups; however, the different spectrum of activity might guarantee a stable pollination service of this plant species.

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CHAPTER 4

Role of nectar amino acids in plant-pollinator relationship: *Gentiana lutea* and social Apidae

4.1 *Gentiana lutea*

Gentiana lutea L. (1753) is a cosmopolitan species belonging to the family Gentianaceae, that mainly grows on calcareous (sub)-alpine pastures (800–2500 m a.s.l.) (Rossi et al., 2014). Its range (Fig. 3) extends from the Pyrenees to Asia Minor (Anchisi et al., 2010).

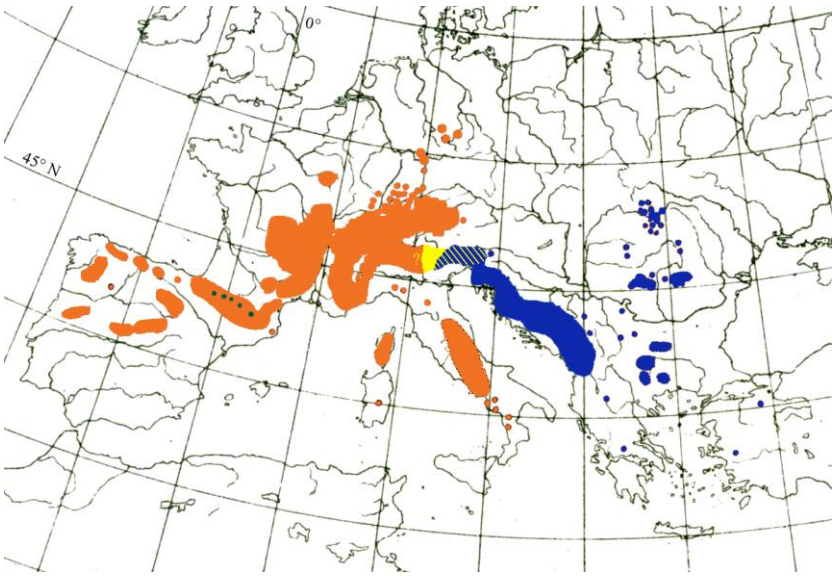

Figure 3. Geographical distribution of *G. lutea* subspecies. Orange, blue, yellow and green refer, to the distribution of subsp. *lutea*, subsp. *symphyandra*, subsp. *vardjanii* and subsp. *montserratii*, respectively. Picture modified from Meusel et al. (1978).

This species, called also "great yellow gentian", is an herbaceous long-lived scapose hemicryptophyte plant that presents an unbranched stout stem, growing up to 2 metres tall. (Rossi, 2012). Basal leaves are glaucous, decussate, lanceolate-elliptic to broadly ovate with 5-7 strong veins; stem leaves are narrower and stalkless (Tutin et al., 1972).

Fertile stems show yellow flowers grouped in pseudo-whorls; the inflorescence develops in basipetal direction and in centrifugal way within pseudo-whorl (Kozuharova, 1994). Each flower shows a split calyx, 10-15 mm long, with 2-7 minute teeth (Pignatti,1982). The corolla is yellow and gamopetalous, with 3-9 deeply engraved lobes. Stigma is bilamellate and anthers are usually free, except for *G. lutea* subsp. s*ymphyandra* where anthers are connate in a tube (Rossi, 2012) (Fig. 4). Five nectaries occur between stamen filaments and corolla attachment point. Flowering begins after 10 years (Yankova et al., 2010) and occurs between June and July. Fruits are many-seeded club-shaped capsules (3-6 cm) (Pignatti, 1982), composed of two carpels and ripening in August. Seeds are circular to elliptic, flattened and winged; dissemination occurs through anemochory (Struwe & Albert, 2002). *G. lutea* is also able to multiply through vegetative propagation. The spreading of rhizome assures population persistence and growth (Hesse et al., 2007), so even large populations are often represented by few individuals (Georgieva, 2007).

Figure 4. *G. lutea* subsp. *symphyandra* flower with connate anthers (Rossi 2012).

Four subspecies of *G. lutea* have been described: subsp. *lutea*, subsp. *symphyandra* (Murb.) Hayek, subsp. *vardjanii* Wraber and subsp. *montserratii* (Vivant ex Greuter) Romo (Tutin et al.,1972; Vender et al., 2010; Wraber, 1986). Distinctive traits have been recently revised by Rossi (2012).

G. lutea subsp. *lutea* corresponds to the general species description. It is distributed in the South European high mountains, from Spain to the North-West part of Turkey, excepted the Balkan Peninsula (Tutin et al., 1972) and the Eastern Alps (Wraber, 1986).

G. lutea subsp. *symphyandra* shows, as peculiarity, the anthers connated in a tube. It is distributed from the Balkan Peninsula to the Eastern Alps (Tutin et al., 1972), with few isolated populations recently signalled (Rossi et al., 2014).
G. lutea subsp. *vardjanii* is present in the South-Eastern Alps (in Italy, Carinthia and Slovenia). It differs from the other subspecies because presents vegetative stemless shoots and yellowish green floral bracts longer than pseudo-whorls (Wraber, 1986).
G. lutea subsp. *montserratii* is endemic to small areas of Pre-Pyrenees and of Central Pyrenees. It is recognizable for the ovate-elliptic corolla lobes, anther filaments longer than anthers and longer flora peduncles than subsp. *lutea* (Vivant, 1975)

4.1.1 Protection and conservation

The species is an important medicinal plant and the unrestrained collection of its rhizome represents its main threat. The species is listed in the "Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora (Habitat Directive) - Annex V", where are included animal and plant species of Community interest whose taking in the wild and exploitation may be subject to conservation measures. In addition, the species is included in the "Council Regulation (CE) No. 338/97 on the protection of species of wild fauna and flora by regulating trade therein - Annex D", that lays down the provisions for import and export, indicates the procedures and documents required for such trade and regulates the movement of live specimens. In Italy *G. lutea* is locally protected by regional regulations. (Rossi, 2012).

4.1.2 Plant-pollinators relationship

G. lutea was generally defined a "incompletely self-compatible" species (Hegi, 1927; Kèry et al., 2000); open corollas, exposed rewards, flower arrangement in a dense inflorescence may facilitate the geitonogamy. However, Rossi (2012) demonstrated that hand-self pollinated individuals show a reduced reproductive output and self-fertilized seeds show a low germination capacity. *G. lutea* is a generalist species. In fact, the

structure of its flowers allows a simple nectar access to numerous taxa that can potentially carry out the pollination service (Ollerton et al., 2007).

Principal pollinators belong to four different insect orders, which present different physiological and energetic necessities: Hymenoptera, Coleoptera, Lepidoptera and Diptera. Pollinators can be divided in dynamic and static pollinators. The firsts visit few flowers of the same plant and fly frequently from a plant to another one, promoting the cross-pollination. Static pollinators instead stay for a long time on the same whorl, promoting geitonogamy.

In the Mt. Grande population of *G. lutea* subsp. *symphyandra*, Rossi (2012) observed that bumble bees were the most pollinator represented group (more than 50% in total) and showed a tendency to became sluggish (Rossi et al., 2014). Some of them in fact were observed staying for a long period on the same flower, walking slowly through floral pseudo-whorls and they did not react when they were disturbed. Consequently, their pollinators role shifted from dynamic to static pollinators. Similar observations were reported in previous studies (Adler, 2000; Jakubska et al., 2005; Herrera et al., 2008) and this behaviour can be included in the so called "drunken pollinator hypothesis" (Adler, 2000). In this hypothesis, the main causes are external factors that contaminate nectar, as yeasts that produce alcohols through nectar fermentation (Ehlers & Olesen, 1997). An other possible explanation is that this behaviour may be caused by endogenous components, as non-protein aminoacids (i.e. β-alanine).

4.2. Nectar as floral rewards

The co-evolution between plants and pollinators resulted in a mutualistic relationship. Entomophilous plant species have evolved attraction and reward mechanisms that ensure repeated visits by the pollinators. In turn, pollinators have developed specific adaptations depending on the different types of flowers, guaranteeing the pollen dispersal from one individual plant to another (Richards, 1997; Dafni et al., 2005). Main floral rewards are pollen and nectar, which contain nutrients such as carbohydrates, amino acids, vitamins and proteins (Proctor et al., 1996). In addition, other both nutritive and non nutritive minor rewards are present. Among nutritive rewards are

flower tissues, food tissues (food scales, food bodies, non fertile pollen and pseudo pollen), stigmatic fluids, fatty oils while non nutritive ones are, for example, nest materials (trichomes, resins, waxes and corolla parts), sexual attractants, shelter and mating sites (Dafni et al., 2005; Strasburger, 2007).

Floral nectar is the primary reward directly consumed by floral visitors (Westerkamp, 1996; Nicolson & Thornburg, 2007). It is an aqueous solution of sugars, amino acids, organic acids, proteins, fats, vitamins, minerals and other minor components; its composition can vary greatly depending on the plant species and environmental conditions (Gardener & Gillman, 2001). Nectar is derived from the phloem sap (Fahn, 1979) and is secreted by a group of specialized cells, called nectaries. Two main types of nectaries can be recognised: extra-floral and floral nectaries. The first ones protect vegetative and reproductive structures from predators and are located in vegetative organs or outer floral parts, and are never involved in pollen transfer (Rossi, 2012). Floral nectaries are instead located within flowers and they are involved in the pollination process (Galetto & Bernardello, 2005). During flower lifespan, the nectar is produced and in same cases absorbed with particular rhythms, usually related to dietary habits of visitors. The nectar production and secretion has several metabolic costs for the plant, which uses more than 30% of the energy producted by daily photosynthesis (Southwick, 1984).

4.2.1. Sugars

Nectar sugar content ranges from 5 to 80% (Davis et al., 1998). Dominant sugars are the disaccharide sucrose and its constituent monosaccharides: fructose and glucose. The proportion of the three sugars tends to be constant within species (Nicolson & Thornburg, 2007), with some exceptions (Herrera et al., 2009). Sugar composition is highly variable among different species, but plants visited by similar pollinators show a similar composition (Petanidou, 2007). Minor sugars, such as sorbitol, melibiose, maltose, and mannitol are usually also present. One of the main factors responsible for maintaining the among-sugars ratio is the enzyme invertase, which catalyzes the

hydrolysis of sucrose before, during and after the nectar secretion (Baker & Baker, 1982).

Nectars that are rich in glucose and fructose are easy to digest and are consumed by an extensive array of mainly non-specialized pollinators (short-tongued bees, wasps, beetles, butterflies and flies), while high-sucrose nectars are adapted to more specialized pollinators, such as long-tongued bees, able to perform sucrose digestion (hydrolysis) (Petanidou, 2007).

4.2.2. Amino acids

Amino acids are the second most abundant category of nectar solutes (Nepi et al., 2012; Nicolson & Thornburg, 2007), although they are found at low quantities (typically 0.002–4.8% organic matter) (Gardener & Gillman 2001), and the biological significance of their presence is still not clear. Also amino acids represent an important alimentary resource that can determine insect choices (Bertazzini et al., 2010; Nicolson & Thornburg, 2007; Petanidou et al., 2006). In fact, nectars of species pollinated by different groups of visitors differ more in their amino acid concentration than in composition (Baker & Baker, 1986; Gardener & Gillman, 2001). Moreover, previous studies support the insect preference for sugar solutions enriched with amino acids (e.g. Rathman et al. 1990; Erhardt & Rusterholz 1998).

Among amino acids, proline is the most abundant in nectars of numerous angiosperms (Gardener & Gillman, 2002; Kaczorowski et al., 2005; Carter et al., 2006; Terrab et al., 2007) and it may represent more than 30% of the total amino acid contents (Nepi et al., 2012). This amino acid constitutes an immediate energy source used by insects in the earliest or most expensive stages of flight (Micheu et al., 2000; Gade & Auerswald, 2002). In fact it is the amino acid which can be metabolised more rapidly producing the greatest amount of ATP (Carter et al., 2006). Probably the presence of proline makes the nectar more attractive to pollinators, increasing their visits and consequently the fitness of the plant (Bertazzini et al., 2010)

4.2.3. Non-protein amino acids

In addition to the 20 classical protein amino acids, even thousands of non-protein amino acids (NPAAs) are present in plants. Of these, about 250 are involved in interactions with bacteria, fungi, plants and other herbivores (Huang et al., 2011; Vranova et al., 2011). Since they are not directly involved in metabolic processes, they can be lethal at high doses, but they may have a whole series of functions at lower doses (Bell, 2003). Their presence has been reported for the first time by Baker and Baker (1971), but the role of these substances is still little known, although they have different ecological and physiological functions in both animals and in plants. These amino acids are not incorporated into proteins and have been considered, at least initially, only as toxic compounds of defence. Some of them block the synthesis or the absorption of protein amino acids. Other ones, having similar chemical structure to the common protein amino acids, can be incorporated into proteins by mistake (Rosenthal, 1991; Taiz & Zeiger, 2013). However, Inouye and Inouye (1980) demonstrated that non-protein amino acids in nectar do not have a deterrent or toxic role. Recent studies showed that the NPAAs have the potential to influence the insect feeding behaviour (Nepi, 2014). NPAAs different effects on insect consumers can be divided into three main categories: a direct action on the nervous system, an alteration of the nutrition rate (phago stimulation), an increase in the activity of the muscles involved in flight (Nepi, 2014).
The amino acid concentration of nectar can vary depending on the age of the flower, but usually GABA and β-alanine are the most represented (Gottsberger et al., 1990; Petanidou & Smets, 1996). GABA acts as an inhibitory neurotransmitter in both vertebrates and invertebrates (Breer & Heilgenberg, 1985), limiting the states of intense stress and collateral damages. In invertebrates GABA receptors are located mainly in muscle tissue and neuromuscular junctions (Bown et al., 2006). Mitchell and Harrison (1984) demonstrated that GABA may also stimulate chemoreceptors sensitive to the perception of the taste of sugar, leading to an increase in power as a result. β-alanine instead seems to be involved in the muscle activity regulation: in vertebrates, and more specifically in humans, β-alanine is the precursor and the only limiting factor of the dipeptide carnosine synthesis, that gives to the muscle tissue more resistance to intense and prolonged efforts (Harris et al., 2006; Artioli et al., 2010). The advantage for the

plant would result from the stimulation of insects movement among different flowers and stems, that means enhanced cross-pollination and more effective pollination service. However, NPAAs long-term effects on pollinators, considering their concentrations found in nectar, are not yet known (Nepi, 2014).

4.2.4. Toxic nectar

Studies on plants-herbivores and plants-pollinators interactions are usually treated separately, however plants are usually submitted simultaneously to the selective pressure of possibly conflicting factors; for example, many species must simultaneously attract their pollinators and defend themselves from attack by herbivores (Adler, 2000). The interactions between herbivores and plants or between pollinators and plants thus contribute both to exert selective pressures on floral traits.

In the nectars of some plant species it is not unusual to find secondary metabolites such as phenols and alkaloids (Baker & Baker, 1983), necessary for the defence from microorganisms and herbivores (Berenbaum, 1995). In fact, bacteria and fungi, for example, can considerably change nectar composition (Herrera et al., 2009) and are often also important plant pathogens (Bubàn et al., 2003). Moreover, yeasts contaminating nectar commonly produce ethanol, which in turn can induce narcotic effects on pollinators like bumblebees (Kevan et al., 1988), wasps (Ehlers & Olesen, 1997) and rodents (Wiens et al., 2008), acting on plant-pollinator interaction. The toxicity of these compounds, however, depends on their dosage, their rate of intake and on the specific sensitivity by visitor consumers (Bown et al., 2006).

Another hypothesis about the presence of these compounds considers the benefit due to a higher pollinator fidelity ("the pollinator fidelity hypothesis"; Baker & Baker, 1975). The presence of toxic compounds would encourage foraging by specialist pollinators (Nepi, 2014). At the same time, the presence of deterrents would reduce or eliminate the visits by nectar thieves, which subtract resources without contributing to the pollination service (Adler, 2000; Manson et al., 2013). A last hypothesis, called "the drunken pollinator hypothesis", was advanced to those cases where the insects presented altered and lethargic behaviour (Adler, 2000).

4.3. Aim of the study

This study is focused on the analysis of nectar composition and on its possible effect on insect pollinator behaviour during flower visits. Floral traits affect resource exploitation by pollinators, influencing search and handling times and also pollen deposition and export, determining fitness differences amongst plants (Karron et al., 2009). Previous studies showed that alterations in sugar composition due to yeasts could affect pollination success (Herrera et al., 2008), while the contribution of non-protein amino acids remains unclear (Nepi, 2014). These compounds can greatly affects visitor behaviour and fidelity, influencing plant reproductive effort.

We considered *Gentiana lutea* L., a generalist long-lived plant visited and pollinated by a wide spectrum of insects, among which bumble bees are the most important and efficient pollinators. Previous studies highlighted that *G. lutea* nectar is hexose-rich and abundant in proline and β-alanine amino acids (Rossi, 2012). Rossi et al. (2014) reported an abnormal behaviour of *Bombus* spp. individuals collecting nectar of this plant. Therefore *G. lutea* represents a suitable model to explore the role of amino acids in the plant-pollinator interaction.

To investigate how nectar composition affects pollinator behaviour, we carried out field experiments in a natural population of *G. lutea* located on Mt. Grande (Bologna, Italy) (Fig. 5) and performed laboratory surveys on bumble bee nectar preferences and behaviour. The aim was to investigate pollinator activity and behaviour, both in nature and in laboratory, in relation to nectar composition.

Figure 5. Study population of *G. lutea* subsp. *symphyandra*, Mt. Grande (Bologna, Italy).

Our results may help to understand the ecological role of nectar components in the light of the complex scenario of plant-animal relationships, contributing to our knowledge about aspects of pollinator behaviour that nowadays still remains rudimentary.

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4.5. Role of nectar amino acid composition in pollinator preference (*Bombus terrestris* L.)

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SUMMARY

Amino acids are the main nectar components after sugars. In addition to protein amino acids (among which Proline is the main one) non-protein amino acids (eg GABA, β-alanine) are also present and sometimes very abundant, but their contribution to floral attraction to pollinators is not clear. *Gentiana lutea* subsp. *symphyandra* is a perennial and generalist plant; in the study area bumble bees are the most important and efficient pollinators. Previous analysis have revealed high concentration β-alanine in *G. lutea* nectar, and field observations indicated an anomalous behaviour in *Bombus* individuals that collected nectar from this plant. To investigate the role of nectar amino acids in plant-pollinator relationship, we analyzed nectar preference under laboratory conditions, using experimental micro colonies of *B. terrestris* fed with artificially produced nectars. Each trial consisted in the preference analysis (by consumption) of 4 different solutions simulating *G. lutea* nectar, 3 of them enriched with β-alanine or proline or both amino acids. Nectars were paired in the 6 possible comparisons (dual choice feeding test). Solutions were administered by syringes and consumption was checked at regular intervals (24, 48, 72, and 96 hours). Trials were performed on *Bombus terrestris* workers and males. The results of the single paired comparisons and average solution consumptions, analysed by Mann-Whitney U-test and Kruskal-Wallis H test, indicated a preference of *B. terrestris* workers for nectars enriched with both amino acids, contrarily to males that consumed less the same solution. Moreover statistical analysis showed for workers and partly for males a clear mortality increasing at the highest concentration. However results suggested that bumble bees tend to prefer nectars enriched in β-alanine, and the preference is influenced mostly by the colony of origin and the kind of paired choice.

INTRODUCTION

Floral nectar evolved as pollinator reward and it has a primary role in plant reproduction (Simpson & Neff, 1983; Perret et al., 2001). Its characteristics contribute to determine plant-pollinator relationships, attracting specific groups of animals. The so called "pollination syndrome" describe the complex of flower features that may result from co-evolution with a specific functional group of animal pollinators (Proctor et al., 1996) and it includes floral traits as reward accessibility, floral morphology and nectar characteristics (Heinrich & Raven, 1972; Baker & Baker, 1983a; Kingsolver & Daniel, 1983; Proctor et al., 1996).

Nectar is an aqueous solution mainly composed of sugars, mono- and disaccharides (glucose and fructose and their combination into sucrose) (Percival, 1965; Nicolson & Thornburg, 2007). However, secondary components as amino acids, lipids, phenols, alkaloids and volatile organic compounds are commonly found in nectar (Kessler & Baldwin, 2007; Nicolson & Thornburg, 2007; González-Teuber & Heil, 2009). All nectar components, including primary and secondary compounds, affect the attractiveness of nectar to pollinators: as a consequence their amount and concentration are often related to a specific pollinator type (Baker & Baker, 1977; Faegri & van der Pijl, 1979; Baker & Baker, 1983a).

Many studies demonstrated that clear differences in nectar sugar composition were correlated with different pollinator classes: for example flowers with a high sucrose/exose ratio are preferably pollinated by humming-birds, Megachiroptera and long tongued bees, while a low sucrose/exose ratio is preferred by passerine birds, Microchiroptera and short-tongue bees (Baker & Baker, 1983b; Kress, 1985; Baker & Baker, 1990; Baker et al., 1998). In addition to nectar sugar composition, also other nectar components can influence pollinators' type. Among them, in the last years several studies investigated the role of amino acids, the second most concentrated solutes in nectars (Alm et al., 1990). Nectar amino acids are more attractive for insects than vertebrates, since the second ones can also gain nitrogen from other sources (Proctor et al., 1996). A unique aspect of the presence of amino acids in nectar is the potential contribution of these compounds to its taste (Gardener & Gillman, 2002). Amino acids have much more diverse chemical structures than sugars and their

concentration may be highly variable, producing a diverse range of tastes (Birch & Kemp, 1989).

Blüthgen and Fiedler (2004) found that different ant species preferred sugar solutions enriched with mixtures of amino acids and preference among seven pairs of single amino acids in sugar solutions differed substantially among ant species. Flesh flies seem to be able to detect single amino acids in artificial nectar and show particular preferences for some of them (Potter & Bertin, 1988). Butterflies showed preferences for nectar with high amino acid concentration (Alm et al., 1990; Erhardt & Rusterholz 1998). Finally, honey bees showed clear preference for solutions enriched with proline and phenylalanine and an opposite behaviour regarding solutions enriched with serine (Inouye & Waller, 1984; Alm et al., 1990; Bertazzini et al., 2010).

In our study we focused on two specific amino acids, proline and β-alanine. Proline is a non-essential protein amino acid (Nicolson & Thornburg, 2007) and it presents the unique ability to stimulate the insect salt cell, increasing the feeding behaviour (Hansen et al., 1998; Wacht et al., 2000). In addition, it is the most abundant amino acid in honey bee haemolymph (Crailsheim & Leonhard, 1997; Hrassnigg et al, 2003) and it is selectively degraded during the initial stages or lift phase of flight (Micheu et al., 2000), resulting in a efficient fuel in rapid, short-term bursts of energy production (Carter et al., 2006). β-alanine, instead, is a non-protein amino acid commonly found in nectar (Nepi et al., 2012; Nepi, 2014) and in general it seems to be involved in muscular activity's regulation as precursor of the dipeptide carnosine. Carnosine is found in both vertebrate and non-vertebrate skeletal muscles and it has been demonstrated that it can increase isometric endurance in humans (Harris et al., 2006).

The aim of this study was to investigate the role of nectar amino acids in plant-pollinator relationship and we analyzed nectar preference under laboratory conditions carrying out dual choice feeding tests on bumble bees. We tested an artificial nectar composition mimicking the natural nectar of the perennial plant *Gentiana lutea*, since previous studies (Rossi, 2012; Rossi et al., 2014) carried out on a population situated on Mt. Grande (Bologna, Italy) indicated bumble bees as its more important and efficient pollinators and described an anomalous behaviour in individuals that collected nectar, particularly rich in β-alanine.

MATERIALS AND METHODS

Study species and experiment conditions

Bumble bee workers and males (*Bombus terrestris* L.) were obtained from commercial colonies (Bioplanet S.c.a., Cesena, Italy) maintained in a climate room at 25 ± 1°C and 40 ± 5% relative humidity (RH), in continuous darkness, fed ad libitum with fresh frozen pollen and sugar syrup. Individuals were collected from 25 colonies under red light and transferred in groups of 15 (separate groups for colony of origin) into small plastic boxes (15 x 9 x 5 cm) perforated along the sides to allow ventilation. They were maintained at 25 ± 1°C and 40 ± 5% RH, in continuous darkness. To uniform samples, very small and very large individuals were excluded from the experiments. Newly emerged bees and old bees were also excluded, basing on colour and lack of hairs.

Artificial nectars solutions

We prepared nectar solutions on the basis of *Gentiana lutea* nectar analysed in 2011 (Table 1). Sugars and amino acids were purchased from Sigma-Aldrich, Milano, Italy.

Table 1. Average concentrations of the main components found in *G. lutea* nectar (Rossi 2012)

Sucrose	Glucose	Fructose	Proline	B-alanine
1.90 mg/mL	177.8 mg/mL	164.9 mg/mL	138.16 mg/L	204.9mg/L

We tested four different solutions: control solution (C), containing only the sugar component, a proline enriched solution (P), a β-alanine enriched one (B) and a solution enriched with both amino acids (P+B). Two different concentrations (C1 low concentration and C2 high concentration, Table 2) were used in order to exclude a preference due to a greater amino acid concentration in the different enriched solutions.

Table 2. Amino acid enriched solution used in the two different concentrations.

concentration	solution	proline	β-alanine	AA final concentration
1	P	138 mg/L	-	138 mg/L
	B	-	205 mg/L	205 mg/L
	P+B	138 mg/L	205 mg/L	343 mg/L
2	P	686 mg/L	-	686 mg/L
	B	-	686 mg/L	686 mg/L
	P+B	343 mg/L	343 mg/L	686 mg/L

Dual choice feeding test

Nectar solutions were paired in the 6 possible comparisons and they were administered by nozzle-cut syringes (5 mL). We checked consumption by weight at 24, 48, 72, and 96 hours and we registered mortality in the same intervals. The mean daily individual consumption of each syringe was calculated on the basis of the number of live insects in a given thesis at check moment.

Data analyses

Differences in consumption and mortality data among the factors solution (4: C, P, B and P+B), thesis (6: C vs P, C vs B, C vs P+B, P vs B, P vs P+B and B vs P+B), day (4) and replication and between the two concentrations within each factor have been analysed by Kruskal-Wallis H-test and Mann-Whitney *U*-test respectively.

RESULTS

Bumble bee workers

The effect of the factors thesis, solution, day and replication on diet consumption and mortality had a different pattern according to amino acid concentration (Table 3).

Table 3. Comparison of bumble bee workers' diet consumption and mean mortality among different factors in both concentrations.

Concentration	Factors	Consumption			Mortality		
		df	Test statistic (H)	*P*-value	df	Test statistic (H)	*P*-value
C1	Solution	3	1.93	0.587	3	1.05	0.790
	Thesis	5	6.001	0.306	5	11.46	**0.043**
	Day	3	13.89	**0.003**	3	165.27	**<0.001**
	Replication	14	76.34	**<0.001**	14	69.83	**<0.001**
C2	Solution	3	14.84	**0.002**	3	0.94	0.815
	Thesis	5	3.62	0.606	5	9.45	0.092
	Day	3	7.07	0.070	3	122.17	**<0.001**
	Replication	9	74.76	**<0.001**	9	204.81	**<0.001**

Bold values represent significant (*P*-value < 0.05) differences in consumption and mortality.

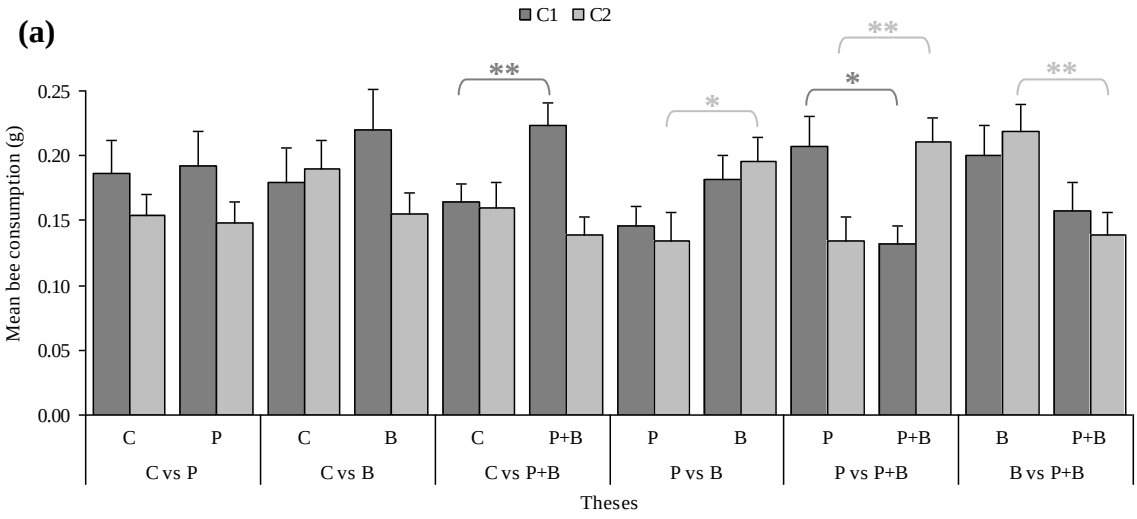
The factor thesis had no significant effect on consumption at both amino acid concentrations (Table 3) but the analysis of solution consumption in each thesis showed significant differences depending on amino acid concentration (Table 4 and Fig. 1a). Particularly, for the low concentration (C1) it was found that the solution P+B was more consumed than the control solution and solution P was more consumed than solution P+B. On the other hand at high amino acid concentration (C2) bumble bee workers consumed more solution B with respect to both solution P and solution P+B as well as more P+B with respect to P (Fig. 1a).

The same factor (thesis) had an effect on mortality only at low amino acid concentration (table 1, fig. 1b). In most of the theses there was a significant higher bee mortality at the high amino acid concentration (table 4, fig. 1b).

Table 4. Comparison (Mann-Whitney *U*-test) of bumble bee workers' diet consumption between solutions in each thesis considering both low (C1) and high (C2) amino acid concentrations. The table reports also the comparison of mean mortality between concentration for each thesis

	Consumption				Mortality	
	C1		C2		C1 vs C2	
Thesis	Test statistic (U)	*P*-value	Test statistic (U)	*P*-value	Test statistic (U)	*P*-value
C vs P	195.5	0.903	770.5	0.777	1300.0	0.095
C vs B	172.0	0.449	658.0	0.306	1314.0	0.162
C vs P+B	1239.5	**0.007**	745.0	0.597	2844.0	**<0.001**
P vs B	654.0	0.160	489.0	**0.003**	2056.0	**<0.001**
P vs P+B	562.0	**0.022**	472.0	**0.002**	2416.0	**0.007**
B vs P+B	600.0	0.054	484.0	**0.002**	2194.0	**<0.001**

Bold values represent significant (*P*-value < 0.05) differences in consumption among the theses.



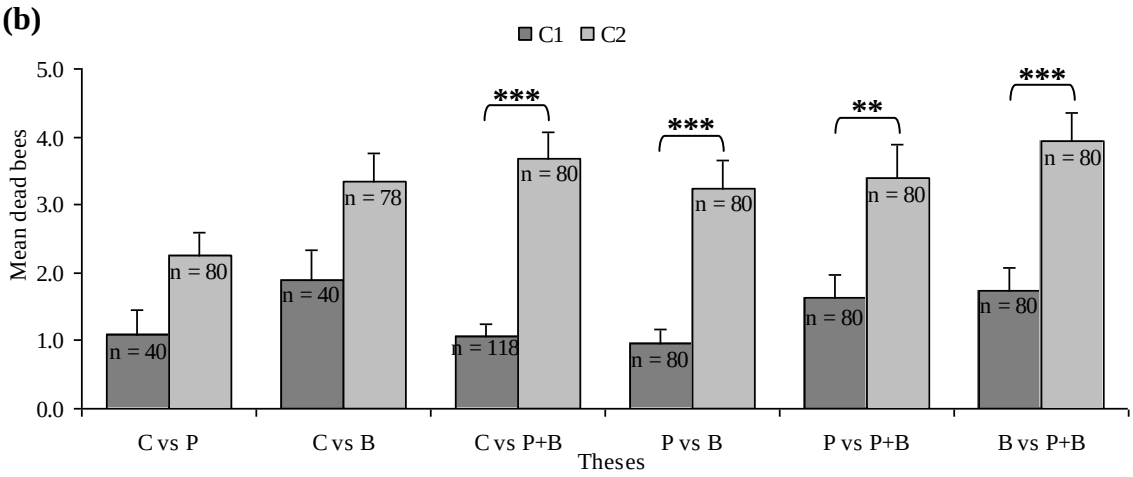


Fig. 1. Bumble bee workers' comparison of diet consumption and mortality data at the two concentrations in the different thesis. **(a)** comparison of consumption between the two solutions of each thesis; n = 40. **(b)** comparison of mortality for each thesis. Significant differences according to Mann-Whitney *U*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The factor solution had no effect on diet consumption by bees at low amino acid concentration, but there was a significant differences between solutions at high amino acid concentration (table 1, fig. 2a) since the solution containing proline (P) was less consumed than all the others, even if significantly only with respect to solution B. Concentration affected only the consumption of the proline solution, whilst was not determinant for all the other solutions.

Bee mortality was never affected by the factor solution for both amino acid concentrations, but in each solution the higher amino acid concentration was associated with a higher mortality (Table 1, fig. 2b).

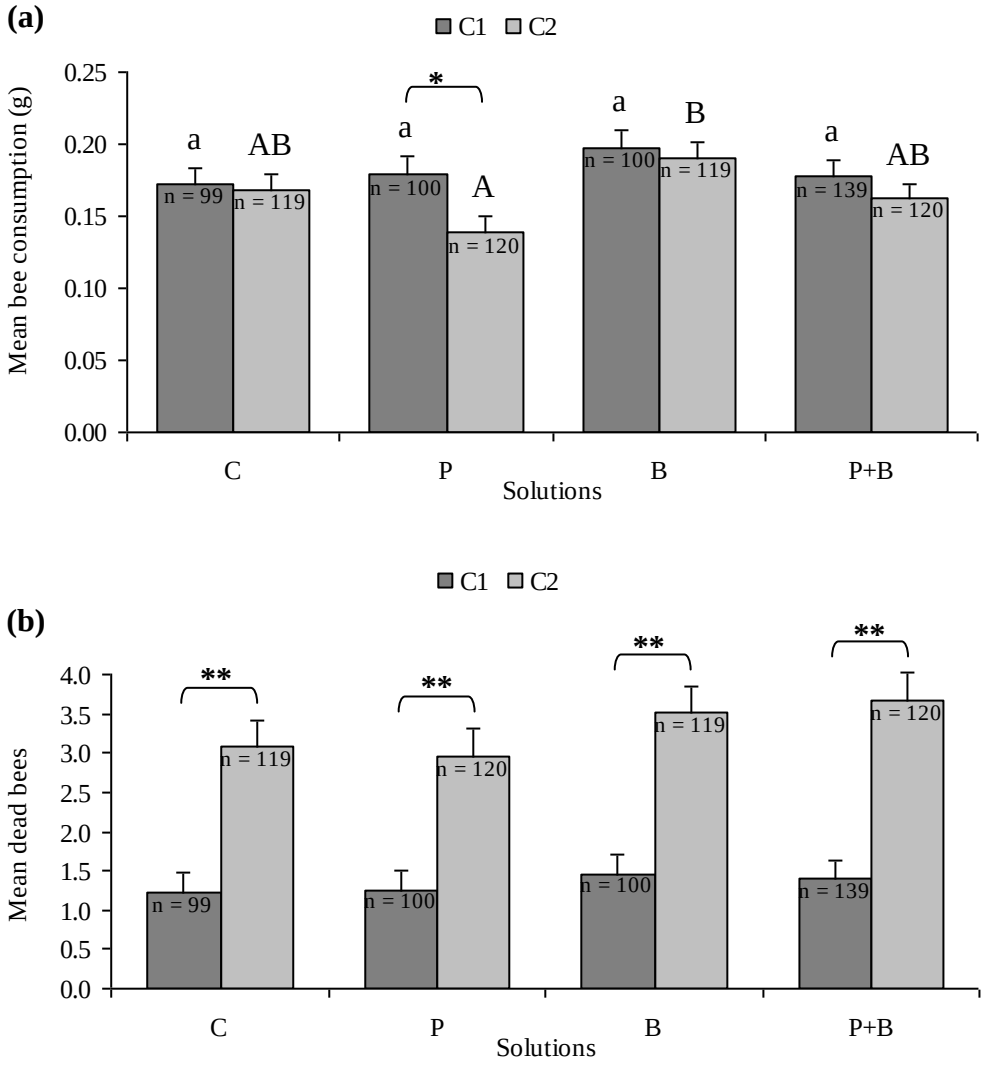


Fig. 2. Bumble bee workers' comparison of **(a)** consumption and **(b)** mortality for each tested solution between the two concentrations. Significant differences according to Mann-Whitney *U*-test: * $P < 0.01$, ** $P < 0.001$. Values marked with different letters were significantly different according to the Kruskal-Wallis *H*-test.

The factor day had significant effect on consumption only at the low concentration (Table 1) where bumble bees workers consumed more diet the day 3 with respect to day 1 (Fig. 3a).

Contrarily, the same factor (day) had an effect on mortality at both amino acid concentration (table 1). Particularly, for both concentrations we found a significant and constant mortality reduction from day 1 to day 3 and 4 (Fig. 3b). Moreover, In all of the days there was a significant higher bee mortality at the high amino acid concentration (fig. 3b).

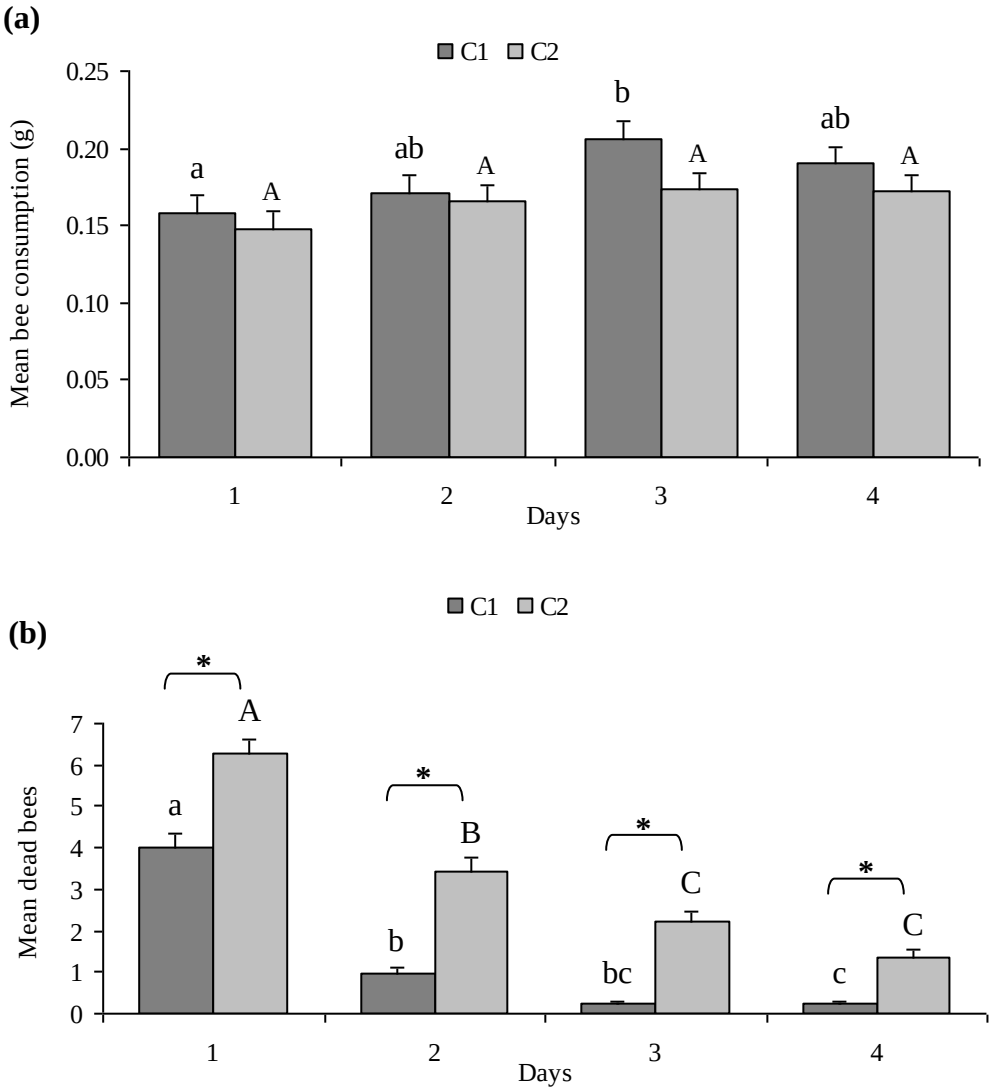


Fig. 3. Comparison of **(a)** diet consumption and **(b)** mortality data for bumble bee workers fed with low (C1, n = 110) and high (C2, n = 120) amino acid enrichment in each day of the experiment. Significant differences according to Mann-Whitney *U*-test: * $P < 0.001$. Values marked with different letters were significantly different according to the Kruskal-Wallis *H*-test.

Finally, the factor replication significantly affected consumption and mortality at both concentration (Table 3).

Bumble bee males

As in bumble bee workers, the effect of the factors thesis, solution, day and replication on diet consumption and mortality of bumble bee males had a different pattern according to amino acid concentration (Table 5).

Table 5. Comparison of bumble bee males' consumption and mortality among different variables in both concentrations (Kruskal-Wallis *H*-test).

Concentration	Factors	Consumption			Mortality		
		df	Test statistic (H)	*P*-value	df	Test statistic (H)	*P*-value
C1	Solution	3	29.93	**<0.001**	3	0.05	0.997
	Thesis	5	0.23	0.891	5	0.10	0.951
	Day	3	6.71	0.082	3	40.21	**<0.001**
	Replication	2	1.64	0.442	2	0.75	0.688
C2	Solution	3	27.52	**<0.001**	3	0.84	0.840
	Thesis	5	10.78	0.056	5	2.68	0.750
	Day	3	18.54	**<0.001**	3	51.73	**<0.001**
	Replication	7	23.61	**<0.001**	7	9.03	0.250

Bold values represent significant (*P*-value < 0.05) differences in consumption and mortality.

The factor thesis had no significant effect on consumption at both amino acid concentrations (Table 5) but the analysis of solution consumption in each thesis showed a certain significant difference depending on amino acid concentration. Particularly, for both concentrations it was found that the solution P+B was less consumed than the control solution. Moreover for the low concentration (C1) results showed that solution B was more consumed than solution P+B.
The same factor (thesis) had no effect on mortality (table 5).

The factor solution had effect on diet consumption by bees at both amino acid concentration (table 5, fig. 4a), since the solution containing both amino acids (P+B)

was significantly less consumed than all the others, excluding the solution P at low concentration. Concentration affected only the consumption of the solutions containing proline and both amino acids.

Bee mortality was never affected by the factor solution for both amino acid concentrations, but in each solution the higher amino acid concentration was associated with a higher mortality, significantly only with respect to solution P+B (Table 5, fig. 4b).

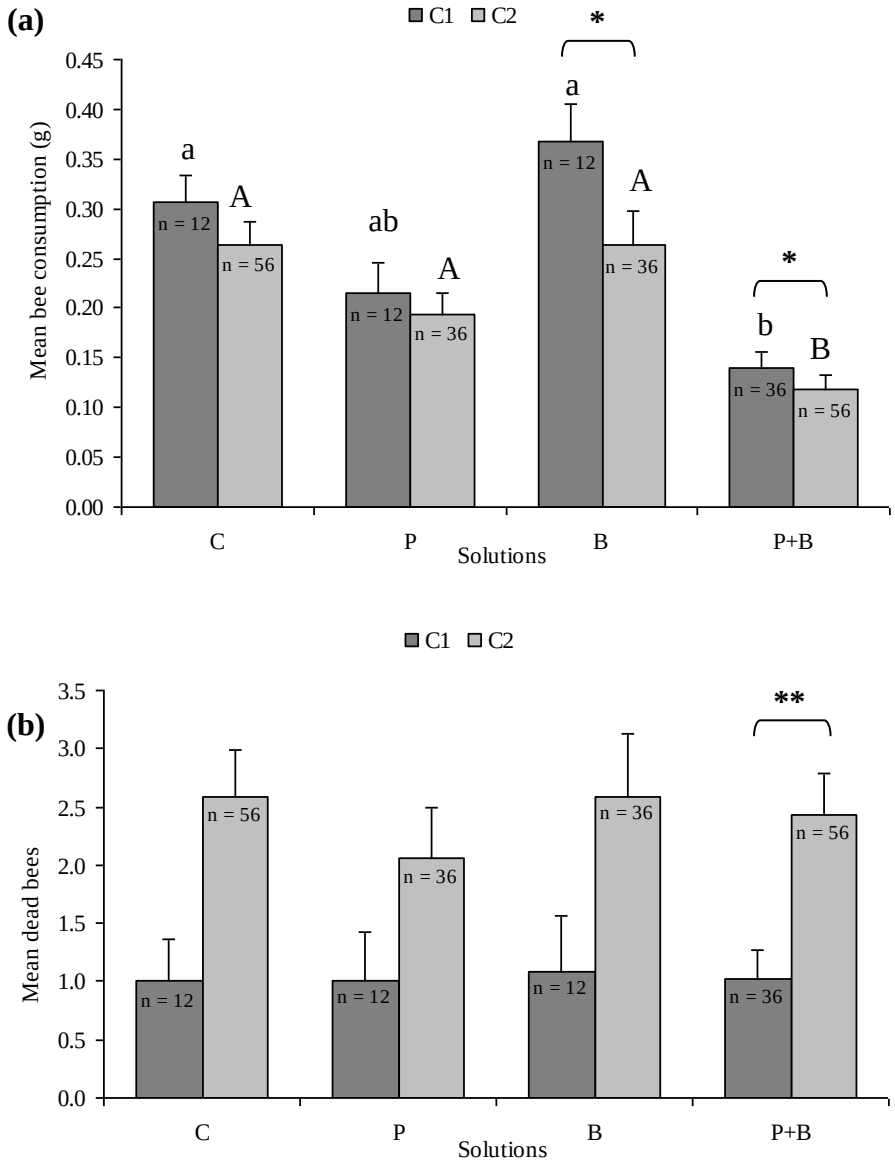


Fig. 4. Bumble bee males' comparison of **(a)** consumption and **(b)** mortality for each tested solution between the two concentrations. Significant differences according to Mann-Whitney *U*-test: * $P < 0.05$, ** $P < 0.01$. Values marked with different letters were significantly different according to the Kruskal-Wallis *H*-test.

The factor day had effect on consumption at the high concentration (Table 5) where male bees consumed more diet the days and 3 with respect to day 1 (Fig. 5a). Concentration did not affect the consumption among days.

Contrarily, the same factor (day) had an effect on mortality at both amino acid concentration (table 5). Particularly, for the low concentrations we found a higher mortality in the day 1 with respect to the others days, and for the high concentration days 1 and 2 showed a higher mortality than days 3 and 4 (Fig. 5b). Moreover in all of the days, excluding day 1 that nevertheless approached significance, there was a significant higher bee mortality at the high amino acid concentration (fig. 5b).

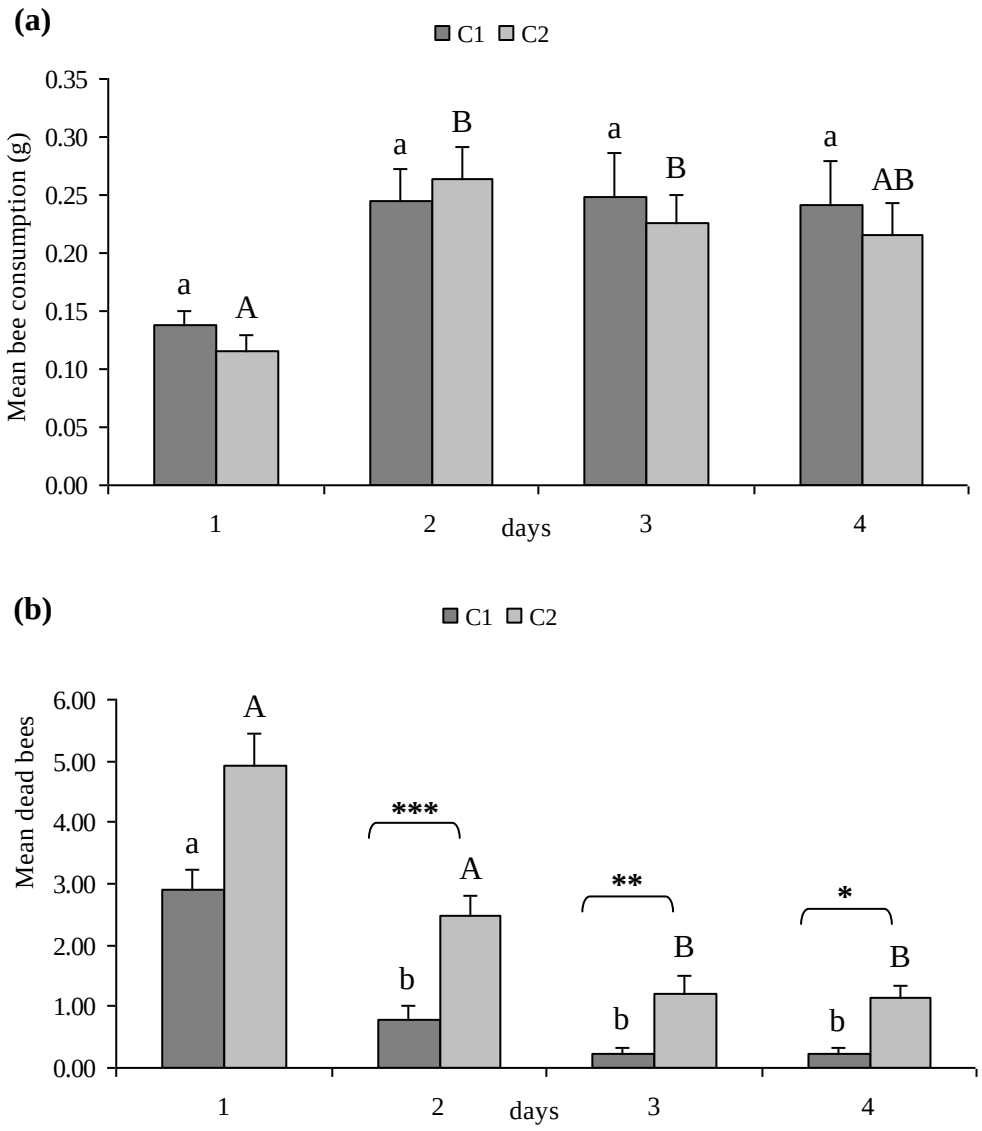


Fig. 5. Comparison of **(a)** diet consumption and **(b)** mortality data for bumble bee males fed with low (C1, n = 18) and high (C2, n = 46) amino acid enrichment in each day of the experiment. Significant differences according to Mann-Whitney *U*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Values marked with different letters were significantly different according to the Kruskal-Wallis *H*-test.

Finally, the factor replication significantly affected only consumption at high concentration (Table 5).

DISCUSSION

Our results demonstrated four different findings: 1) *Bombus terrestris* workers and males showed different amino acid solution preference; 2) the preference was influenced also by the colony of origin of individuals; 3) high amino acid concentration negatively influenced mortality; 4) in general the solution enriched in β-alanine was the most consumed.

Sex-specific preference

Differences in behaviour and in preference based on gender are reported in several studies. In the bombyliid fly *Megapalpus capensis*, males show a preference for complex spots on the ray florets of the orchid *Gorteria diffusa*, contrarily to females (de Jager & Ellis, 2012). Regarding lepidopterans, *Lysandra bellargus* male and female butterflies visit different flowers, depending on sugar and amino acid nectar composition (Rusterholz & Erhardt, 2000), while the hawkmoth *Manduca sexta* show sex-specific differences in foraging behaviour and preferences (Alarcón et al., 2010). In the cricket *Teleogryllus commodus* Maklakov et al. (2008) found that crickets exhibited sex-specific dietary preference in the direction that increased reproductive performance. Concerning hymenopterans, Ne'eman et al. (2006) demonstrated that some species of solitary bees presented differences in foraging behaviour depending on sex. This is probably due to the direct effect of different feeding preferences on different energy intake (Alarcón et al., 2010). In particular, bumble bee males spend a large part of the

day in flight and consequently they have a high energy demand (Bertsch, 1984). Contrarily, bumble bee workers require proteins for somatic maintenance and derive a portion of dietary essential amino acids from free amino acids found in floral nectar (Gardener & Gillman, 2002; Petanidou et al., 2006; Nicolson & Thornburg, 2007). In our study we considered only the female cast of workers and not that of queens, more biologically complex anyway than male cast. In fact, workers develop exclusive structures that require particular constituents, for example wax and venom glands (Landim, 1963; Billen, 1987, Tengo et al., 1991), in addition to a partial ovary development (Foster et al., 2004; Amsalem et al., 2009). Effectively, in our experiment bumble bee workers showed a preference for the solution enriched with both amino acids, while males consumed less the same solution with respect to the other ones.

Effect of colony of origin

Inter-colonies differences in social insects (ants, honey bees and bumble bees) have been previously described by several authors since the introduction of the concept of "superorganism" (Holldobler & Wilson, 2008). Crosland (1989) described intraspecific variation in aggressiveness and kin discrimination ability among colonies of the ant *Rhytidoponera confusa*, and Scharf et al. (2012) demonstrated a collective personality at colony level in the ant *Temnothorax nylanderi*. In honey bees differences among colonies are well documented: several studies describe difference in productivity, temperament, defensive response, hygienic behaviour and pollen hoarding (Pesante et al., 1987; Laidlaw & Page, 1997; Hunt et al., 1998; Guzmán-Novoa et al., 2002; Arathi & Spivak, 2001; Wray et al., 2011). Finally in bumble bees several authors report differences among individual, colonies and possibly among populations on the strength and persistence of innate colour preference and learning speed (Chittka et al., 2004; Raine et al., 2006; Rain & Chittka, 2008; Ings et al., 2009), as well as in foraging behaviour (Evans & Raine, 2014). Therefore our results are in accordance with literature, since the factor colony of origin influenced the preference and consumption of both workers and males.

Effect on mortality of high amino acid concentration

An increased mortality due to a diet rich in protein or amino acids is well documented in different insect taxa. In *Drosophila melanogaster* the ingestion of high levels of protein has deleterious consequences on flies lifespan (Lee et al., 2008) and in the cricket *Teleogryllus commodus* the longevity is maximized on a high-carbohydrate low-protein diet (Maklakov et al., 2008). Regarding social Hymenoptera, Dussutour and Simpson (2009) found that the green-headed ants (*Rhytidoponera* sp.) extract carbohydrates and eliminating proteins from collected foods to improve the macronutrient balance but this removal process shortened life span. Moreover, it has been found that a restriction to high-protein and low-carbohydrate diets in the black garden ants *Lasius niger* decreased worker lifespan (Dussutour & Simpson, 2012). Several studies showed that also honey bees survived longer when fed on high protein:carbohydrate ratios or high concentration of essential amino acids (Pirk et al., 2010; Paoli et al., 2014a,b). Our findings are in accordance with Stabler et al. (2015) that obtained a higher mortality in *Bombus terrestris* individuals fed with high essential amino acid content; in fact in our study both workers and males showed a higher mortality at high amino acid concentration.

Preference for β-alanine enriched solution

The preference for the β-alanine enriched solution instead the proline one could be related to our experiment conditions. Proline has been reported to be stored in the hemolymph as an energy reserve (Chapman, 1998; Micheu et al., 2000) and many insect species may use this amino acid as an energy reserve for flight (Candy et al., 1997; Auerswald et al., 1998; Consoli & Vinson, 2002). We confined bumble bees in little cages avoiding them to fly and consequently the collection of proline could be unnecessary. Contrarily, β-alanine is a precursor of the dipeptide carnosine (Harris et al., 2006) and, like taurine, could be associated to fully functional flight muscles (Whitton et al., 1987). In addition, in honey bee males high concentration of β-alanine was found in retinal interstitial fluid and neuron could use this substance as substrate of

energy metabolism (Cardinaud et al., 1994). Finally, we can hypothesize that the preference at high concentration for β-alanine instead of proline could due to the phagostimulation effect of prolineprotein (Nicholson & Thorburg, 2007; Petanidou, 2007) which can lead to an excessive and deleterious intake of amino acids.

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4.6. Role of nectar chemical composition in plant-pollinator interactions: *Gentiana lutea* L. as model species

Manuscript

SUMMARY

Generalist flowers are visited by a broad variety of insects that may act as pollinators, as occasional visitors, or as pollen or nectar robbers. Moreover among legitimate pollinators the pollination efficiency can be different. Nectar greatly affects visitor behaviour and fidelity, influencing plant reproductive effort. This study is focused on the analysis of floral nectar composition, specifically sugars, in a *Gentiana lutea* L. wild population. We considered also the nectar contamination by pollen and yeasts, that can alter sugar composition and influence insect behaviour. Our results indicate the presence of yeasts mainly in nectar of mature open flowers, accessible to insect visits. Moreover, yeast contamination seems to be related to the presence of pollen in the nectar. Yeasts are probably involved in changes in sugar composition throughout flower lifespan, and our findings suggest that yeast contamination is strictly linked with pollinator's activity. We can thus hypothesize that yeasts are transferred to floral nectar by pollinators, together with pollen.

Our results indicated that nectar modulating the pollination process is not the pure secreted solution. After secretion in fact, nectar in turn is altered by numerous factors, in a complex process of ecological relationships.

INTRODUCTION

Nowadays the study of plant-pollinator interactions and the degree of their specialization is among the most lively and debated issues in plant biology and ecology. Plant pollination systems present a continuum between specialists that depend on a single pollinator taxon, to generalists, pollinated by different animals. In many cases effective pollinators represent only a fraction of the floral visitors' guild, depending on flower morphology, phenology and rewards, and on behavioural responses of visitors

(Waser et al., 1996; Fisogni et al., 2011; Rossi et al., 2014). Floral traits indeed affect resource exploitation by pollinators, influencing search and handling times and also pollen deposition and export, determining fitness differences amongst plants (Karron et al., 2009).

Floral nectar is a primary reward produced by entomophilous plants that is offered to visitors, mostly insects, to entice pollination. It is a chemically complex aqueous solution of which the most important components are sugars, in particular the disaccharide sucrose and its monosaccharide constituents, fructose and glucose. The proportion of these sugars tends to be constant within species (Percival, 1961). In addition, other substances are present: proteins, amino acids (protein or non protein), lipids, phenols, alcohols, alkaloids, and antioxidants (Nicolson & Thornburg, 2007); some of them may have toxic effects that induce behavioural alterations of floral visitors (Adler, 2000; Kerchner et al., 2015). Consequently, nectar can strongly influence pollinator individual behaviour and fidelity to a certain species (Waddington, 2001).

Several hypotheses have been proposed for the possible functions of toxic nectar (Adler, 2000). The "pollinator fidelity hypothesis" suggested by Baker and Baker (1975) considers that toxic nectar could be beneficial by deterring visitors that deliver less intraspecific pollen (Ehlers & Olesen, 1997; Tiedeken et al., 2014) and by increasing pollinator fidelity (Baker & Baker, 1975; Rhoades & Bergdahl, 1981; Wright et al., 2013). The "drunken pollinator hypothesis" suggests that the effects induced on pollinators could mainly be due to exogenous agents, such as yeasts, that produce ethanol that is taken up by visitors (Ehlers & Olesen, 1997). Drinking toxic nectar, pollinators can become "sluggish" and apparently intoxicated. Previous studies reported narcosis and disorientation in bees and bumble bees after drinking toxic nectar (Bell, 1971; Clinch et al., 1972; Kevan et al., 1988). In plants with pollinia or large pollen grains that hamper the flight of pollinators, this effect may reduce their grooming activity, improving pollen transfer between plants (Adler, 2000). Although different hypotheses regarding the adaptive function of toxic nectar have been proposed, no study on toxic nectar has clearly established whether this trait does benefit the plant (Adler, 2000).

Moreover, in nectar are present carbohydrate -metabolizing enzymes (Nepi et al., 2012). Among them, the enzyme invertase is involved in the maintenance of sugar ratio, hydrolysing sucrose into glucose and fructose, before, during, and after nectar secretion (Heil et al., 2005; Nicolson & Thornburg, 2007).

Floral nectar is initially sterile, but once secreted some yeasts and bacterial species may frequently 'contaminate' it (Herrera et al., 2009; Jacquemyn et al., 2013). Air and pollinator insects may transfer yeasts to flower nectar during their visits (Sandhu & Waraich, 1985). The association of yeasts with insect guts is well known too (Good et al., 2014). Nectars contaminated by dwelling yeasts commonly produce ethanol, which in turn can induce narcotic effects on pollinators, in accordance with the "drunken pollinator hypothesis". Nectar yeasts can also alter the composition and concentration of sugars and amino acids in nectar, contribute to the emission of floral volatiles, and warm the nectar compared to the surrounding air. Consequently, alterations in nectar composition due to yeasts could affect pollination success and plant reproduction (Herrera et al., 2013).

Some main nectar yeast species induce fructose synthesis, which has been shown to increase nectar consumption by bumble bees in artificial conditions (Pozo, 2013). Moreover, yeast inhabiting floral nectar may also represent other sort of rewards to bumble bees. Yeasts, following the "toxic nectar hypothesis", could alter bumble bee behaviour to their benefit. In fact, bees disperse yeasts among different flowers. To investigate how nectar composition of the perennial herb *Gentiana lutea* L. affects pollinator guilds, we carried out field experiments in a natural population and performed laboratory analysis. Our main aim was to study nectar chemical composition to investigate the effects of principal components on pollinator behaviours. We focus on sugar composition and on factors that can alter it, like yeasts and pollen grains.

MATERIALS AND METHODS

Study site and model species

Gentiana lutea L. subsp. *symphyandra* (Murb.) Hayek is a long-lived scapose hemicryptophyte. It presents an unbranched (rarely two) stout stem, growing up to 2 meters tall. Basal leaves are glaucous, decussate, lanceolate-elliptic to broadly ovate with 5-6-strong veins; stem leaves are narrower and stalkless (Tutin et al., 1972). In June the plant produces a new sterile or flowering stem. Flowering occurs between June and July. Flowering stems carry up to 10 pseudo-whorls containing numerous pedicellate flowers (about 20). Flower bracts are green and almost equal in length to pseudo-whorls. Each flower shows a split calyx, with 2-7 minute teeth and a yellow gamopetalous corolla with 3-9 deeply engraved lobes. Stigma is bilamellate and anthers are connate in a tube. Five nectaries occur between stamen filaments and corolla attachment point. Four developmental phases of flowers can be recognized: (I) perianth open, anthers closed and stigma unreceptive (II) open flower, one to four dehisced anthers, stigma undivided or hardly bilamellate, (III) open flower, anthers completely dehiscent and stigma bilamellate, and (IV) perianth withered (Rossi, 2012) (Fig. 1).

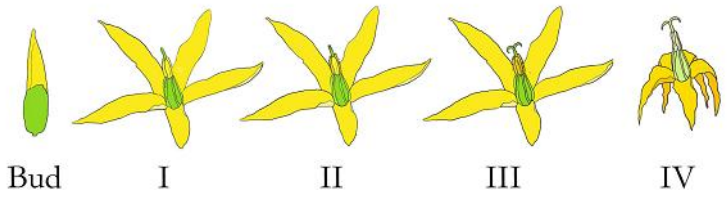

Figure 1. Drawings of flower developmental phases (by M. Albertini, from Rossi, 2012).

Samplings and field observations have been performed in July 2015, in a population situated on the north east face of Mount Grande (Vidiciatico, Bologna), within the Site of Community Importance and Special Protection Area IT4050002 “Corno alle Scale” (44° 8’ 57’’N, 10° 52’ 10’’E, 1380 - 1460 masl). The meadow occurs on a steep slope and is surrounded by a forest of *Fagus sylvatica* L. This population is probably preserved by the steepness of the slope and by the rocky nature of the substrate (Rossi, 2012; Rossi et al., 2014).

Nectar sampling

We sampled nectar from a total of nine *G. lutea* individuals, the sampling was carried out during tree non -consecutive days, starting at the beginning of anthesis and covering the main flowering phenological stages in the population. All manipulated plants and flowers were individually marked with different colour markers, indicating the specific test carried.

We performed six different trials: 1) "invertase": nectar collected from closed flower buds to check the presence of enzyme invertase in pure nectar (not contaminated by pollen); 2) "young virgin nectar": nectar collected from closed flower buds to investigate the composition of freshly secreted nectar; 3) "old virgin nectar": nectar collected from open flowers (phases III and IV), previously emasculated and individually wrapped with NWF (non-woven fabric) small bags during bud phase, to investigate the composition of old nectar not contaminated by self-pollen and inaccessible to pollinators. 4) "contamination by manipulation": nectar collected from not-emasculated, open flowers (phases III and IV), inflorescence wrapped with NWF big bags during bud phase, to investigate the nectar contamination due to manipulation. 5) "contamination by pollinators": nectar collected from open flowers (phase III), emasculated before anthers opening and wrapped with tulle bags about three or four hours before sampling, to investigate the nectar contamination due to insects visits. 6) "control": nectar collected from not-emasculated, open flowers (phase III), wrapped with Tulle bags about three or four hours before sampling. Total volumes of sampled nectar and other information on sample sizes (e.g. number of flowers, number of plants) are reported in table 1.

To collect nectar for "invertase" test we used a micropipette K7501 2 -20 μL (Exacta+Optech GmbH, Germany), samples were then transferred in a single Eppendorf tube. For the other samplings, we used Drummond Microcaps® (3, 5 and 10 μL; Drummond Scientific Co., Broomall, PA, USA). For each sampling we recorded the nectar level on the microcap using a Vernier calliper to calculate the total volume. Nectar samples from different flowers were transferred to distinct Eppendorf tubes filled with 100 μL ethanol. All samples were taken to the laboratory in thermic-bags and kept at 5°C until analyses.

Table 1. Details on nectar sampled volumes and sample sizes for each field trial. Mean nectar volumes are expressed as mean ± SE. ND= not determined.

Data	Invertase	Young virgin nectar	Old virgin nectar	Contamination by		Control
				manipulation	pollinators	
N flowers	>10	20	19	20	2	20
N plants	min. 5	6	4	5	2	6
Total nectar volume (μL)	~100	89.63	72.36	27.31	2.33	40.61
Mean nectar volume (μL)	**ND**	4.48 ± 0.22	3.81 ± 0.38	1.37 ± 0.10	1.17 ± 0.90	2.03 ± 0.17
Nectar volume range (μL)	**ND**	1.68-5.00	0.84-5.00	0.61-2.13	0.27-2.06	1.04-3.00

Nectar analysis

Two analyses were performed on each nectar sample: the determination of nectar sugar composition, and the detection of pollen and yeasts. We analysed nectar sugar composition at the Department of Life Sciences, University of Siena, by isocratic HPLC using a Waters Sugar-Pak I ion-exchange column (6.5 × 300 mm) maintained at 90°C and a Waters 2410 refractive index detector. Water (MilliQ, ph 7) was used as mobile phase at a flow rate of 0.6 mL/min.

To investigate the presence of invertase enzyme on *G. lutea* nectar we diluted 1:20 the nectar sample with a sucrose solution (4.6 mg/mL). We determined the sugar profile at time 0 calculating the concentration of sucrose, glucose and fructose. Subsequently, the solution was incubated at 30ºC and we repeated the sugar determination at 1, 2, 3, 4 and 24 hours.

The number of pollen grains and yeast cells, was detected on an aliquote of 10 μL from each sample of nectar+ethanol (volume approximated to 200 μL). These aliquots, placed on a microscope slide with a glass cover slip, were observed under an optical microscope (total magnification 100×). We registered the number of pollen grains, discriminating between *G. lutea* and non-*G. lutea* pollen, and the number of yeast cells. We calculated the total number of pollen grains and yeast cells for each sample following the expression:

$$n = \frac{N \cdot 20}{V}$$

where N is the aliquot count and V is the nectar sample volume without ethanol.

Data analysis

Data sets were firstly analysed with the Shapiro-Wilk test to check for normality. As data were not normally distributed, we used non-parametric Mann-Whitney test or Kruskal-Wallis test (followed by posthoc multiple pairwise comparisons of mean ranks) to analyse the differences in nectar sugar profile, pollinator behaviour and pollen loads. We used Pearson's correlation to examine the relationships between pollen density, yeast cells density and sugar profile. Statistical analyses were performed with STATISTICA 7.1. software.

RESULTS

Results indicate the absence of the enzyme invertase in G. lutea "pure" nectar. We compared the nectar sugar profile of each trial. "Contamination by pollinators" trial was not considered for analysis due to the low number of sampled flowers (N = 2).

All data on sugar profiles are shown in Table 2. Total sugar concentration was significantly different among trials and the lowest value corresponded to "young virgin nectar" trial. Regarding glucose/fructose ratio (g/f), "young virgin nectar", "old virgin nectar" and "contamination by manipulation" flowers presented similar values and they were significantly different from controls. "Control" flowers presented the lowest fructose percentage (43.10 ± 0.93) and "young virgin nectar" the lowest sucrose percentage (0.07 ± 0.03).

Concerning the presence of pollen grains and yeasts, the amount of *G. lutea* pollen was significantly different among trials ($H = 43.76$, $p < 0.001$): "contamination by manipulation" and "control" trials presented a higher number of *G. lutea* pollen grains than "young virgin nectar" and "old virgin nectar". The amount of pollen belonging to

other species was significantly higher in "control" nectar samples compared to the other trials ($H = 22.44$, $p < 0.001$). The total amount of pollen showed the same pattern as *G. lutea* pollen ($H = 42.27$, $p < 0.001$). Yeasts were absent or significantly less (cell number) in "young virgin nectar" and "old virgin nectar" compared to "control" trial ($H = 41.47$, $p < 0.001$) (Fig. 2).

We found a high number of yeast cells and pollen grains in "contamination by manipulation" and "control" trials (Fig. 2). Moreover, we found pollen and yeast contamination in both sampled flowers (Flower 1 and Flower 2) of "contamination by pollinators" trial (Fig. 3).

Table 2. Nectar sugar profile. Values are expressed as mean ± SE. Values marked with different letters in each row were significantly different according to the multiple comparisons test.

Data	Young virgin nectar	Old virgin nectar	Contamination by		Control	H	p-value
			manipulation	pollinators			
Total sugar/flower (mg)	0.24 ± 0.02 (N=19) **a**	2.10 ± 0.17 (N=19) **b**	1.28 ± 0.09 (N=20) **b**	0.18 ± 0.14 (N=2)	0.46 ± 0.03 (N=20) **a**	64.16	<0.001
g/f	1.04 ± 0.01 (N=19) **a**	0.99 ± 0.01 (N=19) **a**	0.99 ± 0.004 (N=20) **a**	0.67 ± 0.27 (N=2)	1.32 ± 0.05 (N=20) **b**	43.44	<0.001
% glucose	50.89 ± 0.32 (N=19) **a**	49.44 ± 0.17 (N=19) **ab**	49.41 ± 0.12 (N=20) **b**	38.52 ± 10.21 (N=2)	56.18 ± 0.92 (N=20) **c**	45.69	<0.001
% fructose	49.03 ± 0.34 (N=19) **a**	49.86 ± 0.16 (N=19) **a**	50.01 ± 0.12 (N=20) **a**	61.48 ± 10.21 (N=2)	43.10 ± 0.93 (N=20) **b**	41.37	<0.001
% sucrose	0.07 ± 0.03 (N=19) **a**	0.66 ± 0.07 (N=19) **b**	0.58 ± 0.06 (N=20) **b**	0.00 ± 0.00 (N=2)	0.71 ± 0.08 (N=20) **b**	37.83	<0.001

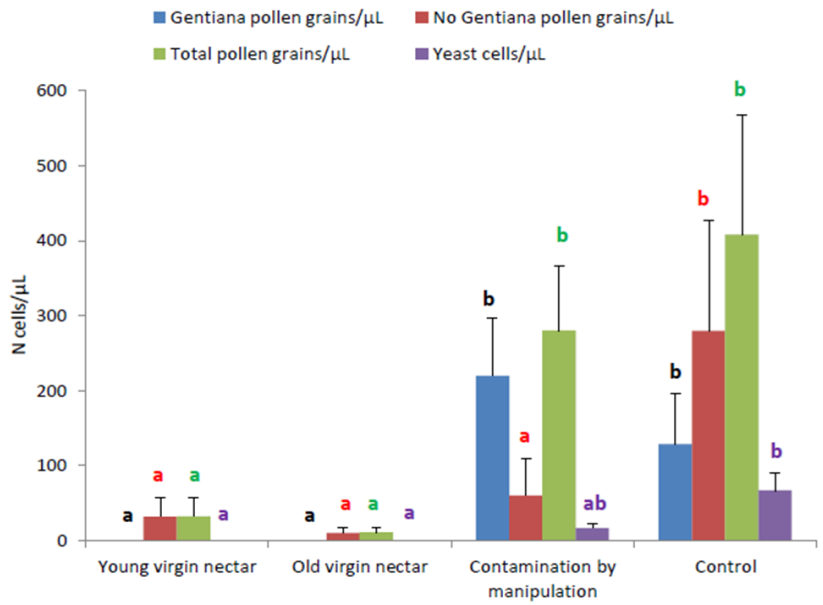

Figure 2. Pollen and yeasts nectar comtamination in the different trials. The number of pollen grains (belonging to G. lutea and to other plant species) and the number of yeast cells per microliter are considered (Mean ± SE). Different letters and colour-letters indicate significant differences according to the multiple comparisons tests. Semple sizes are shown in Table 1.

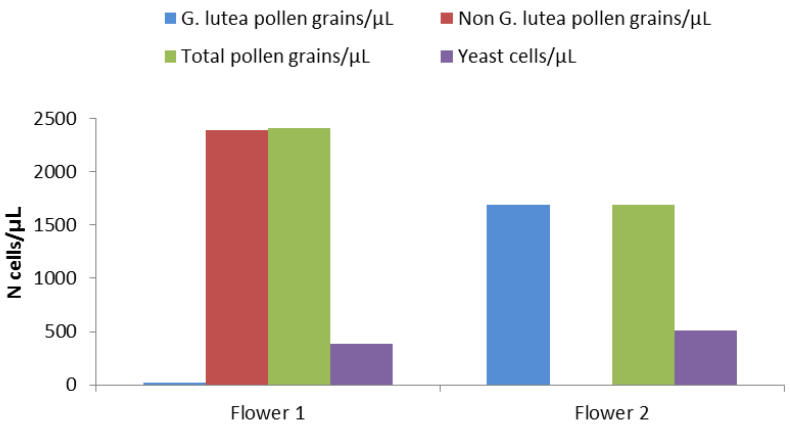

Figure 3. Pollen and yeasts nectar comtamination in the "contamination by pollinators" trial (N = 2 flowers). The number of pollen grains (belonging to *G. lutea* and to other plant species) and the number of yeast cells per microliter are considered. .

We found a significant positive correlation between density of yeast cells and density of total pollen grains (r = 0.8044, p<0.001) (Fig. 4), density of *G. lutea* pollen grains (r = 0.4313, p < 0.001) and density of extraneous pollen grains (r = 0.6673, p < 0.001). We found a significant negative correlation between total sugar concentration on flower and

both density of total pollen grains (r = -0.2523, p = 0.024) (Fig. 5) and density of yeast cells (r = -0.245, p = 0.028) (Fig. 6).

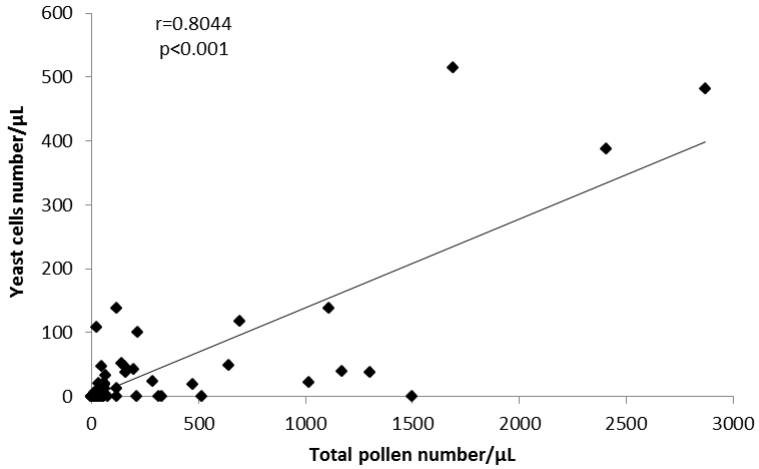

Figure 4. Correlation between yeast cells number/µL and total pollen number/µL. N = 81.

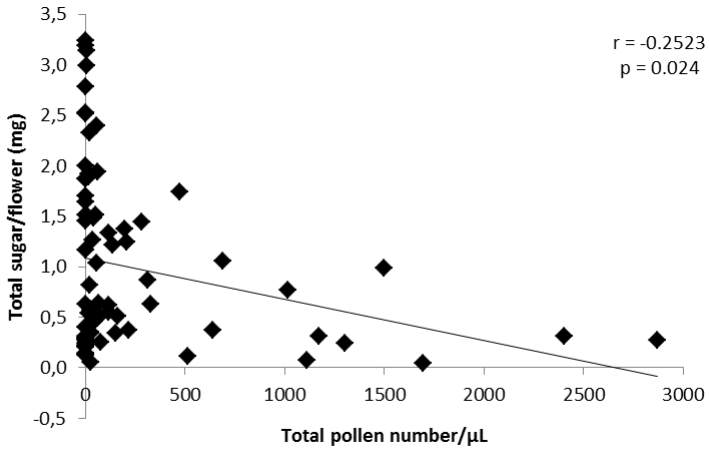

Figure 5. Correlation between total pollen number/µL and total sugar/flower (mg). N = 81.

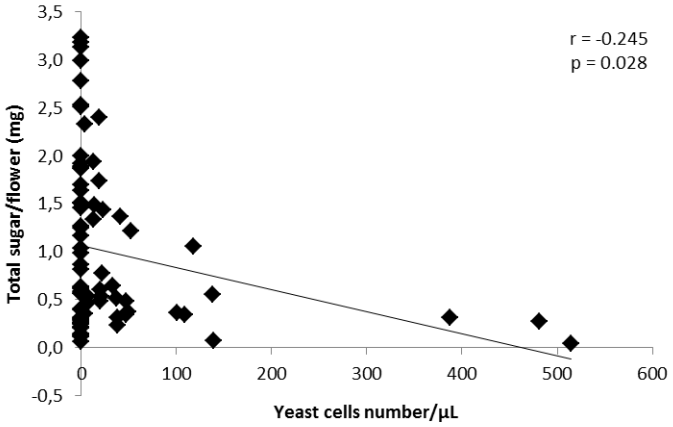

Figure 6. Correlation between yeast cells number/µL and total sugar/flower (mg). N = 81.

DISCUSSION

The dissection of the tripartite relationship linking plants, nectar yeasts, and pollinators offers new angles for deepening our understanding of the ecology of plant reproduction (Herrera et al., 2013).

In this study, we analysed the plant-pollinator system from plant and pollinator point of view. Our observations on *G. lutea* confirm that nectar is the main reward for pollinators (more than 10 µL/flower) (Rossi et al., 2014). Moreover, our analyses indicate that *G. lutea* nectar does not present the enzyme invertase. This means that this plant itself can not regulate the relative proportion of sucrose and the monosaccharides glucose and fructose, prior or during nectar secretion. Then, changes in sugar profile throughout the different floral phases are mainly due to external factors.

Moreover, our data show that "virgin" nectar from young flower buds and "virgin" nectar from open mature flowers present differences in sugar concentration. It is known that when flower buds open, nectar is exposed to the atmosphere and loses water by evaporation (Corbet et al., 1979). The increase of sugar concentration with flower age could be explained by evaporation, resulting from high temperatures and low relative humidity during flower anthesis. Although we didn't study nectar viscosity, we observed that those flowers wrapped to exclude pollinators' visits presented viscous nectar, while the nectar in flower buds had a more aqueous appearance (personal observation). Our data indicate the presence of yeasts mainly in the nectar of mature

flowers, accessible to insect visitors. Moreover, yeast contamination seems to be related to the presence of pollen. Specifically, we found relatively abundant pollen and yeasts in both freely visited flowers and hand-manipulated wrapped flowers. Yeasts seem to be responsible of changes in sugar composition throughout flower lifespan, and our findings suggest that yeast contamination is strictly linked with pollinator's activity. We found the lowest fructose percentage in freely visited flowers (control). The associated glucose to fructose ratio was significantly higher in freely visited flowers compared to newly opened buds and emasculated wrapped flowers, inaccessibile to pollinators. In hand manipulated wrapped flowers the alteration was not significant, but lower than in the nectar samples not contaminated by pollen and yeasts.

Dawson (1932) investigated the selective fermentation of glucose and fructose by yeasts. He found that some yeasts fermented glucose and fructose at equal rates, other ones (including *Saccharomyces cerevisiae*) fermented glucose faster than fructose, while fructose is fermented faster than glucose by some other ones (like *S. pombe* and *S. exiguus*).

Canto and Herrera (2012) investigated the relationship between sugar concentration and yeast density in a wide number of plant species and found that nectar fructose, glucose and sucrose significantly declined with increasing yeast density in certain plant species, but not in others. This indicates that changes in sugar concentration associated with yeast density are to some extent species-specific. To explore this hypothesis, it would be interesting determine which yeast species are found in *G. lutea* nectar.

Our results show that yeast nectar contamination is negligible in flowers not visited by insects (emasculated and wrapped in tulle bag before anthesis): we can thus hypothesize that yeasts are transferred to floral nectar by pollinators, together with pollen. Nevertheless, pollinators are the major - but not exclusive - responsible of yeast and pollen contamination: we found pollen and yeasts also in hand-manipulated but not emasculated flowers, where insects' visits were excluded. In this case the pollen (largely belonging to *G. lutea*) comes mainly from the same flower's anthers, and contamination is principally due to the lack of pollen withdrawal by pollinators.

Also the act of manipulating the flowers during samplings can contribute to the pollen contamination of nectar. Our data indicate that pollinators activity likely leads to pollen contamination (mostly from other plant species) and consequently to yeast transfer.

Anyway, we don't know if yeast spores arrive attached to pollen grains or attached to pollinators bodies. Pozo et al. (2000) described that nectar yeasts as *Metschnikowia reukafii* tend to aggregate in the vicinity of pollen grains fallen into the nectar, leading to positive correlation between the density of pollen grains and yeast density. Although nectar yeast cell density and pollen density vary among plant species, the presence and abundance of pollen in nectar positively affects yeast density. It is reasonable to hypothesize that yeasts may use pollen grains as a growing substrate.

Yeasts could also be responsible for a particular smell of flowers that can act as attractant for pollinators. Moreover, pollen could change amino acid nectar composition, creating favourable conditions for yeast growing. The presence of nectar dwelling microorganisms can have a huge impact on nectar chemistry, especially on sugar composition and concentration (Canto & Herrera, 2012; Vannette et al., 2013). The influence of pollen and yeast in amino acid nectar concentration, temperature and odour is well known (Herrera & Pozo, 2010; Vannette et al., 2013; Golonka et al., 2014). Moreover, it is known that yeasts can produce psychoactive substances from amino acid decarboxylation (like biogenic amine, dopamine or scopolamine) that can affect pollinator behaviour as well (Nepi, personal communication).

This study provides data about *G. lutea* nectar sugar composition, pollen and yeast contamination, indicating that flower nectar quality and composition is influenced by several biotic and abiotic factors, with possible repercussions on both insect visitors and plant pollination. Future investigations (yeast determination and amino acid composition analysis) on the same model species should enable us to better understand the effect of *G. lutea* nectar chemical composition on pollinator behaviour and the consequences for plant fitness.

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4.7. Role of non protein amino acids in nectar: effects on bees behaviour

Manuscript

SUMMARY

Floral and extrafloral angiosperms' nectar is undoubtedly recognized as a valuable energetic alimentary resource for a large variety of animals. Through nectar consumption, insects, small mammals, birds, marsupials and reptiles establish more or less specialized interactions with plants. According to recent studies, nectar mediates interactions that are much more complex than simply alimentary relations. From the more abundant nectar compounds, i.e. sugars and amino acids mainly responsible for its high alimentary value, attention of scientists is moving towards compounds that are not directly connected with its alimentary importance. Among these, secondary compounds appear to play a special role in regulating interaction with other organisms. Although very few is known about their ecological roles in nectar, recently it was proved that secondary compounds may affect the nectar feeders' behaviour interacting with their neurobiology. We addressed a special focus on one class of secondary compounds: the non-protein amino acids. Their presence in floral nectar has been reported since long time but their ecological function has not been investigated. Data from several phylogenetically unrelated species indicate that they may represent a consistent part of the total amino acid content of floral nectar (25-45%) and generally the more abundant ones are γ-amino butyric acid (GABA) and β-alanine. The study of nectar chemistry in a restricted taxonomic context (i.e. the tribe Lithospermeae of the Boragniaceae family) revealed that GABA concentration is particularly high in species with specialist bee- and bumblebee-pollination whilst β-alanine increases in species pollinated by flies and passerine birds. This result suggested to test the effect of an artificial diet consisting of sucrose solutions enriched with GABA and β-alanine on some species of Apoidea. Results show that *Bombus terrestris* and, only partially, *Apis mellifera*, increase their walking activity when fed with the solution enriched with β-alanine at high concentration, while they increase their flying activity with the same solution at low concentration. Moreover, bumble bees greatly increase their survival time when fed

with the solution enriched with GABA. These results push us to further consider the role of β-alanine and GABA in increasing the mobility of insects between flowers and their foraging activity and thus their pollination performances.

INTRODUCTION

Nectar is a chemically complex aqueous solution mainly composed of sugars, in particular the disaccharide sucrose and its monosaccharide constituents, fructose and glucose (Nicolson & Thornburg, 2007). However, the pioneering studies of Baker and Baker (1973, 1975) demonstrated that nectar is more than a simple sugar solution acting as pollinator reward. Nectar is composed also by non-sugar components as amino acids, lipids, phenols, alkaloids and volatile organic compounds (Kessler & Baldwin, 2007; Nicolson & Thornburg, 2007; González-Teuber & Heil, 2009). All these substances affect the attractiveness of nectar to pollinators and their amounts and concentrations are related to the pollinator type (Baker & Baker, 1977; Faegri & van der Pijl, 1979; Baker & Baker, 1983a). Moreover, these components show a large variety of secondary applications not directly related to pollination (Raguso, 2001). For example, scent compounds, in addition to attract pollinators, can also repel antagonists (Galen, 1983; Kessler & Baldwin, 2007); essential oils show clear antimicrobial properties (Lokvam & Braddock, 1999; Kram et al., 2008; Nepi et al., 2011); alkaloids can act increasing plant defence against herbivores (Mithofer & Boland, 2012). In last years, studies have therefore focused on the non-sugar nectar components, and in particular on amino acids, since they are the second most concentrated solutes, and investigated their influence on pollinator attractiveness (Alm et al., 1990).

In addition to the 20 classic protein amino acids, in the nectar were found thousands of non-protein amino acids (NPAAs). Among these, around 250 are involved in interactions with bacteria, fungi, herbivores and other plants (Huang et al., 2011; Vranova et al., 2011). Since they are not directly involved in metabolic processes, they can be considerate as secondary compounds and consequently they can be lethal at high doses, but at lower doses can have numerous different functions (Bell, 2003). Nepi (2014) described three different ways by witch NPAAs can affect the pollinator

foraging behaviour: (i) acting on insect nervous system; (ii) regulating the phagostimulation; (iii) increasing the flight muscles activity. NPAAs concentration and composition vary with the flower age but generally γ-amino butyric acid (GABA) and β-alanine are the most rappresentated (Gottesberger et al., 1990; Petanidou & Smets, 1996; Petanidou et al., 2006; Nepi, 2014).

In this study we focused on two NPAAs: GABA and β-alanine. GABA is known as the principal inhibitory neurotransmitter (Breer & Heilgenberg, 1985), acting to increase the permeability of post-synaptic membranes to chloride ions both in vertebrates and in invertebrates, including insects (Sattelle, 1990; Gardener & Gillman, 2001). In invertebrates, GABA receptors are located peripherally in muscle tissue and neuromuscular junctions, where they are bathed in the haemolymph (Sattelle, 1990; Bown et al., 2006). Von Keyserlingk and Willis (1992) found that their main physiological role was the organization of excitatory events into coordinated muscular contractions. In addition, GABA is involved in the development of the nervous system, promoting neuronal migration, proliferation and differentiation (Owens & Kriegstein, 2002; Bouchè et al., 2003).

On the other hand, less informations are available on the role of β-alanine in insects. This NPAA is involved during sclerotization and pigmentation of adult cuticle (Roseland & Kramer, 1987; Andersen, 2007) and it is the receptor agonist, with taurine, of GABA and mediate inhibitory postsynaptic potentials in insect neurons via increased chloride conductance (Buckingham et al., 1994). In addition, β-alanine seems to be involved also in the biogenic amines system (Farooqui, 2012), important neuroactive molecules of the central nervous system (Roeder, 1994; Monastirioti, 1999). Moreover, β-alanine is a precursor of the dipeptide carnosine and it seems to be involved in vertebrate muscular activity's regulation (Derave et al., 2010): it has been demonstrated that a supplementation with β-alanine results in improved exercise performance, especially during high-intensity exercise in humans (Sale et al., 2010). However, no studies are performed on insect muscle activity.

Our aim was to investigate long-term effects of these two NPAAs on bees' behaviour. Under laboratory conditions we carried out an experiment on bumble bees (*B. terrestris* L.) and one on honey bees (*Apis mellifera* L.), since they are both considered model species in plant-pollinators studies.

MATERIAL AND METHODS

Study species and experiment conditions

Bumble bee workers (*Bombus terrestris* L.) were collected from colonies maintained at 25 ± 1°C and 40 ± 5% relative humidity (RH) in a climate room, in continuous darkness, fed ad libitum with fresh frozen pollen and sugar syrup. Colony were purchased from Bioplanet S.c.a., Cesena, Italy. Individuals were collected from 3 colonies (three replicates) under red light and transferred in groups of 5 into experimental cages composed by a plastic net cylinder (height = 25 cm, diameter = 16 cm) with ends closed with transparent plastic lids, placed horizontally. Than they were maintained at ambient temperature and natural dark-light cycle. Since large variation in body size exists among bumblebee workers, very small and very large individuals were excluded from the experiments. We also excluded newly emerged and old bees, recognizable for the coloration and the hair lack.

Honey bee workers (*Apis mellifera* L.) were obtained from three colonies managed at the CREA-API (Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Bologna, Italy), following standard beekeeping techniques. Forager bees were collected in early summer from three hives (three replicates) using the funnel trap (Medrzycki, 2013). Subsequently honey bees were anaesthetized with a mixture of air and CO_2 (2:3) for 15 minutes and transferred to the same cages used for bumble bees in groups of at least 10 bees (OECD, 1998; EPPO, 2010) and maintained at ambient temperature and natural dark-light cycle for the whole duration of the test.

Artificial nectar solutions

Solutions were prepared according to the concentrations reported in Table 1. We used a control solution (C), containing only sucrose, a β-alanine enriched solution (B) and another one enriched with γ-amino butyric acid (GABA, G). We tested two different concentrations of each amino acid, one natural (NAT), comparable to concentrations of GABA and β-alanine found in floral nectar (Nepi 2014), and one 20 times increased (20X).

Table 1. Amino acid composition of used solutions in both concentrations.

concentration	solution	β-alanine	GABA
1	C	-	-
	B	0.205 g/L	-
	G	-	0.077 g/L
2	C	-	-
	B	4.094 g/L	-
	G	-	1.546 g/L

In the case of bumble bees, the concentration of sucrose in all the solutions was 20% w/v whilst it was 50% w/v for bees since preliminary tests carried out with lower concentrations resulted in high mortality. Solution were administered ad libitum via tip-less syringes.

Sucrose and amino acids were purchased from Sigma-Aldrich, Milano, Italy.

Behavioural observation and data collection

We registered solution consumption, mortality and behaviours during several intervals each day, leaving at least one hour between subsequent interval. Each interval consisted in 5 one-minute observations for each cage. The total number of intervals varied from 23 up to 81 depending on the survival time of different replicates. The observations consisted in recording the number of individuals in a cage that in that moment performed a particular behaviour. The behaviours considered were: walking, feeding, flying, stationary. For honey bees we considered also the occurrence of trophallaxis. Observations continued until the death of the last individual.

Data analyses

Bee survival data were segregated by solutions and analyzed using Kaplan-Meier survival analysis (Hosmer & Lemeshow, 1999). Survival curves were compared using Log rank tests among the three solutions and in pairwise comparison.

Consumption and behavioural data were first analysed for normality using the Shapiro-Wilk test. Since data were not normally distributed, Mann-Whitney U-test was used to assess the significance of differences between the two concentrations of the amino acid enriched solutions and the Kruskal-Wallis H test followed by the pairwise multiple comparison of mean ranks post hoc test to analyse differences among thesis, replicates, colonies.

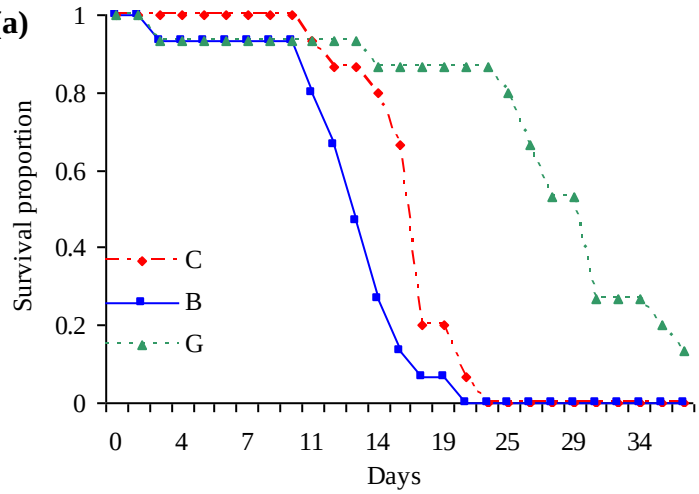
RESULTS

Survival analyses

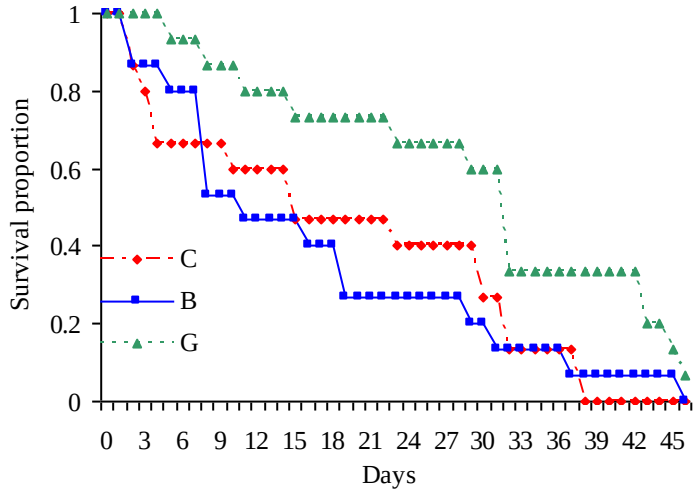
The Log rank analysis showed significant differences between cumulative survival curves of *B. terrestris* fed with solutions 20X (Fig. 1a, Log-rank $\chi^2_2 = 22.182$, $P < 0.001$) and NAT (Fig. 1b, Log-rank $\chi^2_2 = 7.00$, $P = 0.030$).

Bumble bees survived significantly more days when fed with solution G than solution C and B at both concentrations (Table 2). We did not find significant differences in *A. mellifera* survival analyses (Table 2), even if honey bees fed with solution B survived less that the others at NAT concentration (Fig. 1c and 1d).

(a)


Survival proportion
1
0.8
0.6
0.4
0.2
0
C
B
G
0 4 7 11 14 19 25 29 34
Days

(b)


Survival proportion
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G
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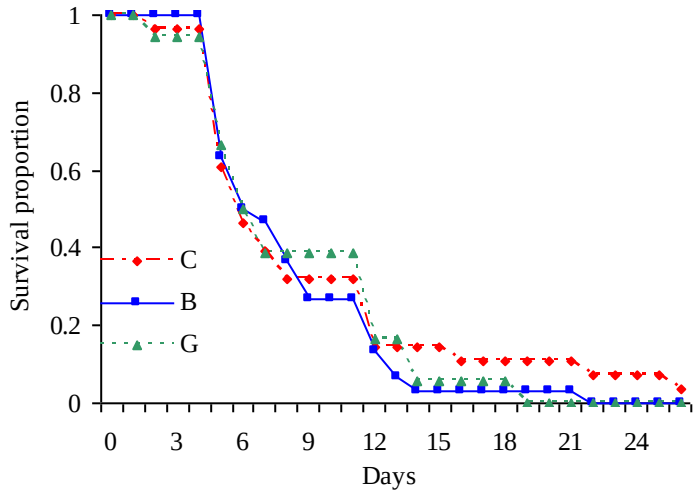
Days

(c)


Survival proportion
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0.8
0.6
0.4
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C
B
G
0 3 6 9 12 15 18 21 24
Days

(d)

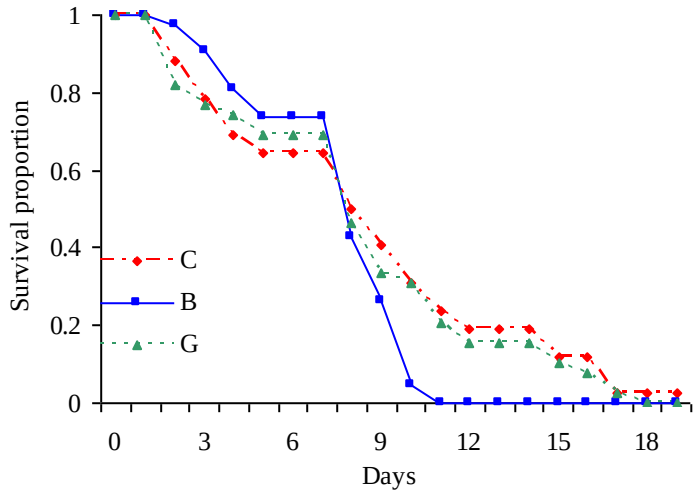

Figure 1. Cumulative proportion of surviving bees fed with the different solutions and concentrations. **(a)** *B. terrestris*, concentration 20X. **(b)** *B. terrestris*, concentration NAT. **(c)** *A. mellifera*, concentration 20X. **(d)** *A. mellifera*, concentration NAT.

Table 2. Pairwise comparisons of survival of *B. terrestris* and *A. mellifera* in the two test concentrations.

Treatments	*B. terrestris* 20X		*B. terrestris* NAT		*A. mellifera* 20X		*A. mellifera* NAT	
	Statistic	P	Statistic	P	Statistic	P	Statistic	P
C vs B	-2.69	**0.007**	0.14	0.886	-0.15	0.884	-1.43	0.151
C vs G	3.40	**0.001**	2.32	**0.020**	0.14	0.888	0.17	0.867
B vs G	3.64	**<0.001**	2.14	**0.032**	0.46	0.645	1.69	0.090

Bold values represent significant (*P*-value < 0.05) differences in bee survival between solutions.

Consumption analyses

The results of consumption analyses showed that bumble bees consumed less solution B than solution C and G in concentration 20X (Fig. 3a), even if the difference was not significant (Kruskal-Wallis $H_2 = 5.48$, $P = 0.065$). Conversely, with solutions NAT we obtained a significant higher value for solution B than solutions C and G (Kruskal-Wallis $H_2 = 14.60$, $P < 0.001$) (Fig. 2a).

In honey bees' experiment, we found difference at high concentration (H_2 = 7.97, *P* = 0.019), where honey bees consumed significantly more solution B than solution C (Fig. 2b), we found no differences in solution consumption in concentration NAT.

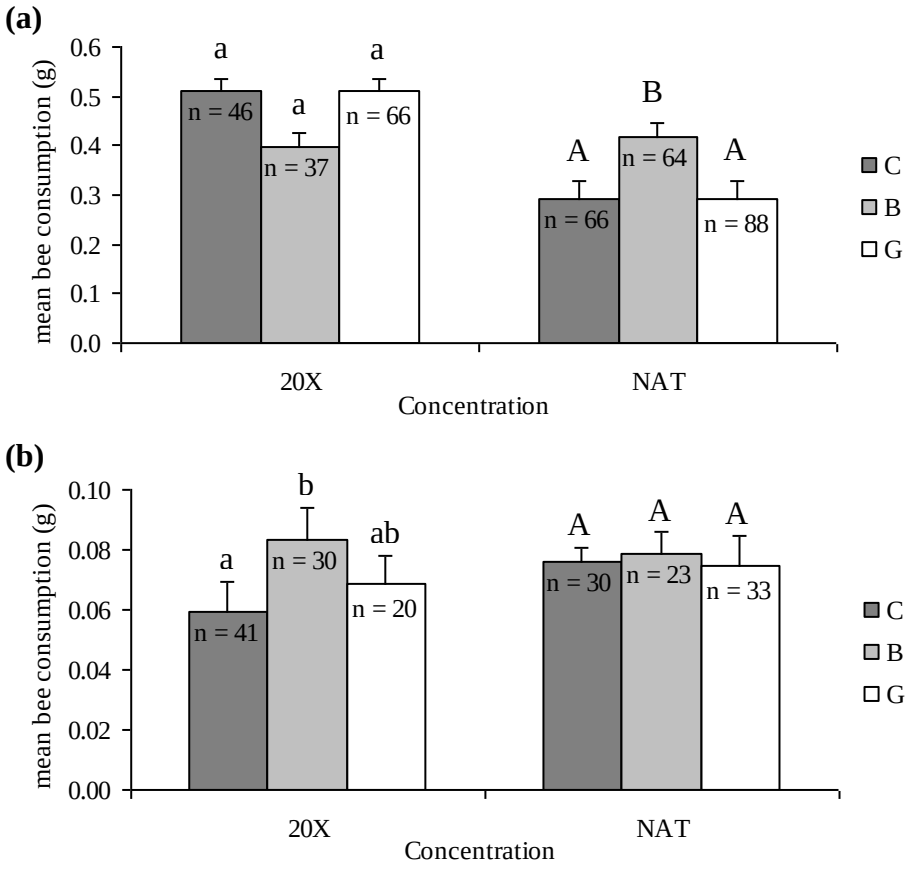

Figure 2. Mean daily bee consumption. **(a)** Bumble bees. **(b)** Honey bees. n = number of measurements. Values marked with different letters were significantly different according to the Kruskal-Wallis H test.

Behavioural analyses

The frequencies' analysis on bumble bee behaviours showed that insects fed with solution B at concentration 20X presented a higher walking behaviour than insects fed with solution C and G (Fig. 3a). Conversely, they presented a lower flying behaviour than those fed with solution C and a lower stationary behaviour than those fed with solution C and G (Fig. 3a).

At concentration NAT, bumble bees fed with solution B showed a higher flying behaviour than those fed with solution C and G (Fig. 3b).

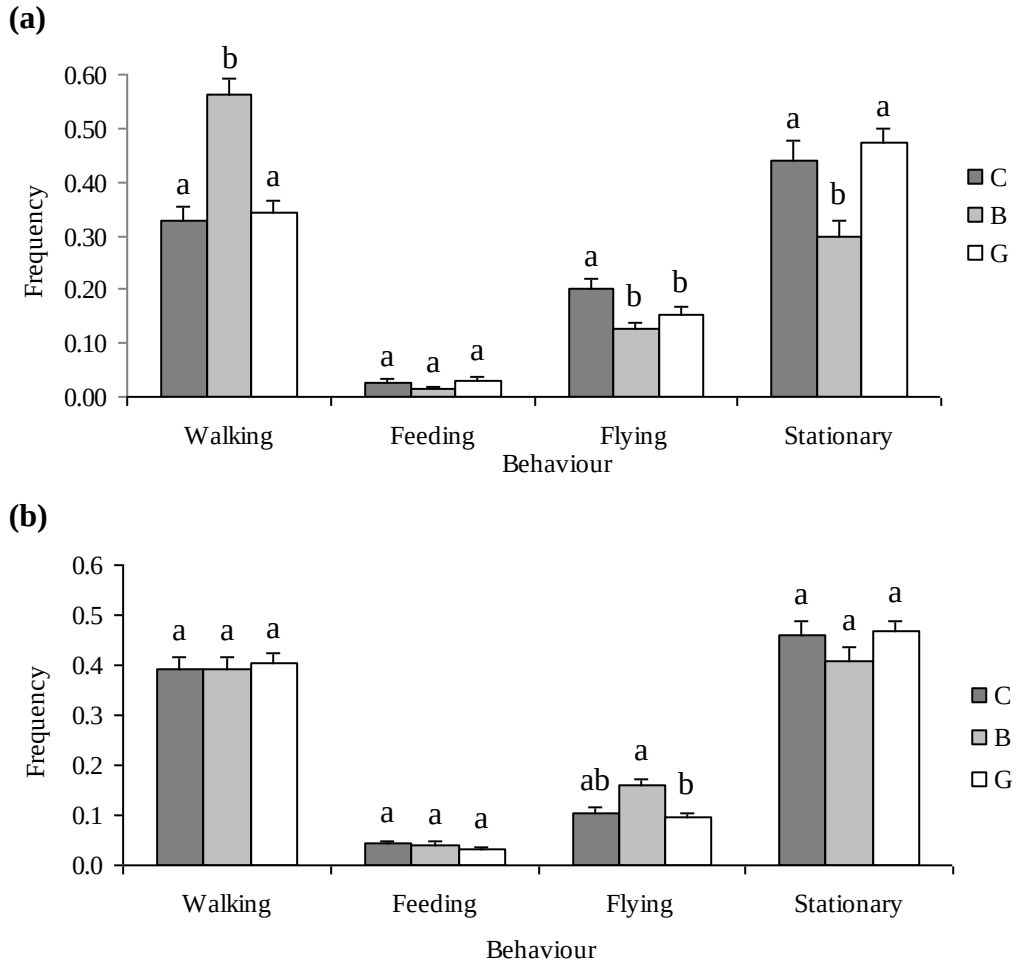

Figure 3. Behaviour frequencies o f bumble bees fed with the three solutions. **(a)** concentration 20X. Number of observations: C = 91 ; B = 77 ; G = 145. **(b)** concentration NAT. Number of observations: C = 154 ; B = 157 ; G = 223. For each behaviour values marked with different letters were significantly different according to the Kruskal-Wallis H test.

Regarding honey bees, insects fed with solution B at concentration 20X presented a higher walking behaviour and a lower stationary behaviour than those fed with solution C (Fig. 4a). At concentration NAT honey bees fed with solution B showed an higher walking behaviour than those fed with solution G whilst there was not significant difference with solution C (Fig. 4b).

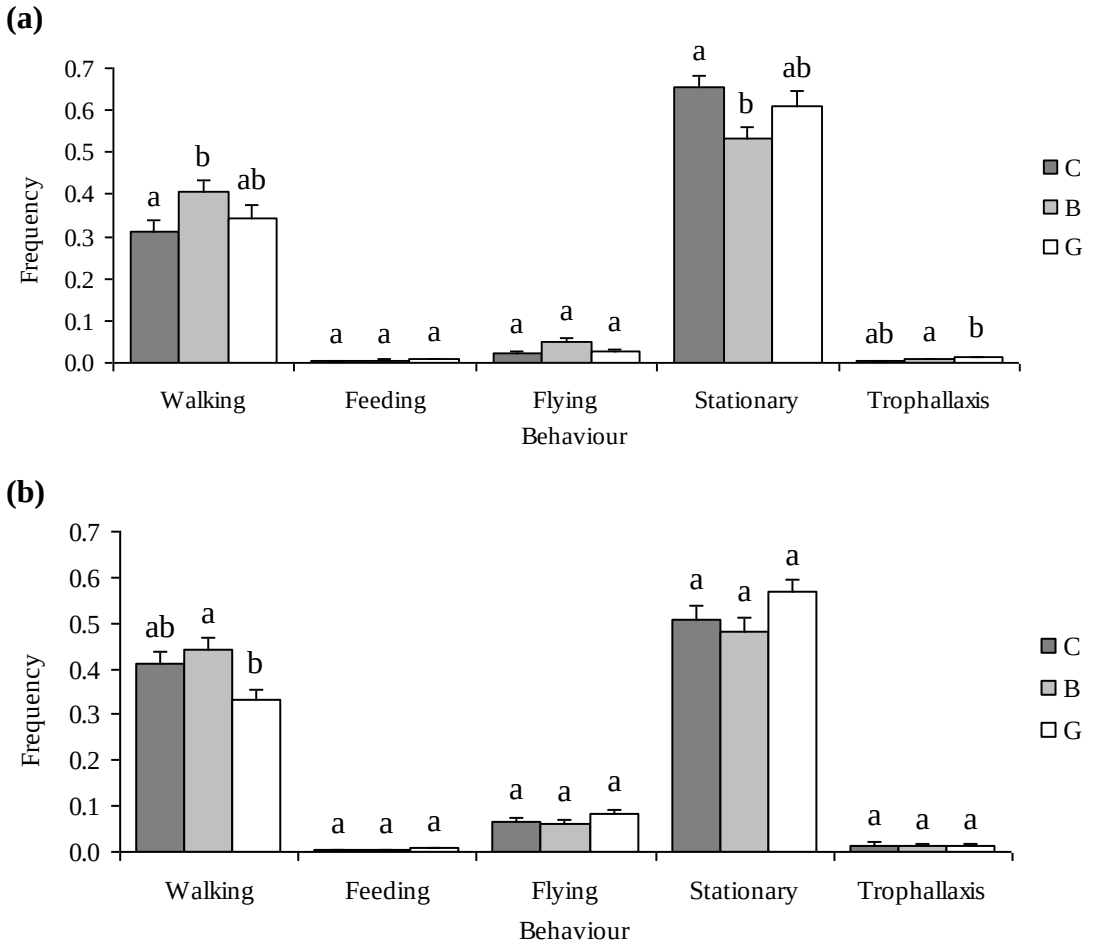

Figure 4. Behaviour frequencies o f honey bees fed with the three solutions. **(a)** concentration 20X. Number of observations: C = 138 ; B = 103 ; G = 70. **(b)** concentration NAT. Number of observations: C = 90 ; B = 66 ; G = 99. For each behaviour values marked with different letters were significantly different according to the Kruskal-Wallis H test.

DISCUSSION

Bees' survival

Our study indicated that a GABA-enriched diet positively affects the lifespan in bumble bees both at natural concentration and at a 20 times higher concentration. This result seems to be in contrast to previous studies. The addiction of essential amino acids to the dietary restriction condition decreased the lifespan of *Drosophila melanogaster* (Lee et al., 2008; Grandison et al., 2009; Emran et al., 2014) and conversely the restriction of

amino acids in the diet of the same species extended its lifespan (Min & Tatar, 2006). The same results were found also in experiments on cockroaches (Hamilton et al., 1990) and crickets (Maklakov et al., 2008). In social insects, similar results were reported for honey bees (Pirk et al., 2010; Archer et al., 2014; Paoli et al., 2014), ants (Cook et al., 2010; Dussutour & Simpson, 2012) and bumble bees (Stabler et al., 2015; Bogo et al., in preparation). However, all the above mentioned studies tested diets enriched in proteins or essential amino acids, not only in non-protein amino acids that are not directly involved in essential metabolic processes (Nepi, 2014).

However, several studies on γ-amino butyric acid showed the healthy role of this NPAA both on plants and animals. In plants, extracellular GABA is involved in communication with other organisms (Shelp et al., 2006), is largely and rapidly produced in response to infections (Chevrot et al., 2006) and acts as protection against oxidative stress (Kinnersley & Turano, 2000; Coleman et al., 2001). In vertebrate and invertebrate animals, GABA is the principal inhibitory neurotransmitter (Breer & Heilgenberg, 1985) and is involved in response to stress (Gronli et al., 2007). In addition, it is an important element in nervous system development (Bouchè et al., 2003). The GABA effects on response to stress and on lifespan could be a possible explanation for bumble bee preference of nectar with high concentration of this NPAA (Nepi, 2014).

Behavioural observation

Regarding bees behaviours, our findings showed a direct effect of β-alanine on the dynamic behaviour of bumble bees and partially on honey bees. It's interesting to notice that the solution enriched in β-alanine with concentration mimicking natural nectar influences positively the flying behaviour of bumble bees, representing a great benefit to the plant. In fact, increasing pollinators' flight, *G. lutea* could indirectly promote bee movements among different plant individuals and consequently favour cross-pollination and allogamy. On the contrary, promoting walking behaviours, plant could promote autogamy since insects would remain on the same whorl pollinating flowers with pollen from the same plant. However, this would happen at amino acid higher concentrations

and it is concevaible that as for other secondary compounds (Manson et al., 2013), β-alanine demonstrate a dose-dependent toxic effect.

Another possible effect of NPAAs is related to the so-called "pollinator fidelity hypothesis" (Baker & Baker, 1975; Adler, 2000) that considers the role of toxic nectar in deterring visitors that deliver less intraspecific pollen (Tiedeken et al., 2014) and in increasing pollinator fidelity (Wright et al., 2013).

In conclusion, these findings push us to further consider the role of β-alanine and GABA in increasing the mobility of insects between flowers and their foraging activity and thus their pollination performances.

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CHAPTER 5

Improvement of bumble bee colonies rearing through biological and technical measures

5.1. Inbreeding

Inbreeding is the sexual reproduction of closely related individual, probable carriers of identical alleles, descendants from one or more common ancestors. In a natural population, the chances of inbred mating tends to increase with the decrease of the population size and with the increase of its reproductive isolation (Thornhill, 1993). A population is a group of conspecific and inter-fecund individuals, which live in a particular geographical area at the same time. It shows a relative reproductive isolation from other populations of the same species. The probability of interbreeding is greater than the probability of cross-breeding with individuals from other areas in fact breeding is substantially more common within the area than across the border (Roughgarden et al., 1989; Krebs et al., 1994). This isolation may depend on the progressive habitat loss or on the presence of barriers, that do not allow partners search during the reproductive periods. This phenomenon is called "reproductive isolation": a collection of behavioural, physiological, ecological mechanisms that prevent individuals of two different populations to mate (Jiménez et al., 1994). Consequently, the heritable variability is circumscribed within the population and can not receive contributions from outside (Campbell et al., 2008). Inbreeding causes a decrease in the fitness of offspring due to a homozygosity increase and an expression of deleterious recessive alleles knows as "inbreeding depression" (Charlesworth & Charlesworth, 1987; Ralls et al., 1988; Barret & Charlesworth, 1991; Lacy, 1993). Inbreeding also produces deleterious consequences for adults, as sperm deformities, sterility and decreased courtship frequency (Frankham, 1995). Therefore a small population with a low genetic variability has less adaptive capacity to environmental changes, pests and pathogens. In these conditions, genetic diversity preservation is a crucial factor in the long-term

conservation of the species (Keller & Waller, 2002; Takahashi et al, 2008) and a selection for mechanisms to avoid mating with close relatives it is expected (Pusey & Wolf, 1996). A widespread strategy to avoid inbreeding is the dispersal of individuals from their natal group; however, in group-living species with low dispersal rates, the ability to recognize and to discriminate kin among conspecifics can constitute an alternative strategy when choosing mates (Blouin & Blouin, 1988; Pusey & Wolf, 1996).

However, we must consider that inbreeding can have also substantial positive effects on the parent's inclusive fitness, by increasing their representation of genes identical by descent in future generations (Parker, 1979; Kokko & Ots, 2006). Inbreeding depression costs and inbreeding kin-selection benefit not necessarily cancel each other. Their balance determines if individuals should avoid or favour mating with their kin (Lehmann & Perrin, 2003; Kokko & Ots, 2006; Parker, 2006; Puurtinen, 20011).

5.1.1. Inbreeding in social Hymenoptera

In those species that live in social groups, the sociality itself inevitably boosts the chances of inbreeding. Therefore inbreeding is both a cause and a consequence of the evolution of sociality. Social arthropods in fact developed a number of mechanisms to prevent inbreeding that have a profound impact on the structure, organization and functioning of populations, by acting on individuals physiology and behaviour (Tabadkani et al., 2012).

Many Hymenoptera species are haplodiploid and their sex determination is regulated by a polyallelic single-locus, following the single-locus complementary sex determination system (sl-CSD) (Duchateau et al., 1994, van Wilgenburg et al., 2006). In normal colonies, derived from outbred mating, fertilized eggs that are heterozygous at the sex-determining locus develop into diploid females, and unfertilized eggs that are hemizygous developed into haploid males. In half of the colonies derived from inbred mating, 50% of the fertilized eggs are homozygous at the sex-determining locus and develop into diploid males (Ayabe et al., 2004) (Fig. 6).

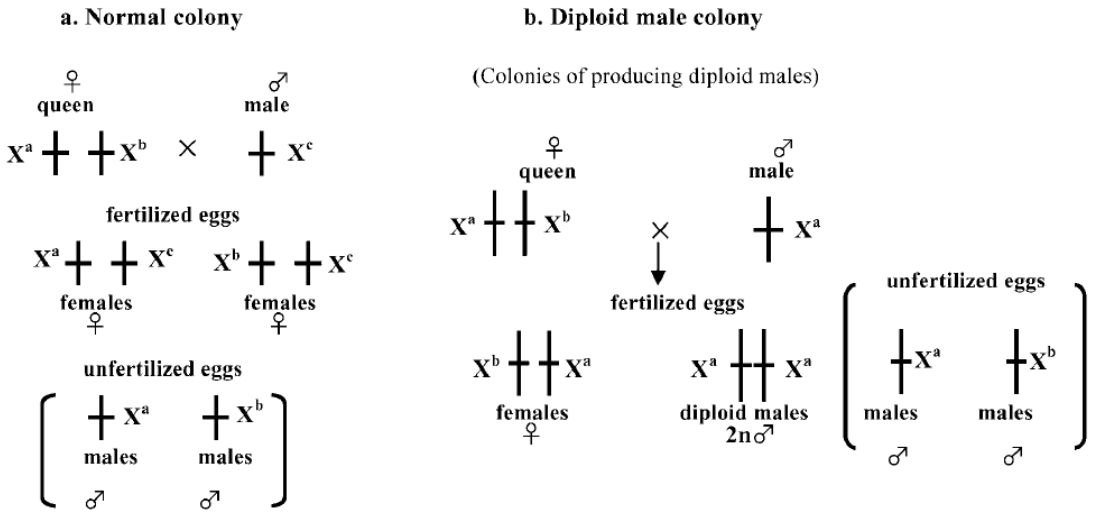

Figure 6. Breeding systemunder single-locus CSD used in the study. (a) Normal colony: fertilized eggs that are heterozygous at the sex-determining locus develop into diploid females, and unfertilized eggs that are hemizygous develop into haploid males. (b) Diploid male colony: half of the fertilized eggs are homozygous at the sex-determining locus and result in sib-mating. They developed into diploid males while the rest develop into diploid normal females (Ayabe et al., 2004).

In principle, haplodiploid species should suffer less from inbreeding than diploid species due to purging of deleterious recessive alleles in haploid males (Henter, 2003). However, the evidence shows that the overall cost of inbreeding is higher in haplodiploid species (Zayed & Packer, 2005).

Since in this situation costs of inbreeding are elevated, haplodiploidy species that reproduce by sl-CSD should present a stronger selection for mechanisms limiting the incidence of inbred mating than species with multilocus complementary sex determination (Bourdais & Hance, 2009). Consequently it is not surprising to observe evolutionary transitions from sl-CSD to non-complementary sex determination in some hymenopteran species as chalcidoid and braconid wasps (Beukeboom et al., 2000). Under field conditions the occurrence of diploid males depends on how many alleles for the sex locus are present in the population. If they are sufficiently large and with a certain variability, their appearance will be very rare; conversely, the genetic drift in small populations can lead to their increase, through a depletion in the number of sexual alleles, and to an increase of homozygosity (Duchateau et al., 1994). An evolutionary strategy adopted by queens of some social Hymenoptera species consists in multiple mating, in order to decrease the possibility to originate individuals homozygous for the

sexual allele and consequently enhances their fitness (Stockley et al., 1993; Cornell & Tregenza, 2007).

5.1.2. Diploid males in *Bombus terrestris*

While in *Apis mellifera* diploid male larvae are recognized and cannibalized shortly after hatching (Woyke, 1965), this does not happen in *Bombus terrestris*, where they can regularly develop until the adult stage since the first brood (Duchateau & Marien, 1995). The presence of wild colonies with diploid males has been observed in several cases (Maebe et al., 2014), although the molecular data for the European continental populations suggest that the rate of inbreeding is not so high (Duchateau et al., 1994).
Previous studies showed that a number of fitness parameters were adversely affected by the presence of diploid males in the colony, (as growing rate, total production of offspring, colony survival ability, colony initiation success) and diploid males themselves showed a weaker immune system than haploid males, while inbred colonies without diploid males did not present any effect (Duchateau et al., 1994; Gerloff et al., 2003; Ayabe et al., 2004; Gerloff & Schmid-Hempel, 2005; Whitehorn et al., 2009).
Consequently the production of diploid males in a colony represents the greatest negative effect of inbreeding, since in nature they are a cost for the colonies in terms of food collection capacity and brood rearing (Duchateau et al., 1994).

5.2. Commercial rearing

The potential value of bumblebees as crop pollinators has been known for a long time and it is proved essential for the pollination of some greenhouse colture, especially tomato. Since 1989, in fact, bumble bees were artificially reared in biofactories, mainly in the Netherlands, to be used for tomato pollination in greenhouses, instead of the more expensive and laborious manual vibrators, which were commonly used in the pollination of this plant in northern Europe (Velthuis & van Doorn, 2006). Currently thousands of artificially reared bumble bee colonies are sold in many countries of the

world. Rearing bumble bees attempts have a long history and firsts date from the beginning of the last century. Sladen, for example, noted some critical stages in bumble bee rearing, as mating and diapause, already in 1912. Since then, many researchers contributed to the artificial rearing, up to the 70s of last century, when the first experiments were successful (Röseler, 1977). In 1987 started the commercial mass rearing, with the launch of the first biofactory in Belgium, the "Biobest", who reared *Bombus terrestris* for tomato pollination in greenhouse. A year later, it was followed by two Dutch companies ("Koppert Biological Systems" and "BBB: Bunting Brinkman Bees"). Then the bumble bee commercial rearing practice extended to Western and South Europe and subsequently it globally expanded (Velthuis & Doorn, 2006). Today there are several biofactories spread throughout the world and they produced and reared annually nearly a million of colonies, of *Bombus terrestris* in Europe and Asia, *B. ignitus* in East Asia and *B. impatiens* in the USA and Canada. One bumble bee colony can pollinate successfully at least 1000 m^2 of tomato in greenhouse (Kearns & Thomson, 2001). The bumble bee turnover is estimated at € 55 million per year, mostly in tomato pollination, with a production of this plant of € 12 billion per year (INEA data 2011). To a lesser extent, bumble bees are used for other crops such as strawberries, peppers, potatoes, eggplant, and also for fruit trees.

5.3. Aim of the study

The progressive and widespread pollinator decline affect many bee species, among which bumble bees. One of the risk factors for wild populations of this social bees is inbreeding, mostly originating from geographic isolation and small size populations. Inbreeding can have severe effects on animal populations, through a decrease in the offspring fitness due to inbreeding depression. In haplodiploid social Hymenoptera it has a further negative effect because of their particular mechanism of sex determination (sl-CSD), that causes the emergence of unviable or sterile diploid males.

We investigated the phenomenon of inbreeding in *Bombus terrestris* colonies reared in captivity, in order to investigate the existence of inbreeding-avoidance systems in this species (e.g. partner choice and kin recognition). Moreover, we determined the

inbreeding risk of our colonies through the calculation of an Inbreeding Risk Index (IRI) and discussed the possible connection with the colonies' reproductive strategies.

The problem of bumble bee decline raises the need of viable solutions to restore or enhance their wild populations. The introduction of artificial nests, described in Chapter I of this thesis, can represent a good solution for some solitary bees, but previous studies and our own experience showed that bumble bees hardly use the artificial shelters. Therefore, the rearing and releasing of colonies must be regarded as nearly the only possible solution for enhancing the population of this social species.

B. terrestris can be considered as a model organism for pollinator studies, since it is a widespread species that easily lends itself to artificial rearing. Although *B. terrestris* laboratory rearing has been carried out since many years, some aspects of the biological cycle are still hard to reproduce in captivity. The aim of this study was then to improve bumble bee artificial rearing, in order to overcome the most critical features, namely diapause and colony initiation.

5.4. References

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5.5. Lab rearing applied method – Bumble bees colony rearing and release.

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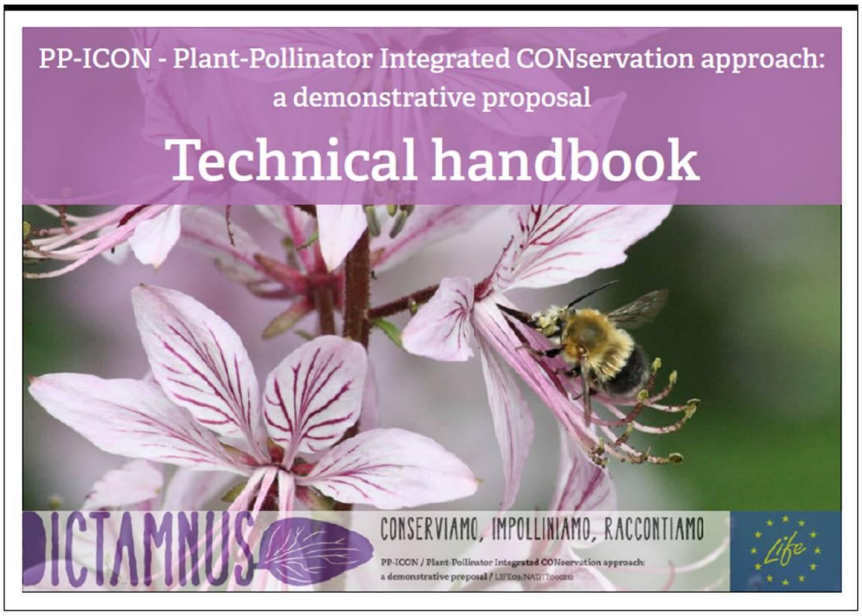

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Authors:
Gherardo Bogo (CRA-API)
Laura Bortolotti (CRA-API)
Antonio Felicioli (DSV)
Alessandro Fisogni (BiGeA)
Marta Galloni (BiGeA)
Mariateresa Guerra (FVG)
Umberto Mossetti (SMA)
Marino Quaranta (CRA-ABP)

Translations and Revision
Catherine Bolton

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4 - GHERARDO BOGO AND LAURA BORTOLOTTI

Bumblebees colony rearing and release

Artificial rearing of bumblebee colonies

Colony originated from wild queens

In addition to the creation of nesting artificial sites, previously described, another way to promote the re-colonization of an environment by bumblebees is by collecting the wild queens, rearing them in captivity and then releasing the ensuing colonies.

Rearing queens artificially makes it possible to increase the diapause survival rate and to establish a colony because it guarantees optimal development conditions such as temperature, humidity, food availability and protection from predators and parasites. The practices described here are applicable to small rearing situations, which are useful primarily for research and

study purposes; these are only a few of the possible methods that can be used.

Wild queen collection

Queens can be collected at two different times of the year: early autumn and late winter. In autumn (between early September and late October) queens can be found on late flowerings, where they forage to accumulate energy stocks to get through diapause. In the Mediterranean area they are readily found on strawberry trees (*Arbutus unedo*), rosemary (*Rosmarinus officinalis*) and peppermint (*Mentha* sp.), but it is sufficient to find a field or garden with nectariferous flowers (wild or cultivated) where the queens can feed.

In early spring (between February and March), however, queens have just emerged from diapause and are easily found on early flowerings. One of the first plants to bloom in our latitudes is loquat (*Eriobotrya japonica*), followed by the large blossoms of *Prunus* fruit trees: almonds (*P. dulcis*), cherry plums (*P. cerasifera*), blackthorns (*P. spinosa*), apricots (*P. armeniaca*), cherries (*P. avium*). In the South, citrus trees can also be considered.

Queens can be collected using an entomological net. Collected queens must immediately be transferred to a perforated container and placed in the dark in a cooled bag, to anaesthetize them slightly to avoid additional stress due to capture and transport.

Bumblebee queen collected in the wild

It is very important not to catch queens that already have full pollen baskets, because this means they already have found a colony.

After collection, the queens are placed in flight cages for 1–2 weeks, with sucrose syrup and fresh pollen (collected by bees), to allow them to absorb the right amount of energy and protein resources for diapause (if they are captured in autumn) or deposition (spring capture). The cages must be placed in a room with a natural cycle of light and dark, and be at ambient temperature (at least 18–20°C). After the flight period, depending on capture time queens will follow two different paths: diapause or colony production.

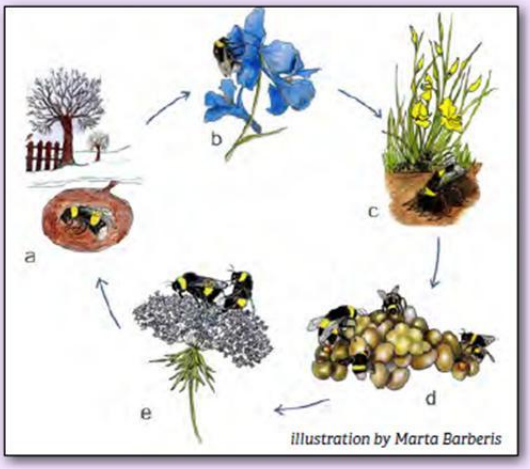
Bumblebees

Bumblebees are hymenopterans belonging to the genus Bombus *(Latreille, 1802), family Apidae. More than 250 bumblebee species have been described. Most of the species are located in the temperate regions of Europe, Asia and America, some are found in the tropics, and a few are found in South America and southern Asia. Moreover, bumblebee species can be found around the Arctic Circle and at high elevations in mountainous regions.*

Bumblebees are eusocial insects like honey bees, but they are defined as "primitively eusocial" because their life cycle shows a solitary phase. Like honey bees, they have only one reproductive individual (the queen) and many sterile workers, all sisters and daughters of the queen, who represent almost all the colony; in the summer months new queens and a certain number of males are produced and they leave the colony to mate. Finally, in late summer mated queens enter into diapause while males and the colony die. The following spring the queens who survive diapause found a new colony.

Bumblebees are haplodiploids: queens and workers are born from fertilized eggs, while males from unfertilized eggs.

Larvae develop inside wax cells that the queen and workers enlarge according to the larvae's increasing size. Bumblebee species are divided into two different categories based on the larval feeding type: pocket makers, including Bombus pascuorum*, and pollen storers, such as* B. terrestris*. In the first group the workers deposit a pollen and nectar mixture at the base of a common larval chamber from which the larvae feed themselves. In the second one pollen and nectar are stored separately in cavities made from the pupal cocoon or specially constructed wax cups*



The bumblebee colony life cycle

a) Diapausing queen
b) Queen foraging on flowers to recover from diapause
c) Queen searching for a nesting site
d) Colony foundation and development
e) Bumblebee mating

Flying cages made of wood and metal mesh, placed in front of a window to allow a natural light/dark cycle

Diapause

In nature queens overwinter deep in the ground, so the artificial diapause must imitate natural conditions. Closed containers are ideal (wood is preferable because it is natural and breathable) filled with gardening soil to maintain the proper humidity.

Containers should initially be placed in an incubator at 15°C (intermediate temperature between ambient and hibernation temperatures) for a week. The function of this period is to induce natural entry into diapause and avoid excessive temperature swings. At this stage it is better to put the food (sugar syrup and pollen) in the container. The food should then be removed from the container and the temperature decreased to 5°C for about 3 months.

When they awaken, the queens surviving diapause are placed in flight cages for about a week under natural conditions of temperature and light/dark cycle. They are fed fresh pollen and sucrose syrup, essential to recuperate energy after diapause and to develop the ovaries to lay eggs.

It is normal that a certain percentage of queens (up to 50%) will not survive diapause, but this is still lower than what would occur in natural conditions.

Colony initiation

To initiate deposition starters are needed in which the queens will be individually positioned. A starter consists of a container (plastic or cardboard) that is small (a 10x5cm base is sufficient) and has perforated sides to let in air and moisture. An absorbent paper substrate and fresh pollen are placed inside it. The substrate will absorb faeces and any excessive condensation; pollen is put in a small container alone or mixed with syrup to form a ball. Starters are closed but have a hole in order to insert a syrup-filled syringe. The syringe should be blunt to allow the queen to suck the content with its tongue.

Starters can be placed into an incubator or in an air-conditioned room. The temperature must be maintained at 28–29°C and the humidity at 60% minimum.

Cuckoo bumblebees

The species of the subgenus Psithyrus, *now included in the genus* Bombus, *are bumblebee social parasites.*
Psithyrus *do not have a worker caste but their queens lay eggs in other species of* Bombus *nests, leaving the task of raising their offspring to host workers. Generally they resemble the host bumblebees, but the hair mantle is not as dense, revealing brilliant abdominal plates. The females do not have a pollen basket and the wing membranes are dark.*
Psithyrus *females come out of hibernation after the host species, waiting until the bumblebee nest has been founded. To locate it they follow the typical smell of the host colony. When they enter a nest, they act calmly, their legs close to their body, to be accepted from the colony. They can be attacked by the host workers but are rarely killed because they have anatomical features allowing them to withstand the attack.*
Subsequently Psithyrus *females hide in the nest material until they acquire the colony's distinctive aroma and will not elicit worker hostility.*
Usually the Psithyrus *queen kills the host colony's queen, but this does not always happen; in some cases it seems that the two queens can live together in the same colony without killing each other.*
When the parasitized nest has a sufficient number of workers, the Psithyrus *female destroys the queen's host eggs and larvae, and reuses the mixture of pollen and wax of the cells to build those in which to lay her eggs. While the host workers rear the* Psithyrus *brood, the parasite female plays no other role in the colony life. New larvae will emerge as parasite adult males and females. Males look like females but are smaller.*
Psithyrus *and* Bombus *have a common ancestor and this type of parasitism may have evolved from conflicts that commonly occur among different species of bumblebee queens (or even the same species) to occupy a nesting site.*

To handle bumblebees safely, lab tweezers (15 cm long) are used, working in a room with red lights. The queens cannot perceive light with a wavelength corresponding to red, seeing it as darkness, and consequently they will not fly.

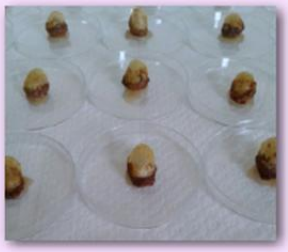
There are several techniques to stimulate queens to lay eggs and they can be used individually or combined.

1. A young pupa, preferably a male pupa, is hot and thus stimulates the queen to lay egg on it. Pupae are taken from already initiated laboratory colonies, then separated from each other, cleaned of excess wax or impurities, and fixed on a support. Pupae must be replaced once a week in case of no deposition.

2. 1–5 newly emerged workers, always taken from initiated colonies.

3. 1–5 newly emerged *Apis mellifera* workers.

4. Two queens placed in the same starter. The strongest queen becomes dominant over the other and will be the only one to lay eggs; the other can be used later to stimulate another queen.



Pupal cocoon fixed with honey bee wax to a Petri dish, ready to be put in a starter as a stimulus for egg laying

If methods 2, 3 and 4 are adopted, a ball of pollen mixed with syrup should be positioned in the container as substrate for the deposition.

Typically, after a month queens that have not laid eggs are discarded. Once the queens lay eggs, they are monitored and once a week the food must be changed and the container must be cleaned. Over time the colony develops and when the first workers emerge it is best to transfer the colony to a larger box (capacity of 6–7 litres) that has been perforated to permit air circulation.

The colonies are given sugar syrup, composed of mineral water, uncontaminated by chlorine and carbonates, and commercial sucrose in a 1:1 ratio. The protein nourishment consists of fresh pollen collected by bees, and then frozen or dried pollen. The first type of pollen is generally given to queens and colonies under development (higher quality); dried pollen is distributed to the colonies that have already started.

Colonies of pocket-maker species

Rearing colonies of pocket-maker species is more difficult and time-consuming. As described above, these species deposit small food masses into pockets located below the common larval chamber and the larvae feed themselves. The stages of farming remain the same as described above, but the pollen administration mode changes. Pollen must be placed in the pockets at least once a day until the first workers emerge; then they accept the pollen and put it into the pockets themselves.

Possible options are to add some newly emerged B. terrestris workers to the brood, which will act as nurses to feed the larvae or allow

Queen of B. terrestris *with a pupa (above) and two queens of* B. pascuorum *within the same starter (below)*

Bumblebee colony at the first stage of development: the queen and the workers of the first brood, pupae and, on them, some egg cups

Queen of B. pascuorum*; the pocket for larval feeding is visible under the pupa*

the queen to go foraging, although this increases the chances of founder predation and death.

Colony release

When releasing colonies in the wild, a certain size is advisable, with at least a dozen workers so they effectively can defend themselves from predators and keep the brood temperature constant. Colony release is carried out in spring, possibly on a sunny day. It is best to place them in an elevated position to impede access by predators and parasites. If they are placed on a pole base, ant glue can also be applied. Before they are released, it is preferable to cover the brood with a layer of cotton wool as insulation.

Colony status can be monitored by observing the adult flight in and out of the nest, or by placing a transparent cover over it to make it possible to observe inside the colony. At the end of the season, nests can be recovered and analysed to look for possible parasites or other clues on colony development to find optimal solutions for the following year.

Bombus terrestris *colony released in the wild*

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5.6. Lack of partner preference system for incest avoidance in the bumble bee *Bombus terrestris*

Manuscript

SUMMARY

Inbreeding is caused by the mating of closely related individuals and produces a decrease in the offspring fitness and deleterious consequences for adults. In haplodiploid social Hymenoptera inbreeding has a further negative effect because of their particular mechanism of sex determination (sl-CSD), that causes the emergence, in half of the founded colonies, of unviable or sterile diploid males. When these males are able to develop until adult stage, as in bumble bees, they represent a huge cost for the colony. With respect to these high inbreeding consequences, a selection for mechanisms of inbreeding avoidance would be expected in bumble bees. Social recognition is one of the most common and efficient system to avoid inbred mating in social insect, but it is poorly studied in bumble bees. In this study we investigated the mating choice between siblings or non-siblings in queens and males of *Bombus terrestris* reared in laboratory. To investigate the role of mating behaviour in mating choice, the tests were performed both in cage and in tunnel. Contrary to what would be expected, we found that *B. terrestris* males and gynes do not show a mating preference for non-siblings compared to siblings (49.3% in non-siblings and 50.7% in siblings) and the mating latency was even shorter for sibling matings than for non-siblings ones.

Key words: *Bombus terrestris* / mating / inbreeding / incest avoidance / kin recognition

INTRODUCTION

In animal populations inbreeding is caused by the mating of closely related individuals and triggers the phenomenon of inbreeding depression, characterized by an increase of homozygosity and the consequent expression of deleterious recessive alleles (Charlesworth & Charlesworth, 1987; Barret & Charlesworth, 1991; Lacy, 1993;

Charlesworth & Willis, 2009). In haplodipoid hymenopterans, inbreeding has further negative consequences due by their particular mechanism of sex determination, which follows the single-locus complementary sex determination system (sl-CSD) (Van Wilgenburg et al., 2006). In social species, as a result of inbred mating by the queen, in half of the founded colonies the 50% of fertilized eggs are homozygous at the sex-determining locus and develop into unviable or sterile diploid males, which represent a cost for the colony itself (Ross & Fletcher, 1986; Liebert et al., 2004; Whitehorn et al., 2009a).

Studies on bumble bee wild populations show that many *Bombus* species suffer from the consequence of inbreeding, mostly due to geographic isolation and small size populations (Darvill et al., 2006; Ellis et al., 2006; Whitehorn et al., 2011; Darvill et al., 2012). In colonies of *B. terrestris* reared in laboratory, after some generations of inbreeding, a decline in the number of worker and male was observed (Beekman et al., 1999). The wide occurrence of inbreeding in bumble bee populations and its high associated costs would suggest the existence of inbreeding avoidance mechanisms in these species.

Dispersal of individuals from their natal group, polyandry and social recognition are among the most common and efficient systems to avoid inbreeding in social insect (Pusey & Wolf, 1996; Tabadkani et al., 2012), but few of them are clearly represented in bumble bees. Recent studies demonstrate in queens of *B. pascuorum* and *B. lapidarius* a dispersal distance of few kilometers (Lepais et al., 2010), while bumble bee males of show higher dispersal ability (Kraus et al., 2009; Wolf et al., 2012). Moreover, only few bumble bee species show polyandry, like *B. hypnorum*, *B. bifarius*, *B. californicus*, *B. frigidus*, *B. huntii* and *B. rufocinctus*, but most of them are monandrous (Estoup et al., 1995; Schmid-Hempel & Schimd-Hempel, 2000; Brown et al., 2002).

Therefore in many bumble bee species the ability to discriminate among conspecifics, with a selection against mating with close relatives, could represent the ultimate strategy to avoid inbred mating. The mechanism of incest avoidance through kin recognition has been described in several social hymenoptera, such as *Apis mellifera* (Getz & Smith, 1986), the Halictine bees *Lasioglossum zephyrum* and *L. malachurum* (Buckle & Greenberg, 1981; Smith & Ayasse, 1987), the Polistine wasps *Polistes fuscatus* and *P. dominulus* (Gamboa et al., 1986; Liebert et al., 2010), and in several species of ants,

among which *Solenopsis invicta* (Keller & Ross, 1998) and *Iridomyrmex humilis* (Keller & Passera, 1993).

Kin recognition for inbreeding avoidance has been studied in few bumble bee species. Foster (1992) found that two species (*B. frigidus* and *B. bifarius*) are able of nestmates recognition and mating avoidance, while two other species are not (*B. californicus* and *B. rufocinctus*). Whitehorn et al. (2009b) observed in gynes and males of *Bombus terrestris* a longer mating latency between siblings compared to non-siblings, and assumed this as an evidence of kin recognition for inbreeding avoidance. However their results do not exclude the possibility of inbred mating in *B. terrestris*.

Mate recognition in bumble bees pass through both behavioral and chemical features (Baer, 2003; Ayasse & Jarau, 2014). Bumble bee males of different species can display four kind of pre-copulatory sexual behaviors to find and attract females: perching, territoriality, nest surveillance, scent-marking and patrolling (Brown & Baer, 2005; Goulson, 2010). In the first males rest on a perch and wait for gynes, which are visually located; in the second they stakes out territory and when a gyne passes they try to grasp her; in the third males guard the entrance of conspecific nests and try to mate with emerging gynes; the fourth is far the most common mating location mechanism in bumble bee males and consists in patrolling along paths where they scent-mark objects (leaves, trunks, fence, rocks, etc) with the secretion of cephalic labial glands that attract conspecific virgin females (Ayasse & Jarau, 2014). *B. terrestris* is an annual eusocial bumble bee species, one of the most widespread and abundant species in the West Palaeartic region (Rasmont et al., 2008). Queens of this species are monandrous (Schmid-Hempel & Schmid-Hempel, 2000) and males show a pre-copulatory patrolling behaviour strategy. When mating occurs in flight cage, however, the above-mentioned behavior has little or no effect on mating success (Djegham et al., 1994; Sauter & Brown, 2001). Previous studies showed that mating success in confinement can be influenced by several factors, like temperature, photoperiod, adult age and size, and male dimension (Tasei et al., 1998; Kwon et al., 2006; Amin et al., 2007; Amin et al., 2010; Amin et al., 2012).

Copulation duration in bumble bee varies among species and across males within species, but in general it is longer than in other social bees and this can be interpreted as

an additional system with which males ensure their own paternity over the offspring (Brown & Baer, 2005).

In this study we compared mating occurrence, mating latency and copulation duration between sibling and non-sibling bumble bee gynes and males, with the aim to investigate the possible presence of a mating preference towards non-siblings as a mechanism to avoid incest.

METHODS

Study species and rearing conditions

We obtained 2215 gynes and 3550 males of *B. terrestris* from 30 second-generation laboratory colonies, reared from commercial ones (Bioplanet S.c.a., Cesena, Italia). Colonies were maintained at 25 ± 1°C and 60 ± 10% relative humidity (RH), in continuous darkness, and fed ad libitum with fresh frozen pollen and sugar syrup. We daily removed newly emerged gynes and males and we kept them in separate plastic boxes of 25x15x14 cm (up to 20 in the same box), according to their gender, date of emergence and colony of origin. Gynes and males were kept in the same climatic room of the colonies, fed with ad libitum fresh frozen pollen and sucrose syrup until they were used for the mating experiment or discarded.

Mating test design

For mating tests we used gynes from 1 to 10 days old and males from 5 to 25 days old, being these the respective ages at which they meet sexual maturity and give the best mating results (Tasei et al., 1998; Amin et al., 2012). We used a total of 517 gynes and 1115 males. When possible, we selected medium or large size gynes and males and in any case we avoided to use very small males, since in *B. terrestris* the size of males has a positive impact on the mating success (Amin et al., 2012).

Bumble bees were mated in groups with a gynes/males ratio of 1:2, which is the one assuring the higher mating propensity (Kwon et al., 2006; Amin et al., 2010). The total number of individuals inside the groups ranged between 12 and 62, according to the number of gynes and males of the proper age interval present at the same time in the same colonies.

We conducted two different mating tests: in the first ("type 1") gynes belong to only one colony; in the other ("type 2") gynes belong to two different colonies. In both cases gynes could choose between twice as many males belonging to their own colony and to a different one, as shown in Table 1. Gynes and males were marked on the thorax with a color tag according to the colony of origin.

Table 1. Number of gynes and males used in the two types of mating. The subscript numbers (1 and 2) indicate two different colonies.

Mating type	Gynes	Sibling males	Non-sibling males
1	n	n	n
2	n_1	n_1	n_2
	n_2	n_2	n_1

Mating tests were performed in two different environmental conditions: i) inside a wooden flying cage (40x40x75 cm) with mash walls; ii) inside a net tunnel (4x2x2 m). In both cases we furnished fresh frozen pollen and sucrose syrup for all test duration. Cage tests were carried out in a climate room, maintained at 20 ± 1°C and 55 ± 5% RH (following Amin et al. 2010) and in natural daylight. Tunnel tests were conducted outdoor, at a mean temperature between 26-29°C and RH of 50-70%.

We performed a total of 47 mating sessions, among which 28 of type 1 (17 in cage and 11 in tunnel) and 19 of type 2 (10 in cage and 9 in tunnel).

Response measures

Mating sessions started when gynes and males were released in group in the cage or tunnel, and they were terminated after one hour, irrespective of the mating success. For

all test duration we constantly observed individuals and we recorded the precise moment of copulation beginning, in order to calculate mating latency (namely the time elapsed between the starting of mating session and the starting of copulation). Each mating pair was removed from cage or tunnel and transferred to an individual transparent plastic box (12x9x9 cm), where it was constantly observed to record the moment of copulation ending, in order to calculate copulation duration. For each couple we recorded the colony of origin of gynes and males and the kind of occurred mating, sibling (S) or non-sibling (N).
For each mating session we calculated the mating rate as the number of mating pairs on the number of gynes which entered the session.

Data Analysis

Data were firstly tested for normality (Shapiro-Wilk test). Differences in mating rates and partner choices between siblings and non-siblings were analysed with Mann-Whitney *U*-test. Timing of copulation latency and duration were analysed by *t* test for independent samples. The influence of the maternal colony of gynes and males in mating success and in partner choice was tested by Kruskal-Wallis H test. All statistical analysis were performed with STATISTICA software (StatSoft Italia srl, 2005). Data are showed as mean ± SE.

RESULTS

Mating rates and partner preference

The percentage mating rates for the two types of mating (1 or 2) and the two environments (cage or tunnel) are shown in Figure 1. We obtained a total mean mating rate of 82.2 ± 0.02 % (n = 412), ranging from 73.1 ± 4.33 % (n = 95) in tunnel with mating type 2 to 86.6 ± 3.25 % (n = 105) in cage with mating type 2. Mann-Whitney *U*-test showed no difference in mating rates between mating types (U = 209.5, P = 0.221),

while showed significant difference in mating environment (U = 170.5, P = 0.033) with a lower mating rate in tunnel (Fig. 1).

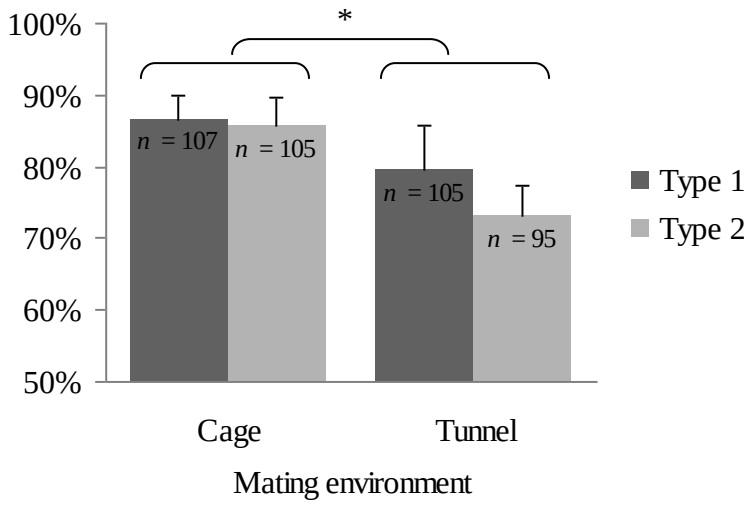

Figure 1. Mean percentage mating rates in the two mating types and environments (cage or tunnel); *n* = number of mating pairs. * = Significant differences according to Mann-Whitney U-test

We analyzed the partner preference by comparing mean mating rates between siblings or non- siblings in both mating types and environments (Figure 2). Mann-Whitney *U*-test showed no statistical differences between siblings or non-siblings mating rates, in none of the tested mating conditions nor in the total mating pairs.

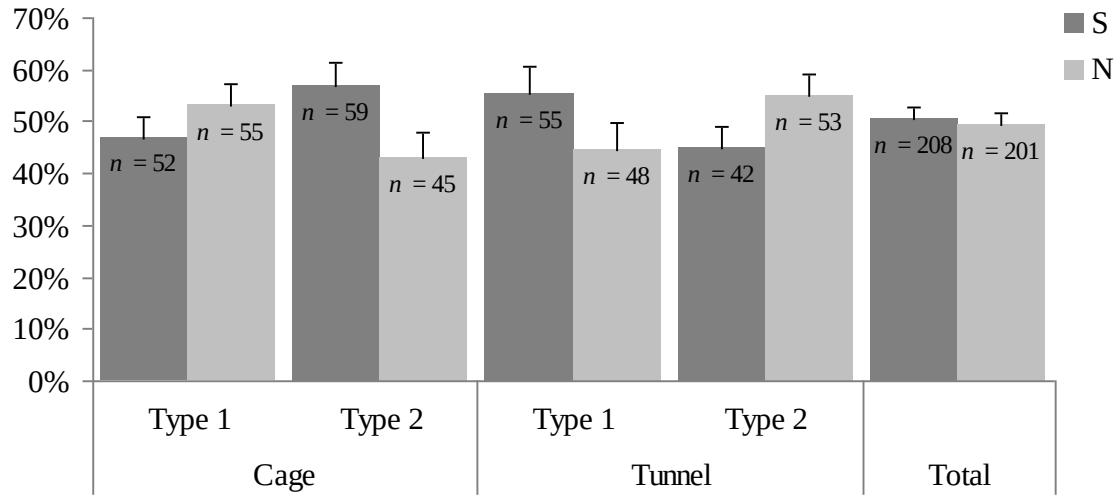

Figure 2 Difference in mean percentage mating rates between siblings (S) and non-siblings (N) according to mating type and environment; *n* = number of mating pairs.

Copulation timing

Considering all the mating sessions together, irrespective of the mating type, the mating environment and the preference (S or N), the mean value of mating latency was 7.84 ± 0.49 minutes (n = 409) and the mean duration of copulation was 26.67 ± 0.43 minutes (n = 403). The comparison of mating latency and copulation duration in the two different environments showed that in cage condition mating started faster (t_{407} = -12.52, P < 0.001) and copulation lasted longer (t_{401} = 13.72, P <0.001) than in tunnel condition (Fig. 3).

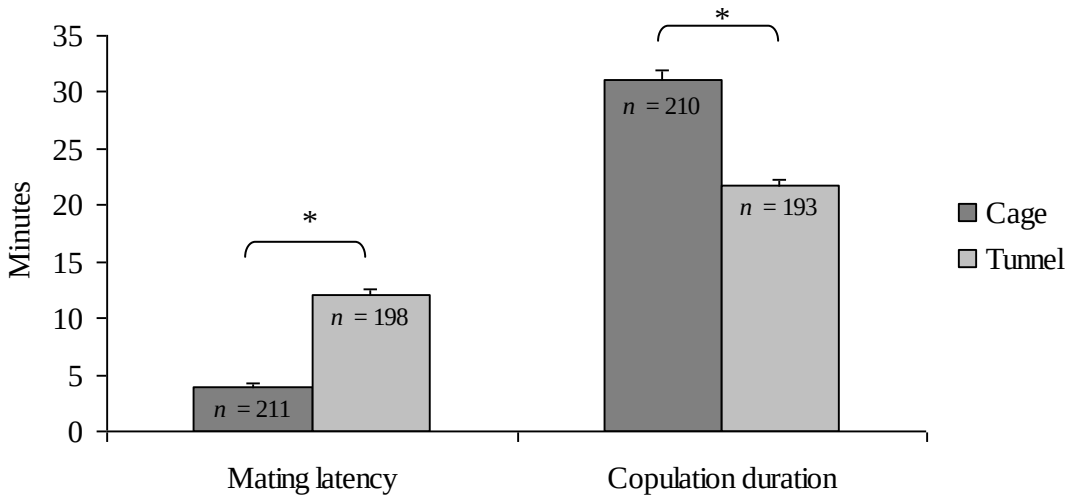

Figure 3. Comparison of mating latency and copulation duration between the two mating environments (mean ± s.e.); n = number of mating pairs. * = Significant differences according to Mann-Whitney *U*-test.

We analyzed the differences in mating latency and copulation duration between S and N matings in the two different mating types and environment by *t* test for independent samples (Table 2). The only statistically significant difference was in the mating latency, which was shorter in sibling matings (10.57 ± 1.10) than in non-sibling ones (13.57 ± 1.19, t_{196} = 2.19, P = 0.030) both in tunnel condition and in the total number of matings (6.97 ± 0.64 and,8.74 ± 0.75 respectively; t_{407} = 2.20, P = 0.029).

Table 2. Mating latency and copulation duration in S and N matings in the two mating environments (mean ± s.e.); n = number of mating. Significant outcomes are shown in bold.

Duration	Space	S	N	*t*	P
Mating latency (min)	Cage	3.82 ± 0.59 (n = 111)	3.85 ± 0.58 (n = 100)	0.85	0.395
	Tunnel	**10.57 ± 1.10 (n = 97)**	**13.57 ± 1.19 (n =101)**	**2.19**	**0.030**
	TOT	**6.97 ± 0.64 (n = 208)**	**8.74 ± 0.75 (n =201)**	**2.20**	**0.029**
Copulation duration (min)	Cage	30.96 ± 0.71 (n = 111)	31.35 ± 0.75 (n = 99)	0.39	0.696
	Tunnel	21.17 ± 0.79 (n = 95)	22.40 ± 0.65 (n = 98)	1.53	0.127
	TOT	26.45 ± 0.63 (n = 206)	26.90 ± 0.59 (n = 197)	0.78	0.439

Effect of the maternal colony

We used Kruskal-Wallis H test to analyze the influence of the maternal colony of gynes and males on mating success and partner choice between siblings and non-siblings. The maternal colony of gynes had no influence neither in the mating success (H_{22} = 32.20, P = 0.074) nor in the partner choice (H_{22} = 32.17, P = 0.075), while the maternal colony of males influenced the mating success (H_{24} = 39.86, P = 0.022) but it had no effect on the partner choice (H_{18} = 20.91, P = 0.284).

DISCUSSION

Partner preference and incest avoidance

The main outcome of this study is the absence in gynes and males of *B. terrestris* of a mating preference between siblings and non-siblings. A previous research on the same species inferred the existence of a mating preference for non-sibling mating, basing on a

shorter mating latency, interpreted as a higher mating propensity, compared to sibling matings (Whitehorn et al., 2009b). However a double choice mating tests was never attempted in this species. Our study is the first one where *B. terrestris* sexuals could choose between siblings and non-siblings for mating and it gives a clear evidence that gynes and males of this species do not use a kin recognition system to avoid incest.

In a similar mating-choice test, Foster (1992) found that gynes and males of *B. frigidus* and *B. bifarius* seems to recognise nestmates of the opposite sex and reduce inbreeding rate (although sibling matings are not totally avoided) while *B. californicus* and *B. rufocintus* did not show this inbreeding avoiding system. The author hypothesize that this variability among species could be linked to the different pre-copulatory behaviour: *B. californicus* and *B. rufocintus* display a nest surveillance behaviour to find female, but since they do not survey their own nest they do not needs nestmate recognition cues; *B. frigidus* and *B. bifarius*, which use patrolling behaviour can frequently encounters their sibling gynes and therefore they need an incest avoiding system.

Following this hypothesis *B. terrestris*, which is a patrolling species, should have a similar nestmate avoidance system, but this is not consistent with our results. Male marking pheromones have been demonstrated to be specie- and subspecie-specific (Rasmont et al., 2005; Coppée et al., 2008), but there are no evidences of its nestmate specificity. On the other hand, if nestmate recognition for incest avoidance would be achieved by male pheromones, our study should have proved. Although cage conditions could have prevented scent discrimination among siblings and non-siblings, due the tight confinement of individuals, in tunnel condition space was large enough to allow the following of chemical cues, and a certain degree of preference should have emerged. Tunnel test was specifically designed to obtain mating results in a more natural condition, where males could display a pre-copulatory behaviour, like patrolling and establishing scent marks, which is not possible in flight cages (Djegham et al., 1994). Instead in our study neither in cage nor in tunnel nestmate recognition system seemed to be used to avoid incest.

In *B. terrestris* nestmate recognition system mediated by cuticular hydrocarbons is used to mark nest entrance and signal nest identity (Rottler et al., 2013), to prevent nest invasion by social parasites (Martin et al., 2010; Blacher et al., 2013), and to reduce the phenomenon of drifting workers (Zanette et al., 2014). No studies have been performed

in bumble bees to detect their possible role in incest avoidance, which has been demonstrated in other insect species (Lihoreau et al., 2007; Lihoreau & Rivault, 2010) and even in some primitively eusocial bees (Smith & Ayasse, 1987). Also closely pre-copulatory behaviours, like male courtship and female rejection seems to have little or no effect on copulation occurrence (Sauter & Brown, 2001), excluding that they convey crucial exchange of information between the sexes which can influence mating success.
In our study we did not directly evaluate the impact of other parameters as adult age and body size on mating success and mating preference, but we observed an influence of maternal colony of males, which in turns can have an influence on adult male characteristics, on mating success, as observed also by other authors (Amin et al., 2012). Contrarily it seems that adult characteristics were not decisive in mating preference, since neither the maternal colony of gynes nor that of males influenced the choice among siblings and non-siblings.

Copulation timing

The duration of copulation can be a system used by insect to adjust their mating investment in relation to their relatedness (Tabadkani et al., 2012). Shorter copulation with siblings than to non-siblings would provide fewer sperm to most closely related partners. In bumble bees longer copulation duration is used by males to manipulate paternity in their own interests, since it promotes effective sperm transfer and allow the application of the so-called "mating plug", a secretion produced from male accessory glands, which is applied to female genital tract to prevent gynes from re-mating (Baer et al., 2000, 2001; Brown & Baer, 2005).
Our mean values for copulation duration in *B. terrestris* are consistent with previous reports (Duvoisin et al., 1999) and no differences are found between sibling and non-sibling mating, indicating the lack of differential investment by males according to gyne relatedness.
The shorter copulation durations observed in our tunnel study may be due to the climatic outdoor conditions, with a temperature up to 29°C, which is known to have a negative effect on mating success (Amin et al., 2010) and could have shortened the

duration of copulation compared to cage conditions, where the temperature was about 20°C. Also mating success was lower in tunnel than in cage, although not significantly, perhaps for the same reason.

Mating latency, calculated as the time elapsed between the release of gynes and males in cage or tunnel and the beginning of copulation, shows a difference between sibling and non-sibling mating, but in opposite way to what expected. Whitehorn et al. (2009b) observed a longer latency in sibling than in non-siblings mating and interpreted this result with the existence in *B. terrestris* of a kin recognition system to avoid incest. In our study the latency values are similar to those of Whitehorn et al. (2009b), but a longer latency was observed in non-sibling versus siblings, and this should lead us to conclude that there is a selection for sibling matings. The most likely explanation is that mating latency is not correlated to mating preference, but can be influenced by other factors. As an example, we observed a shorter latency in cage matings than in tunnel matings, probably because in tighter spaces male are advantaged in finding gynes.

Why not avoid incest?

From our results it can be concluded that *B. terrestris* is a species that do not avoid sibling matings, like *B. californicus* and *B. rufocinctus* (Foster, 1992). However these two species show a nest surveillance per-copulatory behaviour which minimize nestmate encounters and *B. californicus* has a polyandric strategy that further reduce inbreeding risk. *B. terrestris* instead is monoandrous.and display a patrolling pre-copulatory behaviour which does not exclude sibling matings.

Since inbreeding implies serious fitness consequences in bumble bee colonies, we would expect the existence of other mechanisms to avoid mating with close relatives reproductive, such as adult adult dispersal. Queen dispersal has not been studied in *B. terrestris*, although an indirect indication comes from its alien colonization of Tasmania, which was estimated in 300 km in about 10 generations, indicating a high dispersal capacity (Schmid-Hempel et al., 2007). However, the particular context of this expansion is perhaps not representative of the dispersal process in the native range of the species. Recent studies on *B. terrestris* male dispersal indicate that they could carry

out large flight ranges, which can represent an effective strategy for increasing population size and reduce the risk of sibling mating (Kraus et al., 2009; Wolf et al., 2012). On the other hand, in Italy and other European countries, *B. terrestris* is the most abundant and widespread species (Rasmont et al., 2008) and this high concentration, acting as dilution factor in decreasing the possibility of related individuals mating, may be another key factor in incest avoidance.

Finally, the lack of an incest avoiding system in *B. terrestris* could be not interpreted as an evolutionary gap, since inbreeding could have positive effects on parent's fitness by increasing the representation of parental genes in future generations (Kokko & Ots, 2006). In addition, retaining the possibility of sibling mating could represent a protection measure in case of transitory isolation of the population.

Evidences suggest that *B. terrestris* can tolerate high levels of inbreeding, since they were able to spread as an invasive specie originating from few adult individuals (Schmid-Hempel et al., 2007). Moreover in *B. terrestris* inbreeding seems to not affect the immune response of adults (Gerloff et al., 2003) and some inbred colonies grow at similar rate or even performed better than outbred ones, despite the production of diploid males (Duchateau, 1994; Gerloff & Shmid-Hempel, 2005). The possibility to perform indiscriminately inbred and outbred mating, exploiting the advantages of both strategies, can represent one of the key factor for the ecological success of this species.

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5.7. Inbreeding risk in the reproductive strategies of the bumble bee *Bombus terrestris*

Manuscript

SUMMARY

In social Hymenoptera inbreeding can be either a risk or a benefit, depending on the social structure and the environmental conditions. Generally it causes a decrease in the offspring fitness and produces deleterious consequences for adults. In bumble bees and other Hymenoptera species, it has an additional consequence due to their particular mechanism of sex determination (sl-CSD), which brings the production of unviable or sterile diploid males. Nevertheless inbreeding can represents an advantage for the parents, since it favours the transmission of identical genes.

In many insects inbreeding avoidance mechanisms are displayed to prevent matings between close relatives; however these mechanism are poorly represented in bumble bees. The probability of inbred matings within a colony in eusocial insects may depend on the timing of sexuals' emergence and on their sex ratio. In this study, we compared the timing of gynes and males production of 36 colonies of *Bombus terrestris*, by following their development from founding to the emergence of the last gyne. We investigated the probability of siblings mating, due to the simultaneous presence in the same colony of fertile newborn gynes and males, through the calculation of a colony Inbreeding Risk Index (IRI), which considers the fertility overlap of gynes and males and the colony sex ratio in the overlapping period. We were able to divide the colonies in two groups based on the mean value of IRI (above or below 0.581). Colonies with a low IRI show a gyne biased sex ratio and can be assimilated to late switchers according to Duchateau and Velthuis (1998); colonies with high IRI have a male biased sex ratio and can be assimilated to early switchers. The IRI value is strictly correlated with the period of gyne production and with the time elapsed between switch point and gyne point. We concluded that bumble bee colonies display two different reproductive strategies, in term of timing of gynes and males production and sex ratio, which correspond to a different inbreeding risk. Colonies with low IRI have a lower risk to suffer for the consequences of sibling mating, but colonies with a high IRI may be

advantaged in conditions of lack of resources or in situation of isolation, with lack of non-related reproducers.

INTRODUCTION

Inbreeding in animals is defined as the mating of closely related individuals, such as brother-sister or cousins. Inbred mating may affects the fitness of offspring by increasing homozygosity and thus causing the expression of deleterious recessive alleles; this phenomenon is known as "inbreeding depression" (Charlesworth & Charlesworth, 1987; Lacy, 1993; Charlesworth & Willis, 2009).

In haplodiploid hymenopterans with single-locus complementary sex determination system (sl-CSD), such as ants, bees, sawflies and wasps, the costs of inbreeding can be even higher, because of their particular mechanism of sex determination (Van Wilgenburg et al., 2006). In these species inbred matings bring the production of unviable or sterile diploid males, originated by fertilized eggs homozygous at the sex-determining locus. When diploid males are able to develop until adult stage, they would represent half of the diploid offspring of the colony, as it happens in bumble bees (Duchateau et al., 1994; Ayabe et al., 2004). In some cases inbreeding can be an advantage for the parents, since it favours the transmission of their own identical genes in future generations (Kokko & Ots, 2006). Therefore inbreeding avoidance, tolerance or preference in a population is often the result of the balance between benefits and costs (Tabadkani et al., 2012).

In eusocial insects, where males and gynes of the same genetic lineage share a common nest, the probability of inbred mating can be very high, unless inbreeding avoidance strategies are present in the population. Polyandry, drone dispersal and nestmate recognition are among the main mechanisms to avoid inbred mating (Goulson, 2010), but they are largely ineffective in bumble bees. Few bumble bee species are polyandrous (Schmid-Hempel & Schimd-Hempel, 2000; Baer, 2003), but most of them are monandrous, and the studies on mechanism of kin recognition in mating choice gave contrasting results (Foster, 1992; Withehorn et al., 2009; Bogo et al., in preparation). The reproductive strategies of the colonies, in terms of timing of gynes and males

production and sexuals' sex ratio, can be another feature affecting the inbreeding degree of a population.

Bombus terrestris is a very common bumble bee species in the West Palaeartic region (Rasmont et al., 2008). It has an annual life cycle and an eusocial organisation in which mated queens survived to winter in a state of diapause until next early spring, when they emerge and found new colonies. In late summer colonies produce gynes and males (Alford, 1975). The moment when the founder queen starts to lay haploid eggs, from which males emerge, is called "switch point". The last group of diploid eggs laid before or soon after the switch point usually develop into gynes. Duchateau and Velthuis (1988) classified colonies in two groups with respect to the timing of the switch point: "early switchers", when the switch point occurs early in the colony cycle (6-13 days after colony initiation), and "late switchers" when it occurs late (18-32 days after colony initiation).

According to these authors, the two kind of colonies differ both in the timing and in quantity and quality of produced offspring: late switching colonies produce twice as much workers, five times more gynes and half of the males than early switching ones. Thus, early and late switch could correspond to differential reproductive strategies of the colonies, the first male biased and the second queen biased (Beekman & van Stratum, 1998; Duchateau et al., 2004). In this study, we recorded the production of males and gynes in several colonies, to determine the probability of siblings mating due to the simultaneous presence of fertile gynes and males originated by the same colony. We determined the risk of inbreeding through the calculation of an Inbreeding Risk Index (IRI) and we investigated the relationship between the colonies reproductive strategy and their IRI values.

METHODS

Study Species and Rearing Conditions

We conducted the study on 36 second-generation colonies of *B. terrestris*, reared from commercial ones (Bioplanet S.c.a., Cesena, Italy). The whole rearing procedure is

describe in Bogo et al. (submitted). Obtained colonies were maintained in laboratory inside plastic box (25 x 15 x 14 cm), at 25 ± 1°C and 60 ± 10% relative humidity (RH) in continuous darkness, and fed ad libitum with fresh frozen pollen and sugar syrup.

Gynes and Males Emergence and Fertility Periods

From the 36 reared colonies we daily collected newly emerged sexuals, recording for each day their number date of emergence. Collection proceeded at least until the emergence of the last gyne, allowing us to precisely calculate the total number of produced gynes and the period (in days) of gyne production for each maternal colony. On the contrary the number of males registered for each colony was an underestimation of the total produced number since male production continues for several days after the emergence of the last gyne.

We defined the fertility period of gynes and males as the period when they show the highest mating success according to Tasei et al. (1998): 1 to 10 days after emergence for gynes and 5 to 25 days after emergence for males. Then for each colony, we define the "overlapping period" as the time interval (in days) when fertile gynes and males are simultaneously present, and we calculated the number of "overlapped gynes" and "overlapped males" as the number of gynes and males which were fertile in the same time interval.

Calculation of Inbreeding Risk Index

To estimate the risk of sibling mating in each colony, we took into consideration the portion of gynes which are fertile together with their brother, and we calculated an Inbreeding Risk Index (IRI) using the following formula:

$$IRI = \frac{n\ of\ \ overlapped\ \ gynes}{n\ of\ \ produced\ gynes} * \overline{P}_{colony}$$

where $\overline{P}_{colony}$ represents the mean probability that gynes of a colony would mate with their brother during the whole overlapping period. $\overline{P}_{colony}$ is the mean value of all P_{day} values calculated during the overlapping period by the following formula:

$$P_{day} = \frac{n\ of\ \ overlapped\ \ males * 8}{n\ of\ \ overlapped\ \ gynes}$$

where *8* is the maximum number of copulations observed in males of *B. terrestris* (Tasei et al.,1998).

Thus $\overline{P}_{colony}$ is a correction factor that considers the proportion of gynes which can mate with their brothers. When $P > 1$ it is always assumed $P = 1$, and represent the cases in which all the overlapped gynes can copulate with their brothers.

Colony Developmental Data

For each maternal colony we calculated the life span of founder queens from the deposition of the first egg cell until its death, and the timing of the "gyne point" (GP) and "switch point" (SP), namely the day of deposition of the first gyne egg and first male egg respectively. This dates were estimated by subtracting respectively 30 and 26 days (mean duration of preimaginal gyne and male development) from the dates of first gyne and male emergence (Duchateau & Velthuis, 1988).

We calculated the "pre-SP period" and "pre-GP period" as the days elapsed between the deposition of the first egg cell and the SP and GP respectively, and the "SP-GP gap" as the days between the switch point and the gyne point.

Statistical Analysis

Quantitative data were firstly tested for normality (Shapiro-Wilk test) and log transformed when not normally distributed, then analysed by *t* test for independent samples.

To determine which factors best predicted the colony development and the risk of inbreeding, we carried out a correlation analysis. Subsequently we used principal

components analysis (PCA) to explore similarities in colonies' development data according to their IRI, and *t* test analysis to investigate these similarities. All tests are two tailed and the level of statistical significance is $\alpha = 0.05$. Statistical analysis was performed with STATISTICA software (StatSoft Italia srl, 2005).

RESULTS

The mean number of gynes produced by our colonies was 79.6 ± 9.65 (min 2, max 254) and the mean number of overlapped gynes was 62.4 ± 8.78. The IRI values calculated for all colonies varied between 0.117 and 1 and its mean ± SE value was 0.581 ± 0.047 (N = 36). IRI showed a significant negative correlation with both pre-SP period and SP-GP gap (r = -0.633, N = 21, P = 0.002 and r = -0.823, N = 36, P < 0.001, respectively) (Fig. 1).

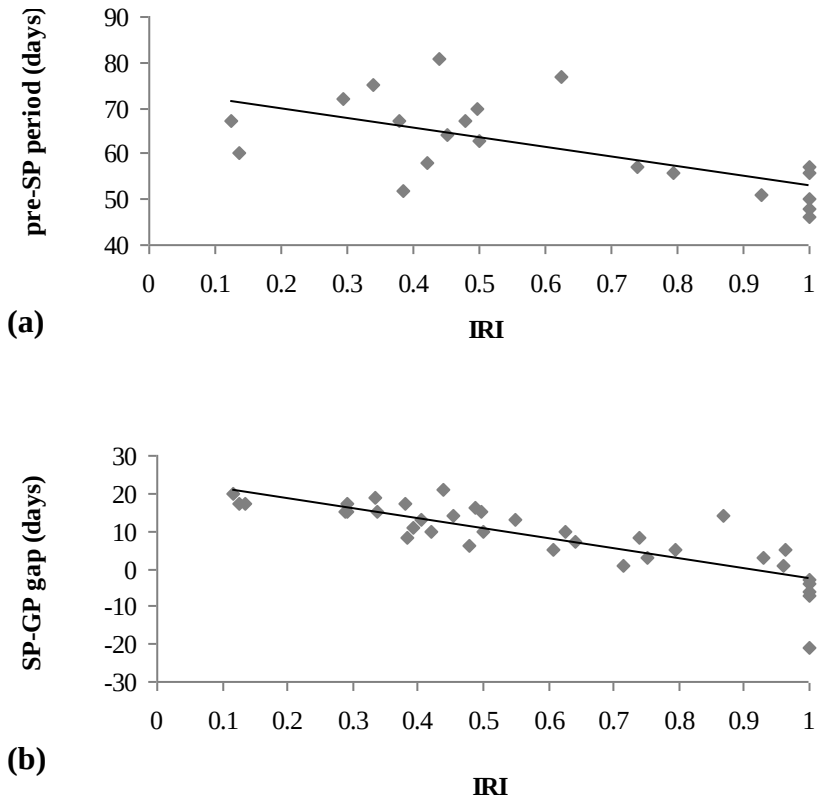

Figure 1. Relationship between IRI and (a) pre-SP period (r = -0.633, N = 21, P = 0.002) and (b) SP-GP gap (r = -0.823, N = 36, P < 0.001)

Basing on their IRI value we were able to divide our colonies into two groups: colonies with IRI < 0.58 and colonies with IRI > 0.58, where 0.58 is the mean IRI value of all colonies. We obtained 20 colonies in the first group (mean IRI ± SE = 0.365 ± 0.028) and 16 colonies in the second one (mean IRI ± SE = 0.850 ± 0.038).

The PCA showed a separation between the two groups (Fig. 2) and it extracted two factors from the colonies' developmental variables, which were positively correlated with the gyne production period and the SP-GP gap, respectively (Table 1). The *t* test analysis revealed significant differences between the two groups in both the PCA factors (factor 1 gyne production period: $t35 = 2.990$, $P = 0.005$; factor 2 SP-GP gap: $t35 = 4.411$, $P < 0.001$).

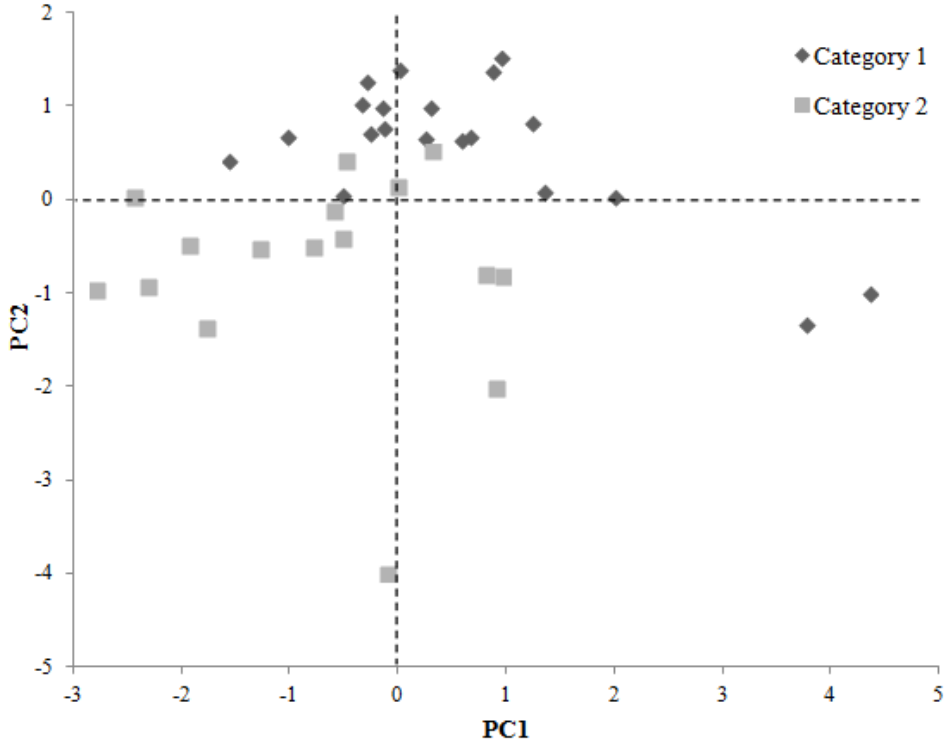

Figure 2. Principal components analysis of colonies' developmental variables.

Table 1. Correlations between PCA factors and colonies' developmental variables and principal components analysis values.

Colonies' developmental variables	PC1	PC2
Gyne production period	0.9574	-0.0459
SP-GP gap	0.4347	0.8581
Eigenvalue	2.2525	1.2558
Variance explained (%)	56.3	31.4

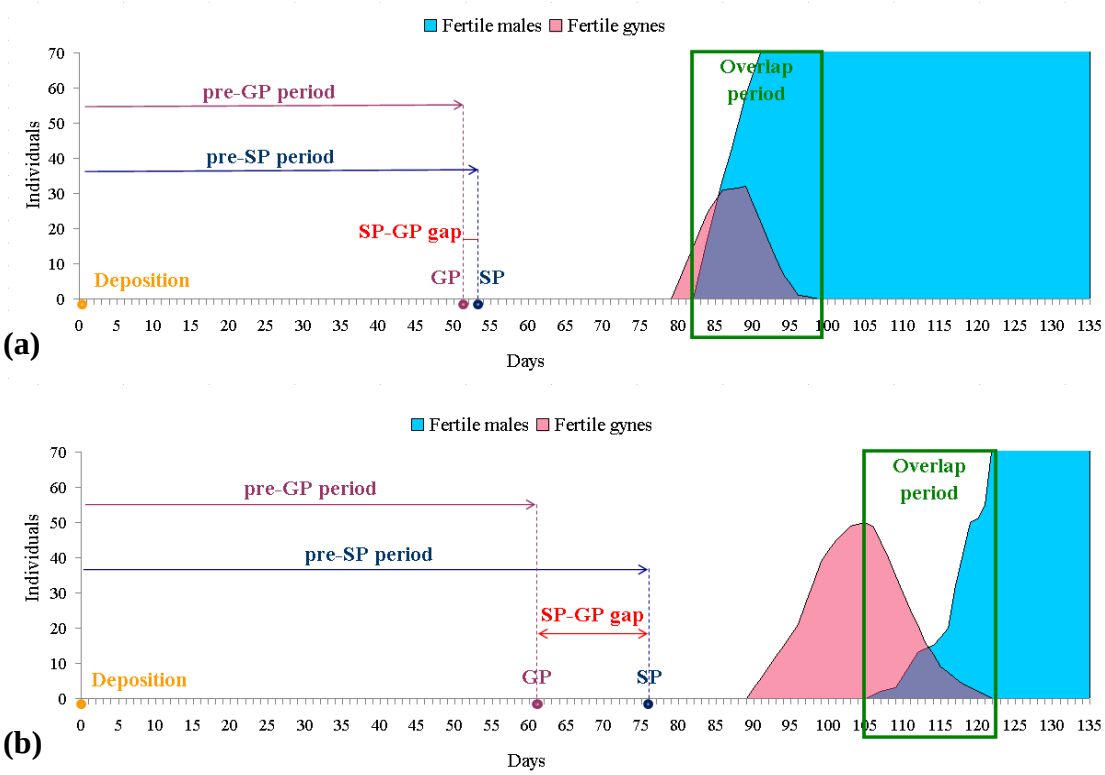

Figure 3. Example of the colony development and the presence of fertile sexuals for a colony a) with IRI<0.58 and b) with IRI>0.58. Description in the text.

Figure 3 shows an example of the developmental characteristics of a colony with IRI < 0.58 (Fig. 3a) and one with IRI > 0.58 (Fig. 3b). Starting from the day of fist deposition, the pre-GP and pre-SP periods and the SP-GP gap are indicated. The coloured areas represent the timing and amount of fertile gynes (pink area) and fertile

males (blue area) for each colony. The green square enclose the overlap period, when fertile gynes and males of the same colony are contemporarily present.

The analysis of developmental data showed that in colonies with IRI < 0.58 the SP occurred later, and consequently the SP-GP gap is higher, than in colonies with IRI > 0.58 (Table 2). Moreover, colonies in the first group produced more gynes, for a longer period and with a lower percentage of overlapped gynes than colonies of the second one (Table 2).

The mean lifespan of the founder queens was not significantly different between the two groups of colonies, but this value was positively correlated with both the pre-SP period and the gyne production period, which in turn were each other positively correlated, as shown in Table 3.

Table 2. Colonies' development carachteristics depending on the IRI categories.

Colony developmental data	**IRI < 0.58 mean ± SE**	**IRI > 0.58 mean ± SE**	***t***	***P***
Founder queen lifespan	79.125 ± 6.594 (*N* = 8)	82.286 ± 7.975 (*N* = 7)	0.3081	0.7629
Pre-SP period	66.333 ± 2.251 (*N* = 12)	55.333 ± 3.037 (*N* = 9)	-3.1591	**0.0052**
Pre-GP period	52.417 ± 1.738 (*N* = 12)	57.000 ± 2.784 (*N* = 9)	1.4646	0.1594
SP-GP gap	14.450 ± 0.878 (*N* = 20)	1.313 ± 2.069 (*N* = 16)	-6.2853	**<0.001**
Number of produced gynes	101.700 ± 13.101 (*N* = 20)	51.875 ± 11.194 (*N* = 16)	-2.8058	**0.0082**
Gyne production period (days)	33.100 ± 2.103 (*N* = 20)	24.500 ± 2.088 (*N* = 16)	-3.0310	**0.0046**
Overlapped gynes (%)	66.491 ± 4.984 (*N* = 20)	96.700 ± 2.180 (*N* = 16)	6.0808	**<0.001**

N = number of colonies.

Significant outcomes are shown in bold.

Table 3. Linear correlation among lifespan of founder queen, SP period and GP period.

Colony developmental characteristics		r	r2	*P*	*N*
Lifespan of founder queen	SP period	0,5317	0,2827	0,0414	15
Lifespan of founder queen	GP period	0,5940	0,3528	0,0196	15
SP period	GP period	0,5370	0,2884	0,0121	21

N = number of colonies.

DISCUSSION

The analysis of the inbreeding risk in colonies of *Bombus terrestris*, based on colony developmental characteristics and timing of production of gynes and males, allowed us to define two categories of colonies, characterised by two different reproductive strategies.

Colonies with a low IFI show a longer pre-SP period and can be assimilated to the "late switchers" define by Duchateau and Velthuis (1988), where the SP occurred at 18-36 days calculated from the emergence of the first workers, which is lower but comparable to our mean value of 66 days, by adding the developmental time of workers, which varies from 21 to 25 days (Duchateau & Velthuis, 1988; Cnaani et al., 2000). Since the pre-GP period is not significantly different between the two kind of colonies, the SP-GP gap resulted higher in colonies with low IFI. The SP-GP gap is the lapse of time when gynes' eggs are laid, thus the longer it is in a colony, the higher is the number of gynes this colony produces. Accordingly, colonies with a low IFI produce a higher number of gynes and for a longer time compared to colonies with a high IFI, as observed by Duchateau and Velthuis (1988) for the late switchers.

On the contrary, colonies with a high IFI can be assimilated to "early switchers" colonies, in which the SP occurs earlier of about 11 days (near to the 14 days of difference found by Duchateau & Velthuis, 1988) and the SP-GP gap is shorter, with a consequent minor number of produced gynes.

Concerning the reproductive strategy, our two colony categories can be ascribed to male biased and female biased ones. Although we did not count the total number of males

emerged in our colonies, we would expect that colonies with a low IRI produce more males than colonies with a high IRI, as observed by Duchateau and Velthuis (1988) for early and late switchers. If we classify our colonies in protandrous (males emerge before gynes) and protogynous (gynes emerge before males) colonies as described by Beekman and Van Stratum (1998), 12 out of the 36 colonies were protandrous, with a SP-GP gap < 4 (because the developmental time of gynes is 4 days longer than that if males). The frequency of protandrous colonies in our study (0.33) was lower than those found by other authors (Beekman & Van Stratum, 1998: 0.44; Duchateau & Velthuis, 1988: 0.42). All our 12 protandrous colonies belongs to the group with IFI > 0.58, although not all the colonies of this group were protandrous, since 4 of them were protogynous, with a SP-GP gap of 7, 8, 10, 14, but showed a high IFI due to the high value of mean $\overline{P}_{colony}$ (high probability of sibling mating).

Thus our high IFI colonies can be roughly assimilated to the protandrous colonies of Beekman and Van Stratum (1998), which produce a lower mean number of gynes and a higher number of males, and had a later switch point than the protogynous ones, leading us to affirm that high IFI colonies (early switchers) show a male biased reproductive strategy, while low IFI colonies (late switchers) a female biased one. The higher frequency of protogynous colonies in our study can account for the overall higher number of gynes recorded in our colonies, compared to the two previously cited studies. Previous studies showed that the different reproductive strategy does not depend neither from the colony size (Beekman & Van Stratum, 1998), nor from the queen life span (Gosterit, 2011). Our results show that the lifespan of the founder queens is positively correlated to both the pre-SP period and pre-GP period, but not with the IRI value. This means that the male biased strategy is not typical of queen with a reduced fitness, as hypothesised by Bourke (1997), although these queens show an earlier SP correlated to the shorter life span. In our study the male biased colonies (with a high IRI) showed a mean queen life span higher than the female biased ones (low IRI), and some of them had the overall most long-lived queen, indicating that the early SP and the consequent male biased strategy, at least in lab reared colonies, is a predetermined feature and not an emergency reproductive strategy. This is in accordance with the idea that variation in sex allocation in bumble bee colonies is under the queen control, as argued by other authors (Bourke & Ratnieks, 2001; Duchateau et al., 2004).

In bumble bee colonies workers can lay haploid eggs, which develop into males (Duchateau & Velthuis, 1989). Usually workers start to lay their own eggs late in colony cycle, namely not before 12 days after the laying of the first gyne egg by the queen, corresponding to our GP (Duchateau et al., 2004). Considering the time required for development and achievement of sexual maturity, these males would hardly meet the gynes produced by the same colony. Anyway, in colonies where the queen dies before the SP and after the GP, aunt-nephew matings can occur, where the percentage of colonies with diploid males is higher (75%) than in case of sister-brother (50%) and nieces-nephew (37.5%) matings. In our study this happened in 4 colonies over 15, where the queen has died before having produced any males (1 colony) or within 3 days after the SP (3 colonies). In these colonies males were produced entirely or mostly by workers, with a possibility of aunt-nephew matings. Nevertheless, all these colonies belong to the group with low IFI, because the gap in the timing of production of gynes and males is large enough. Furthermore, the number of worker-laid eggs which actually develop into adult males is usually low, due to the high rates of egg removal during worker conflicts (Bourke & Ratnieks, 2001).

Conclusions

The IRI of a colony is a predictor of the possibility of incest mating among gyne and male nestmates, but it proved to be also a good descriptor of the colony specific reproductive strategy.

Although a high IRI can be a disadvantage for the colony, due to the negative consequences in inbred colonies, such as the presence of diploid males (Duchateau et al., 1994; Beekman et al., 1999), it can also be part of a reproductive strategy characterized by a male biased sex allocation. Colonies with a high IRI are usually protandrous and have a male biased sex ratio. Since bumble bee males show a high dispersal ability than females (Wolf et al., 2012), males emerged from high IRI colonies will be likely to move away from the maternal colony. This feature, together with the production of a lower number of gynes, reduces the actual probability of sibling matings in these colonies.

On the contrary, low IRI colonies, which are mainly protogynous and produce an higher number of gynes, benefit from a lower inbreeding probability. Even in these colonies, however, sibling matings are never completely excluded, since IRI value is never zero. The different timing in male and gyne production does not bring to avoid sibling mating, since all our colonies produced both males and gynes and a certain degree of overlap in their fertility period is always present, contrarily to what was found by other authors (Gosterit, 2011).

Previous studies demonstrated that *B. terrestris* colonies can tolerate inbreeding without showing a substantial reduction of their performances (Gerloff et al., 2003; Gerloff & Schmid-Hempel, 2005). Therefore in this specie sibling matings could even represent an advantage in case of geographically isolated colonies: when finding a partner from a different maternal colony is difficult, mating with a sibling could represent an ultimate strategy to avoid extinction by transmitting colonial genes to future generations.

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5.8. Effects of pre-diapause queens' weight and pupae's gender on colony initiation in artificially reared *Bombus terrestris* L. (Hymenoptera: Apidae)

Submitted to *Journal of Apicultural Research*

Gherardo Bogo[1,2*], Natasha de Manincor[2,3], Alessandro Fisogni[2], Marta Galloni[2], Laura Bortolotti[1]

[1] CREA – Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Unità di ricerca di apicoltura e bachicoltura, Via di Saliceto 80, 40128, Bologna, Italy.
[2] Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Università di Bologna, Via Irnerio 42, 40126, Bologna, Italy.
[3] UMR 8198 - Evo-Eco-Paleo, Université de Lille, CNRS, F-59000, Lille, France.
[*]Corresponding author. Gherardo Bogo

SUMMARY

Diapause control and colony initiation are among the major problems encountered in the rearing of bumble bee colonies in captivity. In this study, we investigated the diapause survival and the performance of mated and virgin bumble bee (*Bombus terrestris*) queens in relation to the pre-diapause weight. We also tested the effect of pupae's gender on colony initiation by supplying male or queen pupae to stimulate egg laying. During diapause, lighter queens died at higher rates compared to the medium and heavier ones (59%, 17% and 9%, respectively). However, after survival they showed a high performance in laying eggs (100%) and second brood deposition (88%). Unexpectedly, among virgin queens both the heaviest and medium weight queens showed high survival rates (95% and 91%, respectively). We found no significant differences in the egg-laying rates after queens were stimulated whether with a cocoon containing a male pupa (57%) or a queen pupa (55%); however, bumble bee queens stimulated with a queen pupa laid more egg cells (5.5 ± 0.19 egg cells) and developed a first brood larger (10.33 ± 0.43 individuals) than those stimulated with male pupae (4.97 ± 0.19 and 8.57 ±

0.42, respectively). We conclude that lighter queens may have a fitness advantage compared to heavier queens once they have survived diapause. Moreover, our findings highlight the good performance of colonies initiated using queen pupae instead of male pupae.

Keywords: bumble bee; *Bombus terrestris*; diapause; pupae; colony initiation; egg-laying; queen weight; artificial rearing

INTRODUCTION

Bumble bees are annual eusocial insects, whose colonies develop from spring to late summer. The colony is composed by a single queen and several workers (up to 100-200 in some species) (Duchateau & Velthuis, 1988); in the late phase of colony development new queens and males are produced, which subsequently leave the colony for mating. In nature, mated queens survive winter in a state of diapause, inside small cavities (hibernacula), often situated in north-facing banks or slopes. Diapause lasts 6-9 months depending on spring temperature (Alford, 1969). To survive this long period, queens need large fat reserves that are built up prior to diapause (Beekman, Van Stratum, & Lingerman, 2000). Bumble bees, however, show a great flexibility in terms of diapause responses (Estoup, Solignac, Cornuet, Goudet, & Scholl, 1996; Dafni, 1998) and some bumble bee populations show aestivation (De Jonghe, 1986; Gürel, Gösterit, & Eren, 2008) and bivoltinism (Douglas, 1973; Buttermore, 1997). Diapause ends in early spring and queens leave the hibernacula to found new colonies.

Bumble bees are very important pollinators of both spontaneous plants and crops, and their economic importance as pollinators in greenhouses has been recognized for a long time; for these reasons, as well as for experimental and conservation purposes, they are currently reared on a large scale under controlled conditions (Beekman et al., 2000; Velthuis & Van Doorn, 2006).

One of the major problems in rearing bumble bees in captivity is the low diapause survival rate. In bumble bee mass rearing, mated queens are usually stored at 1-5°C for different durations based on the purchasers' demand. Depending on the conditions,

queen survival can vary from about 50 to 90%. After this process, queens can receive a narcosis by CO_2 to stimulate egg laying (Velthuis & Van Doorn, 2006). Previous studies demonstrated the importance of diapause duration (Beekman, Van Stratum, & Veerman, 1998; Gosterit & Gurel, 2009), diapause temperature (Velthuis, 2002; Amin, Suh, & Kwon, 2007) and queen weight (Beekman et al., 1998) on the survival rate. Beekman et al. (1998) indicated 0.6 grams as the lowest weight limit at which queens can survive the diapause and 0.8 grams as the mean weight of pre-diapausing queens and that a higher weight does not influence queens' survival.

Another critical phase in artificial bumble bee rearing is colony initiation, because usually a high number of queens fail to lay eggs. Several techniques have been described to initiate colony rearing in captivity and all include egg laying stimulation, such as placing two queens or 1-5 bumble bee or honey bee workers together (Sladen, 1912; Ptacek, 1991; Duchateau, 1991; Van Den Eijnde, De Ruijter, & Van Der Steen, 1991; Gretenkord & Drescher, 1997) or using a cocoon containing a male pupa (Duchateau, 2000; Yeninar, Duchateau, Kaftanoglu, & Velthuis, 2000; Kwon, Saeed, & Duchateau, 2003).

In this study, we compared the diapause survival and post-diapause performance of mated and virgin bumble bee queens belonging to three weight categories (< 0.6 g; 0.6-0.8 g; > 0.8 gr), and the deposition rate and colony development following stimulation with male or queen pupae. Our aim was to overcome the critical steps of diapause control and colony initiation in *Bombus terrestris* rearing in order to find out the optimal conditions to increase rearing success.

MATERIALS AND METHODS

This study was carried out over three consecutive years (2013-2015) using a total of 897 bumble bee queens, 731 of which were mated and 166 were virgins.

Queen mating and diapause

Queens and males of *B. terrestris* were obtained from commercial colonies (Bioplanet S.c.a., Cesena, Italia), maintained in laboratory at 25 ± 1°C and 60 ± 10% RH, in continuous darkness, and fed with fresh frozen pollen; queens were let free to mate in wooden flight cages (40 x 40 x 75 cm) during a maximum of 5 days under natural light. We used 1-10 days old virgin queens and 5-20 days old virgin males to obtain the best mating result (Tasei, Moinard, Moreau, Himpens, & Guyonnaud, 1998). After mating, males were discarded and only mated queens were kept together inside the cages (up to 50) for a week, fed with fresh frozen pollen and 50% sucrose solution, at ambient temperature and natural dark-light cycle. The 166 virgin queens were put for the same period in the flying cages without males, and treated the same way as mated queens. After a week, all the survived queens were individually weighed and divided into three categories depending on their weight (< 0.6 g; 0.6-0.8 g; > 0.8 g). The choice of these ranges was derived from the results of Beekman et al. (1998). Artificial diapause was induced by moving queens in a plastic box, filled with non-treated topsoil, in a fridge at 15°C for a week (as transition period) and then 3 months at 5°C. After this period diapause survival was checked.

Post-diapause and colony initiation

Diapause was interrupted by removing queens from the fridge and placing them in wooden flight cages (40 x 40 x 75 cm) at ambient temperature and natural dark-light cycle, equipped with fresh frozen pollen and sucrose syrup *ad libitum* to allow the recovery of energy reserves and stimulate ovary maturation. After 7 days survival was checked again ("post-diapause survival").

Mated queens were then placed individually into small plastic boxes (15 x 9 x 5 cm) to start deposition ("starting boxes") and moved in a climate room (29 ± 1°C and 60 ± 10% RH, continuous darkness). Virgin queens were discarded. Each box was perforated along the sides to allow ventilation and equipped with a piece of blotting paper at the bottom, a feeder with fresh frozen pollen (changed three times a week to avoid

fermentation and mould) and a syringe filled with syrup, which was renewed once a week. Boxes were kept constantly clean and blotting paper was changed when necessary.

Egg-laying stimulation

In order to stimulate egg laying, one young pupa collected from commercial colonies was added in each box and replaced with a new one once a week in case of no deposition (Kwon et al., 2003; Gürel & Gösterit, 2008). Young pupae are more efficient in stimulating queen egg laying and they can be discriminated by older ones basing on the colour: 1–2 days old cocoons are whitish and soft, while older cocoons become increasingly greyish with ageing (Kwon et al., 2003).
A total of 368 survived queens were divided into two groups: the first group (n = 188) was stimulated with a queen pupa; the second (n = 180) with a male one. Pupae were fixed with honeybee wax on a plastic Petri dish (6 cm diameter). When queens or males emerged from the pupa they were removed. After four weeks, queens that did not laid eggs were discarded.

Colony development

When the first adult worker emerged, each colony was transferred to a larger plastic box (25 x 15 x 14 cm minimum). Data on colony development were recorded by monitoring each colony three times a week. We registered the following data: the time elapsed from the placing of the queen in the starting box to the first egg cell deposition; the number of egg cells produced in the first brood (bumble bee eggs are laid inside wax cells and the whole progeny can be divided into three broods, easily distinguishable; see Duchateau & Velthuis, 1988); the number of developed larvae and pupae in the first brood; the timing of emergence of the first worker and the starting of second brood deposition (both calculated from the deposition of the first egg); the life span of queens (calculated from queen placing in the starting box until death).

To avoid manipulation, we did not count the exact number of eggs laid in each brood cells, therefore the mean number of eggs per cell in the first brood was estimated as in (1).

$$\frac{\text{n° of pupae} + \text{n° of discarded larvae}}{\text{n° of egg cells}} \quad (1)$$

Thirty colonies were randomly chosen to monitor their development until the emergence of the last queen. We also recorded the total number of newborn queens and timing of the switch point from the emergence of the first individual, calculated by subtracting 26 days (mean duration of preimaginal male development) from the date of first male emergence (Duchateau & Velthuis, 1988).

Statistical analysis

Differences among qualitative data (diapause survival, egg laying, second brood deposition and emergence of the first individual) were analysed with Pearson's Chi-square tests. Quantitative data (number of egg cells, number of larvae or pupae, number of days needed for the first deposition) were firstly tested for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and then analysed by Kruskal-Wallis one-way analysis of variance test, followed by nonparametric multiple comparisons. The *t* test for independent samples was used to analyse differences between pre- and post-diapause weight and colony performance related to the pupa gender. Statistical analysis was performed with STATISTICA software (StatSoft Italia srl, 2005) and R 3.1.2 version (R Core Team, 2014).

RESULTS

In order to discuss the general results on our colony rearing, we compared our data on colony development with bibliographic data, considering only mated queens of all tests pooled together (Table 1). We obtained a total diapause survival rate of 84.81% (n = 620) and a total egg-laying rate of 62.12% (n = 305).

Effect of queen weight on diapause survival and colony initiation

The mean pre-diapause weight of all queens was 0.815 ± 0.004 g. Diapause survival increased with the weight range, since mated queens with the lowest pre-diapause weight (< 0.6 g) showed a significant higher mortality (χ^2 = 10.0841, P = 0.0065) than queens with medium and high weight (Table 2). Also lightest virgin queens showed a higher mortality than medium and high weight queens, even though not significantly (χ^2 = 2.0456, P = 0.3596) (Table 2). The post-diapause survival (recorded during the 7-day flying period inside the cage) did not statistically differ among the three groups. Among the survived mated queens, those with minor weight showed a significantly higher egg laying success (100%) with respect to the other groups. Surprisingly, no significant differences in the egg-laying success were found among the three weight categories if we consider the initial (pre-diapausing) queen number (Table 2).

Table 1. Summary of data obtained from all the tests (only mated queens) compared to bibliographic values (temperature, duration of diapause). All data show the percentage or the mean ± s.e.; *n* = number of queens/colonies. * = Emergence of the first individual calculated from the placing of the queen in the box. ** = mean ± s.d.

Characteristics	Results	Bibliographic data
Diapause survival	84.81% (*n* = 620)	Beekman *et al.*, 1998 5°C, 2 months: 98% (*n* = 40) 5°C, 4 months: 67% (*n* = 28)
Egg laying	62.12% (*n* = 305)	Beekman *et al.*, 1998 5°C, 2 months: 45% (*n* = 18) 5°C, 4 months: 54% (*n* = 15) Gurel and Gosterit, 2008 72.8% (*n* = 70) Gosterit and Gurel, 2009 4.4 ± 0.5°C, 75 days: 82.5% (*n* = 40) 4.4 ± 0.5°C, 105 days: 76.32% (*n* = 38)
Days between queen placement in starting box and first egg deposition	8.511 ± 0.260 (*n* = 305)	Gurel and Gosterit, 2008 11.96 ± 1.31 (*n* = 51) Gosterit and Gurel, 2009 4.4 ± 0.5°C, 75 days: 15.3 ± 1.36 (*n* = 33) 4.4 ± 0.5°C, 105 days: 7.41 ± 0.44 (*n* = 34) **Duchateau and Velthuis, 1988 "early": 14.0 ± 4.8 (*n* = 10) "late": 11.7 ± 5.5 (*n* = 8)

Number of produced egg cells	5.382 ± 0.103 (*n* = 304)	Gurel and Gosterit, 2008 3.39 ± 0.21 (*n* = 51) **Kwon *et al.*, 2003 4°C, 3 months, 1-2 days old pupa 6.14 ± 1.35 (*n* = 7) **Duchateau and Velthuis, 1988 "early": 5.1 ± 1.5 (*n* = 10) "late": 5.4 ± 1.4 (*n* = 8)
Number of workers in the first brood	7.052 ± 0.241 (*n* = 248)	Gurel and Gosterit, 2008 7.55 ± 0.78 (*n* = 29) Gosterit and Gurel, 2009 4.4 ± 0.5°C, 75 days: 11.36 ± 0.96 (*n* = 24) 4.4 ± 0.5°C, 105 days: 10.17 ± 1.02 (*n* = 24) **Duchateau and Velthuis, 1988 "early": 8.8 ± 3.1 (*n* = 10) "late": 10.6 ± 4.0 (*n* = 8)
Days between first egg and first emerged adult	26.248 ± 0.331 (*n* = 226)	*Gurel and Gosterit, 2008 47.00 ± 2.32 (*n* = 30) *Gosterit and Gurel, 2009 4.4 ± 0.5°C, 75 days: 46.33 ± 1.41 (*n* = 24) 4.4 ± 0.5°C, 105 days: 40.08 ± 3.25 (*n* = 24) **Kwon *et al.*, 2003 4°C, 3 months, 1-2 days old pupa 20.5 ± 1.7 (*n* = 16)
Days between first and second brood deposition	17.532 ± 0.260 (*n* = 235)	**Kwon *et al.*, 2003 4°C, 3 months, 1-2 days old pupa 13.1 ± 2.1 (*n* = 16)
Days between first worker emergence and switch point	24.368 ± 2.433 (n=19)	Gosterit and Gurel, 2009 4.4 ± 0.5°C, 75 days: - 5.53 ± 4.35 (*n* = 19) 4.4 ± 0.5°C, 105 days: 8.1 ± 5.37 (*n* = 20) **Duchateau and Velthuis, 1988 "early": 9.8 ± 2.4 (*n* = 10) "late": 23.4 ± 4.6 (*n* = 8)
Number of newborn queens	134.148 ± 11.865 (n=27)	Gurel and Gosterit, 2008 14.38 ± 3.84 (*n* = 16) Gosterit and Gurel, 2009 4.4 ± 0.5°C, 75 days: 8.13 ± 2.67 (*n* = 8) 4.4 ± 0.5°C, 105 days: 12.57 ± 4.94 (*n* = 7) **Kwon *et al.*, 2003 4°C, 3 months, 1-2 days old pupa 89.5 ± 59.6 (*n* = 14) **Duchateau and Velthuis, 1988 "early": 9.5 ± 19.1 (*n* = 10) "late": 55.8 ± 72.8 (*n* = 8)

Table 2. Percentage of queens that survived to diapause and success of consecutive steps of colony development, depending on pre-diapause weight, for mated and virgin queens. *n* = number of queens. Values marked with different letters in each row were significantly different according to the Chi-square test at a, b: P < 0.01; A,B: P < 0.05.

		< 0.6 g	**0.6-0.8 g**	**> 0.8 g**	χ^2	**P**
Mated queens	Diapause survival	42.50% **a** (*n* = 17)	83.06% **b** (*n* = 255)	90.63% **b** (*n* = 348)	10.0841	0.0065
	Post-diapause survival	100% (*n* = 17)	92.55% (*n* = 236)	93.39% (*n* = 325)	0.0972	0.9526
	Egg laying (on post-diapause survived queen number)	100% **A** (*n* = 17)	62.80% **B** (*n* = 130)	59.18% **B** (*n* = 158)	4.3149	0.1156
	Egg laying (on pre-diapause queen number)	42.50% (*n* = 17)	42.35% (*n* = 130)	41.15% (*n* = 158)	0.0649	0.9681
	Second brood deposition	88.24% (*n* = 15)	80% (*n* = 104)	73.42% (*n* = 116)	0.6934	0.7070
	Emergence of the first individual	88.24% (*n* = 15)	79.23% (*n* = 103)	68.35% (*n* = 108)	0.8893	0.6411
Virgin queens	Diapause survival	25.00% (*n* = 1)	91.49% (*n* = 43)	94.78% (*n* = 109)	2.0456	0.3596
	Post-diapause survival	100% (*n* = 1)	88.37% (*n* = 38)	87.16% (*n* = 95)	0.0229	0.9886

The comparison between pre-diapause and post-diapause weight (Figure 1) shows in all cases a significant weight loss during diapause (*t* test, P < 0.001), except for the lighter virgin queens probably due to the low number of individuals.

Results of colony development performances of mated queens belonging to the three weight groups showed statistically significant differences in the number of days needed for the deposition of the first egg and for the emergence of the first adult worker (Table 3). Heaviest queens required more days for first egg deposition (9.18 ± 0.37) compared to the medium-weight queens (7.79 ± 0.36 days; H = 6.5697, P = 0.0374) and for first adult emergence (27.36 ± 0.53 days) compared to the lighter queens (23.33 ± 0.96 days; H = 11.7254, P = 0.0028).

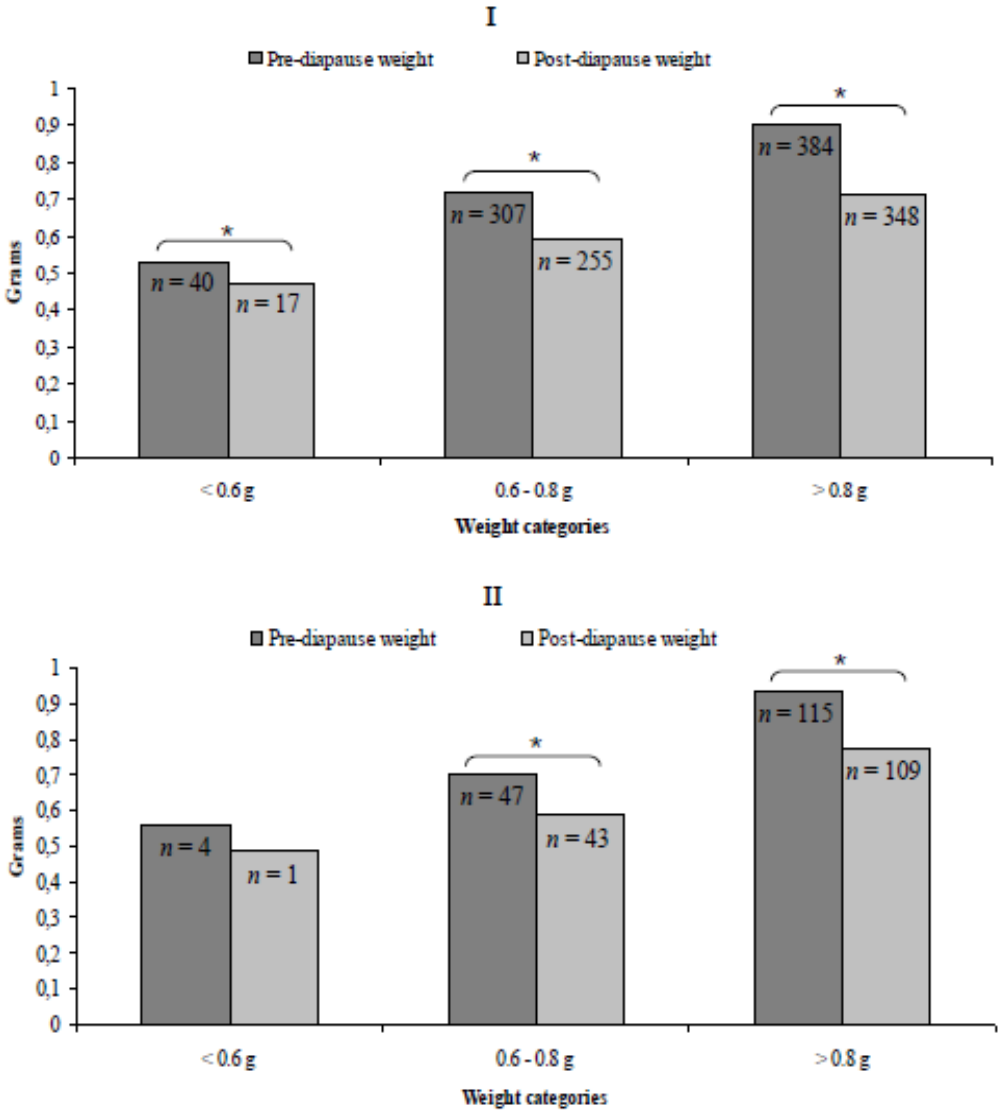

Figure 1. Pre-diapause and post-diapause weight comparison. I) Mated queens. II) Virgin queens. * = significant difference according to *t* test: $P < 0.001$.

Table 3. Colony development performance depending on queen weight. All data show the mean ± s.e.; *n* = number of queens. Values marked with different letters in each row were significantly different after one-way Krukall-Wallis tests followed by non parametric multiple comparison tests (a, b: P = 0.049; A, B: P = 0.007).

	Weight categories			**H**	**P**
	< 0.6 g	**0.6 – 0.8 g**	**> 0.8 g**		
Days between queen placement in starting box and first egg deposition	7.882 ± 1.292 ab (*n* = 17)	7.785 ± 0.363 a (*n* = 130)	9.177 ± 0.373 b (*n* = 158)	6.5697	0.0374
Number of produced egg cells	5.294 ± 0.506 (*n* = 17)	5.527 ± 0.164 (*n* = 129)	5.272 ± 0.136 (*n* = 158)	0.8777	0.6448
Number of discarded larvae	1.588 ± 0.642 (*n* = 17)	2.965 ± 0.369 (*n* = 113)	2.333 ± 0.282 (*n* = 129)	3.8217	0.1480
Number of pupae in the first brood	7.706 ± 1.107 (*n* = 17)	7.229 ± 0.332 (*n* = 109)	6.803 ± 0.358 (*n* = 122)	1.8482	0.3969
Number of total individuals in the first brood	9.294 ± 0.878 (*n* = 17)	9.927 ± 0.421 (*n* = 109)	9.024 ± 0.376 (*n* = 122)	2.6595	0.2645
Estimated mean number of eggs per cell	1.899 ± 0.217 (*n* = 17)	1.945 ± 0.100 (*n* = 109)	1.791 ± 0.087 (*n* = 122)	1.7904	0.4085
Days between first egg and first emerged adult	23.333 ± 0.959 A (*n* = 15)	25.505 ± 0.417 AB (*n* = 103)	27.361 ± 0.530 B (*n* = 108)	11.7254	0.0028
Days between first and second brood deposition	16.733 ± 0.943 (*n* = 15)	17.154 ± 0.376 (*n* = 104)	17.974 ± 0.383 (*n* = 116)	4.2058	0.1221
Queen life span (days)	46.400 ± 5.609 (*n* = 5)	37.043 ± 3.611 (*n* = 69)	38.794 ± 3.903 (*n* = 68)	1.3139	0.5184

Egg-laying stimulation

The analysis of queen egg-laying performance depending on the type of pupa did not show any significant difference (55% and 57% on queen and male pupae, respectively; χ^2, $P > 0.05$), although the production of the second brood and the emergence of adults was slightly higher for queens stimulated with a queen pupa (Figure 2).

Accordingly, the analysis of colony development characteristics shows that queens stimulated with a queen pupa in the first brood produce a higher number of egg cells (5.50 ± 0.19; $t = 2.0028$, $P = 0.0465$), a higher number of pupae (8.29 ± 0.43; $t = 2.9138$, $P = 0.0040$) and a higher number of total individuals (10.33 ± 0.43; $t = 2.9138$, $P = 0.0041$) (Table 4).

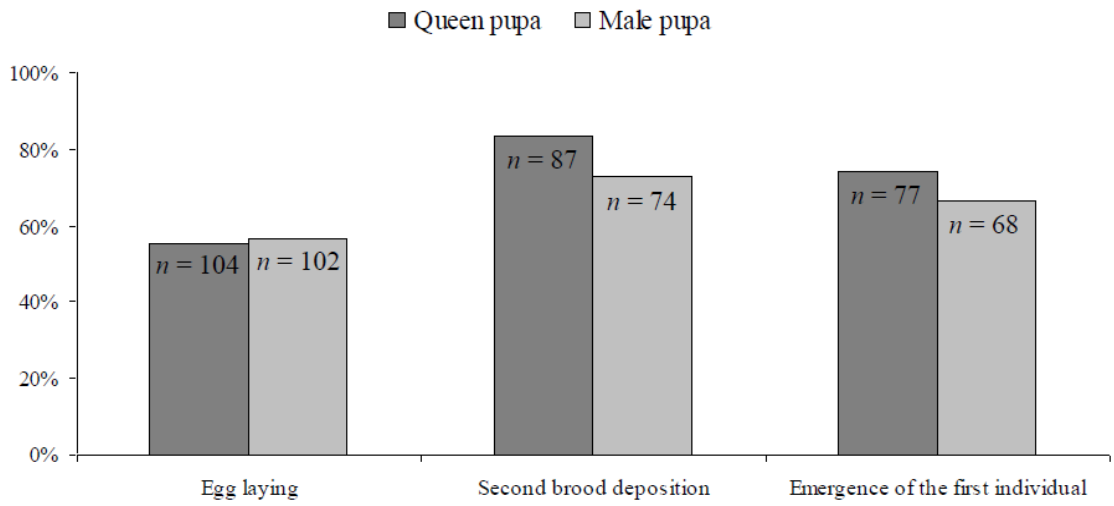

Figure 2. Percentage of queens laying eggs and producing a second brood depending on the gender of pupae (χ^2 test, $P > 0.05$). *n* = number of queens.

Table 4. Colony development performance depending on the type of pupa. All data show the mean ± s.e.; *n* = number of queens. Values marked with different letters in each row were significantly different according to *t* test.

	Pupae gender		***t***	**P**
	queen	**male**		
Days between queen placement in starting box and first egg deposition	7.692 ± 0.420 (*n* = 104)	8.314 ± 0.460 (*n* = 102)	-0.9988	0.3191
Number of produced egg cells	5.500 ± 0.189 **a** (*n* = 104)	4.971 ± 0.193 **b** (*n* = 102)	2.0028	0.0465
Number of discarded larvae	2.349 ± 0.334 (*n* = 83)	2.173 ± 0.306 (*n* = 81)	0.3889	0.6978
Number of pupae in the first brood	8.288 ± 0.433 **A** (*n* = 80)	6.468 ± 0.418 **B** (*n* = 79)	2.9251	0.0040
Number of total individuals in the first brood	10.325 ± 0.428 **A** (*n* = 80)	8.570 ± 0.424 **B** (*n* = 79)	2.9138	0.0041
Estimated mean number of eggs per cell	1.993 ± 0.098 (*n* = 80)	1.782 ± 0.127 (*n* = 79)	1.3150	0.1904
Days between first egg and first emerged adult	24.792 ± 0.557 (*n* = 77)	24.588 ± 0.484 (*n* = 68)	0.2728	0.7854
Days between first and second brood deposition	16.400 ± 0.398 (*n* = 80)	16.800 ± 0.420 (*n* = 70)	-0.6908	0.4908
Queen life span (days)	37.204 ± 4.285 (*n* = 54)	31.673 ± 3.500 (*n* = 52)	0.9956	0.3218

DISCUSSION

Developmental data obtained from our colony rearing are consistent with those found in literature. The main differences are found in the number of newborn queens and in the timing of the switch point, which are higher than the ones found in other studies. These two parameters are strictly linked to each other: when the switch point occurs at an early stage, the number of queens produced is low and conversely when the switch point is in a late stage, the number of queens is high (Duchateau & Velthuis, 1988). Therefore our colonies can be classified as “late switch point colonies”, according to Duchateau and

Velthuis (1988).

Our results confirm the importance of pre-diapause weight for diapause survival, as shown in earlier studies (Horber, 1961; Holm, 1972; Beekman et al., 1998). Accordingly, in our study queens lighter than 0.6 g survived significantly less than the heavier ones, while there was no difference between medium and heaviest queens. Moreover, we found that queen bumble bees lighter than 0.6 g had a higher egg laying success with regard to heavier ones. The size of workers and queens of *B. terrestris* is highly variable, and an overlap between big workers and small queens can be observed. In previous studies, a female adult was classified as queen when its weight exceeded 0.5 g (Bortolotti, Duchateau, & Sbrenna, 2001; Pereboom, Velthuis, & Duchateau, 2003). However, in this study we considered as queens also the females that weighed less than 0.5 g, due to their ability to mate. Our results, in accordance with Beekman et al. (1998), suggest that queen selection by weight is mainly achieved during diapause, since smaller queens have lower survival rates than heavier queens, probably due to low body fat reserves (Hodek & Hodková, 1988). Nevertheless, when lighter queens survive the diapause, their ability to generate a colony is perfectly conserved, suggesting that the physiological features determining who is a "good queen" are not linked with size. In addition, the severe selection operated by our diapause conditions on smaller queens likely determined an increase in queen quality. A further indication is given by the absence of difference in laying success among queens of different size when considering the initial (pre-diapause) queen number. Furthermore, in our study an increase in the weight of bumble bee queens did not positively affect the size of the first brood, as observed by Gösterit and Gürel (2007), but contrarily larger queens required more time to reach the same brood size than smaller ones.

The high survival rate of virgin queens to diapause confirms that mating is not necessary for entering diapause, since also unmated queens are able to survive it (Alford, 1969; Greeff & Schmid-Hempel, 2008). Although we did not continue the observation of virgin queens after diapause, another study showed that under laboratory conditions they are able to develop ovaries and to lay unfertilized eggs (Amsalem, Grozinger, Padilla, & Hefetz, 2015). If such circumstances would happen in nature, they could cause a competition between post-diapausing virgin and mated queens for food resources and nesting site. From a practical point of view, our results imply that in order

to start a new bumble bee colony it is important to select only queens that have certainly mated, since there are no clear differences from the performance of virgin queens both before and after the diapause.

Egg laying in bumble bee queens reared in controlled conditions may benefit from induced stimulation. Gretenkord and Drescher (1997) compared different methods of queen stimulation and found that the most successful consisted in adding some bumble bee workers and larvae to the colony. However, this method may not be practical for large scaled rearing, because of the need to empty several colonies; furthermore, after some days workers start to fight with the queen and need to be removed. The use of a pupa as a stimulus for egg laying seems to be the most practical method, and in several studies a male pupa was successfully used (Duchateau, 2000; Yeninar et al., 2000; Kwon et al., 2003). The choice of male pupae is justified by the fact that they are easy to obtain in large number from old colonies, and the single cocoon can be easily separated from the others (contrarily to worker pupae, whose cocoons are tightly connected to each other). The use of a queen pupa has never been tested before, to our knowledge, although it presents the same feasibility of the male pupae and it is longer available to queen for egg laying, since in bumble bees the pupal development lasts longer for queens than for males (Duchateau & Velthuis, 1988). Kwon et al. (2003) found that queens stimulated by a male pupa fixed horizontally produced more workers in the first brood, probably because the queen had a larger area available to build and incubate the egg cells. We found no difference in the percentage of queens laying on the two types of pupae (55% and 57% on queen and male pupae, respectively), confirming the efficiency of the male pupa; however, the size of the first brood was larger for the queens stimulated with queen pupae. Similarly to the results of Kwon et al. (2003) with the horizontal male pupae, differences were due to the total progeny of the first brood, but we found also a difference linked to the number of egg cells. This is likely due to the size of the queen pupa, whose surface is almost twice as that of the male one. Additionally, by observing the behaviour of males and queens emerging from the pupae, we hypothesize that this result is not only linked to the size of the pupa, but also to the fact that when male bumble bees emerge they damage egg cells laid on the top of the pupa, while emerging queens, as the workers, emerge from the side of the pupa, safeguarding the egg cells. Moreover, if emerging males are not immediately removed

from the box, they start to move around, stepping on and damaging the brood; emerging queens, on the contrary, help the founder queen in taking care of the brood, and is sometimes difficult to distinguish between them (personal observation).

In conclusion, our results indicate that lighter queens, although having a lower diapause survival than heavier ones, show a higher egg-laying rate and a faster development in the first steps of colony growth. Moreover the use of queen pupae is a better choice to stimulate egg laying, compared to male pupae. Our findings can be particularly useful in small scale and experimental bumble bee rearing, including the rearing of wild queens and the further release of colonies in nature.

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CHAPTER 6

General conclusions

This research improved knowledge on the relatively unknown field of plant-pollinator interactions, in ecological context with conservation needs. The findings are relevant for conservation strategies of natural populations of endangered plants and their pollinator communities in many different contexts.

In the development of specific conservation programs towards plants and pollinators, the evaluation of pollinators' efficiency and fidelity is of great importance, in order to focus practical interventions on the effective and best pollinators among the wide spectrum of flower visitors. Also the abiotic factors affecting the dynamics of pollinator communities are worthy of attention and should be taken into account because they could alter the plant-pollinators system, especially under the current global warming scenario. The Chapter 3 of my thesis shows the results of this analysis on the model plant *Dictamnus albus* and the community of its pollinators, with the concrete output of conservation practices.

Since pollinators generally visit flower to benefit from rewards, part of this thesis focused on the key role of nectar composition in modulating pollinator behaviours, with consequent impact on plant fitness. Laboratory results show a noticeable effect of secondary compounds on pollinator mortality. This result needs to be confirmed in the field, where nectar composition depends also on external factors, and where we verified that the action of yeasts can alter the relationship between the sugar constituents and produce toxic substances. A new and significant result obtained in my studies regards the key role of the non-protein amino acid GABA in the increase of bumble bee lifespan, that could have relevant implications in a rearing perspective.

My findings allowed to improve the rearing techniques of *Bombus terrestris*, a worldwide spread bumble bee species, and these outcomes can be very important to set conservation strategies of other bumble bee species and their host plants. The studies on inbreeding problems in *B. terrestris* demonstrated the lack of incest avoidance systems

in this species and highlighting the possibility of sibling mating in isolated populations, for which the conservation actions can be of great importance.

In conclusion, the studies here described provide a complete framework of the conservation problems and the possible solutions regarding plant-pollinator relationships, and open new challenges for future researches.

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