The impact of reared bumblebees and domesticated honeybees towards sympatric wild bees

ir. Laurian Parmentier

UNIVERSITEIT GENT

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

Every kid has a bug period... I never grew out of mine.

E.O. Wilson

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The impact of reared bumblebees and domesticated honeybees towards sympatric wild bees

ir. Laurian Parmentier

Thesis submitted in fulfilment of the requirements for the degree of
Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

De impact van gekweekte hommels en gedomesticeerde honingbijen interagerend met sympatrische wilde bijen

Illustration on the cover:

Honeybee (*Apis mellifera*) and a wild bumblebee (probably *Bombus pratorum*) foraging together on lavender (*Lavendula* sp.)

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Woord vooraf

Hier zijn we dan… het voorwoord van mijn thesis lijkt ook een beetje het sluitstuk van een periode. Ik weet nog goed het moment, de dag dat ik besliste om terug naar het academisch onderzoek te gaan en het blaadje met de projectinhoud met bijhorende aanstekelijke foto van een aardhommel destijds bij ons thuis op de keukentafel lag: “dat zou nog iets zijn voor jou” zei mijn moeder toen. En zo geschiedde. Nu, 4 jaar later zijn we aan het einde gekomen van een boeiende en leerrijke periode van intens onderzoek… echter zoals steeds luidt een einde ook een nieuw begin in. Maar hier wil ik nu toch even ten harte terugblikken op het tot stand komen van dit werk. Een doctoraat maak je immers niet alleen, het is het resultaat van aftoetsen van ideeën binnen een gans team; Ons team, dat noemden we ook wel de ‘Bumblebee group’. Geleid door twee promotoren, elk met hun eigen stijl, hebben ze mij vanaf het begin van mijn doctoraatsonderzoek op aanstekelijke manier gegidst. Ivan, je positivisme en enthousiasme voor wetenschappelijk onderzoek werkt enorm motiverend. Je hebt me niet alleen wegwijs gemaakt in de verschillende aspecten van pathologie, biochemie, diagnostiek,… binnen de wondere wereld van de hommels, maar hebt vooral mijn blik op onderzoek enorm verruimd. Guy, bedankt om mij de kans te geven om dit fantastisch onderwerp te mogen aanvatten binnen je labo, je ervaring te delen en om altijd een antwoord te bieden op vragen (zoals de mailtjes op onmogelijke momenten van de dag – beter gezegd nacht). Beiden van harte bedankt!

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Laurian Parmentier
September 2016

Contents

List of abbreviations

ABPV	Acute bee paralysis virus
ALPV	Aphid lethal paralysis virus
ANOSIM	analysis of similarities
PERMANOVA	permutational multivariate analysis of variance
ANOVA	analysis of variance
bp	base pairs
BQCV	Black queen cell virus
BRSV	Big Sioux river virus
CBPV	Chronic Bee Paralysis Virus
DNA	deoxyribonucleic acid
DWV	Deformed wing virus
dNTPs	deoxyribonucleotide triphosphates
EIDs	Emerging infectious diseases
FAO	Food and Agriculture Organization of the United Nations
GLM	generalized linear model
GLMM	generalized linear mixed model
IAPV	Israeli acute paralysis virus
KBV	Kashmir bee virus
LAB	lactic acid bacteria
LSV	Lake Sinai virus
NCBI	National Center for Biotechnology Information
OTU	operational taxonomic unit
PCR	polymerase chain reaction
R	species richness
SBPV	Slow bee paralysis virus
SBV	Sacbrood virus
SD	standard deviation
SE	standard error
SPSS/R	Statistical Packages
BMV/VdML	Bee macula-like virus (formerly: *Varroa destructor* macula-like virus)
WHO	World Health Organization, a specialized agency of the United Nations

Scope and objectives of the study

Today, biodiversity is under pressure in most parts of the world mainly due to human activity (United Nations 2012). Over the last few decades populations of pollinators are declining severely at global to local scales (Rasmont et al. 1993, Kremen et al. 2002, Pywell et al. 2006, Winfree et al. 2009, Potts et al. 2010). This problem has likely multifactorial causes (Goulson *et al.*, 2015) including changes in climatic conditions, anthropogenic changes of the environment, such as urbanisation and monocultures, agrochemicals, introduction of exotic species and spread of diseases and interactions between them (Ghazoul 2005, Goulson et al. 2008, Potts et al. 2010, Hennig and Ghazoul 2011, Goulson et al. 2012, Vanbergen et al. 2013, McMahon et al. 2015).

Counteracting the pollinator crisis has resulted in management of pollination services, making the use of domesticated honeybee hives and artificial reared bumblebee nests a common practice (Velthuis and van Doorn 2006, Murray et al. 2013). However, managed pollinators can also act as a stressor to native bee populations in decline, for example through competition for food (Goulson and Sparrow 2009, Hudewenz and Klein 2013), spillover of alien pathogens (Meeus et al. 2011, Murray et al. 2013) or spread of non-native gut microorganisms (Natsopoulou et al. 2015). These effects of domesticated bees have been mostly studied on allopatric native bees, for example after introduction of bumblebees and honeybees by intercontinental transports (Stout and Morales 2009, Murray et al. 2013, Goulson and Hughes 2015). Yet, also within their native range, domesticated bees can interfere with wild congeners, while these effects have been far less studied. Therefore, this dissertation will focus on the interaction of domesticated bees (both bumblebees and honeybees) and sympatric native bee fauna.

Chapter 1 introduces the main players, honeybees, bumblebees and wild bees, their pollination services for agricultural and wild plant species. When discussing the multifactorial problem of bee decline, we will focus on the current knowledge about the interaction between domesticated and wild animals, with a special interest in those between allopatric or sympatric domesticated bees and wild bees. Specifically, the impacts of competition and diseases will be described. At the end of the chapter, an overview of Apoidea-associated viruses and protozoan parasites will be given and their spillover will be discussed in the light of a multi-host reality.

While there is mounting literature depicting a risk for spillover of eukaryotic organisms and viruses between domesticated and wild bees, no research has been done on the gut bacterial composition. Gut microbiota is mainly studied in relation with its immune component and nutrient provisioning properties. Currently it is not known if the enclosed breeding system has

impaired the bumblebee microbiota. Therefore, in a first part of this dissertation, we investigated the microbiota of domesticated bumblebees in relation to sympatric wild congeners, employing commercial nests of *B. terrestris* as a study object. In order to unravel the potential differences between managed and wild *Bombus* sp., we will assess their gut microbiota towards composition (chapter 2) and stability (chapter 3). Furthermore, we will investigate if reared bees have an immune-compromised microbiota or if opportunistic bacteria appear in domesticated hosts which could be potential risks for spillover (chapter 4).

In **chapter 2**, a comparison between managed and wild bumblebees of different *Bombus* sp. will be described in terms of diversity, richness and evenness of gut microbiota classified according to OTU (Operational Taxonomic Units). In **chapter 3**, the stability/plasticity of gut microbiota of reared bumblebees under controlled conditions or conditions in an outdoor environment will be investigated. At the end of the chapter, the implications of placing reared bumblebees outdoors towards gut microbiota, especially in relation to non-core *Enterobacteriaceae,* will be discussed.

Given the differences between reared and wild bumblebee gut microbiota and their shift towards *Enterobacteriaceae*, **chapter 4** aims to investigate on the resilience of reared *B. terrestris* microbiota to protect against parasite infections. Therefore we compared foragers collected from nests when placed outdoors with wild bumblebees collected in the same environment. More specifically, the prevalence of parasites *Apicystis* and *Crithidia* in gut microbiota of reared versus wild *B. terrestris* will be assessed and discussed.

In a second part, we then looked at the impact of domesticated bees on sympatric wild bees. As competition for resources and spillover of parasites and viruses between domesticated and wild pollinators are likely to be important factors in the pollinator crisis, we will focus on these drivers and investigate on interactions with apiaries of honeybees, the most abundant domesticated pollinator used worldwide. More precisely the density of honeybees will be assessed towards non-*Apis* pollinators in outdoor environments, with a focus on wild bumblebee species an applying a bioassay tool with *B. terrestris* nests.

In **chapter 5**, we investigate on the potential use of standardized nests of *B. terrestris* as produced in a commercial rearing facility for biological pollination in agriculture, as a bioassay tool, determining environment quality to allow bumblebee development. By assessing their nest development parameters and making a correlation with poor or rich landscape metrics, we will

select useful parameters of these nests. At the end, we will discuss this bioassay as a potential tool investigating on pollinator support (food resources and nesting sites) within any given anthropogenic landscape when combining with conventional monitoring schemes.

In the light of new diseases and their possible link with spillover, in **chapter 6** we investigated on recently described Apoidea-viruses within pollinator networks, and their possible link with hosts of domesticated honeybees. More specifically, the prevalence of Lake Sinai virus (LSV), Bee Macula-like virus (BeeMLV) and Slow bee paralysis virus (SBPV) in different sympatric wild *Bombus* sp. collected in close proximity and at a distance of an apiary will be assessed in different locations in Flanders (North-Belgium).

While in previous chapters we focused on diseases not yet reported in wild bumblebees, in research **chapter 7** we investigated the impact of apiary density and the spillover of established diseases towards sympatric wild bumblebees. In parallel, we also investigated the competition for resources towards sympatric bumblebees and we therefore employed the standard-nest bioassay (chapter 5). In short, we will report on multiple interactions of domesticated honeybees, both top-down and bottom-up drivers, and their impact on the abundance and diversity of sympatric bumblebee and wild bee communities each in proximity and at a distance of apiaries in a spatio-temporal matrix of anthropogenic landscapes in Flanders (North-Belgium).

The final **chapter 8** presents the general conclusions of this thesis. We synthesise the impact of managed honeybees and bumblebees on wild populations at different levels, starting from the spillover of commensally associated gut microbiota and environmentally acquired pathogens to the impact on development parameters and prevalence of other wild species in the environment. We emphasized what our current results could imply for wild and domesticated bee health in general and discussed research directions and future applications.

Chapter 1. General Introduction

1. Domestication of bees

1.1. Honeybees

Honeybees share a long historical relationship with humans as the honey they produce formed an essential part in the diet of our ancestors (DeWeerdt 2015), which has now mostly been replaced by industrialized sweeteners. The honeybee is thought to be native to Africa, western Asia, and southeast Europe (Michener 1974), although its association with man is so ancient that it is hard to be certain of its origins. The earliest signs of prehistoric honey hunting was depicted in a cave painting made around 8000 years ago in present-day Spain (DeWeerdt 2015). Due to its excellent property of collecting nectar and production of honey and other bee hive products for human, it has certainly been domesticated for at least 4000 years (Crane 1990). It has been introduced to almost every country in the world to become now among the most widespread insect species on earth. The European strain of the honeybee, *Apis mellifera mellifera*, appears to be adapted to temperate and Mediterranean climates, and flourishing feral populations occur throughout most parts of Asia, North America, the southern half of South America, and Australia (Goulson 2003b). Since its domestication by humans, major events in this range expansion include its introduction to North-America around 1620 (Buchmann and Nabhan 1996), to Australia in 1826 (Doull 1973), and to New Zealand in 1839 (Hopkins 1911). The African race, *A. mellifera scutellata*, is associated with tropical forests and savannas, and has spread throughout the neotropics and into North America following its introduction to Brazil in 1957 (Goulson 2003b).

Since the shortage of pollinators, the importance of domesticated honeybees has also been directed towards their pollination services of insect-pollinated crops. Domesticated honeybee hives provide large numbers of worker bees and can easily be moved to flowering crops to provide many pollinators. A prime example is the California almond (*Prunus dulcis*), a crop highly dependent on pollination, and each year honeybee hives are massively transported for pollination of almond trees (Brittain et al. 2013). Besides, honeybees are also key to pollinate oilseed rape crops (*Brassica napus*), among a vast number of other insect-pollinated open-field crops worldwide.

1.2. Bumblebees

The potential value of bumblebees as pollinating insects in agriculture has been recognized more recently. Due to their learning capacity they are capable of handling complex blossoms with hidden rewards (Goulson 2010). In comparison to honeybees they visit twice as many flowers per minute and because longue-tongued bumblebees have longer tongues than honeybees, these bumblebees are much better at pollinating flowers with deep corollas (Holm

1966, Goulson 2010). Bumblebees are also able to perform “buzz pollination” or “sonication”. They place their thorax close to the anthers and contract their flight muscles whereby the anthers vibrate and release the pollen (Goulson 2010). Because of their pollination value, hundreds of bumblebee queens caught in the U.K., were introduced by colonialists into New Zealand in 1885 and 1906, to improve seed set of red clover. Four of the species (*Bombus hortorum*, *B. ruderatus*, *B. subterraneus* and *B. terrestris*) became established (Hopkins 1914). Later, *B. terrestris* spread into Israel in the 1960s (Dafni and Shmida 1996), while *B. ruderatus* was introduced to Chile in 1982 and 1983 for pollination of red clover (Goulson 2003b, Velthuis and van Doorn 2006), and by 1994 had spread to Argentina (Abrahamovich et al. 2001).

Following historical successes for pollination services, the potency of rearing bumblebee nests was investigated. In 1987, commercial bumblebee rearing started when a Flemish pioneer, Mr. de Jonghe founded the company Biobest (Westerlo, Belgium) and started the indoor-rearing of buff-tailed bumblebees (*Bombus terrestris*). These reared pollinators were an instant success in the pollination of greenhouse tomatoes in Belgium and the Netherlands. Since then, about one million colonies are produced annually (Velthuis and van Doorn 2006) to be used especially for pollination in greenhouses. As a result, these bumblebee nests have been transported to various other countries to enhance crop pollination. Yet, this has also contributed to invasion of different regions, for example *B. terrestris* has now become established in Japan as feral populations (Dafni 1998). Also, *B. terrestris* arrived in Hobart, Tasmania, in 1992, perhaps accidentally transported in cargo, and has since invaded a substantial portion of the island (Buttermore 1997, Hingston et al. 2002, Goulson 2003b). While initially different companies such as Biobest and Koppert started only with the Palearctic *B. terrestris*, due to the invasive character of this species in its non-native range, there are now several mass-rearing programs in Europe (Velthuis and van Doorn 2006) producing different (local) species to be exported for pollination. For example, a good evolution is that local *Bombus occidentalis* and *Bombus impatiens* are widely used for pollination services in North America (Whittington et al. 2004) and now local *Bombus atratus* and *Bombus bellicosus* are also being produced in Chili and Uruguay (Salvarrey et al. 2013). In China both *Bombus ignitus* and *B. lucorum* are used as a native commercial pollinator, whereas in East Asia (Japan, South Korea) *B. ignitus* is also reared (Mah et al. 2001, An et al. 2007).

1.3. Economic value of pollination by domesticated bees

The total annual economic value of pollination worldwide amounted to $153 billion, which represented 9.5% of the value of the world agricultural production used for human food. Recent estimates suggest that crop pollination by insects underpins $361 billion of crop production worldwide (Lautenbach et al. 2012, Hanley et al. 2015). Next to this, bees contribute to the health of humans as they polinate crops, nuts and foliage producing essential nutrients such as

vitamin A; in a world without bees, models show that global yearly deaths would soon increase 2.7% due to the effects of malnutrition (Smith et al. 2015). A number of bee species are actively domesticated, most notably the honeybee. Indeed, the domesticated honeybee has long been regarded as the most important crop pollinator (Klein et al. 2007).

The total number of beekeepers in Europe was estimated at 620,000 in 2010 (Chauzat et al. 2013). Honeybees are classically used for their pollination of major crops such as almond (*Prunus dulcis*) (Gary et al. 1978), alfalfa (*Medicago sativa*), apple (*Malus* sp.) orchards and rape seed (*Brassica* sp.) (Hanley et al. 2015). The latest estimates for the existence value of protecting honeybees in the UK alone accounted of £1.77 billion/year (Mwebaze et al. 2010), an amount that will exceed a hundredfold worldwide (Hanley et al. 2015).

While domesticated honeybees are mostly used in open-field crops, also bumblebees are regarded as a very important group of pollinators, and domesticated bumblebee nests are most commonly used in enclosed crop production systems (glasshouses and poly-tunnels) (Hanley et al. 2015). An estimation of the turnover in bumblebee rearing industry was around €55 million in 2004 with an annual growth since then (Velthuis and van Doorn 2006). Importantly, about 8% of the agricultural crops have pollen presented in poricidal anthers, and to obtain the pollen an insect has to shake the anthers, which is known as “buzz pollination” (Goulson 2003a). Such crops as those under the insect-pollinated Solanaceae family, including tomatoes (*Solanum esculentum*) (Knapp 2010) and those under the Ericaceae family, such as blueberries, which are important open field crops cultivated in the US and Canada. These crops mainly rely on buzz pollination by bumblebee species (Faegri and van der Pijl 1979, Goulson 2010)) as honeybees are not able to do this and thus cannot efficiently pollinate these crops. The crop value of bumblebee-pollinated tomatoes is €12,000 million in Europe alone (Velthuis and van Doorn 2006). Besides, reared bumblebees pollinate several main horticultural crops such as pepper (*Capsicum annuum*), melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativa*), strawberry (*Fragaria* x *ananassa*), raspberry (*Rubus idaeus*) and orchard fruit such as apple (*Malus domestica*) and cherry (*Prunus avium*) (Stanley et al. 2013).

Despite the huge contribution of domesticated pollinators to fill the need of pollination services a growing body of research indicates that wild pollination services could account for a substantially greater proportion of pollination services than previously thought (Garibaldi et al. 2013, Breeze et al. 2014, Kleijn et al. 2015), even in modern, intensive farm systems (e.g. Winfree et al. (2008)). This is currently prompting the scientific community to suggest that the importance of domesticated bees providing pollination services may have been overestimated at the expense of wild pollination (Westerkamp and Gottsberger 2000, Breeze et al. 2011). Indeed, recent studies reveal that wild bees often supplement domesticated bee pollination resulting in better seed and fruit set in most insect-pollinated crops (Garibaldi et al. 2013).

2. Wild pollinators

2.1.General

About 75% of our crops and many wild flowers require pollination by animals, often by insects (Ollerton et al. 2011). Plant pollinators amongst insects are beetles (Coleoptera), flies (Diptera), wasps, bees (Hymenoptera), butterflies and moths (Lepidoptera). Bees (Apoidea, Anthophila) belong to the Hymenoptera, which is one of the largest orders of insects. They are considered to be the most important group of insect pollinators (Faegri and van der Pijl 1979, Albrecht et al. 2007, Garibaldi et al. 2013). Indeed wild bees constitute a ∼100 million years old monophyletic group that today includes ∼17,500 – 25,000 described species (Engel 2000, 2001, Poinar and Danforth 2006, Michener 2007b, Patiny et al. 2009, DeWeerdt 2015). They originated in early to mid-Cretaceous, roughly in synchrony with the angiosperms (flowering plants) (Danforth et al. 2006).

2.2.Taxonomy and brief overview of wild bees

2.2.1. Phylogenetics

Following the latest insights in classification, the bee species described are classified over about 4 000 genera in 9 families (Danforth et al. 2006, Davis et al. 2010). While the phylogenetic position of some bee families was long subject for debate, a major breakthrough was achieved by (Danforth et al. (2006)) who consolidated the hypothesis of basal position and paraphyly of Melittidae s.l. (sensu latu) excluding the former subfamilies Dasypodaidae and Meganomiidae. The latest phylogenetic cladogram with all families of wild bees (Apoidea Anthophila) is given in figure 1.1. Herunder, we briefly describe all wild bee families, exept the Stenotritidae as this family only occurs in Australia and is out of the scope of this dissertation.

2.2.2. Melittidae

The Melittidae are one of the smallest families of bees (about 200 species in four genera; Michez et al. 2009) and restricted to Africa and the northern temperate zone. Historically, the family has included the Dasypodaidae and Meganomiidae as subfamilies, but recently (Danforth et al. 2006) used a robust cladistic analysis, based on molecular and morphological data, and indicated that Melittidae s.l. (sensu lato) was paraphyletic. Their results suggested that melittid bees constitute a paraphyletic group from which all other bees are derived, and hence proposed to split the traditional family of Melittidae into three distinct families: Dasypodaidae, Melittidae s.st. (sensu stricto) and Meganomiidae.

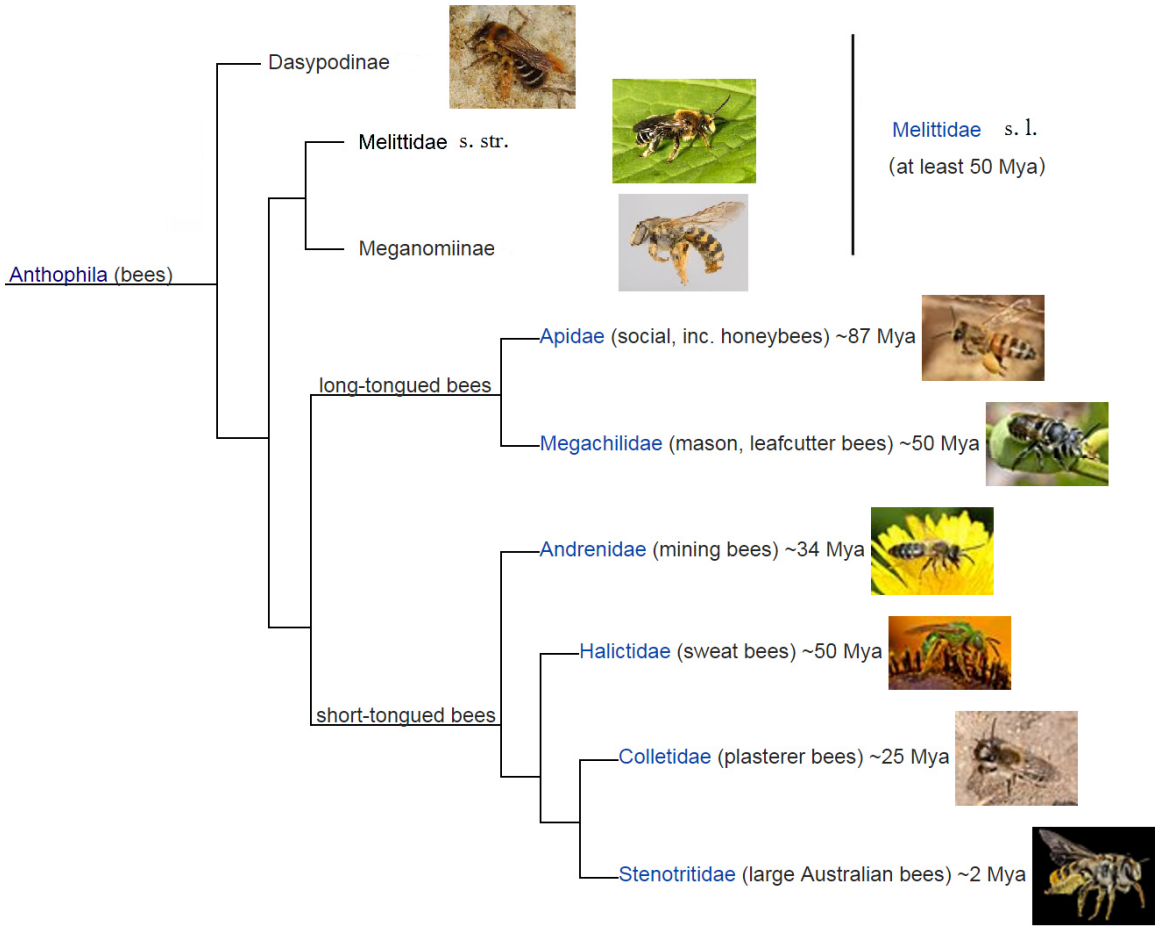

Figure 1.1. Phylogeny and wild bee families (Apoidea Anthophila) (adapted from Hedtke et al. (2013))

So each of the three historical subfamilies is now accorded family status, with Dasypodaidae as the basal group of bees, followed by meganomiids and melittids, which are sister taxa as represented in figure 1.1. (Danforth et al. 2006). In Belgium, the only representative of the genus *Dasypoda* is the species *Dasypoda hirtipes* (Rasmont and Iserbyt 2010-2016), with a distinctive trait of very long hairs on tibia and basitarsus (Peeters et al. 2012). In contrast, the genus *Melitta* is rich in species but morphologically monotonous compared to other melittid bees (Michez et al. 2009). Michez and Eardley (2007) listed 43 species but information about the biology of *Melitta* is limited to a few European species. As far as known, females nest in the soil (e.g. Celary (2006)) and many species are oligolectic, some of which are oil collecting (Michez et al. 2008, Michez et al. 2009).

2.2.3. Short-tongued bees

Phylogenetics (figure 1.1) show a further division in short- and long-tongued bees of which the former there are three families found in Europe, of them, the Andrenidae are most abundant (Peeters et al. 2012).

Andrenidae

The short-tongued Andrenidae (commonly known as “mining bees”) are a large family of solitary, ground-nesting bees. The Andrenidae are further divided in three subfamilies: Andreninae, Oxaeinae and Panurginae (Patiny 2003). Since the species under subfamily Oxaeinae are quite different in appearance, they were formerly regarded as a separate family, but phylogenetic analysis reveals them to be an offshoot within the Andrenidae, very close to the Andreninae as shown in figure 1.1 (Michener 2007a). Generally, the family contains a very large number of taxa, especially among the Panurginae. In the Netherlands alone, 74 andrenid species have been listed (Peeters et al. 2012) of which *Andrena flavipes*, *A. biocolor*, *A. haemorrhoa* and *A. vaga* are also common in Belgium (Rasmont and Iserbyt 2010-2016). The Andrenidae are typically small to moderate-sized bees, and are commonly oligolectic (especially within the subfamily Panurginae). Andrenids are among the few bee families that have no cleptoparasites and most of them have a sting apparatus which is so reduced that they are effectively unable to sting (Michener 2007a).

Halictidae

The Halictidae is the second largest family of Apoidea bees (Danforth et al. 2006) which occur all over the world and are usually dark-colored and often metallic in appearance. They are commonly referred to as "sweat bees" (especially the smaller species), as they are often attracted to perspiration (Michener 2007a, Peeters et al. 2012). Two important genera are *Lasioglossum* and *Halictus* with over 40 species in The Netherlands (Peeters et al. 2012) and Belgium (Pauly 2014). Many species in the subfamily Halictinae are eusocial at least in part, such as *Lasioglossum malachurum* or *Halictus rubicundus* which also occur in Belgium (Rasmont and Iserbyt 2010-2016), with fairly well-defined queen and worker castes (Michener 2007a). Most halictids nest underground, though a few nest in wood, and they feed their progeny by providing pollen clumps, comparable to “pollen storers” in bumblebee species (Peeters et al. 2012). All species of Halictidae are pollen feeders and can be regarded as important contributors to pollination (Michener 2007b).

Colletidae

The family of Colletidae are often referred as “plasterer bees” or “polyester bees”, due to the method of smoothing the walls of their nest cells with secretions applied with their mouthparts; these secretions dry into a smooth, cellophane-like lining. The plasterer bees are further divided into five subfamilies, 54 genera, and over 2000 species worldwide (Michener 2007a). In Europe, only the genera *Hylaeus* and *Colletes* can be found, of which in the Netherlands 25 and 9 species are present, respectively (Peeters et al. 2012). Characteristic of

Hyaleus (or “mask bees”) are the white spots on the forehead which make them distinctive of other genera. All of the colletid bees are solitary, though many nest in aggregations (Michener 2007a).

2.2.4. Long-tongued bees

The long-tongued bees include the family of Megachilidae and Apidae bee taxa of which the latter include the *Bombus* species, which is a focus in this dissertation and will be handled under 2.3.

Megachilidae

The family of Megachilid bees includes the genera *Heriades*, *Hoplitis*, *Coelioxys*, *Lithurgus* and the more common *Osmia* and *Megachile*, the latter also called “mason bees” and “leafcutter bees”, respectively, reflecting the materials from which they build their nest cells (soil or leaves, respectively) (Michener 2007a). Of the Megachilds, the alfalfa leafcutter bees (*Megachile rotundata*) are important crop pollinators, which has been introduced in America for their pollination services (Goulson 2003b). *Osmia* is an important genus in the Netherlands and Belgium with at least 12 species including the common *Osmia bicolor*, which can be observed in early spring (Rasmont and Iserbyt 2010-2016, Peeters et al. 2012). All Megachilid species feed on nectar and pollen, but only a few species are cleptoparasites (Michener 2007a, Peeters et al. 2012). The Megachilidae are a family of only solitary bees whose pollen-carrying structure (called a scope) is restricted to the ventral surface of the abdomen (rather than mostly or exclusively on the hind legs as in other bee families such as the Apidae) (Michener 2007a).

Apidae

The Apidae is the largest family within the Apoidea, with at least 5700 species of bees, which presently also includes all the genera previously classified in the families Anthophoridae and Ctenoplectridae (Danforth et al. 2006). The Apidae include important genera as the honeybees (*Apis* sp.), bumblebees (*Bombus* sp.), carpenter bees (*Xylocopa* sp.), orchid bees (*Euglossini* sp.), stingless bees (*Meliponini* sp.) and cuckoo bees (*Nomada* sp.) (Michener 2007a). The latter typically show cleptoparasitic behaviour of laying their eggs in the nests of other bees, of which some are typical kleptoparasites of *Andrena* species. With over 850 species worldwide, the genus *Nomada* is one of the largest genera in the family Apidae (Michener 2007a) and in the Netherlands, 48 species can be found (Peeters et al. 2012).

2.3. *Bombus* and its biology

2.3.1. General

Among the 250 known bumblebee species (Goulson et al. 2008), *B. terrestris*, also known as the buff-tailed bumblebee, is one of the most studied (Rasmont et al. 2006). *B. terrestris* is an eusocial insect belonging to the superfamily Apoidea. The buff-tailed bumblebee is covered in dense fur and the combination with their big size makes it possible for them to survive in temperate to cool regions (Goulson, 2003), reflecting its natural distribution range which is Europe and West- and Central-Asia (Dafni and Shmida 1996, Goulson 2010). Figure 1.2 represents a phylogenetic overview of bumblebee species which are distributed in Belgium, with the five most commonly found species underlined in blue.

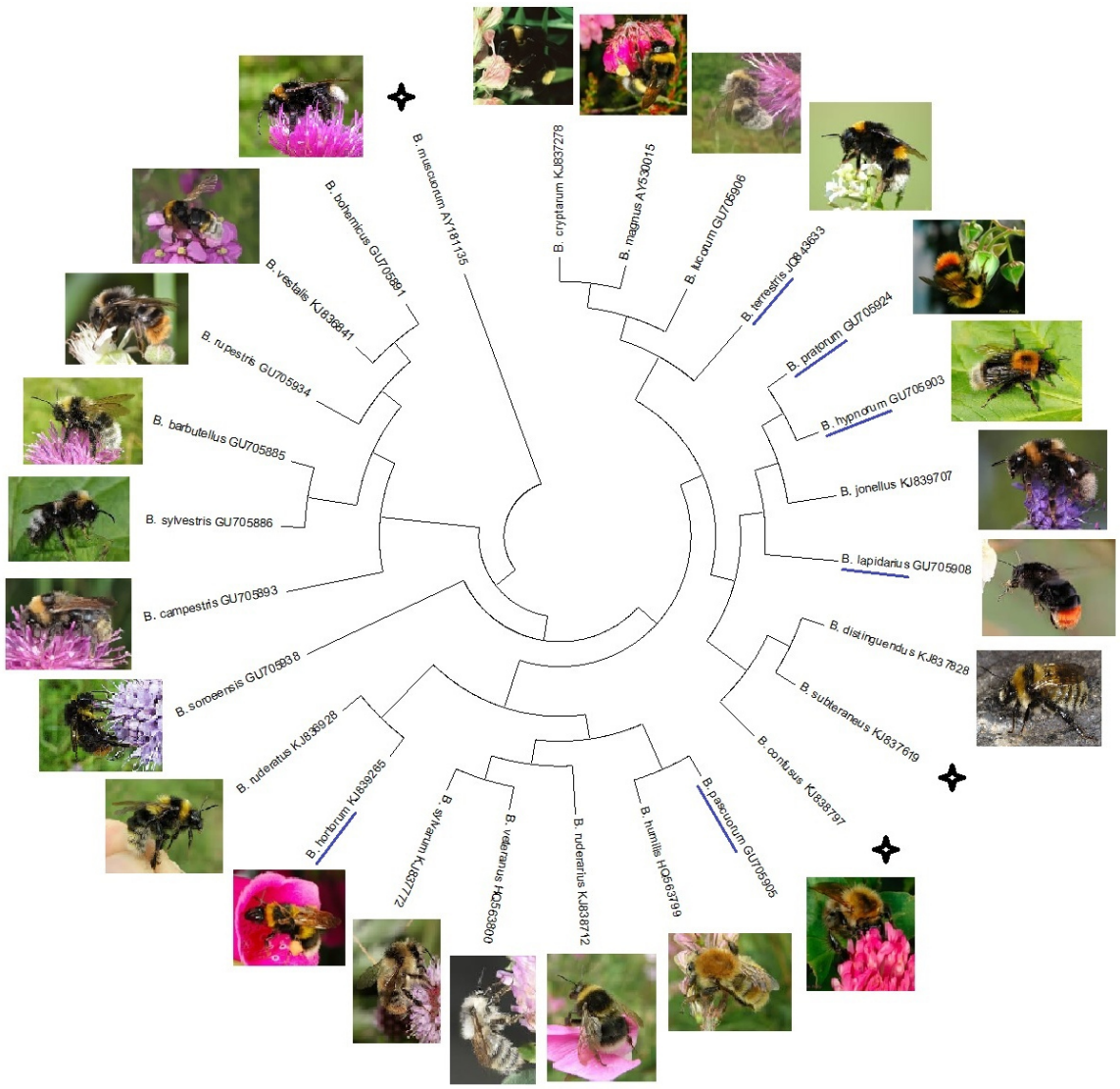

Figure 1.2. Phylogenetic tree of bumblebee species known for Belgium (based on database of Rasmont and Iserbyt (2010-2016) and constructed after alignment in Mega6® using species CO1 sequences; Genbank entry numbers are given); most common species are underlined in blue; species that have become extinct are indicated by ' ✦ ')

In Belgium, *B. terrestris* is one of the five most common bumblebees, which are *Bombus lapidarius*, the red-tailed bumblebee, *Bombus pascuorum*, the common carder bee, *Bombus hortotum*, the garden bumblebee and *Bombus lucorum*, the white-tailed bumblebee (Rasmont and Pauly 2010). Bumblebees are recognizable by their typical colours; although cryptic species also exist. For example, *B. terrestris*, *B. lucorum*, *Bombus cryptarum* and *Bombus magnus* resemble to each other visually and these species are sometimes referred to as the '*B. terrestris* species-complex' (Pauly 1999).

Bumblebees are holometabolous insects or Endopterygoya as they undergo a metamorphosis during their pupal stage which results in adults having huge morphological differences compared to their larval stage. Depending on the way bumblebee larvae are fed, bumblebee species can be divided into pocket makers and pollen storers. In the former group, larvae graze on the fresh pollen that is put underneath the brood clump, whereas the larvae build their own cells from wax and silk in the latter group, whereto *B. terrestris* belongs. Furthermore, a bumblebee nest consists of a queen, workers and drones.

2.3.2. Life cycle based on *B. terrestris*

B. terrestris has an annual life cycle (see Figure 1.3) and a hierarchical nest structure which depends on the sexe and position within the colony (Goulson 2003a). It is an eusocial species, meaning there are overlapping generations and division-of-labor in the colonies. Queens start a new colony after they awake from hibernation in spring (on average February or March in Belgium). After a few days of foraging for pollen and nectar, the queen starts searching for suitable nest sites. Nesting occurs often in old holes of rodents or other species where insulating material such as hair and dry grass is available for nest construction (Gurel et al. 2008, Goulson 2010).

There are 3 important phases in the life cycle of a bumblebee colony: nest initiation, colony growth and a reproduction phase with a switch and competition point which are described briefly here.

Nest initiation

The queen supplies the nest with pollen. She moulds the pollen into a clump which she uses as first brood substrate (fig. 1.3, n°1). In this pollen clump 8 to 16 eggs are laid by the new queen and she keeps the nest warm by shivering (Goulson 2003a). The queen then also produces nectar pots so that she can replenish her energy reserves. In order to incubate her brood, she sits in a groove on top of the clump. During this period, queens generate lots of heat, maintaining an internal temperature of 37-39°C. This enables them to keep a brood

temperature of 30-32°C. The first eggs hatch after about 4 days and the larvae consume the pollen provided by their mother. The young larvae live together within a cavity inside the pollen, also known as the brood clump. After some time, the brood clump breaks up and the larvae build individual cells from silk and wax. During their development they are fed with regurgitated pollen and nectar (Goulson 2010).

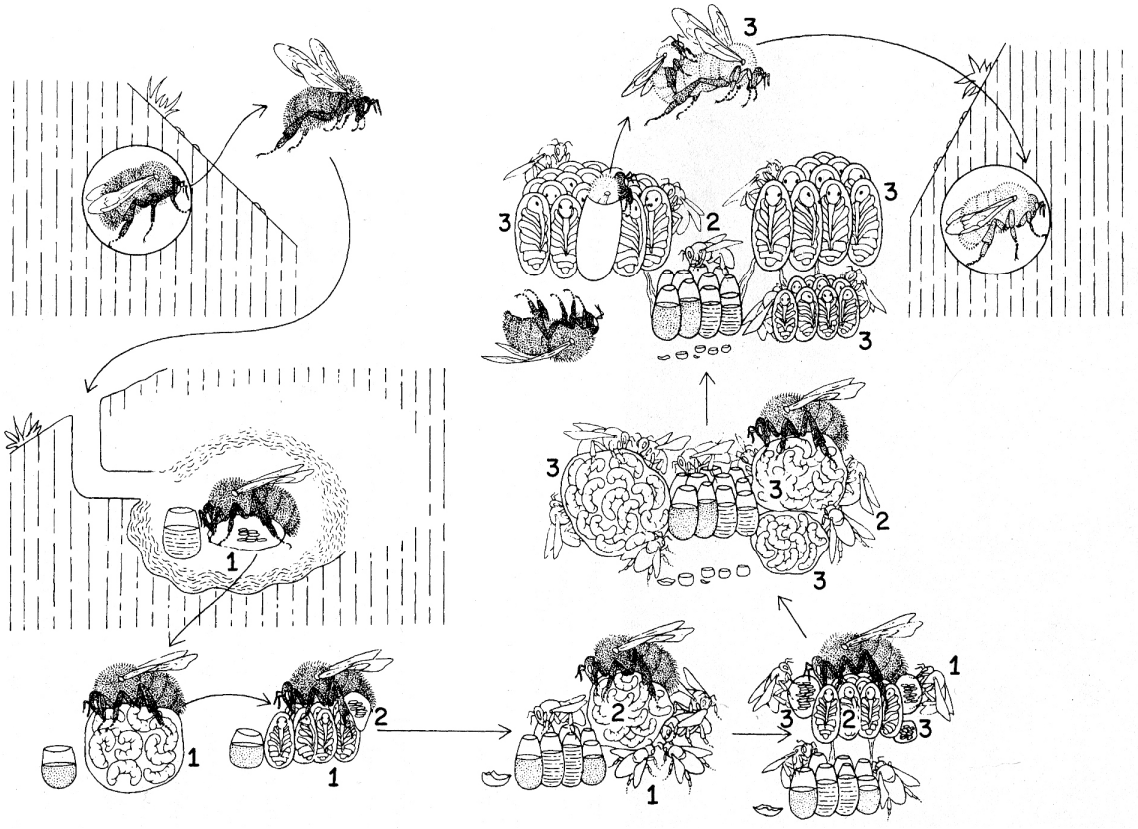

Figure 1.3. Cycle of a bumblebee colony (from Heinrich, 1979). The numbers (1, 2 and 3) represent the successive broods, "3" being the new generation constituted of virgin queens and males

Colony growth

The larvae undergo 4 instars and after about 2 weeks they spin a strong silk cocoon and pupate. As *B. terrestris* is a 'pollen-storer', the larvae develop in individual cells until pupation which takes another 2 weeks for the pupae to hatch. Depending on food supply and temperature the total development period is about 4 to 5 weeks. Larvae that are situated near the centre of the brood clump are kept warmer than those that are located on the outside. Therefore, they become larger and emerge slightly earlier. When the first batch pupates, the queen will collect more pollen and lay new eggs. The new-borns are entirely white at first and for that reason they are called "callow workers". They get their characteristic coloration after 24 hours. The first batch of offspring exists of female workers which collect nectar and pollen, called "foragers" to provide nutrients to the new colony and help to maintain the brood temperature (Gurel et al. 2008). From the next offspring on, some of the new workers will

function as “foragers” and others as “nesters”, which stay in the nest and help the queen with the developing broods (fig. 1.3, n°2). The nest can increase in tenfold within 6 weeks. Colonies of *B. terrestris* can grow up to 350 workers (Goulson 2010).

Reproduction phase

Switch point. When the nest reaches its reproduction phase, the queen will stop producing workers and the nest starts rearing drones and new queens (fig. 1.3, n°3) (Gurel et al. 2008, Goulson 2010). Drones are produced from unfertilized eggs and are thereby haploid, while females are diploid (Goulson 2003a). During the development of eggs within a queen, a diploid cell divides to generate haploid cells called gametes with half of the number of chromosomes. The result is a haploid egg, with chromosomes having a new combination of alleles at the various loci. The development from eggs to adults takes approximately 26 days for males and 30 days for queens (Amin et al. 2012). Both drones and new queens do not play a large role for the colony and are leaving the nest. The young queens leave the nest to forage to build up fat reserves, while the drones leave the nest permanently after about 5 days in search for a mate. These sexuals mate and the young queens go into diapause (Goulson 2010).

Competition point. When the colony continues to grow and reaches a switch point, the queen loosens control to dominate her workers and at a certain point workers begin to compete with their own queen, called competition point. Then, some of the workers will start laying haploid eggs, which results in drones. Also, the queen’s eggs are preyed upon by the workers. Once the drones and the new, young queens have left the nest, the colony rapidly degenerates and the old members of the colony (the males, workers and original queen) die at the end of the cycle. The next year, this colony cycle starts again as the young fertilized queens wake up when the spring temperatures are favorable (Goulson 2003a).

2.3.3. Foraging behaviour and diet

Like the honeybee, *B. terrestris* is a polyectic forager or generalist. All different life stages feed on pollen and nectar (Goulson, 2010). Pollen provides the necessary proteins for the development of ovaries, the production of eggs and larval growth of bumblebees. Nectar acts as an energy source. Both food sources are required for survival (Bailey and Woods 1974). The foraging range of *B. terrestris*, in comparison with other *Bombus* species, is quite large. Experiments with bees marked at the nest (Kreyer *et al.* 2004) and anecdotal observations suggest that species such as *B. pascuorum*, *B. sylvarum*, *B. ruderarius* and *B. muscorum* are mostly remaining within 500 m of their nests whilst *B. lapidarius* forage further afield (mostly < 1,500 m), and *B. terrestris* regularly forages over more than 2 km away from their nests

(Walther-Hellwig and Frankl 2000). However this is a general estimate and foraging distance depends also on other parameters such as landscape elements and size of the nest (Goulson et al. 2004). In an attempt to calculate the average foraging ranges of bumblebee species, Knight *et al.* (2005) used molecular markers and found that the maximum detected average foraging range was greatest for *B. terrestris* (758 m), least for *B. pascuorum* (449 m) and *B. lapidarius* (450 m), and intermediate for *B. pratorum* (674 m) (Knight et al. 2005).

Compared to other bee species, bumblebees are large and mostly covered with fur. As a result, bumblebees are capable of endothermy, which makes them equipped to live and forage in cool conditions (Peat and Goulson 2005, Dafni et al. 2010). They are also capable to endure other servere climat conditions for insects, as they can forage in light rain and wind speeds up to 70 km per hour. In comparison, the bumblebees *B. terrestris/lucorum*, *B. pascuorum* and *B. hortorum* start foraging at lower temperatures than *B. lapidarius* or honeybees (Goulson 2010).

2.4. Pollination service of wild bees

Recent evidence shows that for most crops wild bee pollinators are more effective pollinators than domesticated honeybees and provide the majority of pollination services, suggesting that honeybees supplement, but not replace the pollination services of wild pollinators (Garibaldi et al. 2013). Even for crops predominantly dependent on honeybees, wild pollinators can play an important indirect role by enhancing the pollination efficiency of honeybees through synergetic effects (Greenleaf and Kremen 2006a, b, Brittain et al. 2013). Indeed, Brittain et al. (2013) showed that increased pollinator diversity can synergistically increase pollination service, through species interactions that alter the behaviour and resulting functional quality of a dominant pollinator species. In view of increasing concern about declining numbers of honeybee colonies driven by colony losses and declining number of beekeepers (Potts et al. 2010, van der Zee et al. 2012), wild bees are expected to become increasingly important for pollination of crops in Europe (Breeze et al. 2014). Diverse wild bee communities improve the temporal stability of pollination service delivery and can provide insurance of pollination services under environmental change (Garibaldi et al. 2011, Bartomeus et al. 2013). For example, Holzschuh et al. (2012) showed that that pollination services by wild bees in cherry orchards surpassed pollination by honeybees, and Greenleaf and Kremen (2006b) reported that behavioral interactions between wild and honeybees increase the pollination efficiency of honeybees on hybrid sunflower up to 5-fold. Thus, currently, there is a growing awareness of the importance of wild bees in the pollination, not only of crops, but also of many plants in the ecosystem (Kleijn et al. 2015).

3. Causes of pollinator declines

3.1. Bee declines: current status

While the importance of bees both economically for their pollination service and as ecosystem service providers is increasingly becoming acknowledged, there is mounting evidence that many wild bee species have declined over the last decades in Europe (Patiny 2003, Biesmeijer et al. 2006, Nieto et al. 2014) and the world (Ghazoul 2005, Potts et al. 2010). For example, the bee communities of intensively farmed landscapes have been strongly impoverished (Kleijn et al. 2001, Dupont et al. 2011) and many previously widespread bee species in agricultural landscapes are now only found in nature reserves (Kohler et al. 2008) or (sub)urban refugia (Samnegard et al. 2011). It appears that in northwest Europe the rate of decline of wild bee richness has slowed down in recent years (Carvalheiro et al. 2013). However, as bee communities have become more homogenized during earlier periods of decline (Garibaldi et al. 2011), the reduced rate of species richness decline probably reflects that bee communities are currently dominated by a lower number of more resilient bee species that remained common (Kleijn et al. 2015).

3.2. Drivers of bee declines

Addressing the causes of pollinator declines, many drivers have been brought forward affecting pollinator abundance and diversity and this was observed on different scales (Ghazoul 2005, Potts et al. 2010). The most important drivers described in literature are 1) land-use change leading to a reduced resource availability (Biesmeijer et al. 2006), and the loss and fragmentation of habitats (Goulson et al. 2008, Winfree et al. 2009); 2) use of pesticides (Kevan et al. 1997, Mommaerts et al. 2010, Di Prisco et al. 2013); 3) non-native species and the spread of pathogens (Stout and Goulson 2000, Cox-Foster et al. 2007, Neumann and Carreck 2010, de Miranda et al. 2015), also due to beekeeping practices (Goulson and Hughes 2015) and 4) climate change (Williams et al. 2007, Dormann et al. 2008, Gonzalez-Varo et al. 2013, Vanbergen et al. 2013). Nevertheless, these factors rarely act alone (Didham et al. 2007) and a combination of different stressors such as parasites, pesticides and lack of flowers, is regarded to be more devastating in the light of their global losses (Fauser-Misslin et al. 2014, Goulson et al. 2015).

3.2.1. Loss of habitat and food resources

Since the second half of the 20th century, land use change and agricultural intensification have resulted in the loss and fragmentation of habitat, accompanied by increased use of pesticides and fertilizers (Stoate et al. 2001, Tscharntke et al. 2005). In the UK, Goulson et al.

(2008) depicted the increase of farming productivity by bringing unfarmed areas into production, in modern agricultural landscapes after 1950 as an important driver of forage loss for bees. This practice has led to major losses in grassland in Europe and North America (Fuller 1987, Howard et al. 2003, Hines and Hendrix 2005). For example, in the UK, over 90% of unimproved lowland was lost between 1932 and 1984 (Fuller 1987, Howard et al. 2003). Furthermore, since the introduction of cheap artificial fertilizers, crop rotations with legumes (*Trifolium* spp.) have been almost entirely abandoned (Goulson 2010). Since these leguminoses are highly preferred food sources for example for long-tongued bumblebees, the abandonment of the use of leguminous crops has highly contributed to their observed decline, also in Belgium (Goulson et al. 2005, Rasmont et al. 2006, Goulson 2010).

As bees are entirely dependent on flowers, these altered practices in agriculture leading to reduced forage have inevitably caused negative effects on bumblebees, wild bees and other populations of beneficial insects (Stoate et al. 2001, Goulson et al. 2010). Indeed, a quantitative review investigating the effects of different types of disturbances on bee communities identified habitat loss and fragmentation as the most import negative disturbances for bees (Winfree et al. 2009) and loss of floral resources is thought to be the main driver for bee decline in anthropogenic landscapes (Carvell et al. 2006, Winfree et al. 2011). Since many small landscape elements in the agricultural landscape and beyond such as hedgerows, old fields, scrublands, forests, roadside verges, shelterbeds, borders of streams, green lanes and unimproved and semi-natural grasslands can provide flowers throughout the season, they support a far greater number of foraging bees than modern monoculture landscapes (Stoate et al. 2001, Goulson 2010, Van Rossum and Triest 2012, Scheper et al. 2014)

3.2.2. Use of pesticides

As for honeybees and bumblebees, there is growing evidence that pesticides, especially neonicotinoids have a negative impact on bee growth, reproduction and survival (Wu et al. 2010, Cresswell et al. 2012, Laycock et al. 2012) and could play an important role in the decline of bee species in general (Cresswell et al. 2012, Easton and Goulson 2013). Neonicotinoids can be found in the main food source of bees, i.e. nectar and pollen, with a concentration from 1 to 23 ppb and 1 to 66 ppb, respectively (Goulson 2013), but since they are water soluble, also in glutation droplets on foliage (Blacquiere et al. 2012) and surface waters (Smit et al. 2015). Recently, different studies, with a focus on honeybees, have been performed showing a reduced fecundity of the queen, and reduced learning, foraging and homing ability of the workers (Blacquiere et al. 2012, Gill et al. 2012, Whitehorn et al. 2012, Goulson 2013, Lundin et al. 2015). Since these negative impacts reported, this eventually led to a recent restriction of frequently

used neonicotionoids of clothianidin, imidacloprid and thiamethoxam in Europe (EC 2013a, b, EFSA 2013, Smit et al. 2015, STEP 2016).

3.2.3. Climate change

Also climate change has an impact on the decline of pollinators (Williams et al. 2007, Dormann et al. 2008, Potts et al. 2010, Vanbergen et al. 2013). Climate change can have direct and indirect effects on species, colonies, populations and communities. At landscape level, climate change negatively affects pollinators through repercussions on the direct factors that regulate pollinator populations (Memmott et al., 2007; Iserbyt & Rasmont, 2012), such as the availability of nesting, mating and overwintering sites, changing the temporal activity of bees (Stone and Willmer 1989), changes in phenology, and by shifting climatic niches (Williams et al. 2007). In response to climate change, for many species, geographical ranges are expanding toward the poles, while remaining stable along range edges nearest the equator. Mean elevations of observations for southern species have risen ~300 m since 1974 and mean elevations among northern species in Europe and North America shifted lower (Kerr et al. 2015, Rasmont et al. 2015). This shift is also leading to altered availability of food resources and incidental risk factors (i.e. biotic and abiotic sources of mortality). Year after year, the local bee fauna can change (including local species extinctions) due to variations in local climatic factors such as heat waves and droughts. (Williams et al., 2007; Hegland et al., 2009; Iserbyt & Rasmont, 2012). An indirect effect of climate change is then found in a mismatch in temporal and spatial co-occurrence of plant and pollinator species (Schweiger et al., 2008; Hegland et al., 2009) or in lifecycle (Van Dyck et al. 2015).

3.2.4. Impact of non-native species and spread of pathogens

Since the domestication of honeybee hives and commercialization of reared bumblebees for pollination services, anthropogenic movement of managed bees happened within and outside their natural range. As a result of these transports, negative interactions with native species can occur, such as resource competition or spread of diseases. Indeed, if domesticated honeybee hives and reared bumblebee nests harbor parasites, they may act as a parasite reservoir from which the parasites can spillover to wild bee populations. Different types of parasite spillover can be defined, each with an intrinsic risk, as has been reviewed for bumblebees by Meeus et al. (2011). However, beside spread of diseases, many examples of interactions between domesticated and wild species spanning different sections of the animal kingdom have been reported in the last decades. In the light of this dissertation, an in depth view on possible impacts between native and domesticated species will be given in next section.

4. Interaction of domesticated and wild animals

4.1. General

Since the human population has expanded worldwide, humans have substantially contributed to anthropogenic changes of the environment which influence interspecific interactions over time (Tylianakis et al. 2008, Moleon et al. 2014). Within this larger phenomenon animal domestication can also (in)directly influence ecological interactions. These ecological interactions, such as predation, competition, parasitism and mutualism, are strong selective forces driving the evolution of populations, communities, and ecosystems. Table 1.1 gives an overview of the interaction between domesticated animals and their wild counterparts. In general, two main interactions can be drawn, i.e. 1) competition for resources, mostly for food and 2) spread of diseases. However, some other interactions exist such as behavioral interference or hybridization between species and these interactions can meet each other and interact witch each other. A good example exists in escaped domesticated American minks (*Neovison vison*), selected and reared for their fur, but when such escapees interact with wild mink populations, wild minks are threatened not only by competition for resources but also by hybidisation leading to reduced genetic variability in their offspring and a reduced fitness of the wild mink populations (Kidd et al. 2009).

It is important to distinguish between interactions of domesticated species with wild counterparts within their native range (interaction with sympatric species) and introduced non-native domesticated species interacting with native species (interaction with allopatric species). The latter mostly lead to a more extreme interactions, which will be described below.

4.2. Competition

Usually three conditions must be fulfilled in order to get interspecific competition. There must be overlap (1) in habitat use and (2) in food consumed, and (3) the shared resources must be limited (Tokeshi 1999). Information on overlap in resource use is thus central for the understanding of interspecific competition (Mysterud 2000).

Addressing first to sympatric interactions (introduction of domesticated animals in their native ranges), many examples of competition exist between domesticated and wild animals with a negative outcome for the latter. For example, domesticated sheep (*Ovis aries)* compete with wild chamois (*Rupicapra* sp.) for food resources in the European mountains: where sheep have pastured, no chamois will be pasturing as they typically prefer fresh grass pods (Fankhauser et al. 2008). However there are also few cases reporting neutral interactions. Such an example was reported by Sol (2008) when domesticated pigeons, selected for their flight properties, revert to the wild as feral populations. Then, when competing with wild pigeon (*Columba livia*) populations, they have the physical disadvantage of a tarsus that is disproportionally long for a

Table 1.1. Overview of possible interactions between domesticated and wild animals of different sections

Animal section	Domesticated species	Sympatric wild species	Key effect	Region	Reference(s)
Terrestric mammals	Rinder (*Bovini* sp.), cattle/ox (*Bos Taurus)*	Buffalo, eland, giraffe, kudo antilopes	Diseases (Rinderpest)	Africa	Mack (1970)
	American mink (*Neovison vison*)	Wild American mink (*Neovison vison*)	Competition, Hybridisation	Canada, Ontario	Kidd et al. (2009)
	Dogs (*Canis familiaris*)	Ethiopian wolf (*Canis simensis*)	Diseases (rabies)	Ethiopia	Hughes and Macdonald (2013)
		Mongolian gazelle (Procapra gutturosa), saiga (*Saiga tatarica mongolica*), argali (*Ovis ammon*)	Predation	Mongolia	Young et al. (2011)
	Cats (*Felis catus*)	Birds, wildlife	Predation,	World	van Heezik et al. (2010)
		Wildcats (*F. Silvestris*)	Hybridisation,	Scotland	Yamaguchi et al. (2004)
		Different wildlife	Diseases (*Toxmoplasma*)	USA, Illinois	Fredebaugh et al. (2011)
	Sheep (*Ovis aries*)	Reindeer (*Rangifer tarandus tarandus*)	Behavioral interference	Scandinavia	Colman et al. (2012)
		Chamois (*Rupicapra* sp.)	Competition	European mountains	Fankhauser et al. (2008)
Seafish	Reared Atlantic salmon (*Salmo salar*)	Wild Atlantic salmon (*Salmo salar*)	Competition	Figgjo river, Western Norway	Solberg et al. (2013)
			Hybridisation		Metcalfe et al. (2003) Hindar and Fleming (2007)
		Brown trout (*Salmo trutta*)	Interspecific Introgression		Castillo et al. (2008)
	Salmonids: e.g. catfish (*Diplomystes viedmensis*)	Native fish species	Diseases (*A. tumescens*; *L. salmonis*)	Argentinia, Lake Moreno	Kelly et al. (2009)
				Canada, Europe	Morton et al. (2004)
	Rainbow trout (*Oncorhynchus mykiss*)	Wild rainbow trout	Competition	Lake Huron, Canada	Martens et al. (2014)
		Native perca (*Percichthys trucha*)	Competition	Patagonia, Argentinia	Aigo et al. (2014)
Birds	Selected feral Rock pigeons (*Columba livia*)	Wild Rock pigeons (*Columba livia*)	Competition, positive survival fitness	Europe and N-America	Sol (2008)
Insects	Honeybees (*Apis mellifera*)	Native wild bees (different taxa)	Competition, Diseases	Non-native range	Stout and Morales (2009),
	Alfalfa leafcutter bee (*Megachile rotundata*)	Native Leafcutter bee species	Competition	Non-native range: N-USA, Australia, New-Zealand	Woodward (1996), Goulson (2003b)
	Bumblebees *Bombus terrestris*	Native wild bees	Competition	Non-native range: Tasmania	Buttermore (1997), Stout and Goulson (2000)
	Honeybees (*Apis mellifera*)	Bumblebees	Competition	UK	Goulson and Sparrow (2009)
		Bumblebees	Diseases viruses: DWV	'Europe'	Genersch et al. (2006)
			Nosema	UK	Fürst et al. (2014)
	Domesticated bees (*A. mellifera* and *B. terrestris*)	Wild bumblebees	Diseases (*A. bombi*, *C. bombi*)	UK	Graystock et al. (2014)

terrestrial pigeon, which shows limitations in the use of food resources and, as a result, experience lower survival fitness, leading to the reconstituting of the wild phenotype in feral pigeons. Beside, some typical interactions of allopatric domesticated and native wild species exist, such as those reported for introduced fish species. For example, introduced rainbow trout (*Oncorhynchus mykiss*) in Patagonia resulted in competition for food between native perca (*Percichthys trucha*) fish (Aigo et al. 2014).

4.3. Competition for resources between domesticated and wild pollinators

For pollinating insects there is mounting evidence of allopatric and sympatric interactions between domesticated and wild species. Given the generalist pollination character of *A. mellifera*, honeybees usually visit a hundred or more different species of plants within any geographic region (Pellet 1976, O'Neal and Waller 1984, Roubik 1991, Coffey and Breen 1997, Huryn 1997, Goulson 2003b), and in total they have been recorded visiting nearly 40,000 different plant species worldwide (Crane 1990). Consequently, competition for floral resources has been suggested (Stout and Morales 2009, Hudewenz and Klein 2013) due to (1) niche overlap (Steffan-Dewenter and Tscharntke 2000, Paini and Roberts 2005), and (2) more efficient flower visits, and collecting or depleting of nectar (Schaffer et al. 1983, Gross 2001, Dupont et al. 2004).

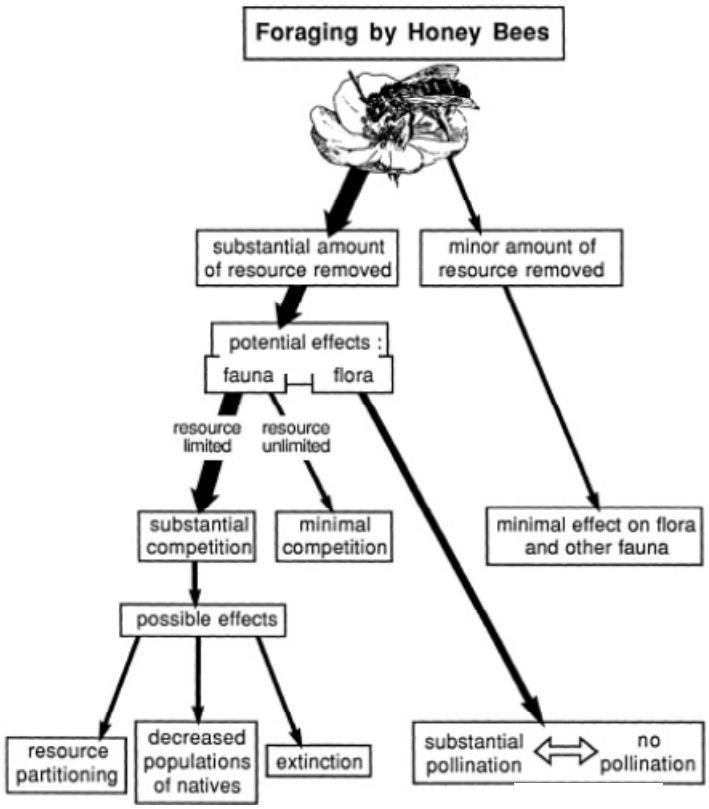

Figure 1.4. Potential effects of honeybee foraging on native plants and animals. Foraging by honeybees will have variable impacts on native flora and fauna depending on the amount of resources removed from flowers. Heavier arrows indicate a potential for stronger effects. Adapted from Huryn (1997)

Allopatric generalist honeybees introduced in their non-native range are likely to have a high interaction potential with native flower-visiting insects and bees (Sugden et al. 1996, Goulson 2003b, Stout and Morales 2009), and floral competition will depend on the amount and quality of resources removed from flowers, and limitation of the forage resource, as represented in figure 1.4 (Huryn 1997). Furthermore, when introduced, their vast number (a normal beehive contains between 30.000 and 60.0000 individuals) compared to small nest structures of less social wild bees will also lead to changes in patterns of abundance, activity and gathering of resources (Huryn 1997, Stout and Morales 2009). However, new evidence also indicate that bumblebees and honeybees do not share randomly their host-plant resources and niche partitioning has been reported (e.g. Leonardht & Blüthgen 2012). Honeybees than rather go for quantity (Leonhardt and Bluthgen 2012), while bumblebees go for quality of pollen (Ruedenauer et al. 2016). Beside for honeybees, interactions have also been noticed for other domesticated bee species outside their native range. The alfalfa leafcutter bee (*Megachile rotundata*), imported for the pollination of alfalfa and other crops (Goulson 2003b), has been observed competing with native leafcutter bees and spreading in N-America, Australia and New-Zealand (Woodward 1996, Goulson 2003b), while managed bumblebees (*B. terrestris*) have been found competing with native wild bees in Tasmania (Buttermore 1997, Stout and Goulson 2000, Hingston et al. 2002, Dafni et al. 2010).

Next to this, sympatric competition between domesticated and wild pollinators can also be expected, yet proof of this is largely lacking. Related to honeybees competition towards wild bees, only a few studies are available. Goulson et al. (2009) examined the size of wild *Bombus* as a proxy for competition between honeybees and four species of bumblebees (Goulson and Sparrow 2009). Beside, one study reports a possible direct competition between honeybees and bumblebees by measuring the impact on developing *B. terrestris* nests (Elbgami et al. 2014). However, this correlative study was achieved in only one location and thus final conclusions cannot be drawn.

4.4. Diseases, spillover and EIDs

One of the major issues arising when domesticated and wildlife animals interact is the rapid spread of diseases. Indeed, when anthopogenic change increasingly brings wildlife into contact with domesticated animals, parasite outbreaks often occur. Herewith transmission of infectious diseases or pathogens from reservoir populations (usually domestic or commercial) to sympatric wildlife populations is known as “spillover” (Daszak et al. 2000). A process termed ‘‘pathogen spillover’’ occurs when pathogens spread from a heavily infected ‘reservoir’ host population to a sympatric ‘non-reservoir’ host population (Daszak et al. 2000, Power and Mitchell 2004).

Pathogen spillover is the major driver of emerging infectious diseases (EIDs) in vertebrates (Daszak et al. 2000) and plants (Anderson et al. 2004). Thus, EIDs can be defined as newly identified species or strains that may have evolved from a known infection, or spread to a new population or area undergoing ecologic transformation, or by reemerging infections (Fauci 2005). In humans, the meticillin-resistant *Staphylococcus aureus* (MRSA) emerging in hospitals, and extremely problematic in that they have become resistant to many antibiotics is a prime example of an EID (Joffe et al. 2011). Generally, EIDs pose a risk to human welfare, both directly and indirectly, by affecting managed livestock and wildlife that provide valuable resources and ecosystem services. Addressing to bees, the movement of pathogens from domestic to wild organisms can lead to spillover of pathogens which can be linked with EIDs. Since EIDs can have detrimental effects on wild bee hosts (Daszak et al. 2000, Power and Mitchell 2004, Hughes and Macdonald 2013), spillover of pathogens is an important threat.

As described above, it is important to consider sympatric from allopatric spillover events, as the latter leads to the introduction of new diseases with possible severe outcomes for the native populations. In mammals, such a historical example can be found in the rinderpest epidemic of the 1890's in Africa; it swept through the whole continent in a time when the domesticated ox was introduced as the principal means of transport. In Africa, for example, the outcome was dramatic as 80-90 percent of cattle, buffalo, eland, giraffe kudo and antilopes died and in South-Africa alone the losses amounted to 21 million cattle (Mack 1970).

Next to this, also sympatric domesticated-wild species interactions can lead to the spread of diseases. Good examples have been reported in the 80ies of 20th century, when the rise of fisheries began and rearing of salmon, rainbow trout and other wild fish species became a common practice. Then, the spread of diseases and parasites between reared salmonids and wild fish species has been reported (Metcalfe et al. 2003, Hindar and Fleming 2007, Castillo et al. 2008, Solberg et al. 2013, Martens et al. 2014). For example, parasitic sea lice (*Lepeophtheirus salmonis*) spread into wild salmon (*Oncorhynchus spp*.) populations when commercially-reared fish escaped from infested salmon farms (Morton et al. 2004) which has been implicated in the demise of wild fish cohorts in both Canada (Morton et al. 2004) and Europe (McVicar 1997, Colla et al. 2006).

Within Apoidea pollinator species, many pathogens could also lead to EIDs and thus pose a major thread, especially when introduced allopatric pollinators carry pathogens that become emergent in native pollinator populations, but also sympatric interactions mediate in the spread of EIDs (Goulson and Sparrow 2009, Meeus et al. 2011, Fürst et al. 2014). This will be discussed in dept under part 6.

5. Overview of Apoidea-associated diseases

In this chapter we focus on the diseases in Apoidea bees. Our aim was not to give a complete overview of all pathogens associated with Apoidea hosts. Parasites such as *Varroa* are only known to to attact honeybee hosts and not bumblebees. Besides, bacterial pathogens such as *Paenibacillus larvae* (American Foulbrood) or *Melissococcus pluton* (European Foulbrood) and other fungal pathogens, are typically associated with honeybee hosts and they were not the focus of this dissertation. For example, *Paenibacillus larvae* is only found sporadically in bumblebees (probably not infective and associated with honeybee collected pollen). As the main subject of this dissertation is to focus on viruses and gut pathogens associated with multiple Apoidea hosts, we focus on viruses and gut associated parasites; We start the overview with Apoidea-associated viruses.

5.1.Viruses

Since around 1960, different viruses have been found and described in the honeybee as host species. Currently, in the honeybee host there exist at least 24 viruses described (de Miranda et al. 2013). In contrast, in bumblebee species and especially wild bees, there are less viruses known (Ravoet et al. 2014). However, this probably reflects more the search efforts and focus on honeybee host as study objects in previous studies. Of the 24 known viruses, some important ones, which will be used in the research context of this dissertation, are summarized in table 1.2.

5.1.1. Poxviridae

Entomopoxvirinae

This virus was discovered in 1982, during a search among flower-associated insects for reservoir hosts of diseases of honeybees. Tiny particles were seen in the haemolymph, in some cells of the body wall, and, most abundantly, in the thoracic salivary glands of three bumblebee species, *Bombus impatiens*, *Bombus pensilvanicus* and *Bombus fervidus* (Clark 1982). Most of the infected bumblebees have severe infections within their hemolymph system. Most of the Entomopoxvirinae were infecting the larval stage and develop slowly when the individual ages. This virus has only been detected in bumblebee hosts, not in *A. mellifera* (Macfarlane et al. 1995).

Table 1.2. Overview of Apoidea-associated viruses

Virus	Abbr.	Family	Host *	Infecting stage reported	Reference(s)
Entomopoxviridae	-	*Poxviridae*	BB.	Larva	Clark (1982).
Acute bee paralysis virus	ABPV	*Dicistroviridae* (AKI-complex)	HB., BB.	Pupae and adult	Bailey et al. (1962), Meeus et al. (2014).
Kashmir bee virus	KBV	*Dicistroviridae* (AKI-complex)	HB., BB., W.b.	Pupae and adult	Bailey and Ball (1991), Anderson (1991), Meeus et al. (2014).
Israeli acute paralysis virus	IAPV	*Dicistroviridae* (AKI-complex)	HB., BB.	Pupae and adult	Singh et al. (2010), Meeus et al. (2014).
Black queen cell virus	BQCV	*Dicistroviridae*	HB., BB., W.b.	Pupae and adult	Bailey and Ball (1991), Peng et al. (2011), Ravoet et al. (2014).
Alphid lethal paralysis virus	ALPV	*Dicistroviridae*	HB., BB.	Adult	van Munster et al. (2002), Granberg et al. (2013).
Big Sioux river virus	BSRV	*Dicistroviridae*	HB.	Adult	Runckel et al. (2011).
Slow bee paralysis virus	SBPV	*Iflaviridae*	HB., BB.	Adult	Bailey and Woods (1974), McMahon et al. (2015).
Sacbrood virus	SBV	*Iflaviridae*	HB., BB., W.b	Larvae, pupae and adult	Bailey et al. (1964), de Miranda et al. (2011), Ravoet et al. (2014).
Deformed wing virus	DWV	*Iflaviridae*	HB., BB., W.b.	Adult	Genersch et al. (2006), Ravoet et al. (2014).
Lake Sinai virus	LSV	Non-classified	HB., W.b.	Adult	Runckel et al. (2011), Ravoet et al. (2014).
Bee (formerly *Varroa destructor*) Malula-like virus	BeeMLV (VdMLV)	Tymoviridae	HB., W.b.	Adult	de Miranda et al. (2011), Rosenkranz et al. (2010), Ravoet et al. (2014).
Chronic bee paralysis virus	CBPV	Non-classified	HB., BB.	Adult	Bailey et al. (1968), Grzeda et al. (2014).

* Abbreviations "BB." = Bumblebee; "HB." = Honeybee; "W.b." = Wild bee species

5.1.2. Dicistroviridae

Different dicistroviridae are present and replicate within bees. Here we discuss the 'AKI-complex, i.e Acute bee paralysis virus, Kashmir bee virus and Israeli acute paralysis virus, Black queen cell virus and some other less frequent viruses.

Acute bee paralysis virus (ABPV) /Kashmir bee virus (KBV) / Israeli acute paralysis virus (IAPV)

These three important viruses are tentatively clustered into the AKI-complex (de Miranda and Genersch 2010). While these viruses cluster together, different symptoms can be observed. For example, infection with IAPV resulted in shivering, whereas bumblebees infected with KBV were quickly dying (Meeus et al. 2014). Bailey and Ball (1991) also report some differences in

phenology. ABPV is mostly active during summer, whereas KBV peaks during autumn. Beside, Bailey et al. (1963) described ABPV as an inapparent infection in honeybee species and in bumblebees (Bailey and Gibbs 1964). KBV was also first described in honeybees, but was later detected in yellow jacket wasps (*Vespula germanica*) in Australia (Anderson 1991). In general, IAPV and KBV can be regarded as multiple-host viruses as they have been detected in honeybees, bumblebees, solitary bees and social wasps (Manley et al. 2015).

An interesting observation related to the needed dose to obtain an infection has been demonstrated for IAPV, in that injection of low particle numbers, as low as 20 particles of IAPV in bumblebees results in death bees after 7 days (Niu et al. 2016), while for oral administration the same effects can be observed, but a high amount of virus particles is needed, resulting in dose dependant effects of infection pathways of the virus (Meeus et al. 2014, Piot et al. 2015).

Black queen cell virus (BQCV)

Black queen cell virus (BQCV) was first isolated from dead queen larvae and prepupae sealed in queen cells whose walls had turned dark brownblack (Bailey and Woods 1977). Diseased pupae are initially pale yellow and have a tough sac-like skin similar to those of sacbrood-infected larvae. The BQCV-infected pupae rapidly darken following death, eventually turning the walls of the queen cell dark brown to black, thus producing the characteristic symptom of BQCV infection. BQCV symptomatic drone pupae have also been observed (Siede and Buchler 2003). BQCV has a worldwide distribution in *Apis mellifera* (Allen and Ball 1996, Ellis and Munn 2005) and persists within the colony as asymptomatic infections in worker bees and brood (de Miranda et al. 2011). Although BQCV can occasionally be detected in *Varroa destructor*, no active vectorial relationship between BQCV and Varroa mites has been observed. More recently, it has also been detected in bumblebee species, while it remains unclear whether a real infection has been observed (Singh et al. 2010, Glover et al. 2011, Gamboa et al. 2015).

Aphid lethal paralysis virus (ALPV)

Aphid lethal paralysis virus (ALPV) is a common intestinal dicistrovirus of several major agricultural aphid pests, associated with aphid population declines (Laubscher and Vonwechmar 1992, van Munster et al. 2002). This virus can be detected infrequently at very low background levels in adult honeybees throughout the year, with a sharp quantitative increase during late summer (Runckel et al. 2011) when bees often feed on honeydew (aphid excreta) during low nectar flows. Latest insight indicates ALPV is rather incidental than truly infectious in bees (de Miranda et al. 2013).

Big Sioux River virus

Big Sioux River virus (BSRV) is genetically closely related to *Rhopalosiphum padi* virus (RhPV; Moon et al. (1998)), a common intestinal Dicistrovirus that uses the plant vascular system to transmit horizontally between aphids (Gildow and Darcy 1990). This virus can be detected infrequently at very low background levels in adult honeybees throughout the year, with a sharp quantitative increase during late summer (Runckel et al. 2011) when bees often feed on plant leaves with honeydew during low nectar flows. However, this virus has only been reported in honeybees so far. Together with ALPV, it is unclear whether these two viruses are incidental or truly infectious in bees.

5.1.3. Iflaviridae

Three important bee viruses have been classified under the Iflaviridae: Slow bee paralysis virus, Sacbrood virus and Deformed wing virus (de Miranda and Genersch 2010).

Slow bee paralysis virus (SBPV)

Slow bee paralysis virus (SBPV) was discovered fortuitously in England in 1974 during propagation experiments with bee virus-X (de Miranda et al. 2011). SBPV is characterised by the paralysis of the front two pairs of legs of adult bees, a few days before dying, after inoculation by injection (Bailey and Woods 1974). Despite its early suggested association with *V. destructor* (Bailey and Ball 1991), SBPV is rarely detected in bee colonies (Bailey and Ball 1991, de Miranda et al. 2010). SBPV can also be detected in larvae and pupae of honeybees, but produces no symptoms in these. Furthermore, this virus has been found in non-*Apis* hosts (Levitt et al. 2013), but only with a low prevalence in bumblebees of *Bombus pascuorum* (McMahon et al. 2015).

Sacbrood virus (SBV)

Sacbrood virus (SBV) was first described by White (1913) and was later proven to be the causative agent of a larval disease named sacbrood (Bailey et al. 1964). Sacbrood virus (SBV) is most commonly seen in diseased honeybee larvae in springtime, but the disease normally clears quickly. The clearest symptoms of sacbrood virus (SBV) appear a few days after capping, and consist of non-pupated pale yellow larvae, stretched on their backs with heads lifted up towards the cell opening, with a saclike larval skin containing a clear, yellow-brown liquid. The virus is also present in adult honeybees, but without symptoms (Lee and Furgala 1967, Bailey 1968, de Miranda et al. 2013). Recently, the virus has also been detected in bumblebee adults, (Singh et al. 2010, Levitt et al. 2013, Reynaldi et al. 2014), while it remains unclear whether a real infection has been observed.

Deformed wing virus (DWV)

The symptoms for deformed wing virus (DWV) are very typical and infected bees have crippled wings and bloated abdomen. Asymptomatic honeybees can also be heavily infected, though with lower titers than symptomatic bees (Lanzi et al. 2006, Tentcheva et al. 2006). The virus is detected in all other life stages of honeybees as well, but without obvious symptoms (Genersch et al. 2006, Lanzi et al. 2006, Tentcheva et al. 2006, Yue et al. 2006, de Miranda et al. 2011). DWV also affects sensory response, learning and memory in adults (Iqbal and Mueller 2007). In 2006, Genersch et al. (2006) reported the discovery of DWV in two bumblebee species (*B. terrestris* and *B. pascuorum*) in Europe, with symptoms comparable to those described in honeybees. When screening both honeybees and bumblebees, DWV is found with a higher prevalence in honeybees, and has been linked with *Varroa* in its transmission (McMahon et al. 2015).

5.1.4. Thymoviridae

Bee Malula-like virus (BeeMLV)

Bee Malula-like virus (BeeMLV), formerly known as *Varroa destructor* Malula-like virus (VdMLV) has been described recently (de Miranda et al. 2015). The differences of viral titers recorded in mite populations suggest that different types of interaction exist between this honeybee virus and *V. destructor*. Currently, the only hosts where the virus has been found are honeybees (de Miranda et al. 2011) and two wild species of *Osmia* collected near apiary sites (Ravoet et al. 2014), but not in bumblebees.

5.1.5. Unclassified

Chronic bee paralysis virus (CBPV)

Chronic bee paralysis virus (CBPV) manifests itself in adult honeybees through two distinct sets of symptoms. One set consists of trembling of the wings and bodies and a failure to fly, causing them to crawl in front of the hive in large masses. They often have partly spread, dislocated wings and bloated bodies as well. The other set of symptoms consists of hairless, greasy black bees caused by nibbling attacks from healthy bees in the colony. They soon also become flightless, tremble and die (Bailey et al. 1968, Bailey and Ball 1991, Ribière et al. 2010). The virus also infects the larval and pupal stages, can be detected in faecal material and is efficiently transmitted through contact and feeding (Ribière et al. 2010, de Miranda et al. 2013).

Lake Sinai virus (LSV)

Lake Sinai virus-1 (LSV-1) and Lake Sinai virus-2 (LSV-2) are two closely related viruses that were identified through a metagenomic sequencing survey of honeybee colonies in the USA (Runckel et al. 2011). Beside, a series of other clades ('LSV 3' until 'LSV 7') has been described recently based on different partial genomic sequences (e.g. Daughenbaugh et al. (2015)), but there is currently no consensus about complex nomenclature for this virus. Yet, according to their genome organization and sequences, it should be placed together with CBPV, in a unique family somewhere between the Nodaviridae and Tombusviridae. LSV-1 is more common than LSV-2, and present throughout the year with a peak in early summer (Runckel et al. 2011). LSV-2 has a very sharp incidence and abundance peak in the late winter with a low incidence and abundance the rest of the year. These viruses have also been detected, with similar incidences and titers, in old European honeybee samples (de Miranda et al. 2013), and more recently in non-Apis bees including bumblebees in South-America (Colombia) (Gamboa et al. 2015) and solitary bees in Europe (Ravoet et al. 2014, Ravoet et al. 2015a). Ravoet et al. (2014, 2015) also reported other clades different from the first described LSV-1 and LSV-2, which will be further discussed in Chapter 6 of this dissertation.

5.2. Protozoa

5.2.1. Trypanosomes and gregarines

Protozoa are single-celled eukaryotes that commonly show characteristics usually associated with animals, most notably with traits of mobility and heterotrophy (Tanada and Kaya 1993). They are classified in several phyla, and most of the protozoa are free-living organisms which can be found in almost every possible habitat. More than 50,000 species have been described so far and about 500 are parasites of insects (Tanada and Kaya 1993). Protozoans are grouped based on their shape and mobility and can be divide into *Gregarines* and trypanosomes.

Gregarines, a group that infects invertebrate hosts, have been placed under the phylum *Apicomplexa*. These are further divided into two groups, the eugregarines and neogregarines with a major difference that the former lacks a vegetative reproduction stage in the host (Kreier and Baker 1991). After infection and entering the host cell, neogregarines undergo multiple divisions, which are called schizogony or merogony. The resulting "merozoites" spread the infection to other tissues in the host until they undergo sexual reproduction. The transmission of

neogregarines then occurs via contaminated food or by cannibalism of infected hosts (Lipa and Triggiani 1996). An important infecting neogregarine in Apoidea bees, is *Apicystis*.

Another group of protozoans infecting bumblebees are classified under the genus *Trypanosoma*. Important members infecting honeybees and bumblebees have long been classified under the genus *Crithidia*. However, since the use of molecular detection techniques, Schmid-Hempel and Tognazzo (2010) found that *Crithidia*-infected bumblebee samples clustered in two different lineages and he introduced a new species called *Crithidia expoeki.* More recently, also Schwarz et al. (2015) found that *C. mellifica* in honeybees clustered into different lineages and described a new parasite under a new genus, named *Lotmaria passim*.

5.2.2. Apicystis

The genus of *Apicystis* belongs to the family of Ophryocystidae in the order of neogregarines. In *Bombus* sp., this neogregarine, formerly known as *Mattesia bombi,* was first discovered in Canada in 1974 and is reclassified in 1988 as *Apicystis bombi.* Worldwide, neogregarines have been reported in beetles (Lord 2007) and in the flour moth *Ephestia kuehniella* (Valigurova and Koudela 2006). Meanwhile, *Apicystis* has been recorded in over 20 *Bombus* sp. (Macfarlane et al. 1995, Jones and Brown 2014, Gamboa et al. 2015), including commercially reared species (Graystock et al. 2013b, Murray et al. 2013, Graystock et al. 2015a) and honeybee hives (Ravoet et al. 2013, Graystock et al. 2014, Graystock et al. 2015a). The life cycle of *A. bombi* shows an asexual and sexual reproduction phase as represented in figure 1.5, leading to the formation of oocysts. When these oocysts (a.) are ingested, sporozoites (b.) start to grow in the intestine and migrate through the midgut wall into the body cavity, further infecting the fat body. When they further grow, they undergo merogony forming meronts (d./e.: Type I meront; asexual phase) until they reach the sexual phase (f.: Type II meronts) and merozoites enter into sporogony producing both micro- and macrogamonts (g./h.) to finally forms a zygote (i.) that grows to produce again oocysts (j./k.) and starting a new infection cycle (Lipa and Triggiani 1996).

When *A. bombi* is infecting bumblebee queens, this neogregarine can have a major impact in that it destroys the fat body due to proliferation. Consequently, it has been suggested that infected spring queens have a lower start-up success (Macfarlane et al. 1995, Rutrecht and Brown 2008). Recently, single and co-infection experiments of *B. terrestris* workers, with feeding *A. bombi* oocysts and injecting RNA virus (DWV), showed that *A. bombi* exhibits both lethal and sublethal effects. Indeed, together with DWV it rather causes a shift from sublethal effects, imposed by a single infection of *A. bombi*, to a lethal effect (Graystock et al. 2016).

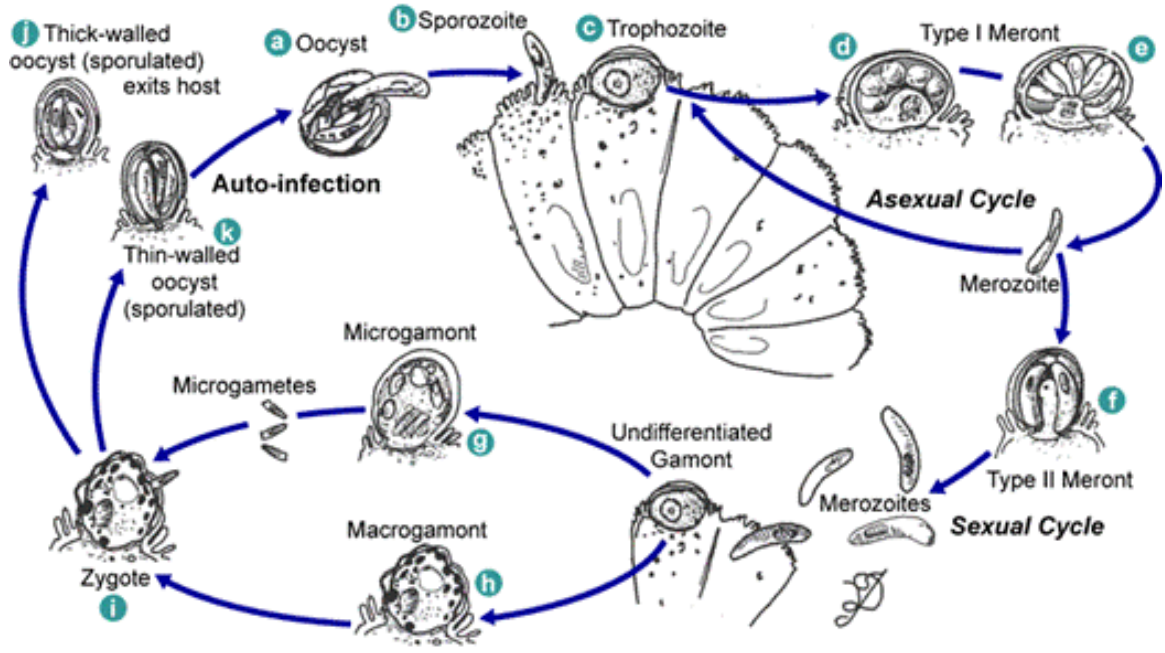

Figure 1.5. Schematic representation of reproduction cycle of protozoan parasites, e.g. *Apicystis* (Cuomo et al. 2012)

5.2.3. Crithidia

Crithidia belong to a genus of flagellate parasites, exclusively infecting arthropods, and mainly insects. Most of these entomogenous flagellates belong to the family *Trypanosomatidae* under the class of kinetoplastea. These parasites are named after one of their unusual features, which is the kinetoplast DNA (kDNA). Unlike nuclear DNA in nature, kDNA comprises a giant network of interlockated mitochondrial DNA, while normally mitochondria are dispersed in the cell. Until a few years ago, literature was dominated by two species, one associated with honeybees, *Crithidia mellifica* (Langridge and McGhee 1967); and one associated with bumblebee hosts, named *Crithidia bombi* (Tanada and Kaya 1993), both having a single host cycle (Schlüns et al. 2010). Schmid-Hempel and Tognazzo (2010) found that after screening a series of *Crithidia*-infected bumblebees, samples clustered in two different lineages "A" and "B" and they split the species introducing *Crithidia expoeki* as a second species (lineages "B"). In honeybees, Schwarz et al. (2015) also found that *C. mellifica* clustered into different lineages and described a new parasite under a new genus, named *Lotmaria passim*. Analyses of new and previously accessioned genetic data show *C. mellificae* is still extant in bee populations, however, *L. passim* is currently predominant in *A. mellifera* globally. Recently, *L. passim* has also been detected in mason bees (*Osmia bicornis* and *O. cornuta*) (Ravoet et al. 2015b).

However, while different trypanosomid species in different hosts have been described now, distinguishing between them is possible applying a strand-specific PCR. Moreover, in bumblebees, it seems that the formerly described “lineage A”, i.e. *C. bombi*, is most frequently observed (Schmid-Hempel and Tognazzo 2010) and is widespread, and Brown et al. (2003a) reported a context dependent virulence in bumblebee queens. Generally, transmission of *Crithidia* between hosts usually happens by ingestion of faeces. Beside, horizontal transmission between colonies can also occur via shared flowers (Durrer and Schmid-Hempel 1994).

5.3. Microsporidia: *Nosema*

Microsporidia are another phylum of parasitic eukaryotic organisms, classified under the fungi. The Microsporidia are obligate intracellulair parasites with a wide range of hosts, including insects (Schmid-Hempel and Stauffer 1998, Wittner and Weiss 1999, Natsopoulou et al. 2015). The best known Microsporidia genus is *Nosema*. For decades, ‘nosemosis’ as a disease of honeybees was exclusively attributed to a single species, *Nosema apis*, which was first described by Zander (1909) in the European honeybee, *A. mellifera*. In 1996, a new species of *Nosema* was first discovered in the Asian honeybee, *Apis cerana*, thus named *Nosema ceranae* (Fries et al. 1996, Higes et al. 2007). In bumblebees, Fantham and Porter (1914) were the first to describe a microsporidium, naming it *Nosema bombi* after the type host *Bombus agrorum*. Hereafter, it was reported that *N. bombi* was found in various *Bombus* sp. in Europe and North America (Macfarlane et al. 1995). Recently, it was discovered that, beside *N. bombi*, bumblebees can also be infected by *N. ceranae* leading to a possible dual infection status of *Nosema* in bumblebees (Graystock et al. 2014).

The main transmission mode for *Nosema* is thought to be trough horizontal transmission, as this parasite produces dormant long-living spores; however there is also some evidence reported for vertical transmission routes (Rutrecht and Brown 2008). Within bumblebee colonies, *Nosema* is transmitted via infected workers, contaminating shared food sources of pollen and nectar. For *N. bombi*, a clear and different pathology has been described in *B. lucorum* (Rutrecht and Brown 2008) and *B. terrestris* (Otti and Schmid-Hempel 2008). In the latter species, it is a systemic disease causing a significant negative impact on the colony development. In *B. lucorum*, the pathology was less pronounced, probably due to the shorter life cycle which did not result in a clear infection, while this can still have a negative impact on the next generation. Beside, *Nosema* has also been detected in other *Bombus* sp., probably damaging many other hosts (Whittington and Winston 2003).

6. Disease spillover and EIDs within Apoidea

6.1. Diseases and spillover within Apoidea hosts

Bees and their nests naturally carry a diverse microbiota including commensal, mutualistic and pathogenic organisms, the latter including protozoans, fungi, bacteria and viruses (Gilliam and Taber 1991, Goerzen 1991, Gilliam 1997, Goulson and Hughes 2015). Some bee parasites, such as Deformed Wing Virus and *Nosema ceranae*, have broad host ranges and are able to infect both honeybees and bumblebees and wild bees whilst others, such as *Paenibacillus larvae*, are seemingly specific to a certain host genus (Genersch et al. 2006, Hamdi et al. 2011, Graystock et al. 2013a). Given these differences, other mechanisms can play in different hosts influencing the population dynamics of their bee hosts, but the extent is still poorly understood (Goulson and Hughes 2015). Moreover, when hosts are brought into contact with new diseases, this can disrupt established host-pathogen interactions, which is likely to occur due to transport of bees for their pollination services to many parts in the world, as has been described supra (part 1.).

The management of important crop pollinators as honeybees and bumblebees leading to the transportation and introduction of domesticated bees carrying a vast range of associated diseases is the basis to generate a spillover of new diseases to their wild counterparts (Goulson and Hughes 2015, Graystock et al. 2015a). Moreover, invasion by non-native parasites has the potential to lead to more dramatic effects since we would expect their novel hosts have little resistance (Daszak et al. 2000, Rosenkranz et al. 2010).

However, since there are differences in transportation routes for domesticated honeybees and bumblebees, and both have different management methods (bumblebee nests are produced indoors while honeybee hives are managed outdoors), both carry a different cocktail of shared and specific associated diseases (Goulson and Hughes 2015). This leads to a complex situation for spillover and EIDs. Here, we will divide spillover events between domesticated honeybees and bumblebees in its non-native range from spillover in their native range.

6.2. Spillover from allopatric domesticated bees to native bees

The best-documented examples of invasions by non-native parasites are in the honeybee (Stout and Morales 2009). The spread of most honeybee parasites has occurred inadvertently as a result of transporting honeybees over long distances around the world (Goulson and Hughes 2015). Being social insects, honeybees live in compact, highly organized and productive colonies consisting of 30,000 up to 60,000 individuals. This social organization and the close interactions among colony members makes them highly susceptible to a variety of infectious diseases,

among which viral and protozoan pathogens are emerging as a serious threat to their health and survival (Chen and Siede 2007, Genersch and Aubert 2010). Indeed, the honeybee diseases, such as chalkbrood, caused by the fungus *Ascosphaera apis*, foulbrood, caused by the bacteria *Paenibacillus larvae*, the microsporidian *Nosema apis*, and the mite *Varroa destructor* now occurs on most parts of the world (Graystock et al. 2015a). Especially the spread of the *Varroa* mite has mediated in the invasion of some major diseases within honeybees (Goulson and Hughes 2015). This ectoparasite was originally associated with the Asian honey bee, *Apis cerana*, which has jumped hosts to the European honey bee *A. mellifera*, a naive host which had little resistance (Goulson and Hughes 2015). Since the 1960s it has spread from Asia to Europe, the Americas and New Zealand (Rosenkranz et al. 2010). Apart from weakening its host (Ellis and Munn 2005), this mite also vectors pathogens such as DWV, and the combined effect of the mite and such diseases is a major contributor to honeybee colony losses in North America and Europe (Rosenkranz et al. 2010, Nazzi et al. 2012). Due to their generalist foraging behaviour, overlapping niches and ranges, honeybees have a good chance to come into direct contact with wild congeners (Manley et al. 2015) leading to the possible spillover of diseases they carried along towards native bees. For example, in the Americas, Singh et al. (2010) detected DWV, BQCV, SVB, IAPV and KBV in bumblebees collected near honeybee apiaries, while the direction of spillover can be bi-directional for multi-host pathogens such as DWV (see 6.3). Also the *Apis*-associated microsporidian *N. ceranae* has been reported to have jumped host to infect conspecific bumblebees found in Patagonia (Plischuk and Lange 2010), China (Li et al. 2012), and recently in *Bombus attratus* in Columbia (Gamboa et al. 2015).

Beside to honeybees, Apoidea-associated parasites are also being redistributed around the globe by the commercial trade in bumblebee colonies, and thus also commercially-reared bumblebee nest can cause spillover towards wild counterparts. The first documented spread of a non-native parasite with commercial bumble bees was of the tracheal mite *Locustacarus buchneri* (Goka et al. 2006). *B. terrestris* imported from Europe to Japan in the 1990s were frequently infested with the tracheal mite (Goka et al. 2001). Although this mite also occurs in Japan, the European race is genetically distinct. In addition to importing *B. terrestris*, queens of the native *B. ignitus*, were sent to Europe to establish a commercial stock, and the established nests re-imported back to Japan have been found to be infected with the European race of the mite and by 2001 mites of the European haplotype were identified in native Japanese bees. Beside, it has been reported that both honeybee (*N. ceranae*) and bumblebee parasites (*N. bombi*, and *A. bombi*) carried by commercially produced bumblebees were infectious in other bumblebees (Graystock et al. 2013b). The potential spread of parasites to wild species has also been investigated by Colla et al. (2006) and Otterstatter et al. (2008) in North America. They monitored wild bumblebee populations near greenhouses for evidence of parasite spillover. In a

pioneering experiment, Colla et al. (2006) showed that bumblebees collected near commercial greenhouses were more frequently infected by those pathogens transmitted at flowers (*C. bombi* and *N. bombi*) than bees collected at sites away from greenhouses, giving a first indication that spillover was possible. A combination of field observations and modeling suggested that waves of *C. bombi* infection can travel outwards from glasshouses containing commercial bumble bees (Otterstatter and Thomson 2008). Beside, in South America Schmid-Hempel et al. (2014) demonstrated that the introduced *B. terrestis* has spread together with protozoan parasite of *C. bombi*, possibly mediating in the disappearance of native *B. dahlbombii*. More recently, in Argentina, Maharramov et al. (2013) investigated the spillover of *A. bombi* from introduced bumblebee nests towards native captured bumblebees. They found that the European haplotype was more common, but did not find final proof of a direct spillover. What is known, however, is that certain parasites indeed have the ability to spillover, and that spillover harbors an intrinsic risk for native species (Meeus et al. 2011).

Today, it should be remarked that mitigation measures are set in bee producing companies (Goulson and Hughes 2015). Beside, the structure of large companies (e.g. Biobest nv.) has changed, with installation of local daughter companies in different parts of the world producing local bee species, which has lead to the reduction of worldwide bee transport. For example: *B. terrestris* has been replaced to local production of *B. ignites* in Japan, and by local production of *B. attratus* in N.-America. While this change has been stated by large companies (For Biobest: personal communication R&D director Felix Wäckers), we have no information how the situation is in smaller companies also producing bees for pollination services. Next to this, the legislation related to bee transport has not been changed, making export of *B. terrestris* from Europe to other parts in the world is still legal with possible negative outcomes as mentioned here.

6.3. Spillover between sympatric domesticated and wild bees

While spillover leading to EID outbreaks are more likely to occur in native allopatric wild hosts (Rosenkranz et al. 2010), also sympatric spillover to wild bees within the natural range of domesticated bees are possible. Less studies are available regarding spillover in its native range, but in a recent study, Fürst et al. (2014) examined geographic patterns of prevalence of *N. ceranae* and DWV in honeybees and wild bumblebees in the UK. Indeed, on proof of its multi host status, these researchers found strong evidence that pathogens regularly transmit between the two hosts but that the majority of flow is from the managed honeybees (which tend to be more numerous) into the wild bumblebees. Recently, also McMahon et al. (2015) investigated the risk of transmission between co-occurring domesticated and wild pollinator species across a wide range of RNA viruses (BQCV, DWV, ABPV, SBPV, SBV, CBPV) and these authors concluded that *A. mellifera* is the preferred source for these viruses in sympatric wild pollinators.

In domesticated bumblebees, Whitehorn et al. (2013) found no evidence of such spillover from commercial bumblebees in the UK, but in Ireland, Murray et al. (2013) reported that bumblebees from commercial hives had markedly higher frequencies of two gut parasites, *Crithidia* spp. and *N. bombi*, compared to adjacent populations, but were free of tracheal mites. The highest prevalence of *Crithida* was observed within 2 km of the greenhouses but the effect was seen up to a distance of 10 km. Later in the UK, Graystock et al. (2014) found *A. bombi*, *C. bombi* and *N. ceranae* to be higher close to greenhouses supplemented with commercially bumblebee nests for pollination than near those that were not.

Generally, what is clear is that the widely reported threats to honeybees is likely to jeopardize the lives of sympatric wild bumblebees (Fürst et al. 2014) and of lesser-known wild bee species (Graystock et al. 2015a). That some of these viruses and parasites are also found in the broader pollinator community suggests the wider environmental spread of these viruses and that domesticated bees intermingle with many other native pollinators as well, resulting in significant potential for interspecific transmission of parasites during shared flower use (Durrer and Schmid-Hempel 1994, Singh et al. 2010, Evison et al. 2012, Levitt et al. 2013).

6.4. Parasite "spillback"

Next to "spillover", there is also the concept of "spillback". The theory behind has been set forward by Kelly et al. (2009) and these authors hypothesize that "parasite spillback" could occur when a nonindigenous species is a competent host for a native parasite. Then the presence of the additional host could increase disease prevalence in native species. According to these authors, and after reviewing the animal-parasite literature they showed that native species (arthropods, parasitoids, protozoa, and helminths) accounted for 67% of the parasite fauna of nonindigenous animals belonging to a range of taxonomic groups. They further showed that nonindigenous species can be highly competent hosts for such parasites and provided evidence that infection by native parasites does spillback from nonindigenous species to native host species, with effects at both the host individual and population scale (Kelly et al. 2009). An example of this principle is demonstrated for domesticated cats and dogs in relation to native felids: Studies of domestic cats and dogs in South America indicate that they are important reservoirs for spillover of *Toxoplasma gondii* (the aetiological agent of Toxoplasmosis) to native wildlife (Fiorello et al. 2006), with the increased ubiquity of *T. gondii* in native felids being attributed to the introduction and increase in abundance of domestic cats since the 16th century (Lehmann et al. 2006). However, recent phylogeographic studies indicate that *T. gondii* has a South American origin in wild native felids (Lehmann et al. 2006, Dubey et al. 2007). If this is indeed the case, then these striking infection patterns are due to spillback, not spillover.

7. Dynamics of multi-host interactions and their impact on wildlife of bees

7.1. Pathogens: defining one-host versus multihost interactions

7.1.1. Virulence and single host-pathogen interaction

To understand the complexity of multi-host interactions, we first look at the pathogenicity of a single pathogen (parasite or virus) and its host (the multicellar insect). In a first step we describe the host-pathogen interaction in a simplified version of reality, i.e a single pathogen in a single host system. Importantly, even within this simplified context, we cannot speak about one fixed virulence, defined as the parasite-induced fitness loss of the host. Indeed, different host individuals will react in a different manner to the different individuals of the same pathogen. Having this idea in mind, we can understand that variability of host and pathogen will change host-pathogen interactions and will have profound effects on the ecology and evolution of the pathogen, but also of the host (Schmid-Hempel and Koella 1994). The drivers of this variability largely depend on the level of ecological organization where variability occurs: variability of pathogens within their individual hosts, variability of host individuals within populations, or variability of hosts and pathogens among populations, i.e driven by genetic, age, social or behavioural differences of the host but also genetic variation of the pathogen (Schmid-Hempel and Koella 1994).

However, disease is not an inevitable outcome of the one host-one pathogen interaction; furthermore, pathogens can express a wide range of virulence in relation to the host. The extent of the virulence, is usually correlated with the ability of the pathogen to multiply within the host and may be affected by other factors (i.e. conditional). Thus, due to difference in virulence within single host-pathogen interactions, some individual hosts will show a higher resistance to pathogens than others.

7.1.2. Prime-host interactions

The variations present in nature that determine the infection success, and the interplay between host resistence alleles with the parasite virulence will determine the development of the infection. Thus, the combination of both host and parasite characteristics creates a host-parasite specificity. As a result, some individual parasite strains can be more infectious to some host strains than others. As an evolutionary consequence, the traits that help a pathogen to exploit one set of hosts makes it less able to attack other hosts, or controversially when the traits that help a host to resist one set of parasites makes it less able to resist others (Kirchner and Roy 2000). We now often see that hosts which are less vulnerable to a pathogen can act in

the transmission to other, less resistant hosts. These hosts can thus be called 'prime host' in the transmission of the pathogen towards other, less resistant hosts.

7.1.3. Multi-host interactions

Given this prime host-one pathogen interaction explained, a challenge in the study of pathogen behaviour and ecology is to expand the theory of one host-one pathogen to a more realistic manner where many pathogens are interacting to many hosts. In a study on multiple parasite and host assemblages (multiple butterfly species interacting with nematodes, microsporidia and fungi), Rigaud et al. (2010) therefore defined the concepts of "multi-parasite hosts" and "multi-host parasites" to avoid confusion with interactions involving multiple genotypes of a single species.

Multi-parasite hosts: Single host species exploited by several concurrent parasite species, either during their whole life cycle or during a given stage within it, at both the individual and population levels. These interactions do not include hyperparasitism, where a parasite is itself the host of a parasite (Rigaud et al. 2010);

Multi-host parasites: Single parasite species exploiting several concurrent host species, for either their whole life cycle or a given stage within it, at both the individual and population levels. This definition refers to parasites that use either multiple sequential host species (complex life cycles) or concurrent host species (Rigaud et al. 2010).

While the concept multi-host parasites and multi-parasite hosts best explains the real situation in nature, epidemiological studies largely overlook the evolutionary consequences because the classical models focus on simpler, single-host systems (Frank 1996). But it is this heterogeneity in different biological interactions within ecosystems that drives the evolution of both host and parasite traits (Schmid-Hempel and Koella 1994), influencing the evolution of transmission patterns, parasite virulence and host exploitation (Gandon 2004). The concept of multi-host interactions can also be used for interactions between bees and their parasites. Indeed, domesticated honeybees, bumblebees and wild bees live in multi-species assemblages (Williams and Osborne 2009, Rigaud et al. 2010) and share several parasites and viruses and they play key ecological roles in their population dynamics (Schmid-Hempel and Koella 1994).

7.2. Mutualistic gut microbiota

Aside from pathogenic interactions, there are also many positive interactions mediated by mutualistic gut microbiota. Indeed, in most insect hosts, the biochemical capabilities of their symbionts extend those of the insect, enabling the insect to occupy new ecological niches. In

these cases, host and microbiota have been coevolved, leading towards a beneficial or ultimately to an obligate interaction (Rosenberg and Zilber-Rosenberg 2011). Apoidea bees, for example, exclusively live on nectar and pollen and have their typical gut microbiota assisting in the fitness and survival (i.e help in digestion and protect against pathogens) (Martinson et al. 2011). Yet, honeybees and bumblebees have recently been shown to harbour a distinct and species poor microbiota, mostly belonging to the Acetobacteriaceae (*Bombella intestini*), Neisseriaceae (*Snodgrassella alvi*), Orbaceae (*Gilliamella apicola* and *Schmidhempelia bombi*), Lactobacillaceae (*Lactobacillus* sp.) and Bifidobacteriaceae (*Bifidobacterium* sp.) (Killer et al. 2010, Killer et al. 2011, Koch and Schmid-Hempel 2011a, Koch et al. 2013, Killer et al. 2014, McFrederick et al. 2014). Moreover, this core set of bacteria was found to be present in different bumblebee species, and similar to, but distinct from bacteria described from the honeybee gut. Significant differences were also observed between the communities of bacteria in the different bumblebee species, while a typical set of "core" bacteria was also common, indicating for a social transmission of typical gut microbiota (Koch and Schmid-Hempel 2011a). This distinct "core set" of gut microbiota may confer protection against parasites (Koch et al. 2013). Next to this, it has also been reported that bumblebees with a deficient gut microbiota show less protection against infection with the gut parasite *Crithidia bombi* (Koch and Schmid-Hempel 2011b). Beside, (Cariveau et al. (2014)) indicated a higher prevalence of *Crithidia* in bumblebees with a lower colonisation of the core gut bacteria *Gilliamella* and a positive correlation between *Crithidia* and the richness of non-core bacteria.

Chapter 2:

Reared bumblebees (*B. terrestris*) harbor a core subset of gut bacteria normally associated with the wild host

This chapter is based on:

16S rRNA amplicon sequencing demonstrates that reared bumblebees (*Bombus terrestris*) harbor a core subset of bacteria normally associated with the wild host (2015).
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1. Abstract

A MiSeq multiplexed 16S rRNA amplicon sequencing of the gut microbiota of wild and indoor-reared *Bombus terrestris* (bumblebees) confirmed the presence of a core set of bacteria, which consisted of *Neisseriaceae* (*Snodgrassella*), *Orbaceae* (*Gilliamella*), *Lactobacillaceae* (*Lactobacillus*), and *Bifidobacteriaceae* (*Bifidobacterium*). In wild *B. terrestris* we detected several non-core bacteria having a more variable prevalence. Although *Enterobacteriaceae* are unreported by non next-generation sequencing studies, it can become a dominant gut resident. Furthermore the presence of some non-core lactobacilli were associated with the relative abundance of bifidobacteria. This association was not observed in indoor-reared bumblebees lacking the non-core bacteria, but having a more standardized microbiota compared to their wild counterparts. The impact of the bottleneck microbiota of indoor-reared bumblebees when they are used in the field for pollination purpose is discussed.

2. Introduction

Bumblebees play an important role in pollination networks, providing an essential ecological service to maintain plant diversity (Goulson 2003a, Goulson et al. 2005) and a commercial service for many agricultural crops (Velthuis and van Doorn 2006). To date there are major concerns towards a global decline of natural pollinators, including bumblebees (Potts et al. 2010), but the impact of different drivers is diverse across geographic locations (Brown and Paxton 2009). Some of these drivers of bumblebee decline could also influence the host microbial community (microbiota). A normal gut microbiota is essential for health and a disrupted gut microbiota (dysbiosis) can invoke a range of diseases (Hamdi et al. 2011). In bumblebees too, intestinal dysbiosis or increased richness of non-core bacteria was associated with higher incidence of infection with the gut parasite *Crithidia bombi* (Koch and Schmid-Hempel 2011a, Cariveau et al. 2014). Indeed a particular set, called the core gut microbiota (Koch and Schmid-Hempel 2011b, Martinson et al. 2011), is specifically associated with bumblebees and honeybees, while absent in solitary bee species (Martinson et al. 2011). Koch and Schmid-Hempel (2011a) performed a cospeciation study sequencing a 16S rRNA library of two core bacteria, *Snodgrassella alvi* and *Gilliamella apicola*. They showed that the resulting bacterial strains per species are rather structured over bumblebee hosts than over geographic locations, thereby underlining an association between the host and their bacteria, predisposing them to possibly evolve a functional dependence.

The microbiota of insects is not only linked with protection against parasites; a vast variety of host-beneficial functions has been reported, including food digestion and detoxification (Engel and Moran 2013). Therefore stressors acting upon the gut microbiota could substantially weaken the bumblebee colony, even further deteriorating their current threatened status.

In this study, we focus on one host species, the buff-tailed bumblebee *Bombus terrestris*, and perform a MiSeq deep sequencing with Illumina and MID technology (multiplex identifier). We compared the gut microbiota of 24 wild buff-tailed bumblebee workers originating from three different environments in Belgium, with *B. terrestris* workers from an enclosed mass rearing system for multiple generations (Biobest). This comparison will allow us to describe how natural the microbiota of these intensively reared bumblebees really is. Furthermore the identification of bacteria in reared bumblebees provides us insights in their host association, because their hosts have been separated from the typical bee-environment and thus excluded of potential horizontal transmission of bee environment-associated bacteria.

3. Materials and methods

3.1. Specimens

24 wild *B. terrestris* workers were collected in three different environments, as described in detail in Parmentier et al. (2014) Location W1 is an urban area with patchy green areas, location W2 is a rural area, while location W3 is an urban area with low abundance of green area. Sampling was performed within the same week in June 2012.

Reared bumblebees were obtained from the bumblebee mass-breeding company Biobest (Westerlo, Belgium). We used 14 workers and each worker was collected from a different colony, each containing approximately 25 workers and one queen.

3.2. Illumina sequencing and taxonomic identification

The whole gut, including the crop, was dissected and stored at -20 °C. The gut was crushed in a 170 µL lysozyme solution (100 mg/ml) and DNA was extracted according to Meeus et al. (Meeus et al. 2013). The V4 region of the rRNA was amplified in triplicate, using the composite 515F and 806R primers designed by Caporaso et al. (2011). The composite primers contain the 16S primer sites, a different nucleotide barcode (on the 806R primer) for each sample and the Illumina adapters sequences that are necessary for the bridge amplification on the Illumina MiSeq flow cell (Caporaso et al. 2011). Amplicons were normalized after quantification of the amount of double stranded DNA with the Quant-iT™ PicoGreen dsDNA reagent (Life Science technologies) on an Infinite M200 microplate reader (Tecan). Samples were mixed at equimolar concentrations and purified using the E.Z.N.A. Cycle Pure Kit and further concentrated with Amicon Ultra-0.5 Centrifugal Filter Device. The equimolar pool was denatured and diluted following Illumina protocols to produce a 8 pM sequencing library. Twenty percent denatured Illumina PhiX Control V3 library was admixed to increase sequence diversity of the final library. Cluster generation and 2x150 paired-end sequencing was performed in one Illumina Miseq flowcell using an Illumina MiSeq Reagent Kit v2. Custom sequencing primers were added to the primers in the kit at a final concentration of 0.5 µM because the adapters on the composite primers do not contain the standard sequencing primer sites. Basecalling and primary quality assessments and de-multiplexing were performed using Illumina's Basespace genomics cloud computing environment.

The complete dataset contained 3,428,218 demultiplexed paired-end reads flagged as 'pass filter' by the Basespace analysis. Sequences were analyzed with the mothur software v.1.31.1 (Schloss et al. 2009), mainly following the standard operating procedure available on http://www.mothur.org/wiki/MiSeq_SOP date 4 March 2014 (Kozich et al. 2013). Before

clustering sequences into OTUs (operational taxonomic units), the complexity was reduced by retaining unique sequences shorter than 275 base pairs and without any ambiguous base pairs. This resulted in 1,747,090 total reads of which 271,532 were unique sequences. Denoising was performed by preclustering all sequences with 1 mutation on 100 base pairs. The UCHIME algorithm (Edgar et al. 2011), with the abundant sequences as reference, identified approximately 9% of the unique sequences as possible chimeras. Furthermore, a large fraction of unique sequences (121,474) only occurred once; these were removed to reduce file complexity, resulting in 2,128 unique sequences of which 4 were excluded because they did not belong to the bacteria domain. Although the amount of unique sequences dropped from 271,532 to 2,218, the dataset still contained the majority of the reads (i.e.1,520,753).

Calculating the distance matrix and clustering with a 0.03 cutoff level resulted in 111 OTUs. A two fold strategy was performed to exclude sequencing errors: first, only OTUs having more than 0.5% of the sequence reads in any sample were kept, and in addition, all sequences not yet reported in bumblebees or honeybees were confirmed by conventional PCR with sequence-specific primers. This procedure resulted in 22 OTUs representing 99.7% of the total reads.

The taxonomic identity of each OTU was revealed by alignment of each sequence with the Bacterial SILVA SEED database. This database (training set) was supplemented with host specific sequences (i.e. host *Apis* or *Bombus*) to improve classification (Newton and Roeselers 2012a). In order to reduce the size of the training set we only included the 805 sequences which were the representative sequence of 99% identity clusters. The identity of each OTU was confirmed by BLASTn of the representative sequences. The get.oturep command in mothur retrieves the representative sequences based on the distance matrix of sequences within one OTU. All representative sequences are provided in a supplementary table S2.1. All sequences confirmed with OTU specific primers were aligned with their representative sequences and identified with the BLASTn algorithm against the non-redundant nucleotide collection and deposited at GenBank (KM030545 until KM030553). Raw Illumina data reads are submitted to the SRA database of Genbank under accession ID SRP050540.

3.3. Verification of new OTUs

To confirm the representative OTU sequence obtained after Illumina sequencing a semi-nested PCR with a universal Eub8F or 984yR primer combined with a OTU specific primer was performed (see Table 2.1). This proved that their presence is not an artifact of random sequencing errors or chimera formation. All OTU specific primers were designed with primer3. A sequence was assigned to a specific OTU if there was a 100% sequence identity with the

overlapping sequence of the representative OTU. The external PCR had 25 cycles with an annealing temperature of 53 °C. The 50 times diluted PCR product underwent an internal touch-down PCR, with 5 cycles starting from 52 °C towards 50 °C, followed by an extra 20 cycli at 50 °C.

Table 2.1 OTU specific primers combined with a universal 16S rDNA primer (Eub8F or 984yR)

Target	Forward primer	Reverse primer
Gamma-E1	TGTCAAGTCGGATGTGAAAT	984yR GTAAGGTTCYTCGCGT
Gamma-E1	Eub8F AGAGTTTGATCMTGGCTCAG	TCACATCCGACTTGACAGAC
Gamma-E2	ACTGCATTTGAAACTGGTCA	984yR
Gamma-E2	Eub8F	ATGCAGTTCCCAAGTTAAGC
Lacto5	Eub8F	CTGTCCTCTTCTGCACTCAA
Firm-S	Eub8F	TCCTGCACTCAAGTCTACCA
Firm-E	Eub8F	GTCTCCCAGTTTCCAATGAC
Lacto3	Eub8F	AGTTTCCACTGCACTTCCTC
Firm -B	Eub8F	GTCTCCCAGTTTCCAATGAC
Gamma_P	Eub8F	CTAGCTTGCCAGTTTTGGAT
Burk	Eub8F	CACTCCAGCTATGCAGTCAC
Actinomycetales	Eub8F	GCTGTGAGTTTTCACAAACG

3.4. Identification of sisters

Sampling of multiple bumblebees from a certain location can contain several sisters. The presence of sisters within a selected locality can influence the comparison of the microbiota among locations. To examine the family relationships, we genotyped the 24 wild bumblebees with 10 microsatellite loci as described in (Maebe et al. 2014). Bumblebee DNA extraction, PCR amplification, capillary electrophoreses and allele scoring were made following the protocol as described in (Maebe et al. 2013). For identification of the possible sisters, we used the program Colony 2.0 (Wang 2004) employing corrections for genotyping errors (5% per locus).

3.5. Characterization of gut microbiota

Samples were normalized to the smallest number of reads for a given sample (n = 16,426). The normalized shared files, generated in the standard operating procedure of the mothur software, were used to generate the diversity calculators and associations, i.e. rarefaction.shared, summary.shared and otu.association. Bacterial evenness (e) was calculated as $e = H/\ln S$, where H is the Shannon index and S is the number of OTUs (Pielou 1966, Mulder et al. 2004). The normalized bacterial abundance is the total number of bacterial reads after normalization. This value cannot be regarded as an absolute quantity, since the total amount of bacteria can differ in different bumblebee guts (Cariveau et al. 2014) and will later on be referred to as relative abundance. Differences of the diversity calculators were determined by

the non-parametric Kruskal-Wallis test in SPSS comparing the specimens from the 3 wild locations and the specimens from a commercial breeding facility.

A multivariate approach with generalized linear models (GLM) in R was followed to compare the relative abundance of different OTUs between the microbiota of reared and wild bumblebees. Again location was chosen as the dependent variable. For multivariate data GLM outperforms distance-based methods in terms of power, not missing low abundant species effects (Warton et al. 2012). Count data with high abundance in combination with zero values often have a negative binomial distribution, with a mean variance plot tending to be quadratic (Warton 2005). The supplementary Figure S2.1 shows the mean-variance plot and the residual vs fits plot showing least pattern for a negative binomial distribution, therefore we ran the manyglm command with family=neg.binom in the mvabund package (Wang et al. 2012). For post-hoc testing again Kruskal-Wallis test were performed in SPSS. In order to improve visualization of the abundance data was transformed log(y/a + 1) with a the minimum possible non-zero abundance, this reduces the dominance of few values with high abundance (Warton 2008).

Nonmetric multidimensional scaling (NMDS) was used to visualize differences in the bacterial community based on a Bray-Curtis similarity matrix of the square root transformed relative abundance of the different OTUs per sample. Clusters of similarity were based on the Bray-Curtis similarity matrix (Primer6 version 6.1.10). Differences in similarity between sisters and non-sister bumblebees were calculated by analysis of similarities (ANOSIM). It calculated a global R statistic which lies between −1 and +1, with high absolute values indicating a large degree of discrimination among groups.

4. Results

4.1. The characteristic phylotypes of wild *Bombus terrestris*

The gut microbiota of 24 wild bumblebee workers foraging in three different locations (W1-W3) was analyzed. In total we identified 23 different OTUs, after OTU picking with 97% similarity. Table 2.2 gives an overview of all the OTUs identified. Their nomenclature is based on the bacterial family to which they belong supplemented with previous nomenclature to show similarity with other studies. The new OTUs were confirmed by PCR with OTU-specific primers (see Table 2.1). For the two closely related OTUs representing *Burkholderiales* and the three closely related OTUs representing *Actinomycetales*, we only identified one bacterial sequence. Also the BifidoX OTU could not be confirmed with PCR because of primer cross reactivity with Bifido 1, 2 or 3.

Table 2.2 Taxonomic identification of OTUs and their closest match in GenBank

Phylum ***Order or Family***	**Name used here (other names)**	**Matching / total basepair first match previously identified in corbiculate bees**	**Association**	**First match not found in corbiculate bees (non-deep sequencing data)**	**References**
Alphaproteobacteria	Alpha1	253/253 JQ673261	Gut *Apis*		(Engel et al. 2012, Moran et al. 2012a)
Betaproteobacteria					
Neisseriaceae	*Snodgrassella* (beta)	253/253 *Snodgrassella alvi*	Gut *Apis* and *Bombus*		(Babendreier et al. 2007, Martinson et al. 2011, Moran et al. 2012b)
Burkholderiales	Burk1, 2[$]			524/532 JQ658329[$] 492/533 HM111030* 470/535 HM108635**	
Gammaproteobacteria					
Orbaceae	*Gilliamella* (Gamma-1)	253/253 *Gilliamella apicola*	Gut *Apis* and *Bombus*		(Babendreier et al. 2007, Martinson et al. 2011, Moran et al. 2012b)
Enterobacteriaceae	Gamma-E1			870/881 CP003938 793/843 AJ971871**	
Enterobacteriaceae	Gamma-E2			794/794 JX860524 726/785 AJ971871**	
	Gamma2	253/253 HM215025	Gut *Bombus*	245/253 NR118490***	(Martinson et al. 2011, Engel et al. 2013)
Pseudomonadaceae	Gamma-P			526/527 KC502873	
Bacteroidetes	*Bacteroidetes*	253/253 JQ388908	Gut *Bombus* and *Apis*		(Koch and Schmid-Hempel 2011a)
Firmicutes					
Lactobacillaceae	Lacto1-Firm5 (Firm-5.	253/253 HM215048	Gut *Bombus*		(Babendreier et al. 2007, Koch and Schmid-

	Lactobacillus (VI))				Hempel 2011a, Martinson et al. 2011, Moran et al. 2012b)
Lactobacillaceae	Lacto2-Firm4 (Firm-4)	253/253 KJ078645	Gut *Bombus* queen		(Babendreier et al. 2007, Koch and Schmid-Hempel 2011a, Martinson et al. 2011, Moran et al. 2012b)
Lactobacillaceae	Lacto3	555/573 HM534759	Crop *Apis*		
Lactobacillaceae	Lacto4 (*Firmicutes* (V))	253/253 JQ388900			(Koch and Schmid-Hempel 2011a)
Lactobacillaceae	Lacto5	581/581 EU753703	Crop *Bombus*		(Mohr and Tebbe 2007)
Streptococcaceae	Firm-S			564/564 KJ186939	
Enterococcaceae	Firm-E			402/402 KJ156978 379/402 AJ971886**	
Bacillaceae	Firm-B	547/550 AJ971921	Gut *Bombus*		(Mohr and Tebbe 2007)
Actinobacteria					
Bifidobacteriaceae	Bifido1 (Killer group 1) *Bifidobacterium actinocoloniiforme*)	253/253 FJ858735	Gut *Bombus*		(Killer et al. 2010, Killer et al. 2011)
Bifidobacteriaceae	Bifido2 (Killer group 2)	253/253 FJ858732	Gut *Bombus*		(Killer et al. 2010, Killer et al. 2011)
Bifidobacteriaceae	Bifido3 (Killer group 3)	253/253 FJ858733	Gut *Bombus*		(Killer et al. 2010, Killer et al. 2011)
Bifidobacteriaceae	BifidoX			247/253 JQ354974	
Actinomycetales	Myc1, 2, 3[$]			491/491 KC128891[$] 474/488 AJ971863**	

All OTUs previously identified in corbiculate bees by non-deep sequencing techniques have their first blast hit in the third column, others in the fifth column.
*abomen of wild bee *Halictus patellatus*; ** *Bombus* sp.; *** *Frischella perrara* from *Apis mellifera*
$ only one of the OTUs was confirmed with OTU specific primers

4.2. Related foraging bumblebee workers have a more similar microbiota

We sampled in three locations (W1-W3). Rarefaction curves reaching a plateau (supplementary Figure S2.2) illustrate that 16,000 sequence reads per sample and 7 specimens per location had sufficient depth. Microsatellite analysis revealed 3 possible sisters at location W1, 2 at location W2, and again 3 at location W3. Although sisters can have large variation in their microbiota and fall within different regions of the non-metric multidimensional scaling plot (Fig. 2.1; open symbols represent sisters), the similarity among sisters is higher (ANOSIM, R = 0.55, *P* = 0.01). Therefore we did not automatically exclude sisters for further data analysis, but we only excluded a sister if her microbiota showed more than 70% similarity with an earlier sampled sister.

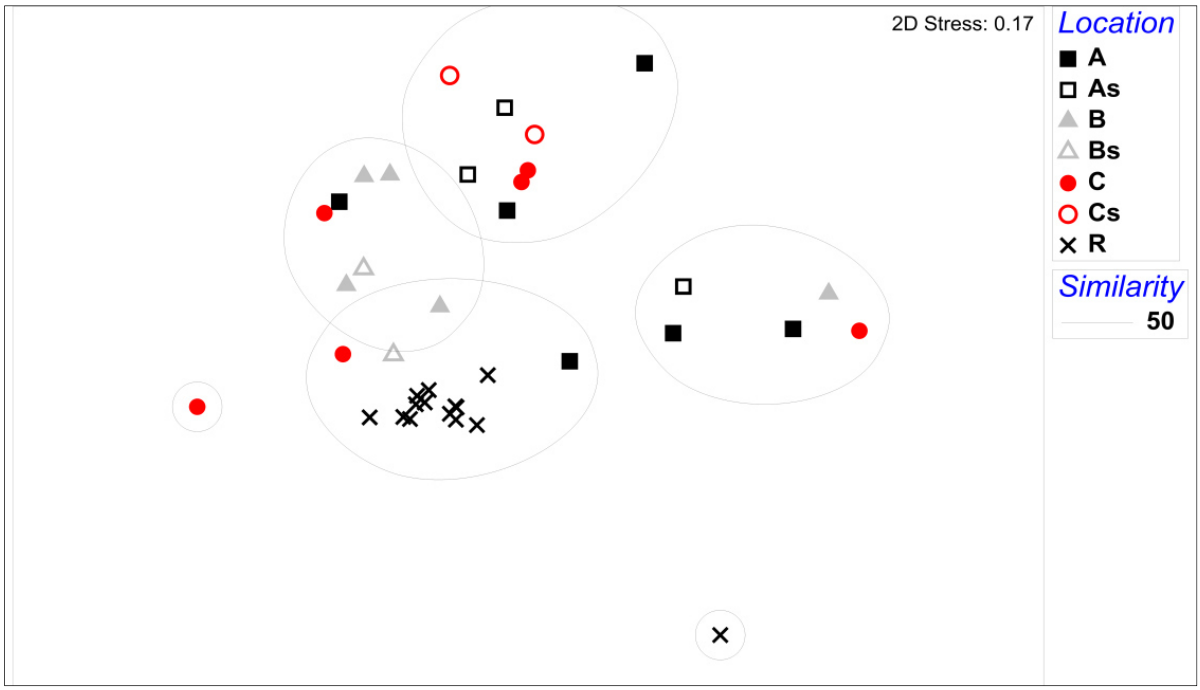


Figure 2.1 NMDS non-metric multidimensional scaling of the bumblebee microbiota of wild (location W1, W2 and W3) and reared (R) *Bombus terrestris.* The open symbols are sister specimens from each location, also annotated by the extra letter s after the location indicator. The circles group samples with a higher similarity than 50% based on the Bray-Curtis similarity matrix

4.3. The microbiota of reared *B. terrestris* is a subset of the wild microbiota

The microbiota of 14 reared bumblebee workers revealed 9 OTUs. Reared bumblebees contained 2 OTUs which we did not retrieve in the wild bumblebees, however these two OTUs (i.e. Gamma-2 and Firm-B) only represented 0.8% of the bacterial sequence reads in reared bumblebees. The lower number of OTUs is not a consequence of a lower number in specimens, as each sampling location of wild bumblebees had more OTUs (Supplementary Figure S2.2).

All other bacterial OTUs (representing 99.2% of the bacterial sequence reads) of reared bumblebees were also retrieved in wild species, although in wild bumblebees these OTUs only represent 40.2% of the total bacterial reads. Figure 2.2 gives an overview of the relative abundance of all OTUs in the three sampling locations (W1, W2 and W3), compared to the relative abundance in reared bumblebees. GLM support a significant difference within these locations (Dev = 173.6, *P* = 0.001), the univariate test are given in the supplementary Table S2.1. Significant pairwise post-hoc tests (Kruskal-Wallis) of all locations per OTU are shown in figure 2.2. We only found differences between the reared bumblebees and specimens collected in the wild. All core bacteria, as defined by Cariveau et al. (2014), were present in the reared bumblebees, except the *Alphaproteobacteria.* Of the latter, only Alpha1 was present in one single wild specimen of our dataset with a low relative abundance. The relative mean abundance of each OTU and the prevalence in wild and reared bumblebees is given in table 2.3.

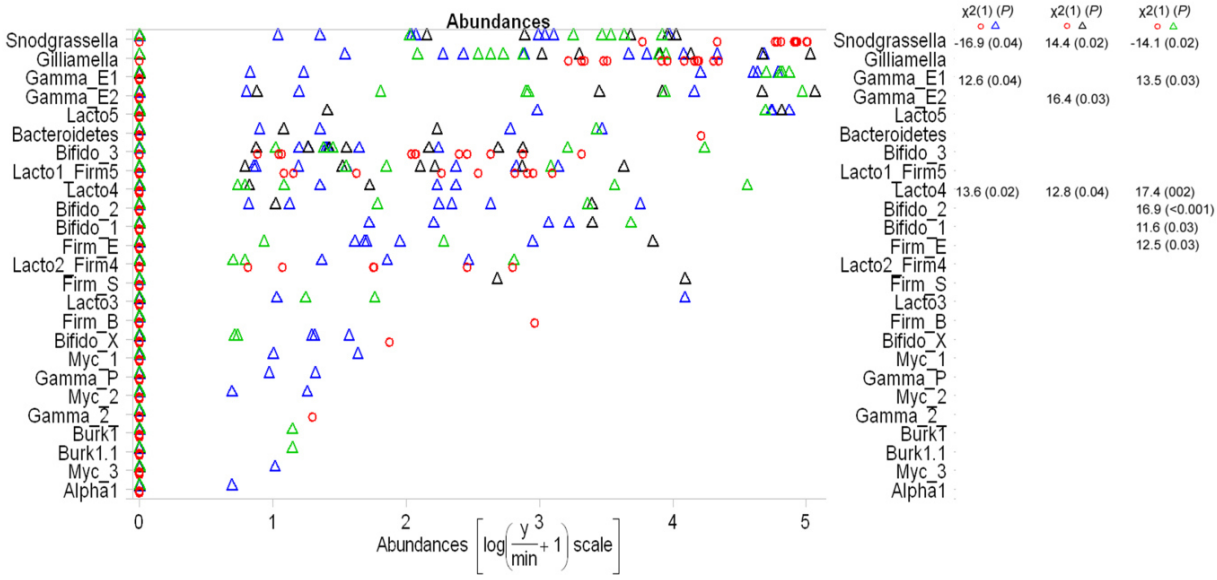


Figure 2.2 The normalized and transformed abundance of the different OTUs of wild and reared *Bombus terrestris.* The wild location A, B, C are represented by a blue, black and green triangle, respectively. The reared bumblebees are represented a line lower by a red circle. On the right side the pairwise Kruskal-Wallis post-hoc test are given for each OTU, but only the significant ones (*P* adjusted < 0.05). No significant values were found between wild locations

Table 2.3: Normalized mean abundance of each OTU and its prevalence in its host *Bombus terrestris*

		Wild specimens		Reared specimens			
Phylum *Order or Family*	Name used here **bold = appear in reared specimens**	Normalized mean abundance (%)	Present in host (%)	Normalized mean abundance (%)	Present in host (%)	Core in honeybees	Core in *Bombus terrestris*
Alphaproteobacteria	Alpha1	0.02	5	0.00	0	Alpha1, Alpha2.1 and Alpha2.2	*Bombella intestini*
Betaproteobacteria							
Neisseriaceae	***Snodgrassella***	10.66	82	57.22	93	*Snodgrassella*	*Snodgrassella*
Burkholderiales	Burk1	0.04	5	0.00	0		
Burkholderiales	Burk2	0.06	5	0.00	0		
Gammaproteobacteria							
Orbaceae	***Gilliamella***	19.19	91	23.63	93	*Gilliamella*	*Gilliamella*
Enterobacteriaceae	Gamma-E1	16.14	36	0.00	0		*Enterobacteriaceae*
Enterobacteriaceae	Gamma-E2	14.43	50	0.00	0		*Enterobacteriaceae*
	Gamma2	0.00	0	0.10	7	*Frischella*	
Pseudomonadaceae	Gamma-P	0.05	5	0.00	0		
Bacteroidetes	**Bacteroidetes**	1.78	27	9.04	14		*Apibacter*
Firmicutes							
Lactobacillaceae	**Lacto1-Firm5**	3.24	68	3.72	64	Lacto1-Firm5	Lacto1-Firm5
Lactobacillaceae	**Lacto2-Firm4**	0.85	23	1.42	43	Lacto2-Firm4	*Lactobacillaceae*
Lactobacillaceae	Lacto3	1.60	18	0.00	0	*Lactobacillaceae*	*Lactobacillaceae*
Lactobacillaceae	Lacto4	3.68	41	0.00	0	*Lactobacillaceae*	*Lactobacillaceae*
Lactobacillaceae	Lacto5	14.06	32	0.00	0	*Lactobacillaceae*	*Lactobacillaceae*
Streptococcaceae	Firm-S	1.67	9	0.00	0		
Enterococcaceae	Firm-E	2.06	32	0.00	0		
Bacillaceae	**Firm-B**	0.00	0	0.69	7		

Actinobacteria						
Bifidobacteriaceae	Bifido1	2.90	27	0.00	0	*Bifidobacteriaceae*
Bifidobacteriaceae	Bifido2	3.22	45	0.00	0	*Bifidobacteriaceae*
Bifidobacteriaceae	**Bifido3**	3.92	77	3.91	79	Bifido3
Bifidobacteriaceae	**BifidoX**	0.29	23	0.22	7	*Bifidobacteriaceae*
Actinomycetales	Myc1	0.07	5	0.00	0	
Actinomycetales	Myc2	0.05	5	0.00	0	
Actinomycetales	Myc3	0.02	5	0.00	0	

We calculated some basic parameters to describe the community richness (sobs = the observed richness and chao = Chao1 estimator), community diversity (the Shannon index), and community evenness (e) (Fig. 2.3). The reared bumblebee specimens had lower parameters (sobs: Kruskal-Wallis $\chi2(3) = 16.5$, $P = 0.01$; Chao1: $\chi2(3) = 7.3$, $P = 0.06$; Shannon index: $\chi2(3) = 19.0$, $P < 0.001$; and e: $\chi2(3) = 10.9$, $P = 0.013$). The lower number of bacterial OTUs in each specimen does not result in a change in relative abundance of bacteria. Only for *Snodgrassella* an increase of its relative abundance was observed (see Fig. 2.2).

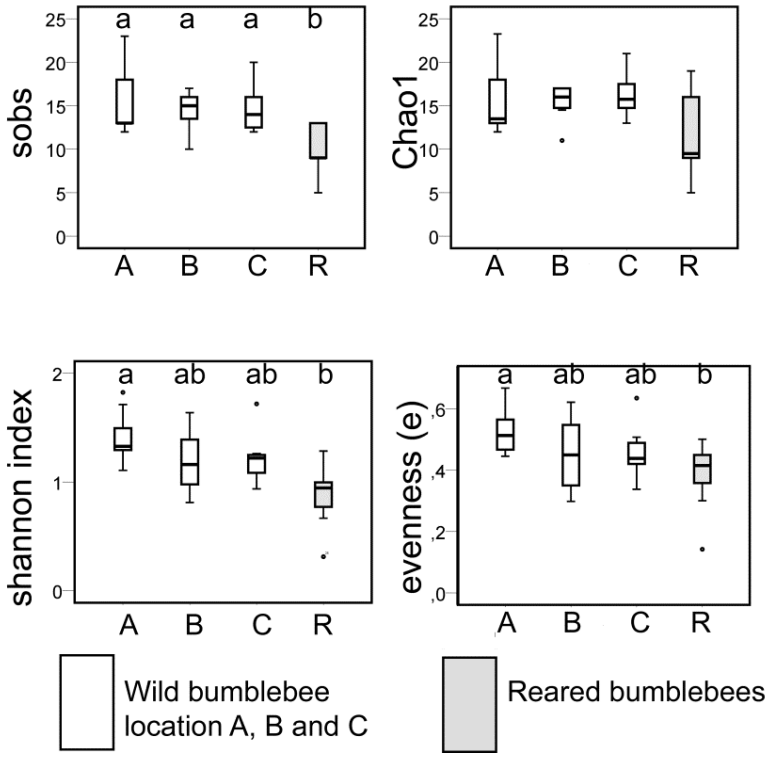


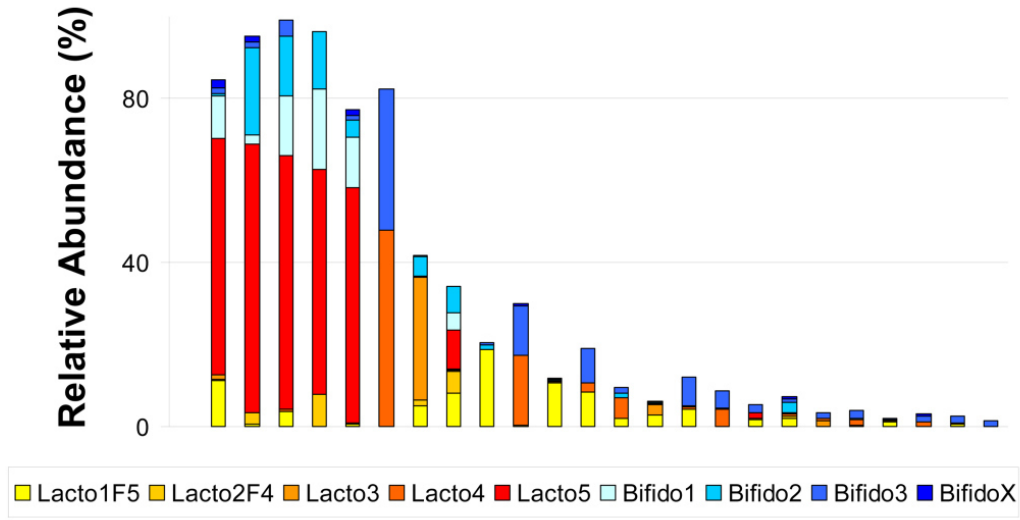
Figure 2.3 The observed richness (Sobs), Chao1 estimator, Shannon index and evenness (e) boxplots of wild specimens collected at location W1, W2 and W3 (in white), while reared bees are represented as R (in grey). Differences by pairwise post-hoc Kruskal-Wallis tests are indicated by a and b (P adjusted < 0.05). The boxplot shows the upper and lower quartiles and whiskers represent the range excluding outliers represented by •

The non-metric multidimensional scaling with similarity matrix overlay (Fig. 2.1) showed that the microbiota of all reared bumblebees, except one outlier, have 50% similarity. As reported above, the reared bumblebees indeed have a different bacterial composition which is confirmed by ANOSIM ($R = 0.24$, $P = 0.002$). The microbiota of reared bumblebees is mainly composed of the core-bacteria and *Bifidobacterium* (Bifido 3) (Fig. 2.3). The outlier is characterized by the presence of a *Bacteroidetes* bacterium. Especially the *Enterobacteriaceae* have a different relative abundance for reared than for wild bumblebees (Fig. 2.2).

4.4. Association between Lactobacillaceae and Bifidobacteriaceae

An association study between the OTUs present in wild bumblebees revealed several associations between OTUs of the *Lactobacillaceae* and the *Bifidobacteriaceae*. These associations were not found in the intensively reared bumblebees. We used a strong Bonferroni correction resulting in a corrected Alpha of 0.00017 (Fig. 2.4b). Mainly the presence of Lacto5 resulted in a higher relative abundance of different bifidobacteria as represented in Figure 2.4a.

A)



B)

	Lacto2F4	Lacto3	Lacto4	Lacto5	Bifido1	Bifido2	Bifido3	BifidoX	Bifido
Lacto1F5	0.01	0.08	-0.21	-0.00	-0.00	-0.10	0.62	0.07	-0.16
Lacto2F4		0.07	-0.14	0.39	0.55	0.62	0.05	-0.02	0.45
Lacto3			-0.08	-.012	-0.11	0.05	-0.17	-0.06	-0.10
Lacto4				-0.18	-0.16	-0.14	**0.78**	-0.12	0.44
Lacto5					**0.85**	**0.78**	0.72	**0.66**	**0.73**
Lacto					**0.76**	**0.72**	0.15	0.58	**0.86**
Bifido1						-0.10	-0.16	0.35	
Bifido2							-0.17	0.26	
Bifido3								-0.13	

Figure 2.4 a. The normalized relative abundance (%) of the different *Lactobacillaceae* and *Bifidobacteriaceae* in wild *Bombus terrestris.* Specimens are ranked from high total *Lactobacillaceae* to low; **b.** Pairwise Pearson correlation coefficients, those in bold and underlined have *P* value below 0.00017

5. Discussion

5.1. The new core bacteria

Deep sequencing of the microbiota of *B. terrestris,* one of the most common bumblebees in Europe, revealed several bacterial taxa known to be associated with corbiculate bees. *Snodgrassella*, *Gilliamella*, Lacto1-Firm5 and Lacto2-Firm4 have been described as core bacteria of *Apis* (Moran et al. 2012a) and the former three are also quite prevalent in *B. terrestris* (see Table 3). The other OTUs, Bifido1, 2 and 3, Lacto3, 4 and 5, and *Bacteroidetes*, are known to be associated with honeybees and bumblebees but with a more erratic occurrence (Koch and Schmid-Hempel 2011a, Martinson et al. 2011). Cariveau et al. (2014) proposed a division in core and non-core microbiota, which can be useful to understand and describe the functionality of the microbiota. Core bacteria are repeatedly associated with individuals of a particular host species or cluster of closely related hosts. If we include deep sequencing data to further ameliorate this subdivision, then bifidobacteria can also be regarded as core bacteria in *B. terrestris,* with the Bifido3 OTU as the most prevalent one. Indeed bifidobacteria have a low relative abundance, but a high prevalence, with only 2 out of the 24 wild specimens having no OTU belonging to the *Bifidobacteriaceae* (data not shown). Our results confirm that *Lactobacillaceae* are core bacteria of *B. terrestris* (23 out of 24 specimens contain lactobacilli, data not shown), with Lacto1-Firm5 as the most prevalent OTU, while the other lactobacilli have a more sporadic occurrence (Table 3).

The OTUs Gamma-E1, Gamma-E2, Gamma-P, Firm-S, Firm-E, Burk1, Burk2 and *Actinomycetales* were confirmed to be present in our samples by OTU specific primers. Sanger sequence confirmation was performed because these bacterial sequences were not yet reported by non-deep sequencing studies. For the latter 5 of these 7 OTUs, the non-detection in previous studies can be explained by the deeper sequencing power of Illumina sequencing. Also for some of these OTUs similar sequences have been found in bumblebee specimens (see table 2). The Gamma-E1 and Gamma-E2 OTUs have a very high relative abundance, which likely should have been picked up during previous sequencing efforts (e.g. Mohr and Tebbe 2006, Koch and Schmid-Hempel 2011a, Martinson et al. 2011). Bias in clone library construction and PCR amplification could explain why these sequences have remained undetected by non-deep-sequencing techniques. The detection of Gamma-E1 and Gamma-E2 is not a local phenomenon, as different *Enterobacteriales* were also present in the 454-sequencing data set of three North American bumblebee species (Cariveau et al. 2014). We therefore argue to regard the yet to be specified genera and bacterial species within the family of *Enterobacteriaceae* as core gut bacteria. Indeed they can be the dominant OTU within the gut microbiota of wild *B. terrestris*, although remain undetected in the reared bumblebees. However their prevalence in bumblebees remains somewhat erratic. This suggests that the environment or other host genetic or physiological

parameters could be more important for their presence. Cariveau et al. (2014) reported a negative association of *Enterobacteriales* presence in the gut and *C. bombi* infection. Aside from this, *Enterobacteriales* have been reported to have a nitrogen fixation function in the fruit fly *Ceratitis capitata* (Behar et al. 2005), and they have been found in different beetles and their larvae where the importance of this bacterial family for concentrating nitrogen for the developing larvae has been debated (Vasanthakumar et al. 2006, Demirci et al. 2013, Podgwaite et al. 2013). For now their role is somewhat ambiguous, but a potential nutritional role should be further investigated.

5.2. The reared bumblebee has a subset microbiota

To date *B. terrestris* are reared in a closed intensive breeding system and so commercially used for biological pollination (Velthuis and van Doorn 2006). Within such a system the ability for horizontal transmission of bacteria is impaired. During colony development nutrition is deposited inside the nest and foragers are unable to leave the nest. Bacterial transmission is only possible between nests in close proximity of each other. Horizontal transmission is still possible when the queens are released for their mating flight in order to ensure a new breeding stock. But the loss of contact with outside bees and flowers could induce a bottleneck in the microbiota of reared bumblebees. Indeed the microbiota of reared *B. terrestris* is a subset of its wild microbiota. There are two bacteria which we did not find in the wild bumblebees: Firm-B occurred in two bees, while Gamma-2 was found in one reared bee. Gamma-2 has also been described as a core-bacterium, mainly because of its presence in honeybees (Moran et al. 2012a), but it is rather scarce in bumblebees, including *B. terrestris* (Koch and Schmid-Hempel 2011a, Koch et al. 2013), and therefore we consider it as the non-core sister of *Gilliamella*. Firm-B can be considered as a non-core bacteria, previously identified with culture dependent techniques in reared blackened bumblebee larvae (Pridal et al. 1997).

The NMDS plot (Fig. 2.1) demonstrated that all intensively reared bumblebees had a similar microbiota, with only one specimen falling outside this group, a specimen having *Bacteroidetes* with a high relative abundance of 93%. All other specimens were dominated by *Snodgrassella* and *Gilliamella.* Also Lacto1-Firm5, Lacto2-Firm4 and Bifido3 were present in reared bumblebees (Table 3). It seems plausible that these bacteria have the potential of vertical transmission (be it with or without the means of contact of two generations within one colony); while for the others (mainly non-core bacteria) horizontal transmission routes from the environment might be more important. However this hypothesis remains to be tested.

The lack of absolute numbers of bacteria restrains us to make informed decisions on the actual bacterial abundance. It remains possible that wild samples harbor low bacterial titer of *Snodgrasella* and *Gilliamella* compared to reared bumblebees, and therefore more exotic bacteria could be

detected in wild bumblebees. Therefore it would be interesting to check for correlations between the abundance of certain bacterial taxa and the absolute titer of the total gut microbiota.

The present data demonstrates that intensively reared bumblebees cannot be regarded as harboring a wild microbiota, as they have a lower bacterial diversity (Fig. 2.3) and a higher relative abundance of *Snodgrassella* (Fig. 2.2). Reared bumblebees are however useful as a simplified model for the microbiota of wild bumblebees which allows to study the interaction of *Snodgrassella*, *Gilliamella*, *Lactobacillus* and *Bifidobacterium*, in a setting with minimal biological variation. This is ideal as a first step of hypothesis testing. The use of reared bumblebees makes the study of bacterial dynamics and interactions in relation with age, nest development or caste more feasible.

5.3. What about bacterial spillover?

Aside from the fact that reared bumblebees harbor a core set of bacteria known to be host-associated, our results also showed they lack bacteria not known to be associated with bumblebees. Therefore when reared bumblebees are placed outdoors for biological pollination purposes, they will not directly spread non-host associated bacteria and thus will not act as a driver of dysbiosis in wild bumblebees. This mechanism of spillover has been described for parasites of managed bees. Indeed domesticated honeybees (Fürst et al. 2014) or reared bumblebees (Murray et al. 2013) can spread parasites and thereby negatively influence the already endangered status of many wild pollinators (Meeus et al. 2011).

Although we do not see a dysbiosis in reared bumblebees, it remains to be investigated if the microbiota changes when the bees are placed outside for their pollination purpose and if the microbiota is suited to prevent viral or parasite infection, like reported for wild bumblebee microbiota (Koch and Schmid-Hempel 2011b, Cariveau et al. 2014)

5.4. Gut colonization of bifidobacteria

Another striking observation was the positive association between *Lactobacillus* and *Bifidobacteria*. This general association in our data exists because of a specific association of Lacto5 with Bifido1, Bifido2 and BifidoX, and of Lacto4 with Bifido3. The association of these specific OTUs is not a consequence of them being present at only one location, as Lacto5 is retrieved from bumblebee samples in all locations. A possible explanation for the associations is that certain lactobacilli are needed to create a suitable environment promoting the growth of bifidobacteria. Studies on human gut colonization dynamics revealed that lactobacilli, among others, are initial colonizers. They are facultative anaerobes and thus reduce the oxygen levels enabling the growth of anaerobic bifidobacteria (Ventura et al. 2012). This common mechanism of oxygen deprivation can

however be performed by a vast majority of the bacteria present in the bee gut. Therefore the specific correlation does not need to be a strict reliance on each other, it could be that the combined drop of certain gut core bacteria allowed for a better relative detection of low abundant bacteria. The observed correlation is indeed between two non-core lactobacilli and the low abundant bifidobacteria.

6. Supplementary data

Table S2.1 List of OTUs and their representative sequences

OTU	Representative sequence
Snodgrassella	TACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGAGCGCAGACGGTTAAATAAGTCAGAT GTGAAATCCCCGAGCTCAACTTGGGACGTGCATTTGAAACTGTGTAACTAGAGTGTGTCAGAGGGAGGTA GAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCTCCTGGG ATAACACTGACGTTCATGCTCGAAAGCGTGGGTAGCAAACAGG
Gilliamella	TACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGT GTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATTGTAGAGGAAGGTA GAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGA CAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAGG
Gamma-E1	TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGAT GTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTA GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA CAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGG
Gamma-E2	TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGATTAAGTCAGAT GTGAAATCCCCGAGCTTAACTTGGGAACTGCATTTGAAACTGGTCAGCTAGAGTCTTGTAGAGGGGGGTA GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA CAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGG
Bacteroidetes	TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTCCGTAGGCGGGTTAATAAGTCAGCG GTGAAAACCTGCAGCTTAACTGTAGAACTGCCGTTGATACTGTTAATCTTGAATAGTATTGAAGTAGCTGG AATGTGTAGTGTAGCGGTGAAATGCATAGATATTACACAGAACCCCGATTGCGAAGGCAGGTTACTAAAT ACCGATTGACGCTGATGGACGAAAGCGTGGGGAGCGAACAGG
Lacto1-Firm5	TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAACGCAGGCGGGAAGATAAGTCAGCT GTGAAAGCCCTCGGCTCAACCGGGGAACGGCAACTGAAACTATTTTTCTTGAGTGCAGAAGAGGAGAGTG GAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGT CTGTAACTGACGCTGAGGTTCGAAAGCATGGGTAGCGAACAGG
Lacto5	TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATG TGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGG AACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTC TGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGG
Bifido3	TACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGGTG TGAAAGTCCACTGCTTAACGGTGGATTGGCGCCGGGTACGGGCAGGCTAGAGTGCAGTAGGGGAGACTGG AATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGC TGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
Lacto4	TACGTAGGTGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGGGAGCGCAGGCGGTCTAATAAGTCTGAT GTGAAAGGTCATAGCTCAACTATGGACGTGCATCAGAAACTGTAAGACTTGAGTACTAGAGAGGGCAGTG GAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACATCAGTGGCGAAGGCGGCTGTCTGGC TAGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGG
Bifido2	TACGTAGGGTCCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGCTCGTAGGCGGCTCGTCGCGTCCGGTG TGAAAGTCCATCGCTCAACGGTGGATTCGCGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTGG AATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGC CGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
Bifido1	TACGTAGGGCGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGGTG TGAAAGTCCACTGCTTAACGGTGGATCGGCGCCGGGTACGGGCAGGCTGGAGTGCGGCAGGGGAGACTGG AATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCGATGGCGAAGGCAGGTCTCTGGGC CGTCACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
Lacto2-Firm4	TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTCTGTTAAGTCTGAAT GTGAAAGCCCTCAGCTTAACTGAGGAAGAGCATCAGAAACTGGCAGACTTGAGTGCAGAAGAGGAGAGT GGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGG TCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGG

Firm-S	TACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTATTAAGTCTGGTG TAAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGA ATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCT GTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGG
Firm-E	TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATG TGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTG GAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGT CTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGG
Lacto3	TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTGTAAGTCTGCTG TGAAAGCCCTCAGCTTAACTGAGGAAGTGCAGTGGAAACTACAAAACTTGAGTACAGAAGAGGACAGTGG AACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTC TGTTACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGG
BifidoX	TACGTAGGGCGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGCTCGTAGGCGGTTCGTCGCGTCTGGTG TGAAAGCCCATCGCTTAACGATGGGTCTGCGCCGGATACGGGCGGGCTTGAGTGCAGTAGGGGAGACTGG AATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGC TGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
Myc1	TACGTAGGGTGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCTGCTG TGAAAACCCGAGGCTCAACCTCGGGCTTGCAGTGGGTACGGGCAGGCTAGAGTGCGGTAGGGGAGATTGG AATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGC CGCTACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGG
Myc2	TACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCG TGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGG AATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGC ATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGG
Firm-B	TACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATG TGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTG GAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGT CTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGG
Gamma2	TACGGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATGATTAAGTTAGGT GTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGGTCGTCTGGAGTATTGTAGAGGAAGGTA GAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGA CAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAGG
Gamma-P	TACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGAAT GTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGTAGTG GAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACTACCTGGA CTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGG
Burk1	TACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGCTATGCAAGACTGAT GTGAAATCCCCGGGCTCAACCTGGGAACTGCATTAGTGACTGCATAGCTGGAGTGCGGCAGAGGGGGATG GAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGG CCTGCACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGG
Burk2	TACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGAG GTGAAATCCCCGGGCTCAACCTGGGAACTGCCTTTGTGACTGCAAGGCTAGAGTACGGCAGAGGGGGGTG GAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAACCCCCTGGG CCTGTACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGG
Myc3	TACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTAGGCGGTTTGTCACGTCGTTTG TGAAAACTCACAGCTCAACTGTGAGCCTGCAGGCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGG AATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGC AGTAACTGACGCTGAGGAACGAAAGCGTGGGTAGCGAACAGG
Alpha1	TACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGGCGGATATTTAAGTCAGGG GTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACTGGATATCTTGAGTATGGAAGAGGTAAGTG GAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGT CCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG

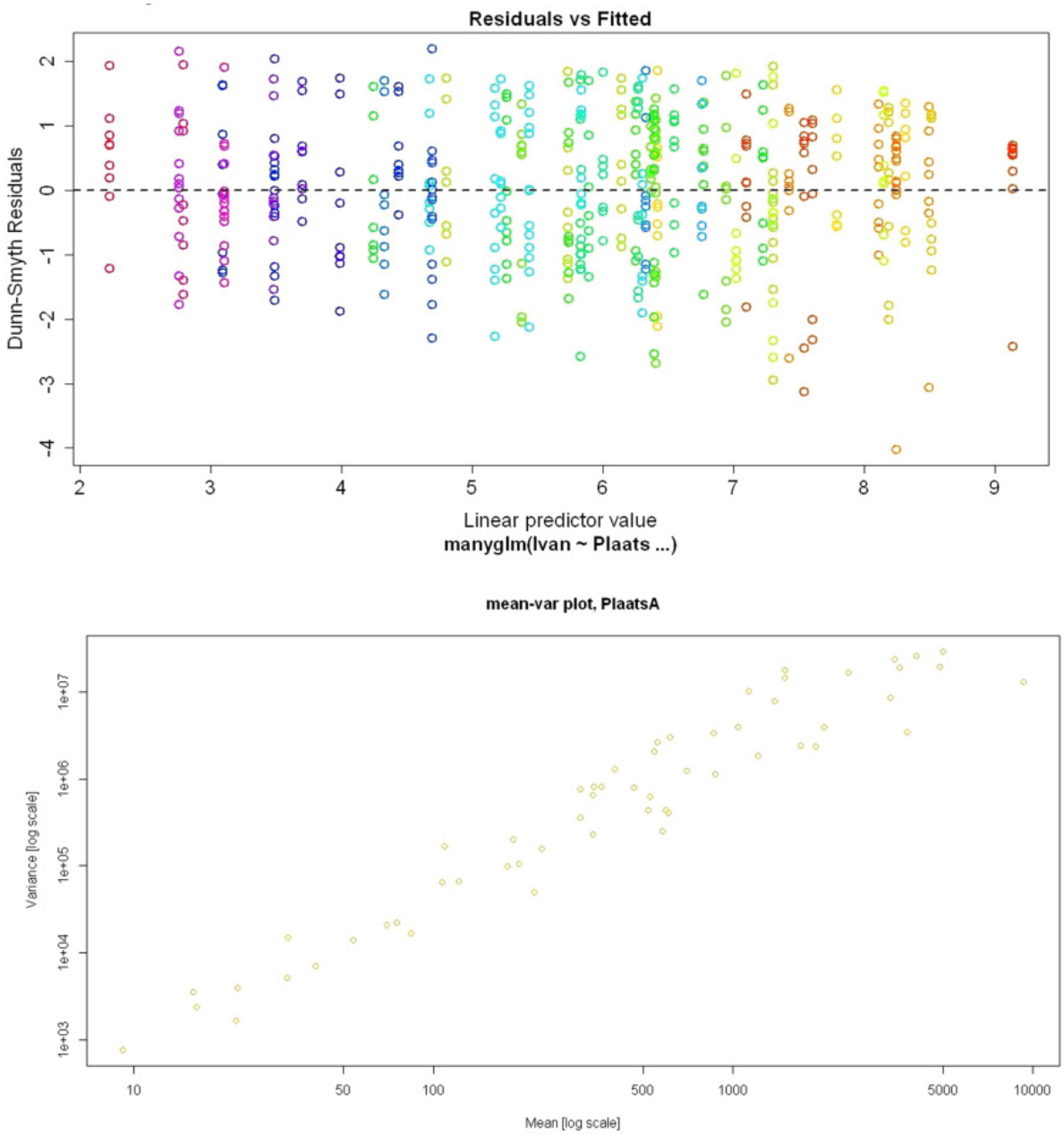


Figure S2.1 The residual versus fits plot and the mean-variance plot of the multivariate bacterial relative abundance data.

Table S2.2 Wald-score after the anova.manyglm function within the mvabund package in R of the normalized abundance of all OTUs comparing bumblebees from three wild location and reared specimens.

Multivariate test		
Df.	Dev.	P-value
34	173.6	**0.001**
Univariate test		
	Wald-score	P-value
Snodgrasella	9.975	0.135
Gilliamella	2.959	0.938
Lacto1-Firm5	0.722	0.938
Lacto2-Firm4	6.791	0.344
Bacteroidetes	0.827	0.938
Bifido3	2.643	0.938
BifidoX	3.127	0.938
Gamma-E1	**24.262**	**0.002**
Gamma-E2	**19.314**	**0.005**
Lacto5	8.146	0.233
Lacto4	**14.456**	**0.027**
Bifido2	**12.652**	**0.041**
Bifido1	6.621	0.344
Firm-S	7.654	0.246
Firm-E	9.788	0.138
Lacto3	7.739	0.241
Myc 1	6.378	0.346
Myc 2	6.372	0.346
Gamma-P	6.382	0.344
Burk1	3.287	0.938
Burk2	3.287	0.938
Myc 3	3.026	0.938
Alpha1	3.025	0.938
Gamma-2	2.073	0.938
Firm-B	2.075	0.938

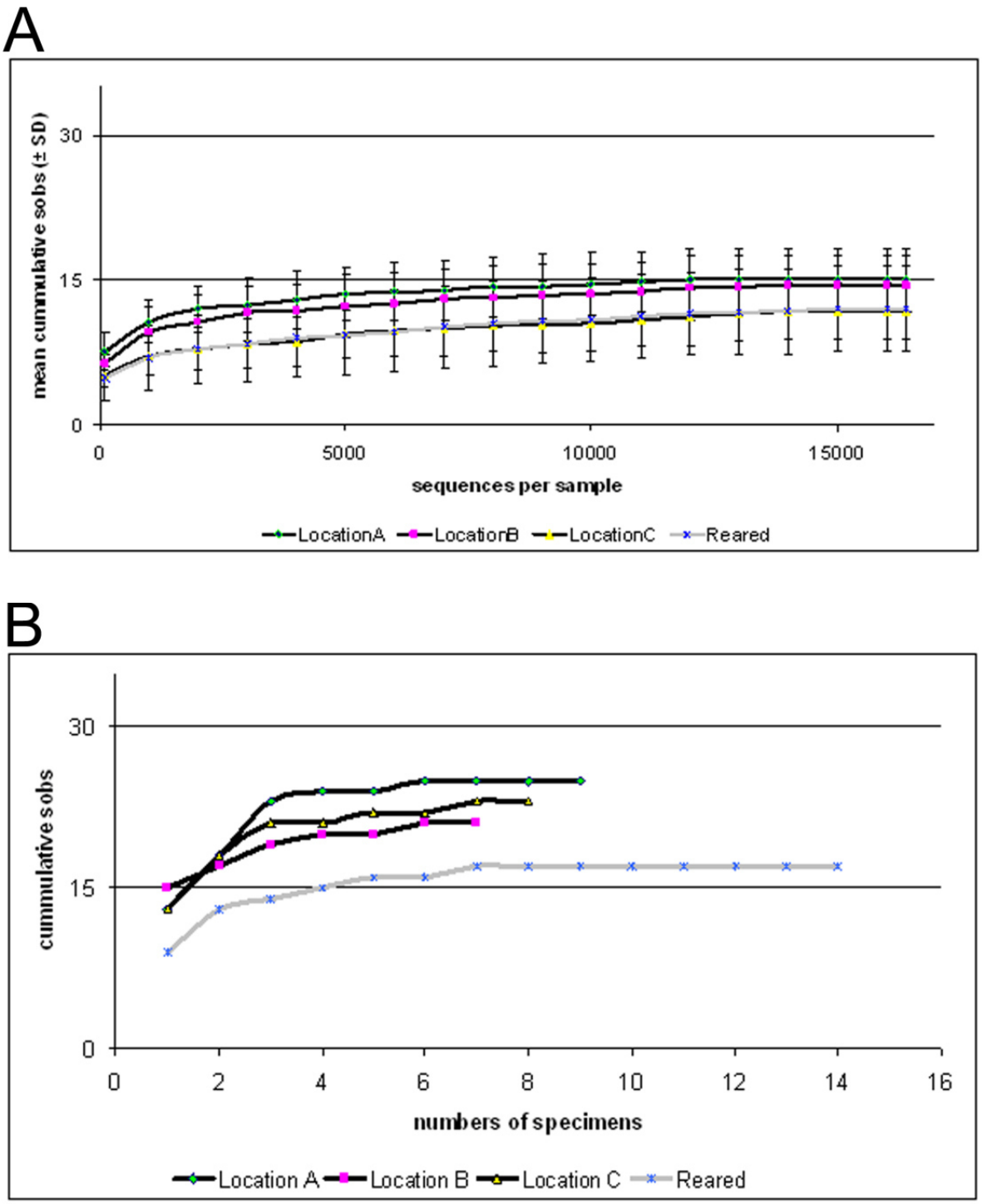


Figure S2.2 a. The number of sequences needed per specimen: the rarefaction curve shows the mean numbers of OTUs per location or breeding facility in function of the reads per specimen (sample); **b.** The number of specimens needed per location: the rarefaction curve shows the numbers of OTUs per location or breeding facility in function of the numbers of specimens analyzed.

Chapter 3:

Plasticity of gut microbiota of reared *Bombus terrestris* nests moved to outdoors

This chapter is based on:

Plasticity in the gut microbial community and uptake of *Enterobacteriaceae* (Gammaproteobacteria) in *Bombus terrestris* bumblebees nests when reared indoors and moved to an outdoor environment (2015).

Laurian Parmentier, Ivan Meeus, Hadi Mosallanejad, Dirk C. de Graaf and Guy Smagghe

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1. Abstract

Bombus nests consisting of one queen, brood, and worker adults, are produced indoors for biological pollination in agriculture. In this study, we investigated the gut microbial community in workers of *Bombus terrestris* when the environment is stable (indoors) or variable (outdoors). When nests were reared indoors under standardized conditions, we identified a small gut microbial community consisting of *Neisseriaceae*, *Orbaceae*, *Lactobacillaceae*, and*Bifidobacteriaceae*, and the age of bumblebee nests and workers did not affect the alpha and beta diversity, confirming a stable microbiota. Secondly, when indoor-reared nests were moved to outdoors, we observed a major shift in the microbial community, especially in the newborn workers fully developed in the outdoor conditions, with a significant colonization of*Enterobacteriaceae*. Our new findings are discussed in relation to host-associated core and non-core bacteria in bumblebees including possible implications for host functioning.

2. Introduction

Insects are in many cases dependent on functions provided by bacterial symbionts (Brownlie and Johnson 2009, Basset et al. 2012), including gut microbiota with beneficial functions in nutrition (Salem et al. 2013) and protection (Dillon and Charnley 1986, 1988). The social nesting behavior of honeybees and bumblebees enables the transfer of gut bacteria over the generations, allowing a close association with the host (Powell et al. 2014). Indeed, social bees, as opposed to solitary bees, generally harbor host-associated bacterial groups, defined as "core set", and the latter are generally found in the gut microbiota of social bees (Kaltenpoth 2011, Koch and Schmid-Hempel 2011a, Cariveau et al. 2014). Core bacteria dominating the gut of social honeybees and bumblebees have been implicated in beneficial functions such as digestion and protection (Klungness and Peng 1984, Forsgren et al. 2010, Koch and Schmid-Hempel 2012, Vasquez et al. 2012, Kwong et al. 2014). Multiple conditions have been reported influencing the bacterial gut community, including the environment, host age, habitat, pathogens and diet (Dillon et al. 2010, Evans and Schwarz 2011, Hu et al. 2014, Yun et al. 2014).

Currently, the buff-tailed bumblebee *Bombus terrestris* is reared indoors for the biological pollination in agriculture. This species is widely distributed and native to Europe, coastal North Africa, and West and Central Asia (Goulson 2010). However, there has been little research on the gut microbial community and the dynamics of reared bumblebees foraging in an environment outdoors.

In this research, we used 16S rRNA Illumina deep sequencing to identify the gut microbial community in workers of *B. terrestris* from reared nests. In a first experiment, we determined the growth and richness of this microbial community when the nests were kept under indoor conditions. Our ambitions were to determine the dominant bacteria in reared nests and the stability of these bacteria in function of the age of the adults and the nest. In a second experiment, the reared bumblebee nests were moved to outdoors to investigate the plasticity of the gut microbial community in the workers of different ages. In summary, the new data found in this study are of use to discuss the relationship between core and non-core bacteria in bumblebees and to generate new insights in the gut microbial stability of reared bumblebee hives when moved to outdoors.

3. Material and methods

3.1. Bumblebee nests and insect labeling

Six nests of *B. terrestris* were obtained from a bumblebee mass-rearing facility (Biobest, Westerlo, Belgium). All colonies contained one queen, new queen brood but without adult workers. The nests were kept in the lab at standard conditions of 28-30 °C and 60-65% relative humidity and

continuous darkness, and they were fed *ad libitum* with artificial nectar (Biogluc®, Biobest) and pollen clumps (Soc. Coop. Apihurdes, Pinofranqueado-Caceres, Spain) as energy and protein sources, respectively (Mommaerts et al. 2006). For the six bumblebee nests, we labeled the queen and then also each newborn worker on a daily basis with a unique marking disc (Bijenhof, Bissegem, Belgium) to the dorsal thorax. The six nests were used for two experiments where in the first experiment three nests were kept indoors under the standard conditions as described above, while in the second experiment the other three nests were moved to outdoors. The experiments were carried out between end of August and late September-early October 2012.

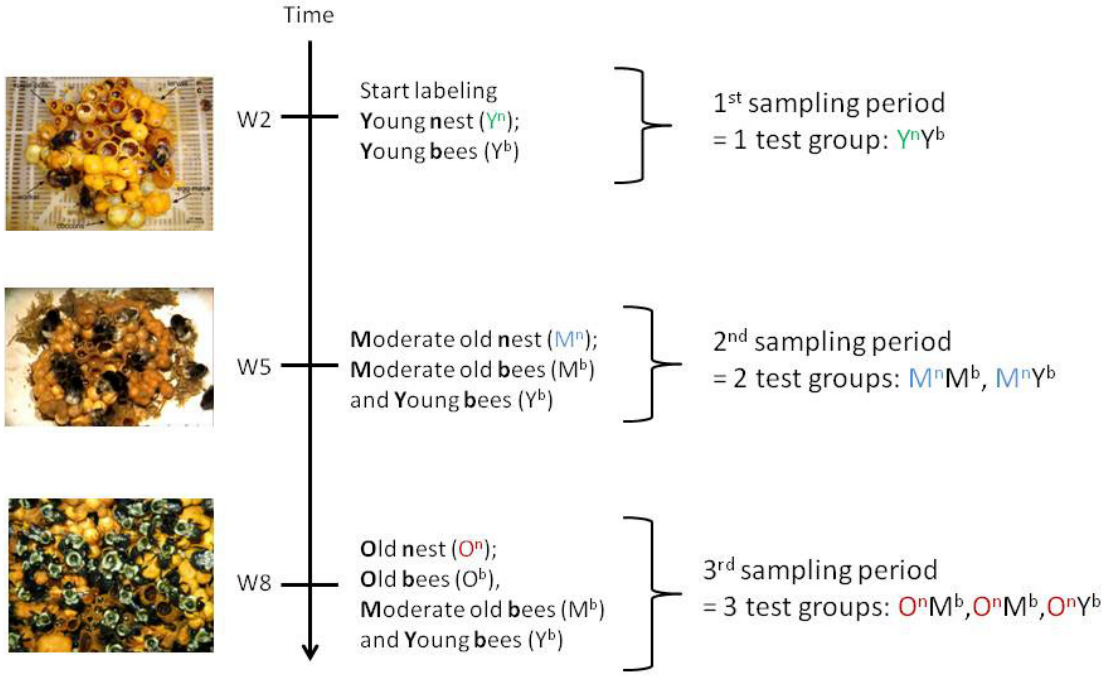


Figure 3.1 Schematic overview of the different sampling periods (i.e. a combination of aging of nest and aging of bees in the nest) as defined in experiment 1 and experiment 2

3.2. Experiment 1: bumblebee nests kept under standard conditions indoors

Three nests were followed for 60 days during the rearing period in the lab under standard conditions. We aimed to investigate the impact of age of the nest and the individual bumblebee worker in the nest. Therefore, we defined 2 age periods of the nest: "Y^n" young nest (4- to 11-day old nest) and "O^n" old nest (40- to 46-day old nest) and 2 age periods of the workers: "Y^b" young bee (4 to 10 days old) and "O^b" old bee (28 to 35 days old). When combining the age period of the nest with the age of the individual workers, we defined three test groups: "Y^nY^b", young nest, young bee; O^nO^b, old nest, old bee; and "O^nY^b", old nest, young bee. In total, we sampled 8 workers per test group. Sampled bumblebees were stored individually at -70°C until dissection of the gut.

3.3. Experiment 2: reared bumblebee nests moved to an outdoor environment

In parallel to experiment 1, we kept the three other nests indoors in the lab so that they developed an initial reared microbiota. When placed outdoors, the three nests developed further under natural conditions during 50 days. The outdoors study site (51°01'32.57"N 3°42'46.10"O) was located in a residential area in the city of Ghent (Belgium) with landscape metrics of an urbanized environment (Parmentier et al. 2014). As shown in Figure S3.2, each nest was placed in a separate polystyrene-box (25 x 35 x 25 cm) with the entrance directed to the east and further treated as described in Parmentier et al. (2014).

In the outdoors experiment, we defined 3 age periods of the nests: "Y^{n}", young nest (4- to 11-day old nest); "M^{n}", middle aged nest (28- to 34-day old nest); and "O^{n}", old nest (40- to 46-day old nest), and 3 age periods of the workers: "Y^{b}", young bumblebee (4 to 10 days old); "M^{b}", middle aged bumblebee (18 to 23 days old); and "O^{b}", old bumblebee (28 to 35 days old). Here, we defined "young" and "old" ages identical as in experiment 1, and the extra "middle" age was introduced in order to sample sufficient forager and nesting bumblebees. We defined a "forager" and "nesting" bumblebee as a worker typically showing a nesting and foraging function, respectively. Thus, in total we defined 5 test groups: "$Y^{n}Y^{b}$", young nest, young bumblebee; $O^{n}O^{b}$, old nest, old bumblebee and "$O^{n}Y^{b}$; "$M^{n}Y^{b}$", middle aged nest, young bumblebee; and "$M^{n}M^{b}$", middle aged nest, middle aged bumblebee. Following our definition, at nest level, initial sampling in the young nests "$Y^{n}Y^{b}$" was achieved under indoors conditions (same as reared hives in experiment 1), whereas sampling in all other test groups was achieved outdoors. In total, we sampled 7 ± 1 nesting bees per test group $Y^{n}Y^{b}$, $O^{n}O^{b}$ and $O^{n}Y^{b}$. In the test groups "$M^{n}Y^{b}$" and "$M^{n}M^{b}$", 6 ± 2 nesting and 6 ± 2 foraging workers were sampled per test group, respectively. Sampled bumblebees were stored individually at -70 °C until dissection of the gut.

To follow and record individually foraging workers leaving or entering the nest, we placed a webcam (Logitech® C920 HD Pro, Lausanne, Switzerland) with a top-view on the entrance of each nest and optimized in "move-detection" function. The flight duration of individual foragers was calculated and we ranked them daily based on the numbers of flights under good weather conditions. We selected foraging workers (foragers) that had at least 5 flights per day under good weather conditions (excluding rainy days and temperatures < 15°C).

3.4. Gut samples processing, Illumina sequencing and identification

The gut, including the crop, was dissected from the different bumblebee workers and transferred individually into a 1.5 ml Eppendorf tube. All equipment used was disinfected with 75% ethanol between each individual dissection. The extraction of bacterial DNA was done as previously described (Meeus et al. 2013), and then the V4 region of the rRNA was amplified in triplicate, using

the 515F and 806R primers elongated with multiplex identifiers (Caporaso et al. 2011). The composite primers include the 16S primer sites, a different nucleotide barcode (on the 806R primer) and the Illumina adapter sequences necessary for bridge amplification on the Illumina MiSeq flow cell (Illumina, San Diego, CA). Amplicons were normalized, mixed at equimolar concentrations, purified and further concentrated as previously described (Meeus et al. 2015) to produce a 8 pM sequencing library. To increase sequence diversity, 20% denaturated Illumina PhiX Control V3 library was admixed. Cluster generation and 2x150 paired-end sequencing was performed on one Illumina Miseq flowcell using an Illumina Miseq Reagent Kit v.2. Custom sequencing primers were added in a final concentration of 0.5 µM (Meeus et al. 2015). Basecalling and primary quality assessments and de-multiplexing were performed at the Miseq sequencing platform of Laboratory for Pharmaceutical Biotechnology (UGent) using Illumina's Basespace genomics cloud computing environment.

The pooled data set contained 3,353,558 double read sequences. A unique barcode was used to separate each sequence from its sample of origin. Sequences were analyzed with the Mothur software v.1.31.1 following the standard procedure (Schloss et al. 2009). In brief, the complexity was reduced by a training set of base pairs before clustering into OTUs (Operational Taxonomic Units). The procedure includes denoising of unique sequences (preclustering all sequences with 1 mutation on 100 bp) and removing of chimeras using the UCHIME algorithm (Edgar et al. 2011). The distance matrix was calculated and clustering with a 0.03 cutoff level resulted in 461 OTUs. To exclude sequencing errors, we only kept these OTUs that had more than 0.5% of the sequence reads in every sample. This procedure resulted in 20 OTUs, representing 99 % of the total reads.

3.5. Classification and verification of new OTUs

The taxonomic identity of each OTU was achieved by alignment of each sequence with the Bacterial SILVA SEED database (training set), supplemented with host specific sequences (i.e. host *Bombus* or *Apis*) to improve classification (Newton and Roeselers 2012b). The identity of each bacterial OTU was achieved by a 2-way strategy. First, BLASTn searches of the representative sequences were achieved. Second, when a 100% base pair alignment was not obtained with representative sequences of *Bombus* hosts of a previous dataset (Meeus et al. 2015) (available at Genbank KM03050545 to KM03050553), a semi-nested PCR with a universal Eub8F or 984yR primer combined with an OTU specific primer was applied (Table 2). All new sequences were deposited at Genbank (KP410382 to KP410393) and raw Illumina data reads of all samples were submitted to SRA database of Genbank under project ID PRJNA270053 (Table 3.1, Table 3.3).

In our study we used a classification system of bacterial OTUs to separate core and non-core OTUs (Koch and Schmid-Hempel 2011; Koch et al. 2013; Cariveau 2014). We defined *Neisseriaceae*,

Orbaceae, *Lactobacillaceae* and *Bifidobacteriaceae* as core families for bumblebees in accordance with Kwong et al. (2014) and Meeus et al. (2015). All other bacteria are regarded as non-core OTUs.

3.6. Statistical analysis

Samples were normalized to the smallest number of reads for a given sample (n = 49,420) using the normalize.shared function and then used to generate the diversity calculators. Calculators "Sobs" (observed number of species), "Chao1" and the Shannon index were used to map the alpha diversity (Schloss et al. 2009). Differences in alpha diversity between pre-defined test groups of reared and outdoor-developed nests were determined by the non-parametric Mann-Whitney U test (SPSS vs. 21). Significance cutoff values were set at 95% ($\alpha = 0.05$). Differences in similarity between test groups (even sampling) were calculated by analysis of similarities (ANOSIM). To visualize differences in the bacterial community (beta diversity), non-metric multidimensional scaling (NMDS) was used based on a Bray-Curtis similarity matrix of the square root transformed normalized relative abundance of the different OTUs per sample (Primer 6 version 6.1.10). An in-dept multivariate analysis was performed in order to evaluate dispersion effects between test groups by using the R package "mvabund" selecting the "anova.manyglm" call. After checking model assumptions by plotting "meanvar" and "manyglm" matrixes, data was further processed as a "negative binomial" distribution, a typical option for abundance count data in the mvabund-package (Wang et al. 2012).

Table 3.2 OTU-specific primers combined with a universal 16S rDNA primer (Eub8F or 984yR)

Target	Primer	
	Forward	Reverse
Alpha-2.1	Eub8F AGAGTTTGATCMT GGCTCAG TGTCAAGT CGGATGTGAAAT	AGTGTGAGAGAGGATTGT GG 984yR GTAAGGTTCYTCGCGT
Gamma-E1	Eub8F AGAGTTTGATCMT GGCTCAG	TCACATCCGACTTGACAGAC
Gamma-E3	CGGTTTGTTAAGTCAGATGT	984yR GTAAGGTTCYTCGCGT
	Eub8F AGAGTTTGATCMTGGCTCAG	ATGCAGTTCCCAAGTTAAGC
Gamma-E4	GAATGGCATCTAAAACTGGT	GAATGGCATCTAAAACTGGT
Bifido-1	Eub8F AGAGTTTGATCMTGGCTCAG	CAGTCTCCCCTACTGCACTC
Bifido-4	GAGTGCAGTAGGGGAGACTG Eub8F AGAGTTTGATCMTGGCTCAG	984yR GTAAGGTTCYTCGCGT CAGTCTCCCCTACTGCACTC
Lacto3	Eub8F AGAGTTTGATCMTGGCTCAG	CTGTCCTCTTCTGCACTCAA
Firm-W	Eub8F AGAGTTTGATCMTGGCTCAG	AGACGGTTATTTAAGTCCGA
Firm-S1	GTGCATTGGAAACTGTTAGA TTAACTGTGGAAGTGCTTTG	GTGCATTGGAAACTGTTAGA 984yR GTAAGGTTCYTCGCGT
Firm-S2	GTGCATTGGAAACTGTTAGA	984yR GTAAGGTTCYTCGCGT
Firm-V	GAGTATCGGAGAGGAAAGTG	984yR GTAAGGTTCYTCGCGT

Table 3.1 Taxonomic identification of OTUs, names used, their closest matching strain, and associated source according to GenBank and papers

Phylum	Family	OTU n°	Name used here (related names)	Basepair match (%) Closest Match Genbank	Best hit(s)	Source associated	Related papers
Alphaproteobacteria	Acetobacteraceae	OTU 43	**Alpha 2.1**	253/253 (100) NZ_AGFR01000021	*Commensalibacter intestini*	Gut insects (*Drosophila*, Butterflies)	
				237/253 (94) KF600322.1	*Acetobacteraceae* bacterium	Floral nectar, associated with gut *Apis mellifera*	Martinson et al., (2011), Cariveau et al., (2014)
Betaproteobacteria	Neisseriaceae	OTU 1	**Snod.** (***Snodgrasella*** sp.; Koch III)	253/253 (100) *KC477411.1*	*Snodgrassella alvi*	Gut *Apis* and *Bombus*	Meeus et al. (2011), Kwong & Moran (2012); Meeus et al. (2015)
Gammaproteobacteria	Orbaceae	OTU 2	**Gillia.** ***(Gilliamella,*** (Koch I,Gamma-1)	253/253 (100) HM215035	*Gilliamella apicola*	Gut *Apis* and *Bombus*	Kwong & Moran (2012); Meeus et al. (2015)
		OTU 8	**Ca. Schmid.** (**Candidatus *Schmidhempelia*** (Koch II,Gamma-2)	253/253 (100) HM215025	Ca. *Schmidhempelia bombi*	Gut *Bombus*	Koch et al. (2011a), Martinson et al (2014), Meeus et al., (2015)
	Enterobacteriaceae	OTU 9	**Gamma-E1**	870/881 (99) CP003938	*Enterobacteriaceae* strain FGI 57	Leaf-Cutter Ant Fungus Gardens	Koch et al. (2011a), Meeus et al., (2015)
	Enterobacteriaceae	OTU 3	**Gamma-E3**	563/564 (>99) JQ522978.1	*Rahnella* sp. Pv5	Bacterial flora of beetles (*P. versicolora*)	Demirici et al. (2013); Podgwaite et al. (2013)
				557/557 (100) JN167946.1	*Yersinia kristensenii*	Wild flowers, associated with *Apis mellifera*	Vasquez et al., (2012)
	Enterobacteriaceae	OTU 22	**Gamma-E4**	294/295 (>99) JX009194.1	Uncultured *Providencia* sp.	Gave fruit fly (*B. correcta*)	
	Pseudomonadaceae	OTU 15	**Gamma-P1**	526/527 (>99) KC502873	Uncultured bacterium	Environmental sample	
Firmicutes	Lactobacillaceae	OTU 4	**Lacto1-F4** (Firm 4)	253/253 (100) KJ078645	*Lactobacillus bombi*	Gut *Bombus* queen related with Firm-4 (*A. mellifera*)	Killer et al., (2014); Meeus et al., (2015)
	Lactobacillaceae	OTU 6	**Lacto2-F5** (Firm 5, Koch VI)	253/253 (100) HM215048	Uncultured Firmicutes bacterium	Gut *Bombus*, related with Firm-5 (*A. mellifera*)	Koch et al. (2011a), Meeus et al., (2015)
	Lactobacillaceae	OTU 340	**Lacto3**	571/572 (>99) JQ009352.1	*Lactobacillus kunkei*	Crop *Apis*	McFred et al., (2010); Vasquez et al., (2012)
	Lactobacillaceae	OTU 27	**Firm-W**	253/253 (100) KF98906.1	*Weissella* sp.	Flower nectar (related with *A. mellifera*)	Anderson et al., (2013)
	Streptococcaceae	OTU 19	**Firm-S1**	254/254 (100) KJ702498.1	*Lactococcus lactis ssp. lactis*	Yak milk dreg	Killer et al., (2014)
	Streptococcaceae	OTU 37	**Firm-S2**	252/252(100) KF599925.1	Uncultured *Enterococcus*	Bee bread food stores (*Apis*)	Anderson et al., (2013)
	Enterococcaceae	OTU 13	**Firm-E**	402/402 (100) KJ156978	*Enterococcus faecium*	Gut *Bombus*	Koch et al. (2011a); Meeus et al. (2015)
	Bacillaceae	OTU 18	**Firm-B1**	547/550 (99) AJ971921	*Bacillus* sp. Bt 35	Gut *Bombus terrestris*	Mohr & Tebbe (2007); Meeus et al. (2015)
	Veillonellaceae	OTU 58	**Firm-V**	261/262 (>99) KJ082047.1	*Veillonella dispar*	Environmental sample	
Actinobacteria	Bifidobacteriaceae	OTU 5	**Bifido-1** (Koch IX; Killer 1)	549/555 (99) FJ858733.1	*Bifidobacterium bombi*	Gut *Bombus*	Killer et al. (2010); Koch et al. (2011); Meeus et al. (2015)
	Bifidobacteriaceae	OTU 11	**Bifido-4** (Killer 4)	543/570 (95) FJ858737.1	*Bifidobacterium bohemicum*	Gut *Bombus*	Killer et al., (2011)

4. Results

4.1. Workers of reared nests harbored a small microbial gut community of stable composition

In total, we sampled 24 nesting bumblebee workers from three reared nests, having representative samples of bumblebees in three age groups (Y^nY^b, O^nY^b, O^nO^b). Rarefaction curves (Supplementary figure S3.1 in the supplemental material) illustrate that an asymptote was achieved for 49,000 sequence read per sample and 7 samples per test group, indicating sufficient sequencing depth. After OTU picking procedure, the microbial community of the reared workers (experiment 1 and initial reared bumblebees in experiment 2) clustered in 13 OTUs. Figure 3.2a shows the relative abundance of each OTU per test group.

Most of the sequences are classified under known core OTUs (Table 3.1), i.e. *Snodgrasella* (Snod.) classified under *Neisseriaceae*, *Gilliamella* (Gillia.) and Candidatus *Schmidhempelia* (Ca. Schmid.) belonging to *Orbaceae*, Lacto1-Firm4, Lacto2-Firm5, Firm-B1, Firm-E, Firm-W, which are all *Firmicutes*, and Lacto3 classified under *Lactobacillaceae*, and Bifido-1 belonging to the *Bifidobacteriaceae*. A very small number of the reads belonging to Gamma-E1 and Gamma-E4 (both < 0.5%), was classified under *Enterobacteriaceae*.

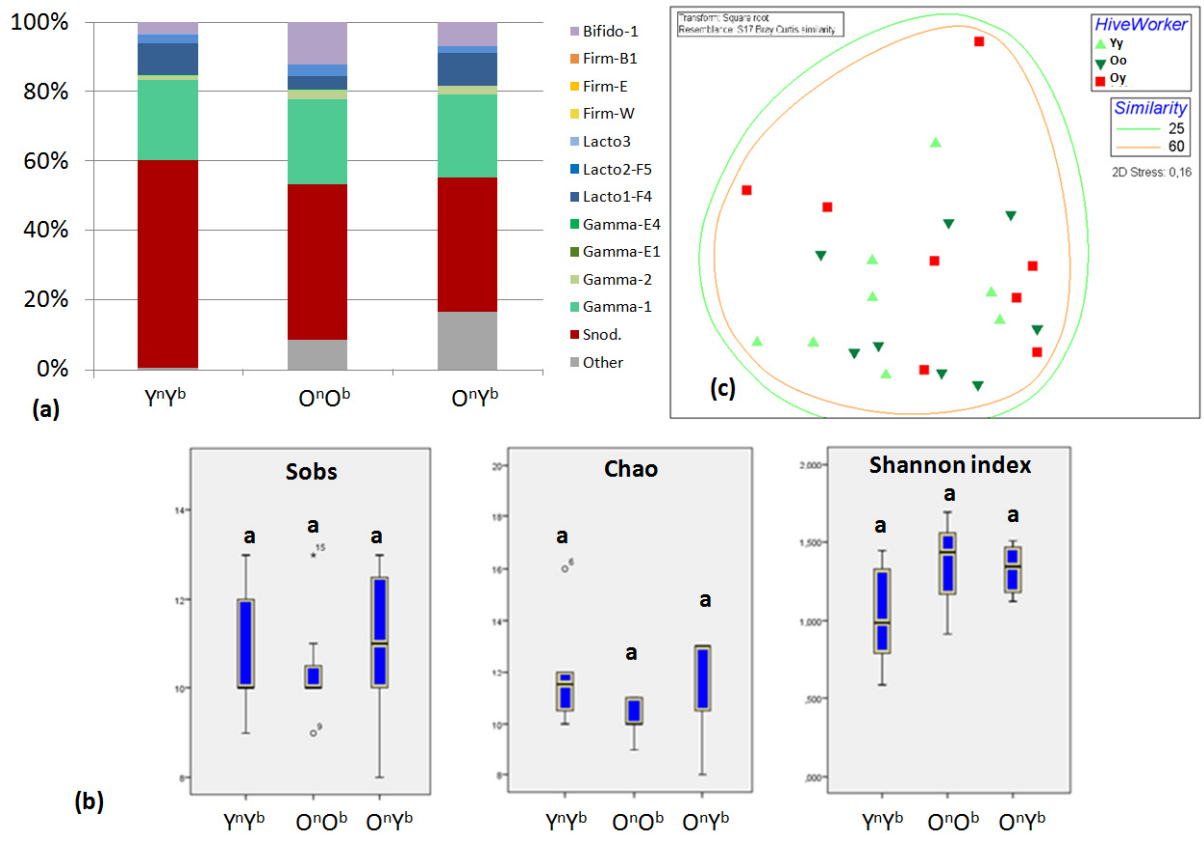


Figure 3.2 a. Relative abundance of all OTUs comparing groups Y^nY^b, O^nO^b, and O^nY^b of the reared *B. terrestris* nests; **b.** Alpha diversity box plots; **c.** NMDS plots. P =0.05; Mann-Whitney U

Table 3.3. P values (Mann-Whitney U test, a = 0.05) of alpha diversity indicators Sobs, Chao, and Shannon index between test groups Y^nY^b, O^nY^b, and O^nO^b for lab-reared hives

	Sobs		Chao		Shannon index	
	Y^nY^b	O^nO^b	Y^nY^b	O^nO^b	Y^nY^b	O^nO^b
O^nO^b	0.382	–	0.051	–	0.442	–
O^nY^b	0.382	0.105	0.505	0.065	0.234	0.999

When looking at alpha diversity calculators such as community richness (Sobs and Chao1) and diversity (Shannon index) (Figure 3.2b), no differences were observed between the test groups in the reared nests (Mann Whitney U test; P values are given in Table 3.3). Besides, looking at the 2-dimensional NMDS plots that visualizes beta diversity (Figure 3.2c), no differences between test groups (ANOSIM, R = 0.11, P = 0.51) were observed, which was also confirmed by multiple univariate test statistics (all P values > 0.05, Table 3.3). However, an in-depth multivariate analysis (Manova results using "mvabund" package, Table 3.4) estimated that there exist small differences (Dev = 22.4, P = 0.045) between the groups, indicating small dispersion effects between young and old bumblebee workers.

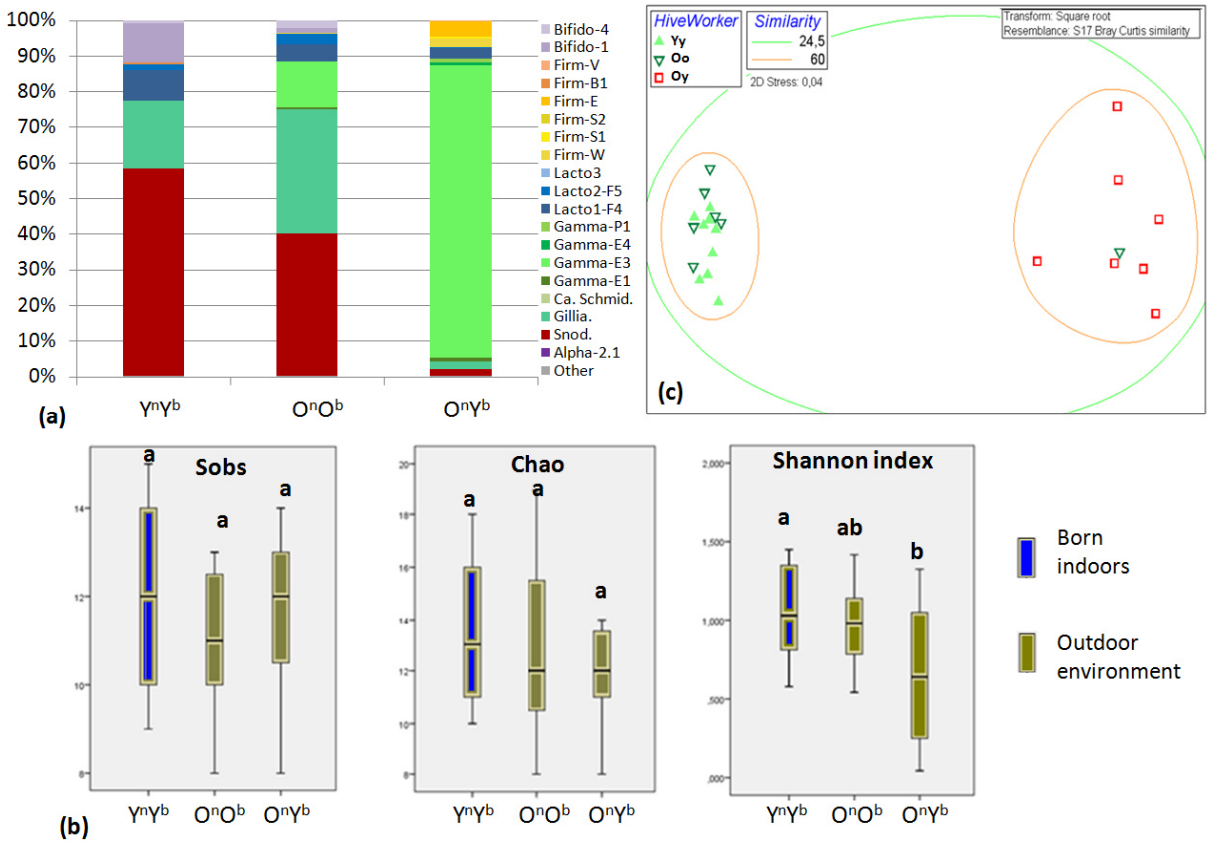


Figure 3.3. a. Relative abundance of all OTUs comparing test groups Y^nY^b, O^nO^b, and O^n Y^b of reared *B. terrestris* nests moved to an outdoors environment; **b.** Alpha diversity box plots (Sobs, Chao1, and Shannon index); **c**. NMDS plots. P =0.05; Mann-Whitney U

Table 3.3 P values and Wald test scores of gut microbiota OTUs, multivariate and univariate comparison between test groups Y^cY^b, O^cO^b, and O^cY^b of indoor reared hives (P > 0.05; mvabund package. anova.manyglm test)

Multivariate test		
Df.	Dev.	P-value
21	22.632	**0.045**
Univariate test		
	Wald-score	P-value
Bèta	6.740	0.116
Gamma-1	0.077	0.981
Gamma-2	0.152	0.981
Gamma-E2	2.344	0.726
Lacto1-F4	1.224	0.751
Lacto2-F5	0.251	0.981
Lacto3	2.344	0.726
Bifido-1	2.770	0.497
Other	6.539	0.116

4.2. Newborn workers showed a gut microbiota shift with a major colonization of *Enterobacteriaceae* when the nests were moved to outdoors

In the second experiment, reared nests were moved to outdoors, and we sampled again 24 nesting bumblebee workers for the 3 test groups with sufficient depth (Supplementary figure S3.2). The microbial community in the initial reared workers Y^nY^b developed a typical microbiota as seen in experiment 1 (Figures 3.1a and 3.2a) but workers from the nests moved to outdoors shifted completely after 6 weeks and new OTUs were taken up (Figure 3.2a). Data analysis revealed 20 bacterial OTUs as summarized in Table 3.1. Looking at alpha diversity (Figure 3.2b), we detected no differences in community richness (Sobs: P = 0.306 and Chao1: P = 0.507), but a significant difference in community diversity (Shannon: P = 0.002) in the group of young workers born in the old nest (O^nY^b). Indeed, the evenness of the gut microbiota is different in these young bumblebees due to the major uptake of new OTUs, especially Gamma-E2. Multivariate statistics (anova.manyglm) demonstrated a clear dispersion effect between the test groups (Dev = 585665, P = 0.001). For the beta diversity (Figure 3.2c), as represented in the NMDS plot (STRESS = 0.04), we saw a clear separate cluster (ANOSIM, R = 0.99, P = 0.001), grouping all workers that were newborn in the old nest (O^nY^b).

When looking at differences between foraging and nesting workers from the nest moved to outdoors, we sampled 14 bumblebees with an equal number of foraging and nesting workers (test groups M^nY^b and M^nM^b) with sufficient depth (Supplementary figure S3.3). Although we observed a

significant difference between the age categories M^nY^b and M^nM^b (anova.manyglm; Dev = 204923; P = 0.023), both univariate and multivariate statistics calculated no significant differences between foraging and nesting workers or between nests of both ages (univariate: all P values > 0.05, multivariate: anova.manyglm; Dev = 89929, P = 0.289; see Table S3.1 in the supplementary material).

Finally, when comparing reared nests with nests that were moved to outdoors, we observed 11 additional OTUs in the gut microbiota, here identified as Alphaproteobacteria (Alpha-2.1), *Enterobacteriaceae* (Gamma-E3, Gamma-E4, Gamma-P1), *Lactobacillaceae* (Lacto-3, Firm-S1, Firm-S2, Firm-E, Firm-B1, Firm-V) and *Bifidobacteriaceae* (Bifido-4). Especially, one OTU had become very dominant, namely Gamma-E3 with a BLASTn match that is close to the environmental bacteria *Rahnella* sp. (Genbank JQ522978.1, > 99 % identity) and *Yersinia kristensenii* (Genbank JN167946.1, 100 % identity) (Table 3.1). Remarkably, we observed a dominance of *Enterobacteriaceae* in all shifted profiles. Indeed, considering all age groups in the old nests outdoors, NMDS visualization showed a cluster of all young bumblebees (O^nY^b) together with one old bumblebee (O^nO^b) and two bumblebees of middle age (O^nM^b) that all showed shifted profiles (ANOSIM, R = 0.99, P = 0.005, data not shown). Moreover, a relative dominance of *Enterobacteriaceae* correlated with a drop in the relative abundance of the core OTUs, as was clear in the individual profiles of all age groups (Supplementary figure S3.4).

5. Discussion

In this study we used Illumina deep sequencing technology of the 16S rRNA gene to investigate the gut microbial communities of bumblebee workers in nests reared indoors under controlled conditions in the lab in comparison to nests moved to outdoors after an initial indoor rearing period. We demonstrated that, in reared nest, the gut microbial community is stable over 6 weeks and all age groups are composed of a small number of identical core bacterial taxa (OTUs) classified under *Neisseriaceae*, *Orbaceae*, *Lactobacillaceae* and *Bifidobacteriaceae* which can be grouped under core bacteria in bumblebees (Table 3.1). This observation is in agreement with Meeus et al. (2015) who found that reared *B. terrestris* nests showed a subset of core bacteria compared to wild bumblebees. Therefore, our in-depth analysis confirms a homeostasis of OTUs and negligible inter- or intra-nest variability when bumblebees are fed pollen and artificial nectar *ad libitum* and the kept under constant conditions. A homeostasis in gut microbiota of artificially reared, well-fed bumblebees has already been reported, even when bumblebees were primed with diseases (Koch et al. 2012). Therefore, our results indicate that age of the nest and worker is of minor importance when assessing the core microbiota in the gut of bumblebees under controlled conditions.

Second, we investigated in-depth the gut microbiota of reared nests moved to an outdoors environment and observed that the alpha diversity calculators showed no significant differences, whereas the beta diversity calculators differed significantly compared to reared nests. Here we discuss our results with a study on social and herbivorous (pollen-feeding) *Cephalotes varians* ants (Hu et al. 2014). In this study, a comparison between laboratory-reared and field-caught workers revealed significant differences in the beta diversity of the gut microbiota. However, it should be remarked that, although the alpha diversity did not change between the bumblebee nests that were moved to outdoors and those that were kept indoors in our experiments, the outdoor-developed bumblebees showed extra OTUs, i.e. Firm-B1, Firm-E, Firm-W, Lacto3, Gamma-E1 and Gamma-E4. Therefore, a possible increase in alpha diversity outdoors could be hidden because the extra OTUs are small and dispersed, as was also the case in the *C. varians* study (Hu et al. 2014).

In this study we observed that the profiles can change, mainly due to the colonization of Gamma-E3. A first hypothesis might be that the new bacteria were already present in low numbers in the nest of reared bumblebees, but that they were suppressed due to constant rearing conditions. Indeed, the outside environment itself with different nectar and pollen sources and other less optimal conditions is very different from the constant rearing conditions indoors promoting a small and stable microbiota (Meeus et al. 2015). In agreement, a shift in the gut bacterial diversity has been demonstrated in starving desert locusts *Schistocerca gregaria* compared to normal fed individuals (Dillon et al. 2010). Although, a second hypothesis remains possible as our results indicated that most of the new OTUs, including Gamma-E3, have been linked with environmental sources (BLASTn results shown in Table 3.1). Thus, the colonization of Gamma-E3 can also be caused by the uptake of natural bacteria from the environment, probably by foraging workers.

Because the foraging workers had a more direct contact with the new outdoor environment compared to nesting workers, we expected that the former would have a microbiota that shifted earlier compared to the latter. However, in this study we showed that the microbial community in the foraging workers did not shift earlier than the nesting workers of the same age but that the shift was mainly observed in the new workers (indifferent of their function) born in the old nest. We speculate that, when the workers had an established microbiota typically present in the young or middle aged nest, they were less susceptible to take up new microbiota. Nonetheless, we believe that the foraging bumblebees can still bring the new bacteria from the outdoor environment into the nest, and this in turn resulted in a new inoculation in the old nest, that produced the newest generation of bumblebees.

Importantly, we observed that shifted profiles showed a high abundance of *Enterobacteriaceae* at the expense of the core bacteria *Snodgrassella* sp. and *Gilliamella* sp. (Figure 3.2a). We showed that the *Enterobacteriaceae* bacteria are not exotic and most of the OTUs

correspond with Genbank entries linked with hosts as *Apis*, *Bombus* and other insects (Table 3.1) (Killer et al. 2011, Anderson et al. 2013, Engel et al. 2013). We believe this is an important observation because *Enterobacteriaceae* are not regarded as core gut microbiota in social bees. However, it should be remarked that both Cariveau et al. (2014) and Meeus et al. (2015) also reported that the relative abundance of *Enterobacteriaceae* can be dominant (relative abundance up to 90%) in some wild *B. bimaculatus* bumblebees and wild collected *B. terrestris*, respectively. Nevertheless, an *Enterobacteriaceae*-dominated gut microbial community can have its influence on the fitness of the bumblebee host or its resilience against pathogens (Cariveau et al. 2014). Indeed, a disruption in the homeostasis of mutualistic core-bacteria has been linked with dysbiosis and associated with pathogens (Hamdi et al. 2011, Koch and Schmid-Hempel 2012) and this may have happened also in the young workers in the old nests that were moved to outdoors. However, one should take care before making this conclusion not investigating variations in functions between individual bacteria species classified under the same family as was also reported by Hu et al. (2014) in the case of *C. varians* ants. In our case, the large uptake of *Enterobacteriaceae* does not necessarily indicate a state of dysbiosis but can also contribute to a host nutritional function, as has been reported in other insects including fruit flies and beetles (Behar et al. 2005, Vasanthakumar et al. 2006). Indeed, our BLASTn searches indicated close matches with *Rahnella* sp. (> 99 % identity) and *Yersinia kristensenii* (100 % identity). The latter bacterial species have been isolated from wild flowers from a nature reserve and are associated with *Apis mellifera* (Vasquez et al. 2012), while the former has been isolated from the gut of the willow leaf beetle *Plagiodera versicolora* (Demirci et al. 2013). *Rahnella aquatilis* has also been isolated from the gut of other beetles such as longicorn beetles (Park et al. 2007, Podgwaite et al. 2013) and Vasanthakumar et al. (2006) described that this bacterium plays an important nitrogen-fixing role for developing larvae in the southern pine beetle *Dendroctonus frontalis.* Thus, we believe that the large uptake of *Enterobacteriaceae* can also contribute to a host nutritional function in bumblebees. However, further research is necessary before firm conclusions can be drawn. Today, there exist no specific studies dealing with the regarded non-core *Enterobacteriaceae* in bumblebees or other bee taxa. Since these bacteria are present in the gut of European as well as North American bumblebee species, further research is needed in this family, in relation to host functioning, next to a further interest in the functions of mutualistic core and environmental non-core bee gut microbiota.

6. Supplementary data

Table S3.1. P-values and WALD scores of gut microbiota OTUs. multivariate and univariate comparison between age and foraging versus nesting bees; significant values are bolded (α = 0.05; mvabund package. anova.manyglm test)

Multivariate test			
	Df.	Dev.	P value
Nester-Forager	12	89929	0.289
Age	11	204923	**0.023**
interaction N-F:age	10	28701	0.485

Univariate test						
	Nester-Forager		Age		interaction N-F:age	
	Wald-score	P value	Wald-score	P value	Wald-score	P value
Alpha-1	305.347	0.920	381.636	0.902	45.934	0.966
Bèta	9885.124	0.728	51082.689	0.082	5340.098	0.710
Gamma-1	2293.259	0.769	18309.114	0.082	3975.332	0.710
Gamma-2	0.340	0.999	3.358	0.986	0.000	1.000
Gamma-E1	0.000	0.999	0.393	0.986	0.000	1.000
Gamma-E2	63463.857	0.159	84772.925	0.082	15181.672	0.444
Gamma-E3	10.124	0.920	9.945	0.985	0.894	0.966
Gamma-P	1.019	0.999	0.342	0.986	11.457	0.966
Lacto1-F4	4483.930	0.769	41138.911	0.082	99.239	0.966
Lacto2-F5	3714.108	0.769	4.556	0.986	297.041	0.966
Lacto3	0.100	0.999	13.817	0.985	18.452	0.966
Firm-W	1176.526	0.769	4258.491	0.279	140.308	0.966
Firm-S1	423.181	0.915	339.631	0.911	13.502	0.966
Firm-S2	0.000	1.000	0.000	1.000	0.000	1.000
Firm-E	575.057	0.915	487.717	0.890	24.277	0.966
Firm-B	326.023	0.915	349.358	0.911	24.938	0.966
Firm-V	1.386	0.986	1.694	0.986	0.000	1.000
Bifido-1	2921.056	0.769	2879.439	0.462	28.350	0.966
Bifido-2	314.043	0.915	844.182	0.814	1.073	0.966
Other	34.874	0.920	44.782	0.985	3358.007	0.710

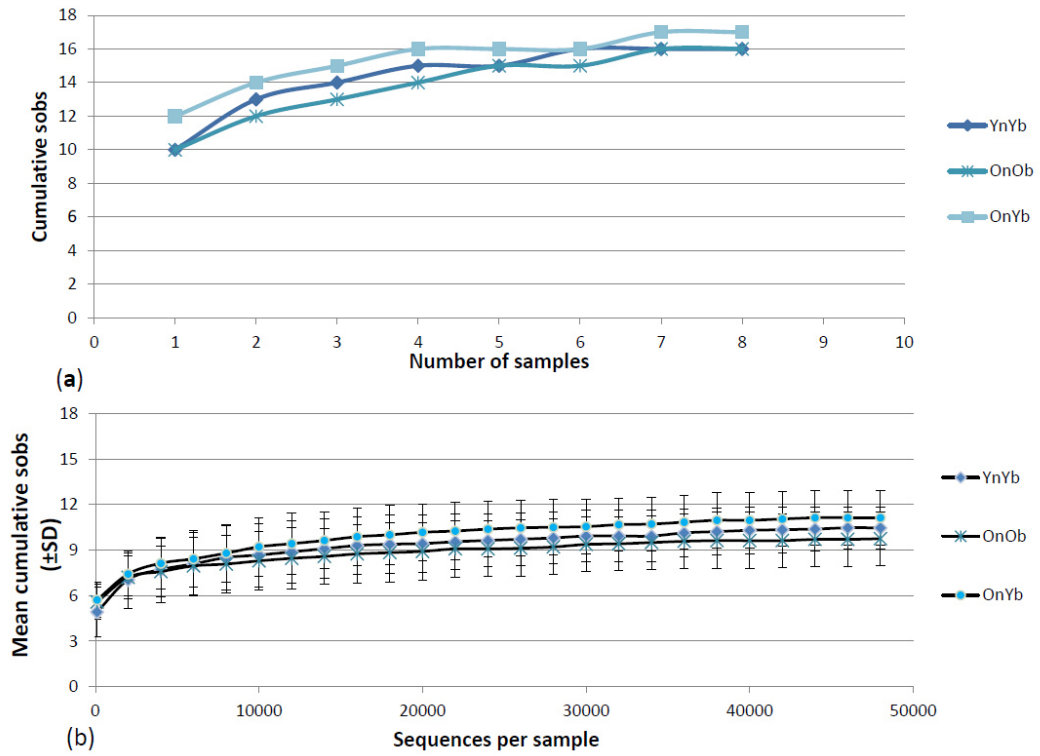


Figure S3.1 Reared nests. (a) Cumulative Sobs plotted versus number of samples; (b) Mean cumulative Sobs plotted versus number of sequences per test group

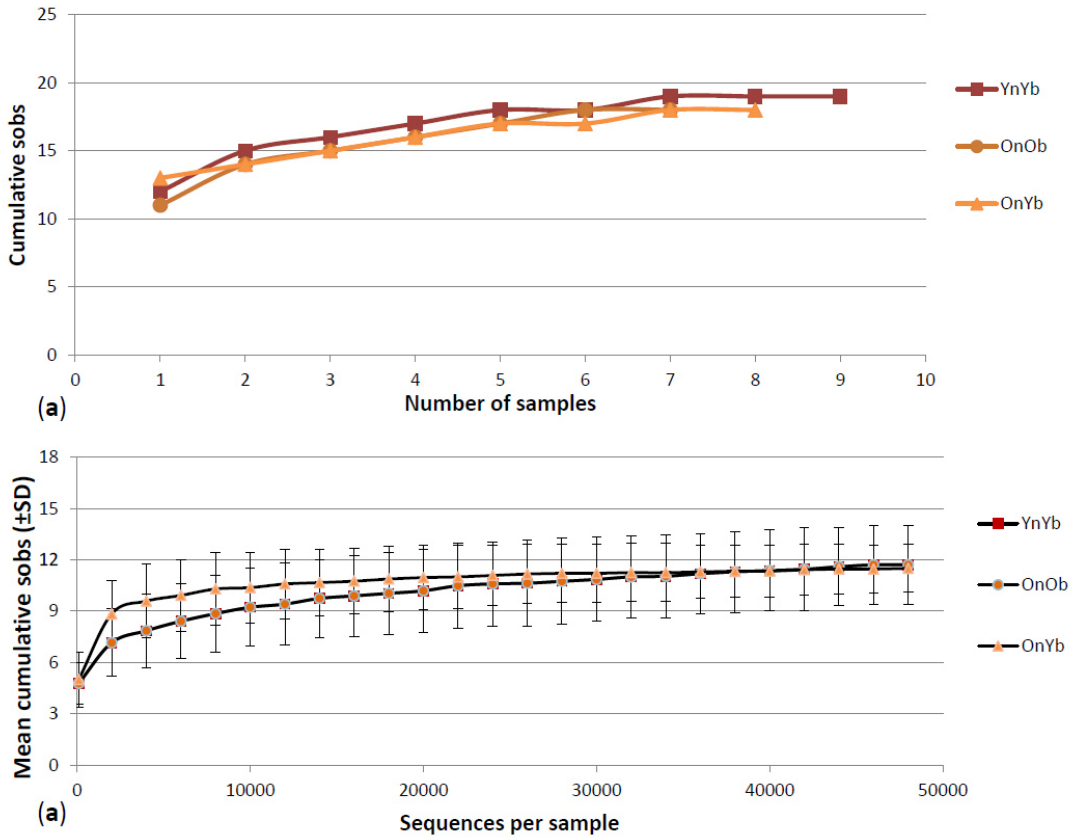


Figure S3.2. Reared nests moved to outdoors. (a) Cumulative Sobs plotted versus number of samples; (b) Mean cumulative Sobs plotted versus number of sequences per test group

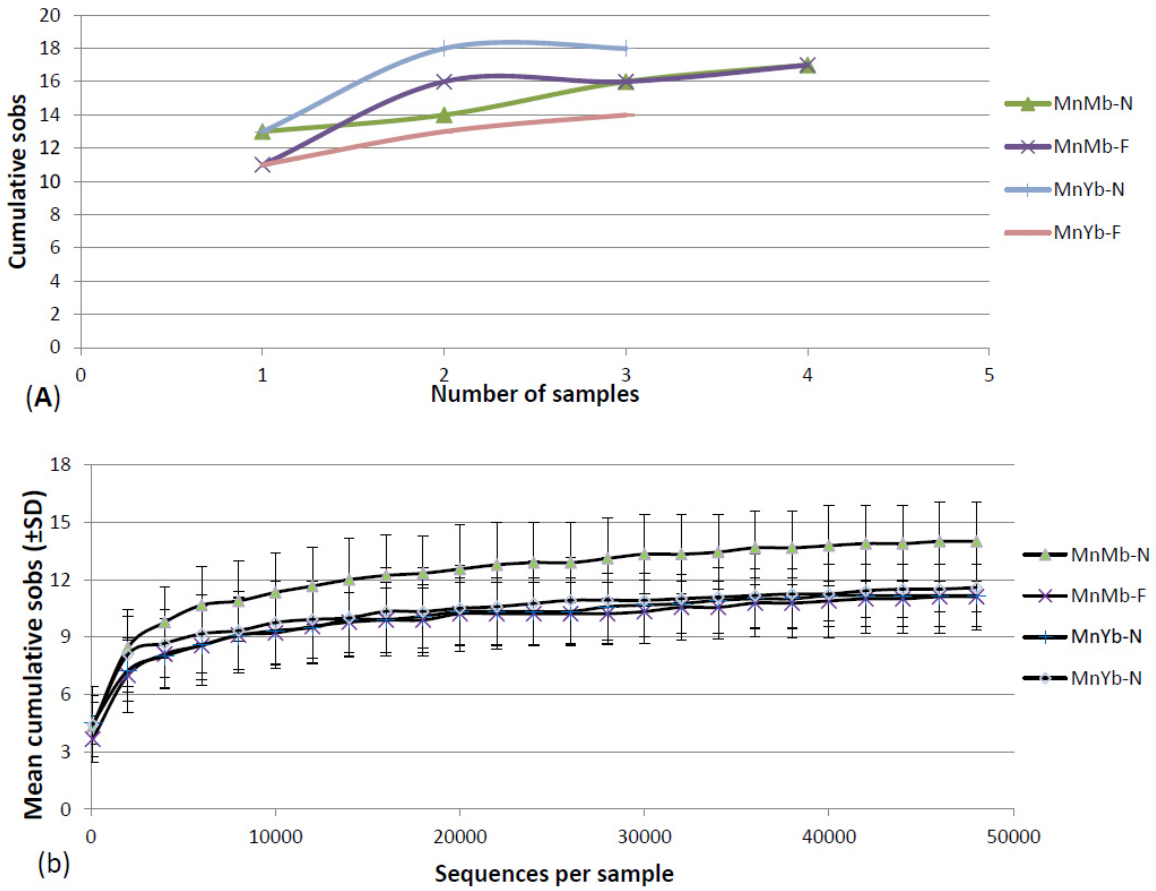
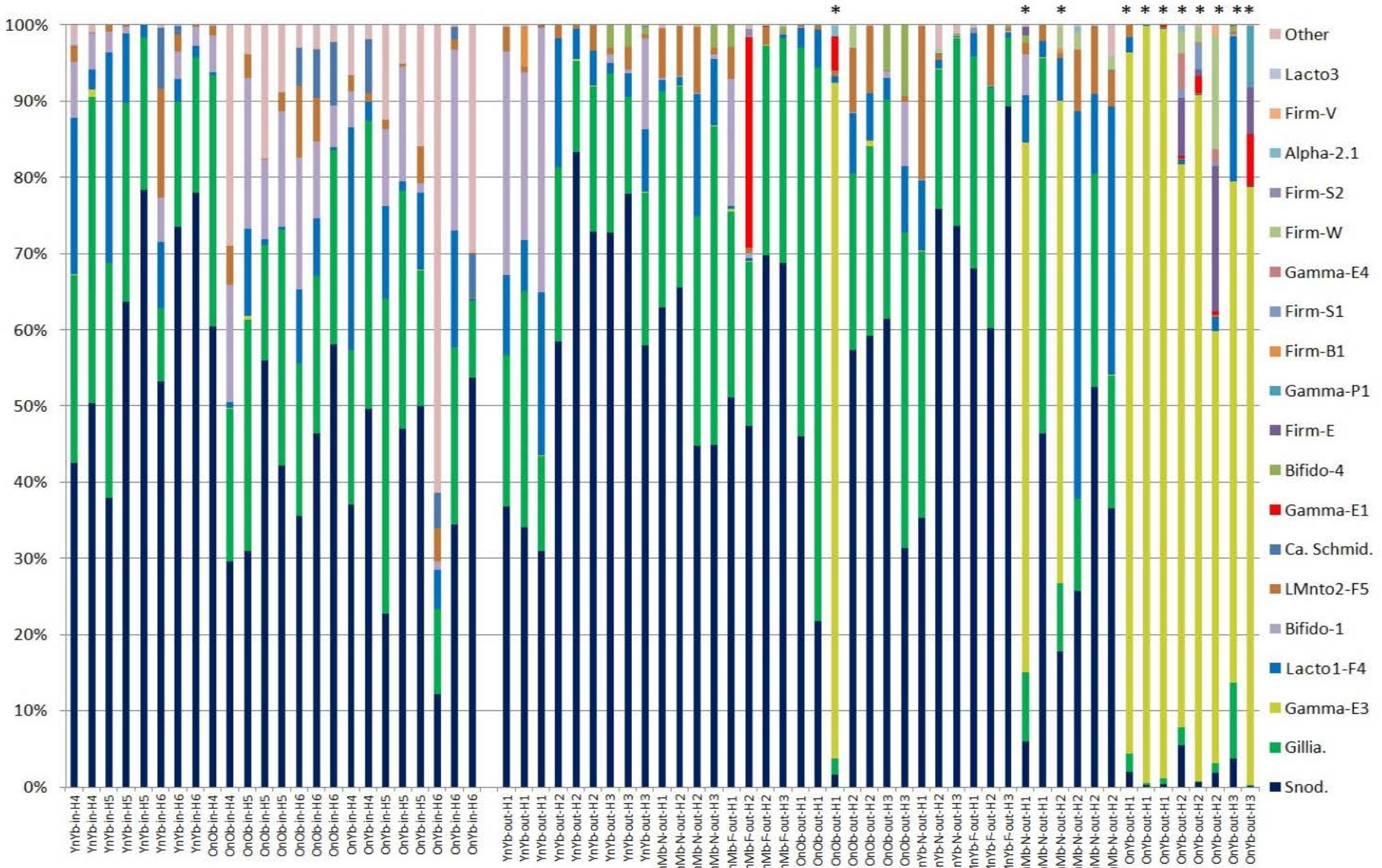


Figure S3.3. Forager (F) versus nesting (N) bumblebee workers. (a) Cumulative Sobs plotted versus number of samples; (b) Mean cumulative Sobs plotted versus number of sequences per test groups M^n Y^b and M^n M^b

Figure S3.4. Relative abundance of OTUs of individual samples of all test groups; shifted profiles are indicated with an asterisk



Chapter 4 :

Impact on gut parasites of “bottleneck” microbiota of reared bumblebees foraging outdoors

1. Abstract

Currently, the buff-tailed bumblebee *Bombus terrestris* is reared indoors for the biological pollination in agriculture, and application both in greenhouses and open field crops is growing. As there is an ongoing debate that the use of managed bees could spread pathogens towards wild counterparts, the application of managed bumblebees need further investigation towards stability of associated pathogens. While commercial breeders claim that these reared nest are free of viruses and (gut) associated parasites, in a previous study (Parmentier et al. 2014) we indeed confirmed that that the bottleneck gut microbiota of these reared nest is stable under indoor conditions, while shifing when placed outdoors for several week. In this study, we investigate on two important gut pathogens, *Crithidia* and *Apicystis* of reared bumblebee nests (*B. terrestris*) after being placed outdoors for several weeks. We screened samples of these nests and compared with wild samples collected (of *B. terrestris*-complex) in the proximate environment. We obtained a significant higher abundance of *Crithidia* in the reared samples but not for *Apicystis*. Our observations are discussed in relation to the bottleneck microbiota of reared bumblebee nests and in view of a possible spillback mechanism of gut pathogens playing here when interacting with wild bees.

2. Introduction

Currently, the buff-tailed bumblebee *Bombus terrestris* is reared indoors for the biological pollination in agriculture, especially for greenhouse crops. There is an ongoing debate related to the use and impact of managed bees, especially in relation to bee transports. As demonstrated in chapter 1, due to intercontinental transports, contact of allopatric managed bees and wild bees can lead to spillover of diseases which can further mediate in the emergence of EIDs (Power and Mitchell 2004, Otterstatter and Thomson 2008, Dafni et al. 2010, Meeus et al. 2011, Graystock et al. 2013b, Graystock et al. 2015a). Consequently, there is a current consensus in literature that such intercontinental transports should be avoided in favor of employing local bee species for pollination services (Meeus et al. 2011, Maharramov et al. 2013, Goulson and Hughes 2015).

Related to domesticated bumblebees, it is supposed that, when these nests are only used in closed greenhouses, that the risk of a sympatric spillover should be low (Graystock et al. 2015a). However, it has been demonstrated that bumblebees can escape from greenhouses (Colla et al. 2006, Dafni et al. 2010, Goka 2010) leading to competition (Inari et al. 2005) and pathogen spillover (Dafni et al. 2010, Goka 2010) in native wild populations. For example, In Ireland, it has been found that the infection level of gut parasites *Nosema bombi* and *Crithidia* in conspecific wild bees was significantly higher within a distance of 2 km from greenhouses compared to a distance of 10 km, demonstrating a possible spillover from commercially used *B. terrestris* nests (Murray et al. 2013). Moreover, there is a current trend of using commercially-reared nests for pollination in open-field crops, leading to inevitable interactions between domesticated and wild species. Thus, sympatric spillover of parasites is also likely to occur, and when happening repeatedly within sympatric pollination networks, this can lead to a disturbance of local host-parasite dynamics.

Since previous studies stressed on the negative outcomes of spillover from domesticated nests , mitigation measures are increasingly been put forward (Goulson and Hughes 2015) and domesticated bumblebee nest are screened to be parasite-free after indoor-rearing. However we advocate that the introduced domesticated bees can still act as a reservoir for parasites or viruses when foraging outdoors due to a higher uptake and proliferation of pathogens in these domesticated hosts. This possibly further leading to a repeated sympatric spillover to wildbees, a process also referred as spillback (Kelly et al. 2009, Goulson and Hughes 2015). Indeed, in chapter 2 we showed that although the gut microbiota of reared *Bombus* species share a core of socially transmitted gut microbiota seen in wild bumblebees (Meeus et al. 2015, Billiet et al. 2016), the diversity and richness of reared bumblebees was lower compared to wild species leading to a “bottleneck microbiota”. Moreover, in chapter 3 we investigated on the stability of this bottleneck microbiota and showed that, when reared bumblebee nests are placed outdoors, their core gut microbiota hugely shifts with

a major uptake of *Enterobacteriaceae*, which was especially seen in the newborn workers when the nests were about 8 weeks old (Parmentier et al. 2015). The bottleneck microbiota of reared bees, or shifted microbiota after nests were placed outdoors could impair the immune competence of reared bees. Indeed, it has been shown that bumblebees with a deficient gut microbiota are less protected against infection with the gut parasite *C. bombi* (Koch and Schmid-Hempel 2011b) and the variation in gut microbiota was correlated to the variation in resistance to *C. bombi* (Koch and Schmid-Hempel 2012). Moreover, Cariveau et al. (2014) also suggested a positive correlation between the richness of non-core gut microbiota and gut parasites of *C. bombi*.

Here we speculate one of the consequences of this "bottleneck microbiota" of reared bumblebees could lead to a higher level of gut parasites compared to wild bumblebees. Therefore, we setup an explorative experiment to compare the pathogen prevalence of foragers of reared bumblebee *B. terrestris* nests placed outdoors with bumblebee foragers captured in the same environment. We used placed these nests in a spatial matrix of different landscapes and screened for the presence of two important gut parasites, i.e. *Crithidia bombi* and *Apicystis bombi* (Schlüns et al. 2010, Koch and Schmid-Hempel 2011b, Maharramov et al. 2013) after foraging 6 weeks outdoors and compared pathogen load within sympatric wild collected specimen of the same species.

3. Material and methods

3.1. Study sites and sampling *B. terrestris* bumblebees

We selected for 6 locations in provinces East Flanders and Flemish Brabant (Flanders, Belgium) and further searched for 2 study sites (a. and b.) in each location to obtain a total of 12 study sites, as represented in figure 4.1. In each study site, we placed two (standardized) commercial *B. terrestris* hives which served to sample bumblebees after the nest developed for 6 weeks in the environment. We sampled two foragers per nest, by observing the nest entrance, and trapping a bee entering or leaving the nest. In parallel, we also sampled wild bumblebees of *B. terrestris* in the neighborhood of the reared bumblebee nests. To ensure that we did not collect foraging bees from the nests as wild bumblebees, we closed the nests before collecting wild bees of the *B. terrestris*-species complex. We collected 2 foraging bees from the domesticated nests and 2 wild bees which were put immediately in a box with dry ice to ensure a direct chilling of the bees. After sampling, the bumblebees were chilled and put in the ultra freezer at -80 °C until further processing.

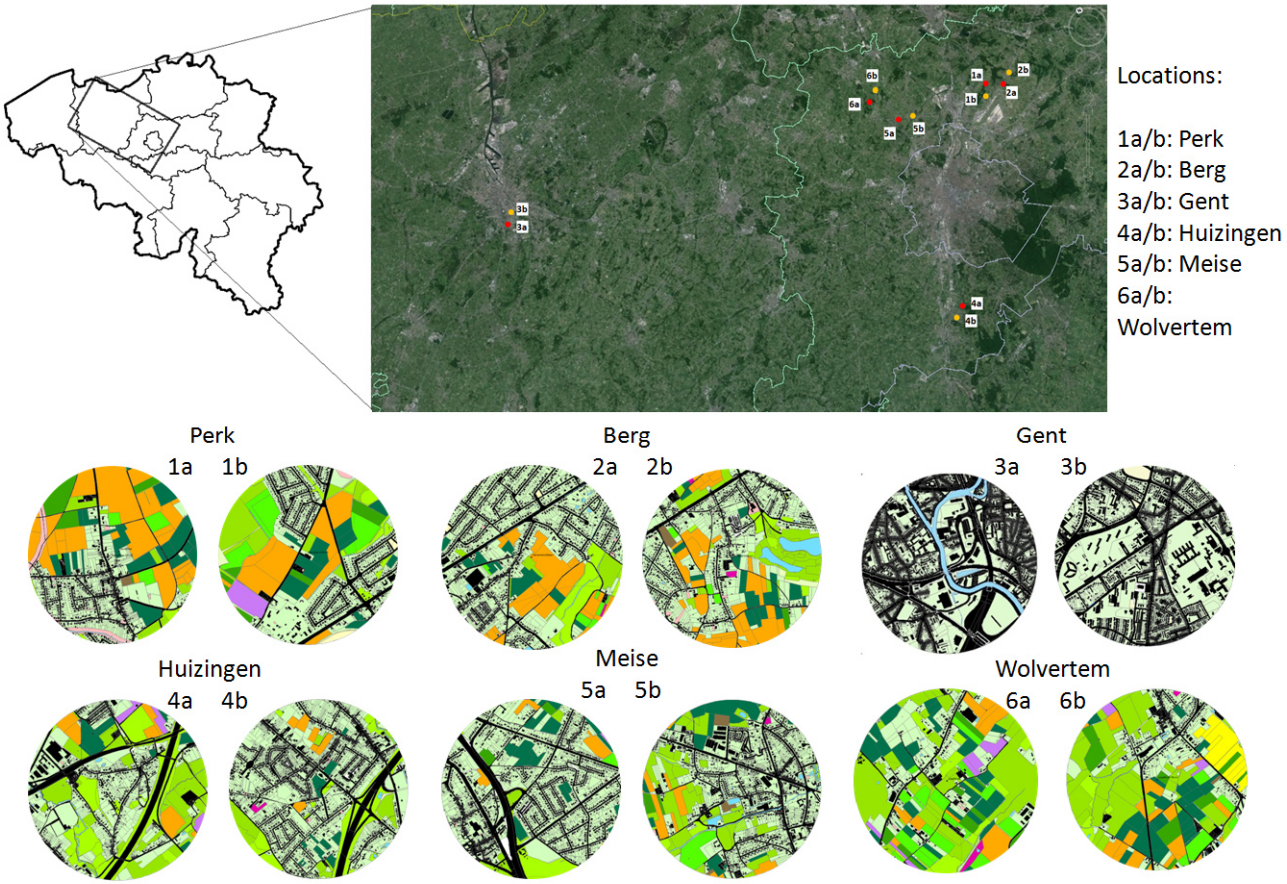


Figure 4.1. Locations and study sites selected in our test setup. In each location, we selected two study sites where two standard domesticated *B. terrestris* nests were placed. After 6 weeks 2 foraging bees were sampled from each nest (n = 24) , and in their neighborhood also 2 wild *B. terrestris* (n = 24)

3.2. Parasite detection

Each sampled bee was processed individually, we removed the thorax from the body which was put in Eppendorf tubes together with 800 µl RTL lysis buffer G, 3 stainless steel beats of 2 mm and 1 of 5mm and a 100 µl of zirkonia beats. Tissue disruption was achieved by beat beating for 2 min at 30 Hz and 1 min at 20 Hz using a TissueLeaser II device (Qiagen®). After centrifuging at 17,000 g for 3 min until disrupted tissue parts were spinned down, 200 µl of the supernatant collected to be used in the DNA extraction protocol according manufacturer's specifications (DNA extraction; DNeasy® Lipid Tissue Kit, Qiagen®). At the end of the procedure, we obtained a total of 200 µl DNA in RNA-free water. Extracted samples were stored at -20°C prior to molecular detection of parasites.

We used a multiplex PCR based screening method on DNA extracts for detection of protozoan parasites. All PCR reaction mixtures contained 2 µM of each primer; 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 1.25 U Taq DNA polymerase (Qiagen) and 1 µl of DNA extract. We used the combined broad range primer sets NeoF CCAGCATGGAATAACATGTAAGG – NeoR GACAGCTTCAATCTCTAGTCG and SEF CTTTTGGTCGGTGGAGAT – SER GGACGTAATCGGCACAGTTT to screen for *Apicystis* and *Crithidia* and

generating a 260 bp and 420 bp fragment, respectively (Meeus et al. 2010). Positive *Crithidia* samples were sequenced to confirm infections strain of *C. bombi*.

3.3. Statistics

Comparing differences infection level of *Apicystis* and *Crithidia* in domesticated versus wild bumblebees, we run statistics in R using the Welch Two Sample t-test in package "stats".

4. Results

4.1. *A. bombi* and *C. bombi* infections

Results of a multiplex PCR on 24 individual samples of domesticated and wild collected *B. terrestis* from 6 locations are visualized in figure 4.2.

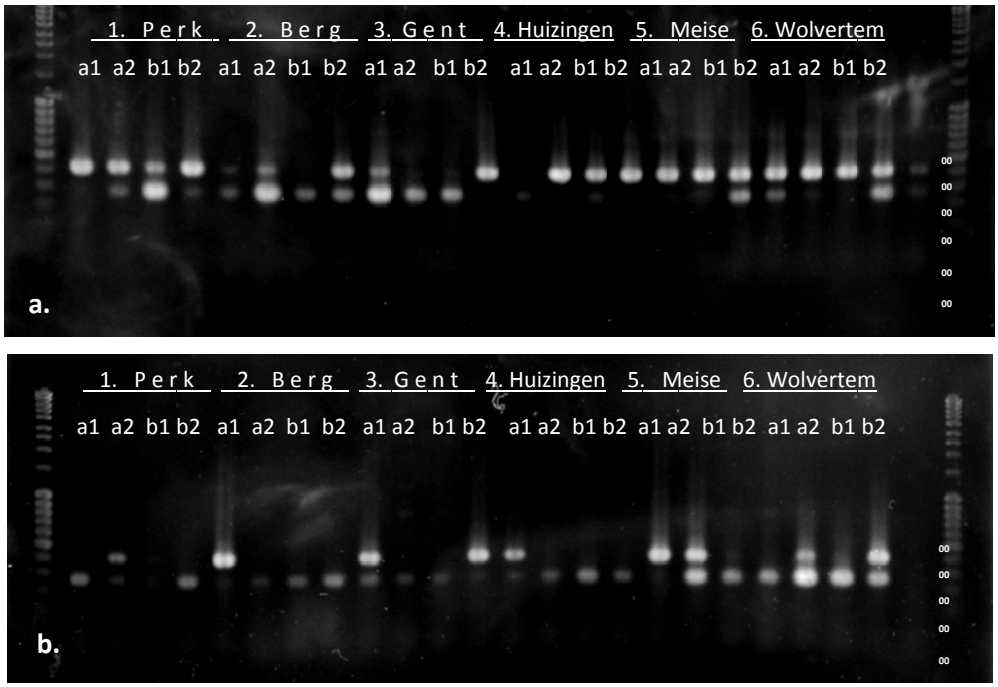


Figure 4.2. PCR results of *Crithidia* (420 bp) and *Apicystis* (260 bp) infections visualized on a 1,5% agarose gel. Shown are samples from domesticated *B. terrestris* (**a.**) versus wild *B. terrestris* bumblebees sampled within the same environment, in 24 study sites (12 locations) (**b.**); note the visual difference of *Crithidia*-infection level in domesticated vs. wild bumblebees

When comparing infection rate of *C. bombi*, we obtained a significant higher infection in domesticated versus wild bumblebees (P = 0.008; unpaired t.test), while no difference was seen between these groups for *A. bombi* infection rate (P = 0.242; unpaired t.test), as summarized in table 4.1. While a clear difference was obtained when looking at individual *C. bombi* or *A. bombi* infections, total infection rate showed no difference between domesticated and wild collected bumblebees (P = 0.209; unpaired t.test).

Table 4.1. Infection rate of *C. bombi* and *A. bombi* infections in individual samples of domesticated and wild *B. terrestris* bumblebees

Parasite	Nest/Wild	Infected (n =24)	%	P-value
C. bombi	Nest	18	75	**0.008**
	Wild	9	38	
A. bombi	Nest	13	54	0.242
	Wild	17	71	

5. Discussion

5.1. Impact of a "bottleneck' gut microbiota of reared bumblebees

We explored the impact of domesticated reared bumblebees having a core but "bottleneck" microbiota towards pathogen resilience when nests are placed outdoors. Focusing on foraging bumblebees, we screened individual samples from 24 nests placed in 12 different study sites for gut pathogens of *C. bombi* and *A. bombi.* After 6 weeks foraging in the outdoor environment, we showed that *C. bombi* had a significant higher prevalence in comparison to wild bumblebees of the same *B. terrestris* species complex, captured in the same environment, but no effect was seen for the neogregarine *A. bombi*. Thus, domesticated reared bumblebees with a "bottleneck" microbiota foraging outdoors can show a different resilience, both negative and neutral towards gut infections. Yet, it has been described that especially *C. bombi* infections can lead to clear detrimental effects on the fitness of bumblebees (Schlüns et al. 2010, Koch et al. 2012). Indeed, it has been shown that this gut-infecting parasite impacts on survival of *B. terrestris* queens over hibernation, colony founding, and the subsequent reproductive fitness of colonies (Brown et al. 2003b, Yourth et al. 2008). Beside, infection of workers of *B. impatiens*, results in lower foraging efficiency (Gegear et al. 2006). Moreover, domesticated bees infected with *C. bombi* have been suggested to mediate as reservoirs to the spread towards wild bees (Colla et al. 2006, Li et al. 2012, Sachman-Ruiz et al. 2015) as well as sympatric bees (Murray et al. 2013, Graystock et al. 2014, Graystock et al. 2015a).

5.2. Reared bumblebees outdoors mediating in "parasite spillback"?

The observation of a higher *C. bombi* load in domesticated hosts can have important implications towards wild sympatric congeners, possibly leading to a spillback towards the latter when there is shared contact, i.e. direct or indirect via shared flowers (Singh et al. 2010, Graystock et al. 2015b). Indeed, it has been suggested before that spillback of diseases is a neglected concept and

mediates in 67% of the outbreak of animal diseases (Kelly et al. 2009), as explained in chapter 1. In a recent review on mitigating measures in the anthropogenic spread of bee parasites, also Goulson and Hughes (2015) hypothesized that managed bees, entirely free of parasites on arrival at a site, may become infected with parasites from wild bees, which may then spillback into wild bees. Indeed, it has been shown before that that commercial bumblebee nests can become infected (e.g. for *Nosema*, *Apicystis* and *Crithidia*) while these diseases were not detected initially (Maharramov 2015). Here, we add that the prevalence of at least one important disease, *C. bombi*, known to impact on bumblebee host fitness, can be higher in comparison to wild congeners. As we did not screen all nests before placing outdoors to be parasite free (but stated by the breeder company – personnal communication with Biobest, and confirmed in previous experiments), in this exploring experiment we cannot give final proof of a possible spillback. Yet, we showed a first indication that this principle could have played here and in the light of ongoing and increased use of native commercial reared nests in open field crops, this is an important observation which need further in-depth investigation, also in a wider spatio-temporal matrix of different stressors and landscapes (Sydenham et al. 2014, Goulson et al. 2015). This will also be investigated more in depth within research chapter 7.

Chapter 5:

Commercial bumblebee nests to assess an anthropogenic environment for pollinator support: a case study in the region of Ghent (Belgium)

This chapter is based on:

Commercial bumblebee hives to assess an anthropogenic environment for pollinator support : a case study in the region of Ghent (Belgium) (2014).
Laurian Parmentier, Ivan Meeus, Lore Cheroutre, Veerle Mommaerts, Stephen Louwye and Guy Smagghe

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1. Abstract

Anthropogenic changes of the environment influence the distribution and abundance of pollinators such as bumblebees and have been proposed as one of the main causes in their worldwide decline. In order to evaluate the impact of expanding anthropogenic landscapes on supporting pollinator potential, reliable tools are needed. *B. terrestris* is one of the most abundant bumblebee species in Europe, and these bumblebees are known as generalist pollinators of not only wild flowers in nature but also of crops in agriculture. For more than two decades, these bumblebees have been commercially mass reared for biological pollination in greenhouses. In this project, we placed commercial hives of the bumblebee *B. terrestris,* containing one queen and 40 workers, in three different locations in the region of Ghent (Belgium), and the performance of these hives was followed during a 4-week period in spring 2012. In parallel, we determined the floral richness and diversity index in the chosen study sites. The sites consisted of a rich urban environment with patchy green areas opposed to an urban environment with poor landscape metrics; a third rural study site showed average positive landscape metrics. The results demonstrated that the hive biomass and numbers of workers increased significantly in the rich compared to the poor environment, providing a mechanism to discriminate between study sites. In addition, the bumblebee-collected pollen showed that the flowering plants Salix spp. and Rosaceae/Prunus spp. are dominant food sources in all anthropogenic environments during early spring. Finally, the results are discussed in relation to the optimization of the experimental setup and to the use of commercial bumblebee hives in assessing local pollinator support within any given environment.

2. Introduction

Over the last few decades, traditional environments have been changing rapidly in most regions due to urban, industrial and agricultural expansion (United Nations 2012). This has widespread implications for wildlife and plant populations at international, national, regional and local scales (Kremen et al. 2002, Pywell et al. 2006, Winfree et al. 2009, Potts et al. 2010). While these impacts have been relatively well-studied for species such as birds and large animals, little is known about the impact on bees (Gallai et al. 2009, Cameron et al. 2011). Because bumblebees and honeybees are wide-range pollinators, they are not only essential in ecosystems, but also of crucial importance for seed and fruit production in many agricultural crops (Fuchs and Muller 2004). In addition, commercial bumblebee nests have been applied for the last two decades for pollination services in greenhouse crops, particularly tomatoes (Velthuis and van Doorn 2006). Given their considerable importance, the apparent global decline of pollinators has led to growing concern (Goulson 2003a, Ghazoul 2005, Goka 2010, Goulson 2010, Potts et al. 2010, Szabo et al. 2012).This problem is complex and seems to be a result of several causes, including pollution and the use of agrochemicals (Mommaerts et al. 2010, Whitehorn et al. 2012), anthropogenic landscapes (Goulson et al. 2010, Verboven et al. 2012, Wojcik and McBride 2012) and a shortage of flowering plants and food sources (Pywell et al. 2006, Cameron et al. 2011).

To develop good conservation strategies, it is essential to know which environmental elements support pollinator development. To date, different studies compared anthropogenic environments by counting the abundance of pollinators and calculating pollinator densities. Henning and Ghazoul (2011) demonstrated that plant diversity and floral abundance in urban environments promote pollinating flower visitors. The urban parameters “extent of green area” (any patch of vegetation within the urban matrix) and “edge density” (the boundaries between urban land use types) were found to be important determinants of pollinator abundance and flower visits. But the situation seems to be more complex when focusing on individual bee species in urban environments. In this respect, Banaszak-Cibicka and Zmihorski (2012) concluded that a city can be a very important habitat for bees but not for all bee species because there are winners and losers. Similarly, Wojcik and McBride (2012) investigated the numbers of individual pollinator visits by different bee species on *Eschscholzia californica* poppy patches in urban or wild land environments. The major conclusions were that in wild land, resource patch size and density correlated well for all bee species, and that in urban environments (cities) with similar resource mosaics (edge density and green area) the larger-bodied bumblebees (e.g. *Bombus vosnesenskii*) were less abundant as compared to the smaller-bodied ones and less social bee taxa. Generally, there is a consensus that cities can have a good

distribution of resources, but results, based on pollinator counts, indicated that an urban environment can have different effects on the abundance of pollinator species.

Currently, insights obtained by counting the numbers of pollinators are crucial, however these lack the capacity to identify whether a certain environment has the potential to support more pollinators. When looking for a tool among different experimental setups to measure this potential for pollinator support, bioassays based on field experiments are probably the best placed to obtain the most realistic data (van der Steen 2001). An example has recently been found in the use of hives of the honeybee *Apis mellifera*, using these workers as small flying samplers to detect heavy metal contamination in the environment (van der Steen et al. 2012). However, there are several disadvantages with honeybees as for example the high cost of honeybee hives, the non-standardized size of honeybee hives, the appearance of honeybee diseases and varroa mites leading to uncontrolled losses of workers, and the accumulation of contaminants in the produced honey products. Some other examples of bioassays exist with flying insects, for example, butterflies (*Danaus plexippus, Lycaena melissa*) have been released in open field to screen for toxicological effects by the Lepidoptera-specific *Bacillus thuringiensis* toxins (Mattila et al. 2005, Peterson et al. 2006).

In this study, we report on the use of commercial bumblebee nests, that are produced for biological pollination and that are standardized to contain one queen and 40 workers, for assessing local pollinator support in an environment of study. We selected 3 pre-defined anthropogenic environments in the region of Ghent in Belgium, namely two urban zones, one close to the city centre and an industrial harbor site, and a third rural location out of the city centre. The urban study sites were chosen so that our setup consisted of a rich environment with patchy green areas opposed to an environment with poor landscape metrics. In each study site, bumblebee nests were placed and the performance of these nests was followed in the respective environment. Different parameters as “nest biomass increase”, “numbers of living workers”, “numbers of dead marked workers”, “numbers of pupae”, “numbers of queen pupae” and “difference in numbers of full sugar pots” were scored with the aim to identify those that are significant in determining the environment of study. In parallel, we determined the floral richness and the Shannon-Wiener diversity index (SWDI) in the chosen study sites in the beginning of May 2012, and this was carried out together with an analysis of pollen samples as collected by foraging bumblebees.

3. Materials and Methods

3.1. Determination of study sites

For the 3 locations, we selected a circle as our study site with the five nests in the centre and a radius of 1000 m as the average foraging distance (Fig. 5.1). This choice was based on Walther-Hellwig and Frankl (2000) and Knight et al. (2005), reporting that 663 m is the average foraging distance for *B. terrestris* and 758 m the minimum estimated maximum foraging range, respectively.

The study sites were characterized by using 3 landscape parameters: "extent of green area", "edge density" and "flower diversity". The "extent of green area" and "edge density" followed the method of Henning and Ghazoul (2011) and calculated in ImageJ (Schneider et al. 2012). Green area included meadows, grassland, gardens and parks (but not forests and uniform farmlands), and was calculated as the proportion of green area (m^2) to the total area (m^2). We did not discriminate between the types of land use adjacent to green area patches. Edge density (m/ha) was defined as the edge length of green areas divided by its total area. The patch density (per 100 ha) = n / a, with n = numbers of patches; a = area. In our analysis we used a resolution of 5 m.

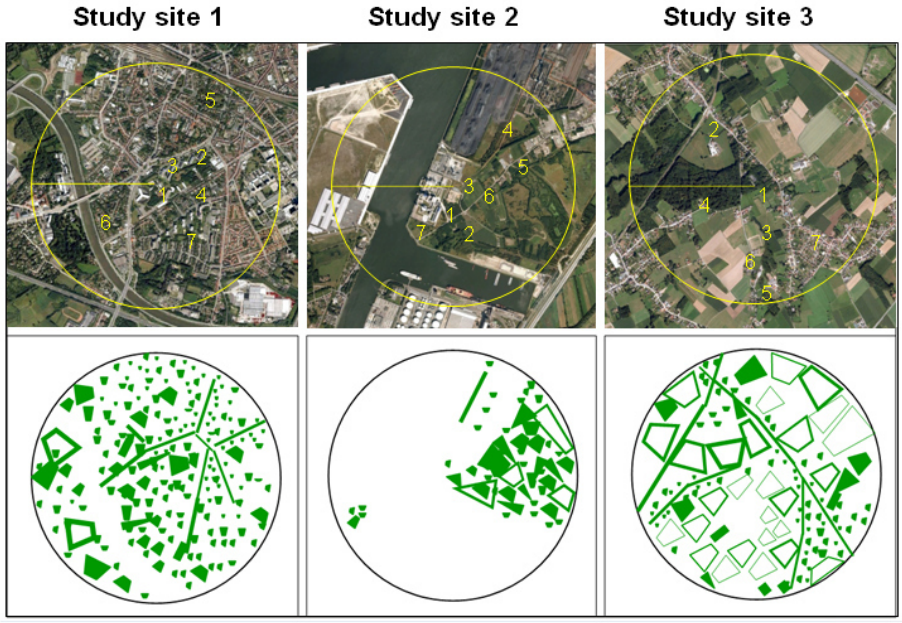
Since foraging behavior of bumblebees is also under the influence of a spatial distribution of the flowering plant species in that landscape (Walther-Hellwig and Frankl 2000), we calculated for each study site also the flower plant biodiversity with use of the SWDI (Hennig and Ghazoul 2011). In each study site, 7 areas of 50 m² (Fig. 5.1) were chosen *at random* and in these we determined the flowering plant species in herb, shrub and tree layers to species level and also made a total count of the different flowering plant species as presented in Table S5.1. With these data the SWDI was calculated in accordance with the method as described by Van Geert (2010): $SWDI = \sum (P_i \times \ln P_i)$, with P_i representing the relative abundance of one flowering plant species compared to all species present in the same area, and "i" representing the area and ranging between 1 to 7.

Study site 1: rich environment in city centre. The first study site, situated at 50°58'28.67"N 3°48'25.62"O (Fig. 5.1), is located in a residential neighborhood close to the city centre of Ghent. In this neighborhood, most houses have a small garden wherein a variety of flowering plants are growing. This environment was also characterized with landmarks such as public parks and small shrubberies containing trees and flowerbeds. The calculated percent of green area was 21.24% and the edge density was 86.0.

Study site 2: poor environment in industrial zone. The second study site is situated at 51°09'10.24"N 3°47'21.65"O, and is an industrial zone in the seaport of Ghent. This environment was composed of only about a quarter of green area, containing trees and flowering plants and therefore poor in food (Fig. 5.1). Other typical landscape traits were larger buildings and a sea canal. The green area percentage and edge density were 11.33% and 46.8, respectively.

Study site 3: rural environment. A third, rural test locality, situated at 51°01'32.57"N 3°42'46.10"O, is a rural area with agriculture, forestry and urban activities, and located outside of the city of Ghent. Typically, this study site is an open landscape structure with longer edges, composed of streets with houses and gardens as well as bigger fields with monocultures. There were also some small forests in this test area. The percent green area of total area was 21.43% and edge density was 40.8.

Figure. 5.1. Overview of the 3 pre-defined anthropogenic environments in the region of Ghent in Belgium with study site 1: rich environment close to city centre; study site 2: poor environment in an industrial area in seaport; study site 3; rural area with agriculture, forestry and urban activities and outside of city centre. The study sites were defined by a circle with a radius of 1000 m and with the 5 nests in the centre. N° 1-7 represent the 7 areas of 50 m² per study site where the flowering plant species presence was determined. For each study site, the landscape parameters of green area and edge density were calculated as described in the M&M



	Study site 1	Study site 2	Study site 3
Green area (km²)	0.38	0.25	0.56
Perimeter green area (km)	32.69	11.69	22.85
% green of total area	21.24	11.33	21.43
Edge density (per 100 ha)	86.0	46.8	40.8

3.2. Bioassay with commercial *B. terrestris* bumblebee nests

3.2.1. Origin of bumblebee nests and marking of workers

Fifteen nests of *B. terrestris* were obtained from a commercial bumblebee mass-rearing (Biobest, Westerlo, Belgium). All nests contained one queen and 40 workers, and the nests were fed with pollen and artificial nectar *ad libitum* (Mommaerts et al. 2009). Prior to the field experiment,

each bee was marked with a color specific to the study site. In addition, all pollen and artificial nectar was removed from the nests before placing the nests outside.

3.2.2. Placement of bumblebee nests in each study site

In the centre of each study site we placed 5 bumblebee nests. The nests were placed in a polystyrene multi-nest-box (80 x 35 x 25 cm) where each had room for a maximum of 3 nests (Fig. S5.1.). The performance of these nests was followed during a period of 4 weeks from mid April 2012 to mid May 2012. During the experiment, the multi-nest-boxes were covered with black plastic for reasons of insulation and protection against rain. All nests were placed with their entrance to the East. In study sites 1 and 2, a collateral placement was applied: the multi-nest-box with 2 nests was placed in front of the other multi-nest-box with 3 nests. In study site 3, all nests were placed next to each other in a linear arrangement.

3.2.3. Assessment of performance of bumblebee nests during experiment

For all nests, their foraging activity was checked visually at day 3 after the start of the experiment in order to confirm that all colonies were active. The following 6 nest parameters were measured during the experiment: “nest biomass increase”, “numbers of living workers”, “numbers of dead marked workers”, “numbers of pupae”, “numbers of queen pupae” and “difference in numbers of full sugar pots”. All the parameters were checked at the beginning and the end of the experiment, except for “numbers of dead marked workers” which was recorded on a weekly basis and then dead workers were removed from the nests. For “numbers of workers” we counted marked and unmarked workers separately where unmarked specimens included both workers newly developed in the nest during the experiment and wild bumblebee workers that entered the nest.

3.2.4. Statistical analysis

For all data, normal distribution of variances was confirmed by an independent F-test ($\alpha = 0.05$), and then the means ± SD were analyzed by an independent-sample *t*-test ($P = 0.05$) using SPSS vs. 21.0. Calculation of the test power was conducted using a statistical power calculator (DSS Research, Arlington) with the option of ‘two sample–average’ for analysis of study sites 1 and 2, and ‘one sample–average’ for analysis of study site 3.

3.3. Determination of bumblebee-friendly flowering plants in each study site

To determine the bumblebee-friendly flowering plants as present in each study site, we compared the list of flowering plants as we observed per study site (Table S5.1) with a list of bumblebee-friendly flower plant taxa as reported before by Lemmens et al. (2012). Total counts of plant species were grouped and presented per plant family.

3.4. Analysis of pollen as collected by foraging bumblebees in each study site

Here we analyzed the pollen that was collected by the foraging workers of the nests in each study site. To achieve this, at the beginning of May 2012, 10 bumblebees returning to their nests were collected *at random* with a sweep net in each study site, and the pollen loads were scraped from their corbicula and stored individually. Afterwards, the collected workers were released. For pollen identification, we used the protocols of Reille (1998) and Van der Ham et al. (1999), and then analyzed under a light microscope with use of the identification key of Beug (2004).

4. Results and Discussion

4.1. Assessment of bumblebee nest performance in a rich versus poor study site (sites 1 and 2)

In the study sites 1 and 2, the 5 nests were placed with one multi-nest-box containing 2 nests, in front of the other multi-nest-box, containing 3 nests. Although not predicted, only the data of the 2 front nests could be used because the 3 back nests did not develop well (data not shown). Here it was of interest, with use of the front nests, that the parameters “nest biomass increase” and “numbers of living workers” were significantly higher (P=0.013; t=8.65; df=2 and P=0.047; t=4.46; df=2, respectively) for site 1 with a rich environment as compared to site 2 with poor landscape metrics (Table 5.2). The “numbers of dead marked workers” did not differ significantly between the two study sites (P =0.97; t=-0.41; df=2), and the parameters “new worker pupae” and “difference in numbers of full sugar pots” were just below the statistical thresholds (P=0.11; t=2.71; df=2 and P=0.13; t=2.54; df=2, respectively). While the parameter “difference in numbers of queen pupae” was different between the rich and poor study site (P = 0.01), we note that typically these larger pupae were only observed in well developed nests after 4 weeks of foraging.

Table 5.2. Overview of the 6 parameters in the assessment of the performance of the bumblebee nests in the 3 study sites. For the study sites 1 and 2, only the data of the 2 front nests are given because the 3 back nests did not develop well. For the study site 3, the parameters are based on the 5 nests placed next to each other in a linear placement. Data are presented as means ± SE

Study sites (Number of nests)	Nest biomass increase (g)	Numbers of living workers	Numbers of dead marked workers	Numbers of pupae	Numbers of queen pupae	Difference in numbers of full sugar pots
Study site 1 (n = 2)	562 ± 60	238 ± 5	15 ± 16	143 ± 11	32 ± 5	25 ± 21
Study site 2 (n = 2)	180 ± 17	147 ± 28	15 ± 6	122 ± 2	0	-22 ± 15
Study site 3 (n = 5)	190 ± 77	77 ± 50	28 ± 10	61 ± 56	0	-12 ± 12

4.2. Assessment of bumblebee nest performance in a rural location out of the city centre (site 3)

In study site 3, the 5 bumble bee nests were placed next to each other. With these data (Table 2), we calculated the statistical power and a type II statistical rate (ß =0.8) for all nest development parameters. For "nest biomass increase" and "number of living workers" we obtained a power of 18.5 g and 62.7 workers, respectively, which indicates that these two parameters can be used to discriminate between anthropogenic environments. However, it should be remarked that both parameters are not independent, as we calculated a correlation of 0.82 between them.

For the parameters "number of dead marked workers" and "number of pupae", we obtained a lower power of 12 workers 69.8 pupae, respectively, which suggests that these parameter are less useful to discriminate between sites.

The parameter "difference in number of full sugar pots" had a power of 40 sugar pots and we believe this may be used to differentiate between environments with large differences in landscape metrics. For instance, we obtained a negative value for this parameter when we have an environment with lower landscape metrics (*i.e.* study sites 2 and 3) as opposed to a positive value for this parameter in study site 1 with higher landscape metrics.

For the last parameter, "numbers of queen pupae", we could not calculate the power as we did not see development of queen pupae in study site 3, and so we believe that the parameter "difference number of queen pupae" is less useful to discriminate between sites. However, the emergence of queen pupae is also indicative for the presence of pupae of drones (males). Drones, as reported by (Goulson 2010), leave the nest after a few days and do not return. As a consequence, it is recommended that the bumblebee nests assessments are stopped before the moment that drones start emerging because we selected "nest biomass increase" as a parameter discriminating between sites. In late-switching nests no drones have already emerged when the first queen pupae appear. However, certain environmental conditions or nest features can induce early-switching, and in these cases drones emerge earlier and prior to the appearance of queen pupae (Duchateau and Velthuis 1988, Goulson 2010). Although this early-switching and drone-producing behavior was not recorded in any of the nests in our experiment – perhaps because we worked with commercial bumblebee nests known for their high daughter queen-producing potential –, we believe that it is best to use the appearance of queen pupae as the moment for stopping the assessment experiment.

4.3. Presence of bumblebee-friendly flowering plant species in study sites

As shown in Table S5.1, the values for the SWDI were 3.657, 3.281 and 3.405 for study sites 1, 2 and 3, respectively. So, for the 3 study sites, similar SWDI values were calculated as there were many overlapping flowering plant taxa (50.27%). Subsequently, only a minor proportion of detected

flowering plant taxa was unique per study site: 6.49% for site 1, 1.08% for site 2, and 4.32% for site 3. In addition, as shown in Figure 5.2, species of the different bumblebee-friendly plant families of *Saliaceae, Rosaceae, Ranunculaceae, Laminaceae, Geraniaceae, Ericaceae* and *Brassicaceae* were abundantly present in all 3 study sites.

4.4.Analysis of pollen collected by foraging bumblebees

Over all pollen loads collected, identification confirmed the presence of pollen from the plants groups of *Prunella, Ranunculaceae*, *Salix* and *Sorbus,* and within the *Sorbus* group, pollen was found from the *Rosaceae* and *Prunus* type. But it was clear that most pollen originated from the *Sorbus* and *Salix* groups across the 3 sites. For site 1, pollen of *Sorbus* and *Salix* was present in 70% and 30% of the pollen loads, respectively; and for site 2 in 20% and 90%, and for site 3 in 80% and 30%. These data indicate that *Prunus*, *Rosaceae* and *Salix* species are important food sources for *B. terrestris* bumblebees foraging in the 3 environments in the time period under study from mid April 2012 to mid May 2012.

We noticed that in the study sites with poorer landscape metrics (study sites 2 and 3), we typically identified more than one type of pollen in one load. In study site 2, the pollen load consisted in 20% of the cases of a mixture from *Sorbus/Prunus/Rosaceae* and *Prunus/Prunella*; in study site 3 this was the case for 30% of the loads (*Sorbus*/*Prunus*/*Salix, Sorbus*/*Prunus*/*Ranunculaceae* and *Sorbus/Rosaceae/Salix*). Indeed this phenomenon was not surprising and it has been reported that bumblebees and honeybees are flower consistent (Goulson et al. 2002, Grüter et al. 2011), but this behavior is lost with lower landscape metrics.

4.5. Flower plants as food source for bumblebees

In order to find out the flower plants that the bumblebees visited, we compared the list of flowering plant species in the 3 study sites (Table S5.1, Fig. 5.2) with the data of the pollen analysis. The high importance of the pollinator-friendly plant families *Salix* and *Rosaceae*/*Prunus* was observed in every location. In addition, in study sites 1 and 3, we also found pollen belonging to the Ranunculaceae family in the corbicula of foraging bumblebees. In study site 2, pollen belonging to the Lamiaceae family was also collected. Indeed, these families represent early blossoming plant species that are all pollinator-friendly. The fact that we found only a few plant families (3 per study site) as a food source in a certain time period seems not to be an uncommon phenomenon. In comparison, a pollen analysis study with honeybees held by Girard et al. (2012) in the vicinity of blueberry monocultures during a foraging period after blossoming (July) showed that *Brassicaceae*, *Trifolium* L. spp. and *Vaccinium macrocarpon* were the 3 dominantly-collected plant taxa in the pollen loads.

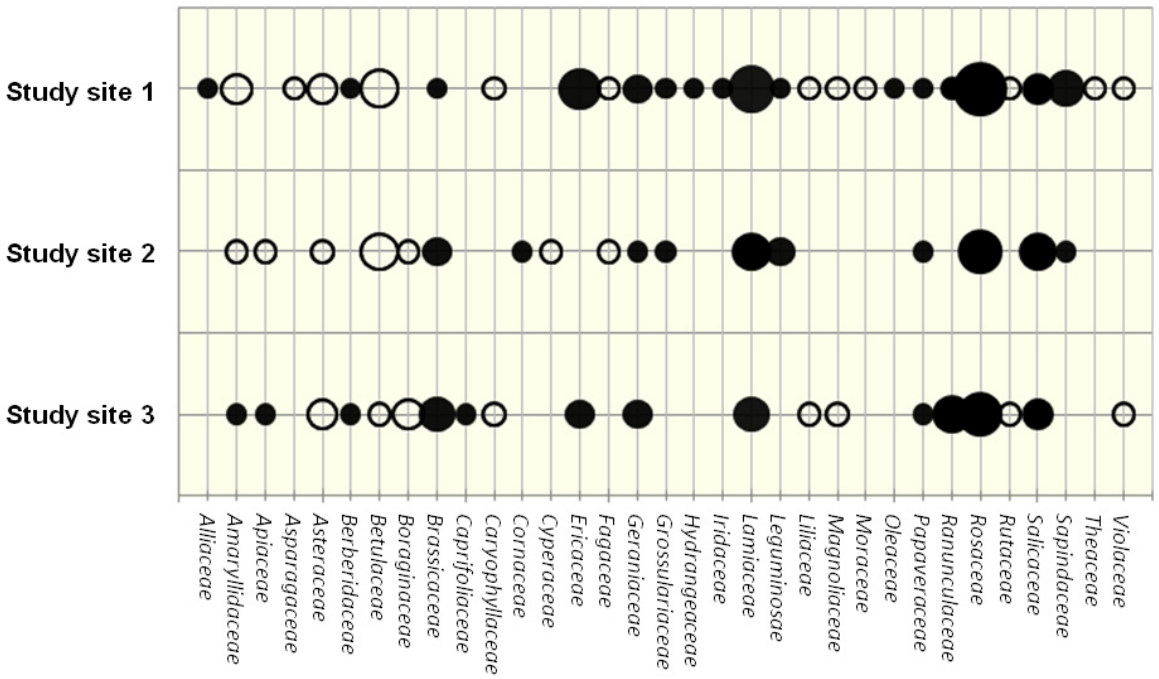


Figure 5.2. Schematic overview of the presence of bumblebee-friendly flowering plant species per family for each study site. The diameter of each circle represents the total numbers of flowering plant species per family; the filled circles indicate bumblebee-friendly families while open circles unfriendly families based on (Lemmens et al. 2012)

Our results indicated that the analysis of pollen loads as collected by the foraging bumblebees is in relation to their floral environment and so can be used to screen an anthropogenic environment for flower plant preferences of the bumblebees. But it should also be remarked here that the timing of the experimental setup is important. The surplus in certain food sources (i.e. plant families) is a temporal phenomenon with changing food resources and this may result in the loss of a stable pollinator presence. To have good insights in a local area, successive periods of analysis in different seasons should be conducted as was also suggested by van der Steen et al. (2012).

5. Conclusions and Perspectives

The data of this first study with commercial nests of bumblebees (*B. terrestris*) as placed in 3 anthropogenic environments with different landscape metrics, demonstrated the potential of such bumblebee nests as a bioassay of the environment. Indeed the parameters “nest biomass increase” and “numbers of living workers” enabled the evaluation of nest performance and could discriminate between a rich and poor environment. This agrees also with the experiments and recommendations by van der Steen (2001) and Goulson et al. (2002). However, we recognize here that the setup with a “poor” versus “rich” environment needs to be enlarged before drawing final conclusions. Furthermore, the protocol needs to be optimized so that the nests are placed in a linear formation.

Second, the pollen analysis demonstrated that the flowering plants *Salix* spp. and *Prunus* spp. are the dominant food resources in all anthropogenic environments during early spring.

Another major result of this study is that we believe that the data on bumblebee nest performance in combination with conventional pollinator counts can generate information on pollinator densities and food resources of the environment under study. In other words, the combination of both analyses can provide better insights to what extent an environment has the potential to support extra pollinators. For example, the situation that the bumblebee nests demonstrate a good nest performance and pollinator counts are low, indicates that the presence of natural pollinators in that environment is too low in relation to the available food resources. In the other situation that the bumblebee nest performance is low, then the environment is not suited to support extra bumblebees. In the case where pollinator counts are high, then there is probably a competition for food resources by pollinators that are naturally present in the environment of study. In the latter case, we expect that there exists a natural equilibrium between numbers of flowers and pollinators present. In the situation that bumblebee nest performance and pollinator counts are both low, this suggests an unfriendly environment for pollinators with low food resources and a lack of appropriate nesting places. In such environments, it is recommended to enhance both flower recourses and nesting places. As a final conclusion, we believe that standardized bumblebee nests, as commercially available for biological pollination in agriculture, can be useful tools, in addition to the traditional counting of pollinators, for the assessment of local pollinator support in anthropogenic environments and then to take measures for conservation strategies.

6. Supplementary data

Table S5.1. List of flowering plant species, with their counts (n) and the Shannon-Wiener diversity index (SWDI), in each of the 3 study sites

Plant family	Species/ subspecies	Study site 1		Study site 2		Study site 3	
		n	Pi*LN(Pi)	n	Pi*LN(Pi)	n	Pi*LN(Pi)
Alliaceae	*Allium ursinum*					1	-0.05425
Amaryllidaceae	*Narcissus narcis*	2	-0.11901	2	-0.12207	1	-0.05425
	Galanthus nivalis					1	-0.05425
Apiaceae	*Daucus carota*	1	-0.07188				
	Heracleum sphondylium			1	-0.07387		
Asparagaceae	*Hyacinthoides non-scripta*					2	-0.09139
Asteraceae	*Taraxacum officinale*	5	-0.21571	2	-0.12207	5	-0.17191
	Bellis perennis	3	-0.15679			5	-0.17191
Berberidaceae	*Mahonia aquifolium*	1	-0.07188			2	-0.09139
Betulaceae	*Corylus avellana*	1	-0.07188	2	-0.12207		
	Betula sp.			4	-0.19279	1	-0.05425
	Betula pendula tristis					1	-0.05425
	Alnus sp.			2	-0.12207	2	-0.09139
Boraginaceae	*Symphytum officinale*	1	-0.07188	1	-0.07387		
	Myostosis	1	-0.07188				
Brassicaceae	*Cardamine pratensis*	3	-0.15679				
	Lunaria annua	2	-0.11901			1	-0.05425
	Capsella bursa pastoris	1	-0.07188	1	-0.07387		
	Brassica napus			1	-0.07387		
Caprifoliaceae	*Lonicera sp.*	1	-0.07188				
Caryophyllaceae	*Stellaria holostea*	1	-0.07188			1	-0.05425
	Silene flos-cuculi	1	-0.07188	1	-0.07387		
Cornaceae	*Cornus sp.*			1	-0.07387		
Cyperaceae	*Carex sp.*			1	-0.07387		
Ericaceae	*Erica sp.*	1	-0.07188			1	-0.05425
	Azalea sp.	1	-0.07188			1	-0.05425
	Pieris japonica					1	-0.05425
	Rhododendron					1	-0.05425
Fagaceae	*Quercus petraea*			1	-0.07387	3	-0.12207
Geraniaceae	*Geranium dissectum*	1	-0.07188	2	-0.12207	1	-0.05425
	Geranium robertianum	1	-0.07188			1	-0.05425
Grossulariaceae	*Ribes sanguineum*			1	-0.07387	1	-0.05425
Hydrangeaceae	*Hydrangea macrophylla*					1	-0.05425
Iridaceae	*Crocus sp.*					1	-0.05425
Lamiaceae	*Lamium album*	3	-0.15679	2	-0.12207	1	-0.05425
	Lamium purpureum	2	-0.11901	3	-0.16058	1	-0.05425
	Glechoma hederacea	3	-0.15679	5	-0.22033	3	-0.12207
	Lamium maculatum					1	-0.05425
	Lamiastrum galeobdoron					1	-0.05425
Leguminosae	*Laburnum*			1	-0.07387	5	-0.17191

	anagyroides						
	Cytisus scoparius			1	-0.07387		
Liliaceae	*Tulipa sp.*	2	-0.11901			2	-0.09139
Magnoliaceae	*Magnolia sp.*	1	-0.07188			2	-0.09139
Moraceae	*Morus alba pendula*					1	-0.05425
Oleaceae	*Ligustrum sp.*					1	-0.05425
Papaveraceae	*Chelidonium majus*	1	-0.07188	1	-0.07387	1	-0.05425
Ranunculaceae	*Ranunculus acris*	3	-0.15679			1	-0.05425
	Ranunculus ficaria	2	-0.11901				
	Anemone nemorosa	3	-0.15679				
Rosaceae	*Prunus serotina*	1	-0.07188	2	-0.12207	3	-0.12207
	Prunus spinosa			1	-0.07387		
	Prunus serrulata	1	-0.07188			4	-0.14855
	Prunus sp.	1	-0.07188	1	-0.07387	2	-0.09139
	Malus sp.	1	-0.07188	1	-0.07387	1	-0.05425
	Crataegus sp.					1	-0.05425
	Chaenomeles japonica					2	-0.09139
Rutaceae	*Skimmia japonica*	1	-0.07188			1	-0.05425
Salicaceae	*Salix sp.*	1	-0.07188	2	-0.12207	1	-0.05425
	Salix alba			1	-0.07387		
	Salix vitellina					1	-0.05425
	Populus sp.	1	-0.07188	1	-0.07387		
Sapindaceae	*Acer sp.*			4	-0.19279	3	-0.12207
	Aesculus hippocastanum					2	-0.09139
	Aesculus cornea					1	-0.05425
Theaceae	*Camellia japonica*					1	-0.05425
Violaceae	*Viola sp.*	1	-0.07188			2	-0.09139
Total n° flowering plant families (n° species)		**27 (47)**		**17 (28)**		**20 (34)**	
SWDI		**3.657**		**3.281**		**3.405**	

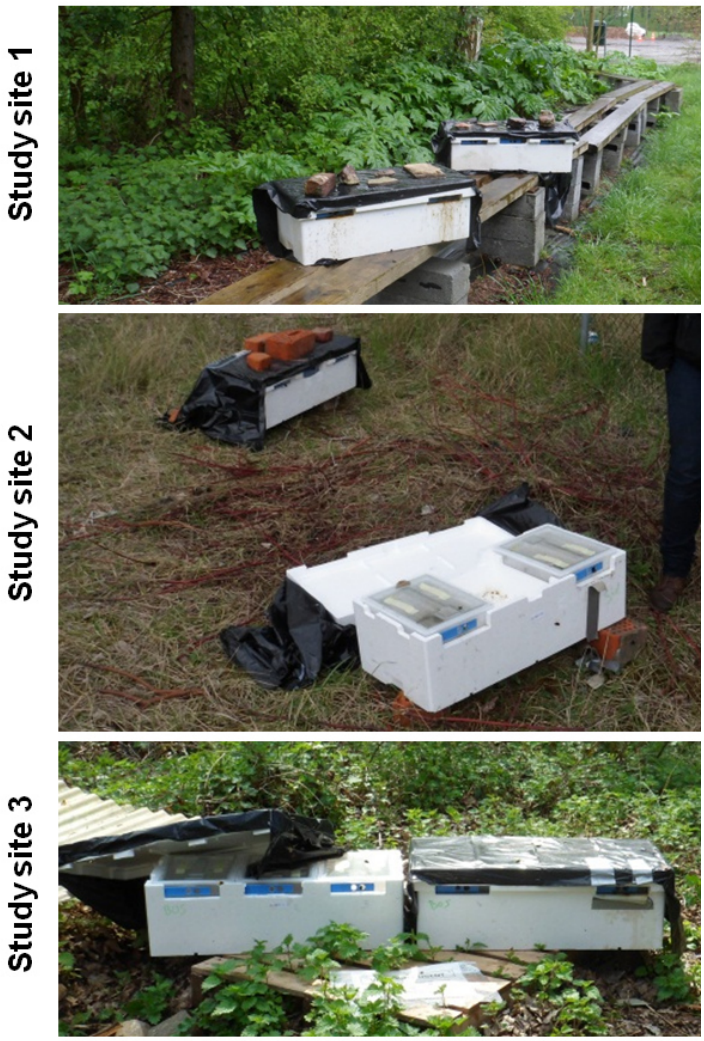


Figure S5.1. Placement of bumblebee nests in each of the 3 study sites

Chapter 6:

New RNA viruses in wild bumblebee hosts

This chapter is based on:

Varroa destructor Macula-like virus, Lake Sinai virus and other new RNA viruses in wild bumblebee hosts (*Bombus pascuorum*, *Bombus lapidarius* and *Bombus pratorum*) (2016).
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1. Abstract

Pollinators such as bumblebees (*Bombus* spp.) are in decline worldwide which poses a threat not only for ecosystem biodiversity but also to human crop production services. One main cause of pollinator decline may be the infection and transmission of diseases including RNA viruses. Recently, new viruses have been discovered in honeybees, but information on the presence of these in wild bumblebees is largely not available.

In this study, we investigated the prevalence of new RNA viruses in *Bombus* species, and can report for the first time Bee Macula-like virus (BeeMLV; formerly known as VdMLV) and Lake Sinai virus (LSV) infection in multiple wild bumblebee hosts of *Bombus pascuorum*, *Bombus lapidarius* and *Bombus pratorum*. We sampled in 4 locations in Flanders, Belgium. Besides, we confirmed Slow bee paralysis virus (SBPV) in wild bumblebees, but no positive samples were obtained for Big Sioux river virus (BSRV). Secondly, we screened for the influence of apiaries on the prevalence of these viruses. Our results indicated a location effect for the prevalence of BeeMLV in *Bombus* species, with a higher prevalence in the proximity of honeybee apiaries mainly observed in one location. For LSV, the prevalence was not different in the proximity or at a 1.5 km-distance of apiaries, but we reported a different isolate with similarities to LSV-2 and "LSV-clade A" as described by Ravoet et al. (2015), which was detected both in *Apis mellifera* and *Bombus* species. In general, our results indicate the existence of a disease pool of new viruses that seems to be associated to a broad range of Apoidea hosts, including multiple *Bombus* species.

2. Introduction

Bumblebees are regarded as important pollinators of wild flora (Goulson and Darvill 2004, Goulson 2010) and of many crops including tomatoes, cucumbers and other top fruit (Velthuis and van Doorn 2006). Despite their importance, pollinators are declining worldwide (Ghazoul 2005, Potts et al. 2010). In the last decades, different RNA viruses have been described in honeybees, such as Black queen cell virus (BQCV) (Peng et al. 2011), Deformed wing virus (DWV) (Genersch et al. 2006, Evison et al. 2012), Sacbrood virus (SBV) (Singh et al. 2010, Reynaldi et al. 2014) and Slow bee paralysis virus (SBPV) (Bailey and Woods 1974). However, these so-called honeybee pathogens have recently also been reported in solitary bees (Ravoet et al. 2014), bumblebees (Fürst et al. 2014, McMahon et al. 2015), and also in non-Apoidea hosts as *Vespula* (Evison et al. 2012) species.

Because some important pollinators including honeybees and bumblebees are polylectic foragers, they share common food plants (Singh et al. 2010, Rohde et al. 2013). Therefore, a possible indirect transmission route of pathogens for different bee taxa has been described by means of contact with shared contaminated flowers (Singh et al. 2010). Besides, a bi-directional transmission between honeybees and bumblebees remains possible (McMahon et al. 2015). Probably these RNA-viruses share multiple pollinator hosts, pointing to an interconnected network of RNA viruses within and among a range of pollinator species (Fürst et al. 2014, McMahon et al. 2015). It therefore seems that these RNA viruses pose a threat for different Apoidea species and other members of the pollinator community (McMahon et al. 2015).

Studies performed to elucidate the decline in honeybee colonies (Runckel et al. 2011, Granberg et al. 2013) has resulted in the discovery of new viruses, including Lake Sinai virus (LSV) (Runckel et al. 2011), Big Sioux river virus (BSRV) (Runckel et al. 2011), SBPV (Bailey and Woods 1974, de Miranda et al. 2010) and Bee Macula-like virus (BeeMLV) (de Miranda et al. 2015), formerly known as *Varroa destructor* Macula-like virus(VdMLV) (de Miranda et al. 2011). Recently, these viruses have also been found in solitary bees (Ravoet et al. 2014), but none, except SBPV, have been reported in European bumblebees. Despite that RNA viruses seem to be generally present in Hymenopteran hosts, data on new RNA viruses related to widespread pollinators as bumblebees are still lacking.

In this study, our first aim was to screen whether BeeMLV, LSV, BRSV and SBPV are present in wild bumblebee species, and secondly to investigate on the effect of distance to honeybee apiaries on the prevalence of these RNA viruses in bumblebees. Therefore, we selected four locations in the provinces of East- and West-Flanders (Belgium) and sampled wild bumblebees in the close proximity and at a distance of 1.5 km from an apiary of honeybees (*Apis mellifera*). Selecting three abundant species for wild bumblebees, we focused on *B. pascuorum*, *B. lapidaries* and *B. pratorum.*

3. Material and methods

3.1. Defining study sites close to an apiary (Api-near) and at a distance (Api-far)

We sampled bumblebees in the provinces of East- and West-Flanders (Belgium) in 2013, each containing two coupled study sites (4x2 study sites in total) (Suppl. Fig. S6.1). We designed our locations to have a study site close to an apiary (Api-near) and at a 1.5 km-distance (Api-far). The choice of an Api-near site was made based on the distribution of apiaries in Flanders, Belgium. A distribution map of beekeepers (registered at the Federal Agency for the Safety of the Food Chain, Brussels, Belgium) and verified by contacting the beekeepers, was used to generate an actual distribution map of beekeepers and actual locations of honeybee hives. This map was used to pinpoint the Api-near sites (screened to be rich in apiaries) and to search for the Api-far study sites, with a minimum number of apiaries in the neighbourhood. We selected a radius of 750 m as maximum forage distance, as described previously (Parmentier et al. 2014) to define a distance of 1.5 km between the Api-near and Api-far study sites. Around the Api-near sites we counted a mean of 6.5 ± 2.6 honeybee hives per km^2 compared to 0 ± 0 for the Api-far sites. In Belgium the mean number of honeybee hives per km^2 is 3.6 (Chauzat et al. 2013), which is in between our two extremes.

3.2. Sample collection and RNA extraction

In all locations we sampled individual *B. pascuorum*, *B. lapidarius* and *B. pratorum* in equal numbers per study site (Api-near and Api-far), with a total of 28, 80 and 18 samples per species and per location, respectively. Simultaneously in the Api-near sites, we sampled honeybees in 26 hives. We obtained samples of 30 randomly selected bees per hive. As representative bumblebee species, we selected *B. pascuorum*, *B. lapidarius* and *B. pratorum* because they were generally present in our study sites. Caught bumblebees were transferred in individual tubes which were put immediately on dry ice and stored at -70°C at the end of the sampling day. Honeybees were pooled whereas bumblebees were crushed individually for 5 min after adding 4 ml or 700 µl Qiazol® (Qiagen Benelux, Venlo, the Netherlands), respectively, and zirconia (0.1 mm) and stainless steel (1 mm) beads. A total of 500 µl of supernatants was centrifuged at 17,000 g for 3 min. Next, 900 µl of Qiazol was added to 100 µl supernatant and the protocol was followed according to manufacturer's instructions (RNeasy Lipid Tissue; Qiagen Benelux, Venlo, the Netherlands). The RNA was eluted from the column in 50 µl RNA-free water. Honeybees sampled from each hive were pooled in groups of 10 honeybees and this was done in triplicate. In each tube 4 ml Qiazol® was added for bead beating and further processed as described for individual bumblebee samples.

3.3. MLPA analysis and reverse transcriptase PCR

Initially, we screened bumblebee and honeybee samples for a range of known positive-sense single stranded RNA viruses, by employing multiple ligation-dependent probe amplification (MLPA) using the RT-MLPA kit (MRC-Holland, Amsterdam, the Netherlands). The MLPA technique is capable to detect multiple viruses at once with only a minor loss of sensitivity compared to strand-specific PCRs (De Smet et al. 2012, de Miranda et al. 2013). We used probes designed for the detection of positive-sense single-stranded RNA of the following four viruses: BeeMLV, LSV, SBPV, and BSRV (De Smet et al. 2012, Ravoet et al. 2014). The composite probes contained aside from the virus specific part, a stiffer region to differentiate the length of the probes and a primer region to amplify the probes. Probe amplification was performed by 5' FAM-labeled primers. Fragments were separated by capillary electrophoresis and aligned using an intern standard (GS500 LIZ). Fragment detection was achieved by a calibrated fluorochrome reader (Genetic service Unit UZ Ghent, Ghent University, Ghent, Belgium) and sample processing by employing the Peak Scanner vs.2 software, selecting option "Sizing default – Primer present".

For sequence analysis, positive MLPA samples were selected and further used. Reverse transcriptase was performed on initial RNA with random hexamer primers with the Revert AidTM First Strand cDNA Synthesis Kit (Invitrogen, Merelbeke, Belgium). All strand-specific PCR reactions contained: 1.5 mM $MgCl_2$; 0.2 mM dNTP; 1.25 U Hotstar Taq DNA polymerase (Qiagen), 1 to 5 µl cDNA product (300-500 nmol of RNA) and 2 µM of each primer. We used specific primers described by Ravoet et al. (2015) for LSV BRSV, SBPV and BeeMLV. The following cycling conditions were used: 95°C for 5 min; 37 cycles of [94°C for 30 s, 53-56°C for 30 s, 72°C for 45 s]; final elongation 72°C for 10 min; hold at 4°C. Elongation temperatures were set at 53°C for LSV, 54°C for BRSV and SBPV or 56°C for BeeMLV; virus-specific primers used are given in Table S1.

3.4. LSV infection confirmation in *Bombus*

To confirm a possible infection of LSV in *Bombus*, we organized a second sampling of 15 *B. pascuorum* in 2015 to screen for LSV in inner tissues. We selected two study sites with a higher prevalence of LSV based on the MLPA results. Randomly collected bumblebees were immediately dissected; we removed the gut and sampled the inner body parts (fraction inner body) and the remaining fraction (outer body part + inner rest fraction). Samples were further processed as described above and we first applied a strand-specific PCR (based on primer set described by Ravoet et al., 2015). To confirm weakly positive samples, a semi-nested PCR was developed combining the forward LSV-deg-F primer by Ravoet et al. (2015) and a specific reversed LSV-Nest-R primer (Table S6.1) generating a 385 bp fragment. The following cycling conditions were used: 95°C for 5

min; 37 cycles of [94°C for 30 s, 56°C for 30 s, 72°C for 45 s]; final elongation 72°C for 10 min; hold at 4°C.

3.5. Sequence analysis and phylogenetics

Each virus positive with MLPA was amplified by RT-PCR, and related sequences were retrieved from GenBank with the BLASTn algorithm and aligned with BioEdit. Prior to the alignments, outgroups were chosen based on phylogenetic similarities with other viruses. More specifically, DVW (AY292384.1) was chosen as outgroup for BeeMLV and SBPV based on de Miranda et al. (2010), whereas CBPV (AF461061.1) was chosen as outgroup for LSV (consensus between Cook et al., 2013 and Ravoet et al., 2015). For LSV, distinct geographical origins and strains are available in Genbank and were added to our phylogenetic analysis. Alignments were imported in MEGA6 and phylogenetic trees were constructed based on the neighbor-joining method and generating bootstrap probability scores (n = 1000). For LSV we focused on the RdRP gene variability which encodes for an RNA-dependent RNA polymerase that is more conserved in the different LSV strains than the ORF1 and ORF2 capsid genes genes (Ravoet et al. 2015a).

3.6. Statistics

We used generalized linear mixed models (GLMM) for binomially distributed data (SPSS statistics vs. 22), defining location as random effect, to explore the overall relationships of virus prevalence between study sites (Api-near vs. Api-far), *Bombus* species and the interaction between both. We observed no interaction between the two main fixed factors (location and *Bombus* species) (all P-values > 0.9; data not shown), therefore we omitted the interaction in further GLMM modeling. P-values were generated and given for the four viruses kept in analysis.

In addition to individual GLMMs, we summed the prevalence of all viruses to obtain proportions per sample and to test for coinfections between viruses. The multiple-kind lottery model of Janovy et al. (1995) was used to calculate the theoretical distribution of the pathogens. The parasite presence determined in 63 analyzed bumblebee specimens was used to calculates the expected pathogen distribution or the number of colonies infected with 0 to 3 pathogens. By means of a Pearson Chi-square test ($P < 0.05$) with SPSS vs. 22, we compared if the observed pathogen distribution differed from the theoretical distribution. The same approach was followed to infer which interaction between pathogen pairs occurred within this multi-pathogen host system (Janovy et al. 1995).

4. Results and discussion

4.1. Detection and density distribution of new RNA viruses in *Bombus* spp.

In this study we report on four less-studied RNA viruses BeeMLV, LSV, BRSV and SBPV, and for the first time on the presence of BeeMLV and LSV in multiple wild *Bombus* hosts. LSV was also recently detected in single host of *Bombus atratus* in South America (Columbia) (Gamboa et al. 2015). We employed MLPA, a method first used to detect multiple viruses in honeybees and recently applied for virus detection in bumblebee samples (McMahon et al. 2015). We applied an individual PCR and virus-specific primers (Table S6.1) to generate virus fragments, confirming selected positive MLPA samples both for honeybees (VdML: KT956841; SBPV: KT956843; LSV: KT956845) and bumblebees (VdML: KT956842; SBPV: KT956844; LSV: KT956846) after Genbank searches, i.e. BeeMLV (HQ916350.1, 96% identity), LSV (KM886904.1, 88% identity) and SBPV (EU035616.1, 100% identity). We did not detect BRSV in any screened honeybee hive (n = 29) or bumblebee specimen (n = 123). We found the highest prevalence for LSV and BeeMLV in *Bombus* hosts, which was 21.1 % and 12.2 %, respectively. However, no significant differences between *Bombus* species were observed after statistical analysis (*Bombus* as factor: all P-values > 0.67; GLMM). SBPV was found in only 4.1% of the samples, a result that is in agreement with McMahon et al. (2015) who obtained an average prevalence of 5 % in a recent UK survey.

We analyzed the density distribution of multiple viruses within individual bumblebees to understand a possible link between virus prevalence. Looking to positive samples for 3 viruses (BeeMLV, LSV and SBPV), we found a small but significant correlation ($\chi^2 = 8.039$; $P = 0.045$). Looking to dual virus distributions, we found a correlation between LSV and SBPV ($\chi^2 = 9.601$; $P = 0.008$), but not between LSV and BeeMLV ($\chi^2 = 0.583$; $P = 0.747$) or BeeMLV and SBPV ($\chi^2 = 3.648$; $P = 0.161$).

Thus, when we detected LSV or SBPV, the probability was higher that both viruses co-occurred within the same specimen. We looked if this co-occurrence could be explained by a preferred presence in a certain host species and therefore re-calculated the effects for *B. pascuorum*; the only host sampled with enough power. We obtained again a significant effect between LSV and SBPV ($\chi^2 = 79.739$; $P = 0.01$) indicating no difference in density distribution between *Bombus* species. In comparison, Ravoet et al. (2015) who screened RNA viruses in honeybees, reported also co-infections between LSV and BQCV within a single host. This may indicate that after an LSV infection, it is more likely to be infected with a second virus within multiple *Apoidea*-hosts and particularly SBPV in screened *Bombus* sp.

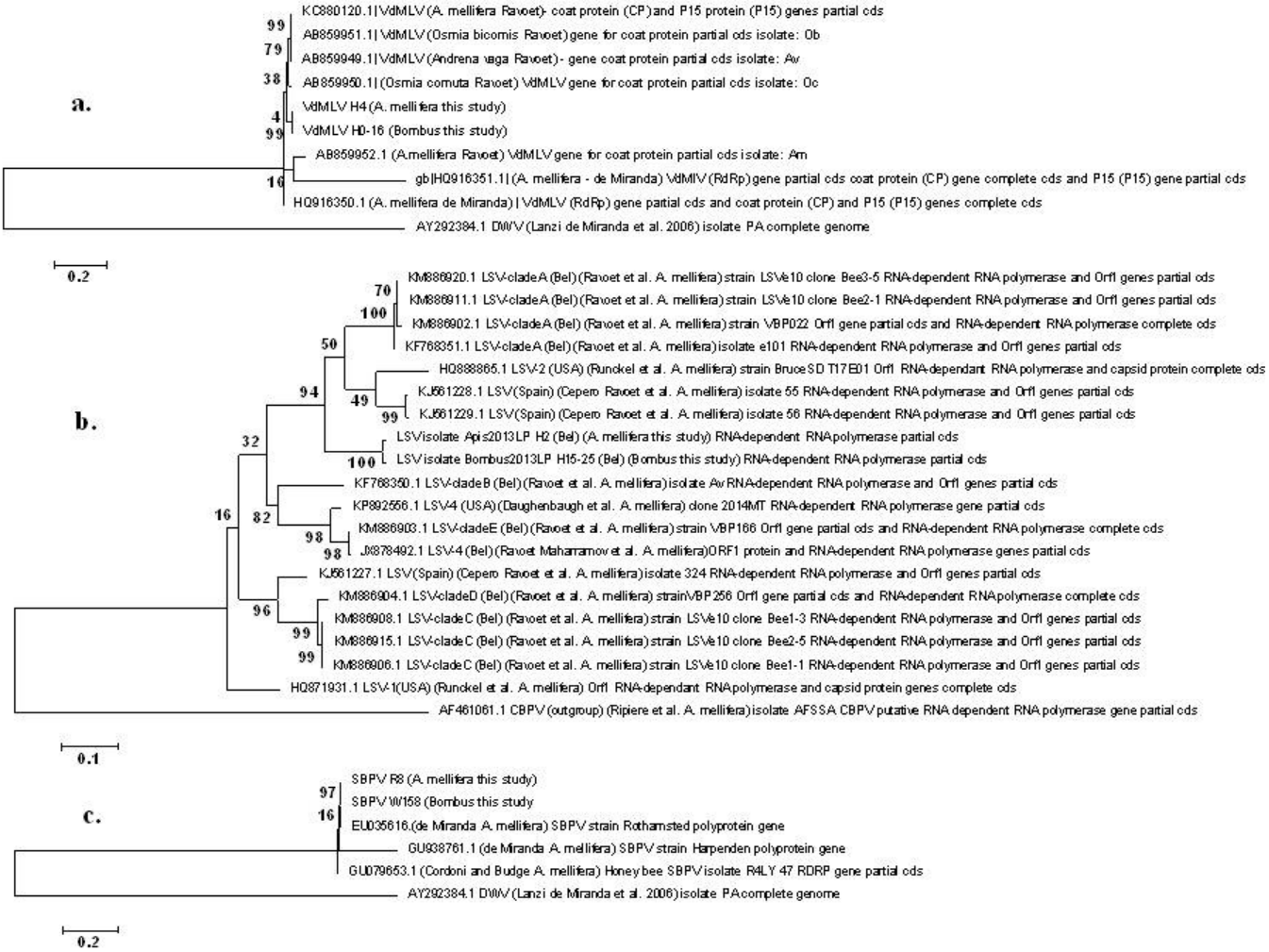

Figure 6.1. Phylogenetic analysis of the 3 viruses, BeeMLV (**a.**), LSV (**b.**) and SBPV (**c.**), as detected in the 3 wild *Bombus* species in the proximity of apiaries and in the honeybee (*Apis mellifera*) samples taken from the apiaries. Phylogenetic trees were constructed using the neighbor-joining method. Each added sequence is indicated by its Genbank accession number. For LSV, different strains were added: LSV-1 (USA, HQ871931), LSV-2 (USA, HQ88865), LSV-4 (Belgium, JX878492), a selection out of 5 different LSV clades in a Belgian survey (here named as: LSV-clade A till LSV-clade E) (KM886902-KM886920 and KF768350-KF768351) (Ravoet et al., 2015), and strains found in Spanish *A. mellifera* populations (KJ261227-KJ261229) (Cepero et al., 2014)

Table 6.1. Presence of RNA viruses detected in wild bumblebees (*Bombus* sp.) in the proximity of apiaries (Api-near) and at a 1.5 km distance (Api-far)*

	BeeMLV		LSV		SBPV		BRSV	
Bombus sp.	Api-near	Api-far	Api-near	Api-far	Api-near	Api-far	Api-near	Api-far
B. lapidarius	(14 ; 1)	(14 ; 0)	(14 ; 1)	(14 ; 0)	(14 ; 0)	(14 ; 1)	(14 ; 0)	(14 ; 0)
B. pascuorum	(40 ; 10)	(40 ; 3)	(40 ; 14)	(40 ; 12)	(40 ; 3)	(40 ; 1)	(40 ; 0)	(40 ; 0)
B. pratorum	(9 ; 0)	(6 ; 1)	(9 ; 0)	(6 ; 0)	(9 ; 0)	(6 ; 0)	(9 ; 0)	(6 ; 0)
Total *Bombus* sp.	(63 ; 11)	(60 ; 4)	(63 ; 14)	(60 ; 12)	(63 ; 3)	(60 ; 2)	(63 ; 0)	(60 ; 0)
Statistics Api-near vs. Api-far °	**0.011**		**0.67**		**0.76**		**--**	

* Results are presented as (sample number ; number positives); ° P-value, $\alpha = 0.05$, GLM.

4.2. BeeMLV prevalence in *Bombus* sp. in proximity of apiaries

When comparing viruses in wild bumblebees sampled in the Api-near and Api-far sites (Table 6.1), we observed a higher prevalence for BeeMLV in *Bombus sp.* when collected in the proximity of the honeybee apiaries (P = 0.011; GLMM). Importantly, we observed a location effect in the BeeMLV prevalence, with a higher prevalence in the proximity of honeybee apiaries mainly observed in one location. Indeed, in one location, BeeMLV was very abundant in the Api-near (90 %) versus the Api-far study site (20 %), whereas in other locations the virus prevalence was low (average of 7.5 ± 5 %) or totally absent (Figure 6.2). Because of the low prevalence of BeeMLV in the other locations, the magnitude of the influence of apiaries remains unresolved and other environmental parameters could also be influential. The location effect on BeeMLV distribution is recently described for honeybee hives with either few or many colonies in an apiary infected, and a bimodal distribution pattern was seen in Autumn, i.e. the season with the highest BeeMLV prevalence (de Miranda et al. 2015).

In our study we did observe an infection of the same BeeMLV strain (sequence identity 100%) in *A. mellifera* and *Bombus* samples (Figure 6.1.a), which can suggest a possible transmission between the two hosts. Generally, little information is available about the spread of this virus within and between hosts. According to recent information, the *Varroa* mite is considered to be a primary host for BeeMLV (de Miranda et al. 2011, de Miranda et al. 2015). It is known that this ectoparasite is capable to infect honeybees as it delivers the virus directly into the hemocoel by puncturing the integument during nourishment (Rosenkranz et al. 2010). Therefore this parasite could be important for the transmission within honeybees. However as this parasite has not been reported to feed on bumblebees no direct transmission from the mite to bumblebees would seem probable. BeeMLV has also been found in solitary bees, such as *Osmia cornuta* and *Osmia bicornis*, suggesting a common transmission pathway. One of the possibilities is a spread of this virus by means of shared contact with contaminated flowers (Ravoet et al. 2014) which has also been shown as a transmission pathway for other RNA viruses (Singh et al., 2010).

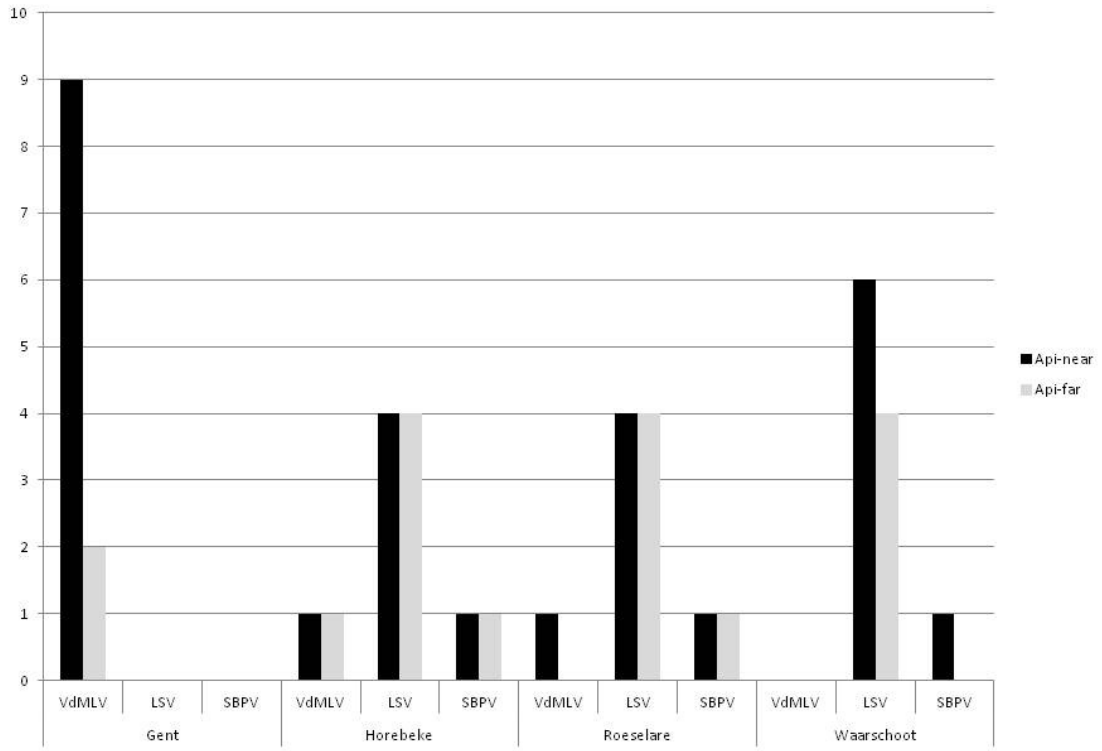

Figure 6.2. Prevalence of the 3 studied viruses BeeMLV, LSV and SBPV over the 4 locations, and in the 2 study sites per location, namely close to an apiary (Api-near) and at a distance of 1.5 km from this apiary (Api-far)

4.3. Prevalence, phylogeny and infection of LSV in *Bombus* sp.

For LSV we did not retrieve a higher prevalence in *Bombus* species when collected in the close proximity of the honeybee apiaries (all P-values > 0.67). LSV has first been described as a honeybee virus (Runckel et al. 2011), but our results did not indicate that the presence of honeybees influenced the presence of this virus in wild bumblebees. Multiple reservoirs for LSV are known, as it has also been found in solitary bee species of the genera *Andrena* and *Osmia* (Ravoet et al. 2014). The phylogenetic data for LSV points towards different strains with high sequence divergence (Figure 6.1.b). We indicated the closest relation to LSV-2 (Runckel et. Al., 2011) and the strains found ("LSV-clade A") in *A. mellifera* by Ravoet et al. (2015). However, this closest match resulted in low similarities, between 74 % and 80% for LSV-1 and LSV-2, respectively. Indeed both the *Bombus* (Bombus_2013LP_H1,5-25) and *Apis* (Apis_2013LP_H2) LSV sequences cluster apart from described LSV strains, as shown in Figure 6.1.a, with a bootstrap probability score of 92% between the closest branch clustering LSV-2 and "LSV-clade A" sequences. Currently, only a few LSV strains, in essence LSV-1 and LSV-2, have been described based on complete genomic sequences in the USA (Runckel et al. 2011), and Ravoet et al. (2015) reported different LSV clades (here named A-E) in *A. mellifera* hives in a Belgian survey. For LSV, a series of other clades has been described based on different partial genomic sequences (e.g. Daughenbaugh et al., 2015), but there is currently not a consensus

about the complex nomenclature for this virus. Whereas BeeMLV and SBPV are members of the *Iflaviridae* family, the LSVs are still unclassified, but they are related to the *Anopheline*-associated C virus (AACV) and Chronic bee paralysis virus (CBPV) (Cook et al., 2013). They have a different genome organization, leading to the proposition of the genus Sinaivirus (Kuchibhatla et al. 2014). Thus, it seems that multiple clades are present within the LSV cluster (containing LSV-1, LSV-2, LSV-4, LSV-5, LSV-6, LSV-7, LSV clades A-E and new isolates in this study) and different clades can infect multiple hosts. Currently, no pathological data of this virus is present and infection studies with the virus for different clades are not available and would be useful to further untangle this complex into different viral species.

In this study, we report for the first time LSV in *Bombus* sp. Therefore we wanted to reassure that the LSV presence represented a real infection. For this we randomly collected 15 extra wild *B. pascuorum*. We then creened the inner fat body fraction and the remaining body fractions (outer body part + inner rest fraction) separately, generating 2 x 15 samples per bumblebee. Of these samples, only 2 samples, represented by the same bumblebee were unambiguously positive for LSV after a first screening with a strand-specific PCR (based on primer set described by Ravoet et al., 2015). Since the inner body tissue of this bumblebee was also positive, we thus confirmed a real LSV infection. Some samples had a very weakly band probably because of low LSV presence. In addition, we performed a semi-nested PCR. This resulted in 4 positives out of 15 (~27%) samples in the inner body fraction (Genbank n° KT956847) and 7 positives out of 15 samples (~47%) of the remaining body parts (Genbank n° KT956848 - KT956849), respectively (Figure S6.2). Since we observed a difference in virus prevalence, we speculate that the use of specific primer sets for screening the whole "LSV-complex" presents difficulties. Indeed, it will be difficult to design universal primers because of the sequence diversity between different isolates and clades of LSV. Probably universal primer sets miss the opportunity to detect all positive samples. Thus the sequences we picked up could reflect the used primer set rather than the actual diversity of LSV presence. Secondly, we conclude, as well as for other RNA pollinator viruses, that tissue selection is an important parameter when performing prevalence studies. As tissue tropism of the virus can influence the outcome of virus detection, we suggest to screen for inner tissue to confirm a real infection.

4.4. Prevalence and phylogeny of SBPV in *Bombus* sp.

Finally, when looking to SBPV, we observed a low prevalence, i.e. 1 out of 28 samples of *B. lapidarius* and 15 out of 80 samples of *B. pascuorum*. Phylogenetic analysis (Figure 6.1.c) derived from a positive *Bombus* sample and *A. mellifera* hive, showed 100% sequence identity based on 886 bp, and these sequences clustered together with the 'Harpenden' strain (GU938761.1; 82% identity)

and the 'Rothamsted' strain (EU035616; 100 % identity) found in honeybees (de Miranda et al. 2010) and also, but in a separate branch, with isolate (GU079653.1; 99% identity) found in honeybees sampled in the UK. McMahon et al. (2015) also reported that SBPV in *A. mellifera* and *B. pascuorum* clustered together, while those from *B. terrestris* clustered separately. Besides, in sympatric populations of *A. mellifera* and *Bombus* species, it has been found that at least for some RNA viruses, including ABPV and SBPV, the prevalence can be higher in bumblebees than honeybees questioning the transmission direction between species (McMahon et al. 2015).

5. Conclusion

In this study, we provided data of some new Apoidea viruses, that have initially been described in honeybees, in wild *Bombus* species of *B. pascuorum*, *B. pratorum* and *B. lapidarius*. Of the 4 viruses, BeeMLV and LSV had the highest prevalence. We found that there is only a link between *A. mellifera* hives for BeeMLV, although a location-effect may play a role here. For LSV, we could report for the first time an infection in *Bombus* hosts based on a specific screening of inner body tissue. We believe that screening of specific inner tissue is a requisite to confirm a virus-infection within a host. We further confirmed the complex taxonomical situation of this virus complex and reported a new isolate both in *A. mellifera* and *Bombus*. More information about transmission routes and pathological data of this virus is needed, and would be useful to further untangle this complex into different viral species.

As these new viruses are not only present in honeybees and differences in prevalence and strains can be found, different transmission routes are possible, probably in a network via contaminated flowers (Singh et al. 2010), honeybees (Meeus et al. 2011, Peng et al. 2011, Fürst et al. 2014, Reynaldi et al. 2014), honeybee parasites (de Miranda et al. 2015, Ravoet et al. 2015a), bumblebees (McMahon et al. 2015) and other solitary bees (Levitt et al. 2013, Ravoet et al. 2014).

6. Supplementary Information

Table S6.1. Virus target and specific primers used

Target	Primers	Sequence (5'-3')	Size (bp)	Reference
BSRV	BSRV-4714F	RGTGCAGCTTTATGCGTTGCC	519	(Runckel et al., 2011)
	BSRV-37R	CCGCTGTTGAGAATAAGGATATCCAGG		
LSV complex	LSVdeg-F	GCCWCGRYTGTTGGTYCCCCC	578	(Ravoet et al., 2013)
	LSVdeg-R	GAGGTGGCGGCGCSAGATAAAGT		
LSV-semi-nested	LSVdeg-F	GCCWCGRYTGTTGGTYCCCCC	385	(Ravoet et al., 2013)
	LSV-Nest-R	TGTCAGTGTGTGAGCATGATG		This study
SBPV	SBPV-F8156	GATTTGCGGAATCGTAATATTGTTTG	868	(de Miranda et al., 2010)
	SBPV-B9023	ACCAGTTAGTACACTCCTGGTAACTTCG		
BeeMLV	BeeMLV-F	ATCCCTTTTCAGTTCGCT	438	(Gauthier et al., 2011)
	BeeMLV-R	AGAAGAGACTTCAAGGAC		

Table S6.2. Presence of 'honeybee viruses' BeeMLV, LSV, SBPV and BSRV in the selected apiaries (*Apis mellifera*) at the 4 locations *

Apiary	BeeMLV	LSV	SBPV	BSRV
Gent	(5 ; 1)	(5 ; 1)	(5 ; 1)	(5 ; 0)
Waarschoot	(7 ; 4)	(7 ; 2)	(7 ; 1)	(7 ; 0)
Horebeke	(4 ; 2)	(4 ; 1)	(4 ; 1)	(4 ; 0)
Roeselare	(10 ; 6)	(10 ; 2)	(10 ; 6)	(10 ; 0)
Total	(26 ; 16)	(26 ; 8)	(26; 11)	(26 ; 0)

* Results are presented as: (sample number ; number positives)

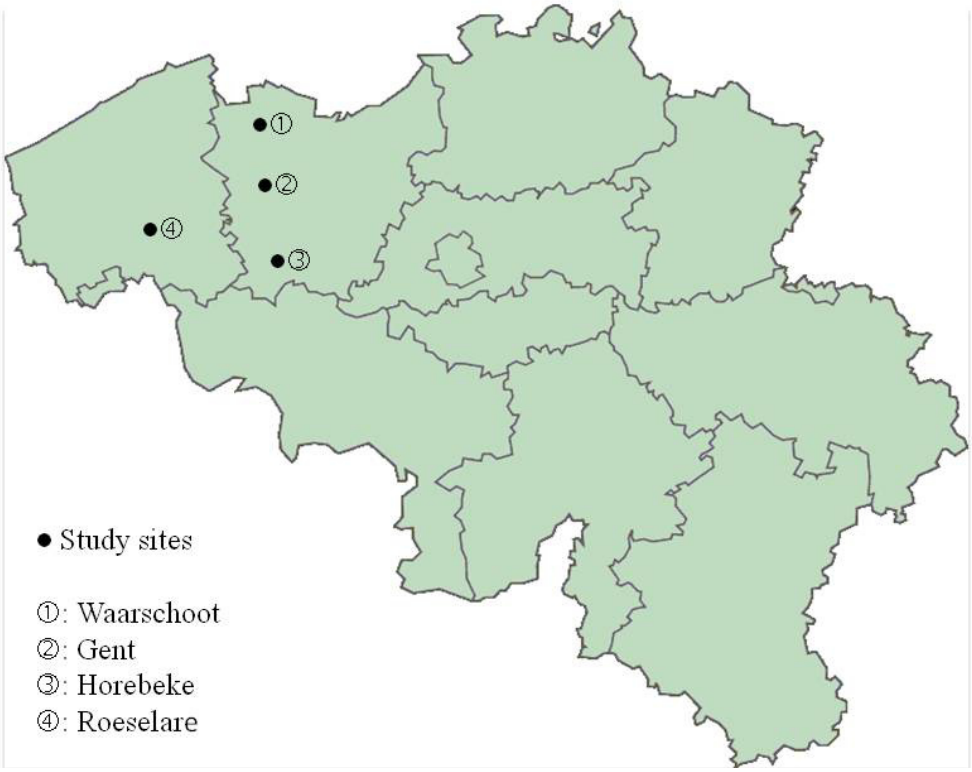

Figure S6.1. Locations defined in this study; each location was designed to have a study site near an apiary (Api-near) and a site at a distance of 1.5 km from this apiary (Api-far)

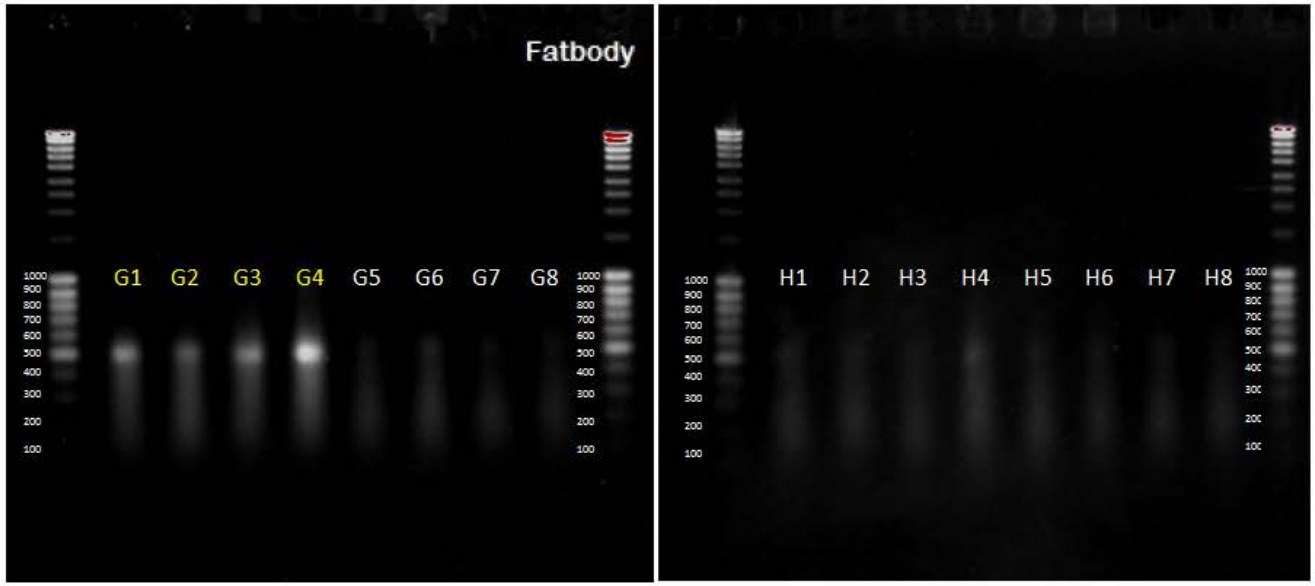

Figure S6.2. Overview LSV positive results in fat tissue and other body parts after screening on 15 wild collected *Bombus pascuorum* samples in 2 locations. Threshold for unambiguous positive samples after this nested PCR was set as clear full bands

Chapter 7:

Domesticated honey bees impact on multiple drivers of sympatric wild bees

This chapter is based on:

Domesticated honey bees impact on multiple drivers of sympatric wild bee ecology

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1. Abstract

Wild pollinators are currently in decline on a local to global scale. Counteracting this phenomenon has led to the use of the pollination services of domesticated bees, primarily in anthropogenic ecosystems. Last decades hives of honeybees (*Apis mellifera*) have been used intensively to pollinate a growing area of crops. Yet, there is mounting evidence that managed honeybees may affect the health of wild bees, as reported in the non-native range after introduction of honeybees. Here we demonstrate that honeybee domestication correlates with top-down and bottom-up drivers of sympatric wild bees populations. We found a positive correlation between apiary presence and honeybee-associated parasite and virus prevalence in wild Bombus pascuorum. Competition for resources in proximity of apiaries was increased, as reared Bombus terrestris nest development and survival was lower. Finally, we counted lower abundance of non-*Apis* bees in apiary dense study sites. Therefore we discuss possible actions to ameliorating beekeeping practices in relation to wild sympatric bees, basically advising less distortion of natural host-parasite interactions and host-resource competition.

2. Introduction

Insect pollination is key for up to 80% of the plant species (Gallai et al. 2009, Garibaldi et al. 2013), and the mutualism between plants and pollinators has led to a tremendous biodiversity (Bascompte and Jordano 2007). Yet, this biodiversity and many plant-derived ecosystem services are under threat, as wild insect pollinator populations including bees are declining on a global to local scale (Kremen et al. 2002, Winfree et al. 2009, Potts et al. 2010, Tscharntke et al. 2012). For crop pollination, losses are counteracted by employing managed bees (Woodcock et al. 2013). Especially the honeybee is well known for its commercial pollination potential, increasing yield in 96% of animal-pollinated crops. However, in comparison to pollination based solely on honeybee visitation, wild insects are enhancing fruit set twice as much as they pollinate crops more effectively (Garibaldi et al. 2013). Furthermore, massive domestication of honeybees, to be transported to the crop fields, can impact native sympatric bees (Goulson and Sparrow 2009). Indeed it has been suggested based on proxy measurements that honeybees can compete with wild bees, this because of niche overlap (Steffan-Dewenter and Tscharntke 2000, Paini and Roberts 2005), more efficient flower visits, and collecting or depleting floral resources (Schaffer et al. 1983, Gross 2001, Dupont et al. 2004). Although these proxy measurements are valuable they only are indicating a potential competition between honeybees and native bees. To determine threats on long-term survival of native bee species fecundity, survival or population density needs to be assessed (Paini 2004). Furthermore the above-mentioned interactions of domesticated honeybees (*Apis mellifera*) with non-*Apis* species have been mainly described when introduced outside their native range (Schaffer et al. 1983, Roubik 1991, Huryn 1997, Goulson 2003b, Dupont et al. 2004, Paini 2004, Stout and Morales 2009), while in their native range host plant preference and habitat differentiation between different bee species is thought to be coevolved and thus competition is less obvious.

Here we specifically look at how honeybee domestication can influence bumblebee development and parasite prevalence in its native range. Domestication can impact two major drivers in population dynamics of wild bees: top-down by the spread of diseases; or bottom-up processes influencing foraging success by competition for flower resources. Related to the latter, Goulson and Sparrow (2009) examined the thorax size of wild *Bombus* as a proxy for competition between honeybees and four species of bumblebees. Yet, when there is an overlap in habitat niches and resources between Apoidea bees (Steffan-Dewenter and Tscharntke 2000), a fitness impact on bumblebee nests can be expected. Here we assess competition by measuring bumblebee nest development in urban and semi-urban landscapes. We use a coupled study design, pairing study sites with a similar landscape but being different in their apiary density. A similar approach, only studying one paired location indicated a potential competition between honeybee domestication and *B. terrestris* (Elbgami et al. 2014). These

results are preliminary as based on a single correlative study, especially considering the potential confounding effects of both spatial and temporal variation. For example, different studies have provided contrasting evidence related to competition due to honeybees (e.g. Thomson, 2006 versus Tepedino et al., 2007 (Colla et al. 2006, Tepedino et al. 2007)), illustrating the risks in drawing conclusions from a single correlational study (Stout and Morales 2009). This apparent incongruence seems to be the result of substantial variability of correlational data (Thomson 2006) stressing the importance of randomizing spatio-temporal confounding factors, which can be achieved employing a paired study design in a matrix of variable locations.

Beside to a possible resource competition, presence of apiaries could influence natural host-parasite associations. Honeybees could act as a reservoir for pathogens influencing top-down processes driving of wild bee ecosystems. Indeed, it has been reported that honeybees and wild pollinators host the same multi-host pathogens (Genersch et al. 2006, Singh et al. 2010, Peng et al. 2011, Evison et al. 2012, Graystock et al. 2013a, Levitt et al. 2013, Ravoet et al. 2014) suggestioning potential spillover events. When random flower patches are studied, honeybee density is a significant predictor of virus prevalence in bumblebees (Fürst et al. 2014, McMahon et al. 2015). However, knowing the communication skills of honeybees, with foraging distances up to 14 km, foragers are preferentially allocated to highly rewarding flower patches (Couvillon et al. 2014). Thus the predictive power of honeybee density could be linked with the specific flower patches attracting different honeybees from a wider environment. The relationship between apiary density and parasite or virus prevalence in wild bees remains unstudied. A positive relation can be expected as the majority of the foraging trips take place within one km of the honeybee hive (Eickwort and Ginsberg 1980, Couvillon et al. 2014, Seeley 1995).

Here we assess if honeybee domestication interplays with both top-down and bottom-up drivers of sympatric wild bee population dynamics. Therefore we search for correlations between apiary density and (i) parasite and virus prevalence in wild bumblebees (*Bombus pascuorum*), (ii) bumblebee nest development and survival of reared *Bombus terrestris* nests and (iii) bee diversity and abundance. In our study locations we implemented a coupled design, each which contained an apiary dense study site (ADS) and a study site harboring few apiaries (apiary sparse study site, ASS) in anthropogenic landscapes. In order to correct for spatio-temporal differences in pollinator assemblies, different landscapes (urban to semi-rural) were assessed over different study periods, but each pair of coupled study sites within locations were chosen have the same landscape metrics.

3. Material and methods

3.1. Location selection and defining coupled study sites

3.1.1. Paired design of locations with apiary dense and apiary sparse sites (ADS and ASS)

As an experimental backbone in this study, we defined the coupled study site set up. Therefore, we selected locations each with two study sites, i.e. one harboring many apiaries (ADS = apiary dense study site) and one study site with few apiaries (ASS = apiary sparse study site). These coupled ADS-ASS sites within each location were separated by 1.5 ± 0.1 km. The distance had two rationales. First, the distance between the locations needed to be large enough to ensure that the sampling populations (i.e. measurement of pathogen prevalence, nest development and bee assemblages) were different. Second, the two study sites should only differ in relation to the amount of apiaries and honeybee hives present. All other parameters should be as equal as possible, i.e. paired design. Therefore the distance between the two study sites was kept to a minimum and the two sites were chosen to have similar landscape metrics.

3.1.2. Landscape metrics analysis and statistics

Landscape metrics analysis (Suppl. dataset S7.1) was done in Geographic Information Systems (ArcGIS, ESRI v. 10.2.2). For each study site we identified several habitat types in an area with a 750 m radius. We defined different land covers: 1) impervious areas including buildings, streets and other hardened surfaces; 2) semi-natural habitats encompassing permanent biodiverse grassland, biodiversity valuable land and forests; 3) arable land with mainly insect pollinated crops encompassing orchards of fruit, nut-producing trees and legumes; 4) arable land mainly encompassing fodder crops; 5) grassland encompassing biodiversity poorer grasslands; 6) artificial green areas mainly encompassing gardens, public green areas such as parks and 7) rest fraction encompassing railways tracks, riverbeds, etc. We then analyzed all locations to meet our prerequisite of equal landscape metrics between coupled ADS-ASS study sites in each experiment over years 2013 and 2015. Permanova statistics (adonis call in R package Vegan) were run separately in each experiment to test if coupled ADS-ASS sites showed equal landscape metrics. For details see supplementary dataset S7.1 - part 1 for the experimental setup regarding pathogen (virus and protozoa) prevalence, supplementary dataset S7.1 - part 2 for the setup regarding measurements of competition, and supplementary dataset S7.1 - part 3 for setup regarding pantrap and transect walk monitoring. Following this study design resulted in correlated errors between locations. Therefore, a random factor ‘location’ being the variation across the sampling locations was included into the statistics of different experiments.

3.2. Top-down impact: pathogen prevalence

3.2.1. Virus and parasite prevalence

Within each ADS and ASS study site 10 *B. pascuorum* individuals were caught (n = 2 x 5 x 10). *B. pascuorum* is the most abundant and wide spread bumblebee species within our sampling locations, together with *B. terrestris*. Bumblebee foraging ranges are variable, mainly depending on forage availability, and bumblebee species. *B. pascuorum* is considered as a 'doorstep foragers' with anecdotic evidence of foraging ranges within 500 m (Walther-Hellwig and Frankl 2000, Goulson 2003a). Therefore, we focused on this species following our set up with a mutual distance of 1500 m between ADS and ASS sites. *B. pascuorum* can be taxonomically distinguished with quick color keys (Prŷs-Jones & Corbet, 2011). For *B. pascuorum* some confusions may arise with *B. muscorum* and *B. humilis* while the two latter species have no spikes of black hairs on the upper part of their abdomen, previous monitoring in Belgium (Rasmont and Iserbyt 2010-2016) revealed that these two bumblebee species are very rare in our sampling sites and if present with very low numbers (0, 2 and 23 counts for *B. humilis*, *B. muscuorum* and *B. pascuorum*, respectively; see Suppl. dataset S7, table S7.3.1.).

3.2.2. Diagnostics

Each caught bee was transferred to a separate container with a cotton saturated with 50% sugar water and stored at -70°C at the end of the sampling day. We performed RNA extraction on a total bee, after crushing in Qiazol (700 µl) with zirconia (0.1 mm) and stainless steel (1 mm) beads for 5 minutes in the Bullet Blender Homogenizer (Next Advance, Inc.). 500 µl of supernatants was centrifuged at 17.000 g for 3 minutes. 900 µl of Qiazol was added to 100 µl supernatants and the protocol was followed according to manufacturer's instructions (RNeasy Lipid Tissue; Qiagen). The RNA was eluted from the column with 50 µl RNA-free water. To check the infection status of commercial bumblebee colonies (Biobest, Belgium) we sampled three pools of four bumblebees in 4 ml Qiazol for bead beating. Thereafter the same procedure was followed beginning with the centrifugation of 500 µl supernatant.

In each apiary study site we screened the honeybee hives located at the center of the study site, i.e. 5, 7, 3, 3 and 10 hives. We sampled three pools of 10 bees in 4 ml Qiazol, following the same procedure as described above.

Reverse transcriptase was performed on 5 µl of RNA with random hexamer primers with the Revert AidTM First Strand cDNA Synthesis Kit (Thermo Scientific). All PCR reactions contained: 1.5 mM $MgCl_2$; 0.2 mM dNTP; 1.25 U Hotstart Taq DNA polymerase (Qiagen) and 1 µl cDNA product. The sequence of the primers is given in supplementary table S7.2.1. For microsporidia and protozoa detection we

confirmed 4 positive specimens for each parasite (i.e. *A. bombi*, *C. bombi*, and *N. bombi*) by Sanger sequencing (LGC genomics).

Virus detection was performed by MLPA (Multiplex ligation-dependent probe amplification) with virus specific primers to prepare cDNA and probes to detect the virus (see supplementary information Table S1 for sequence information). We followed the protocol described as the BeeDoctor technology using the *SALSA RT-MLPA* kit (MRC Holland) (De Smet et al. 2012). We used 500 ng of RNA to be reverse transcribed (Revert AidTM First Strand cDNA Synthesis Kit; Thermo Scientific) and screen for viruses from the AKI complex (acute bee paralysis virus, Kashmir bee virus and Israeli acute paralysis virus complex), DWV complex (deformed wing virus (DWV), Kakugo Virus and Varroa destructor Virus-1), BQCV, and sacbrood Virus (SBV) (De Smet et al. 2012). We used FAM labelled primers to amplify ligated probes and differentiated their length by capillary electrophoresis (*Genetic Service Unit*; Ghent University). Probe length calculation and thus virus identification is performed with Peak Scanner vs.2 software (Applied Biosystems).

3.2.3. Statistics

We implemented a generalized linear mixed-effects model (GLMM) to test multivariate and univariate statistics. Each response variable, being presence-absence data of the different parasites and viruses, was treated as a binomial error distribution with a logit link function. We used R studio with R package lme4 version 1.1-10 (Bates et al. 2015) and had two fixed factors apiary (presence-absence) and the count data from the pan trapping of *Bombus*. Here we take advantage of the paired setup, where apiary is a binary main factor, and location as a random factor. Multivariate effects of pathogens were visualized by a PCoA (betadisper call in R package Vegan).

3.3. Testing impact on bottom-up driver

3.3.1. *Bombus terrestris* nests as tool assessing competition

In each of coupled ADS-ASS study site (n = 2 x 8) we placed 3 bumblebee colonies (*B. terrestris*) obtained from a commercial breeder Biobest (Westelo, Belgium), as a monitoring tool for competition using the nest parameter 'biomass increase', which is a measure for nest fitness parameters including fecundity (number of queens) and survival (number of dead versus newborn workers) of the bumblebee nest (Parmentier et al. 2014). Nests were put into a polystyrene box for isolation and protection against rain. In total we placed 48 nests outdoors, harbouring on average 45 workers (± 19.9 SD). Three colonies were allocated randomly to each study site, within a distance to the centre of 100 ± 50 m.

3.3.2. Testing impact of pathogens on *Bombus terrestris* nest development

We screened nests (total = 45; n = 3 per location) before placing outdoors in different locations (2 x 6 ADS-ASS) and they showed no single infections of Apoidae-associated protozoa *Crithidia*, *Apicystis*, *Nosema* and viruses DWV, SBV, BQVC, and of the AKI-complex.

Prior to the experiment, we also tested if there were no confounding impacts of pathogens on nest development during the 6 weeks foraging outdoors in a separate experiment (see methods and analysis in supplementary dataset S7.2). The nest development parameter 'biomass increase' was chosen based on earlier experience (Parmentier et al. 2014). When inferring nest 'biomass increase', to total pathogen load per hive, we observed no effect (multivariate statistics: Res.df = 23, Dev = 2.88, P = 0.58; and all univariate statistics: P > 0.45). After this validation analysis, parameter 'biomass increase', was thus calculated by measuring nest weight before placing and after foraging for 6 weeks outdoors and used as a measure for competition in coupled ADS-ASS sites.

3.3.3. Statistics

We implemented a generalized linear mixed-effects model (GLMM) to test multivariate and univariate statistics in R package lme4 version 1.1-10 (Bates et al. 2015). The response variable, being the nest 'biomass increase' in GLMM was treated as a log-normal error distribution based on earlier observations of bumblebee nests following a logarithmic development (Crone and Williams 2016). We defined three factors, combining apiary (presence-absence) with count data from the pan trapping of *Bombus* and *Apis*. We defined in the model *Bombus* counts and *Apis* counts as fixed factors; While these factors were initially added, they were omitted in a less complex model with better AIC score (delta 4).

3.4. Testing impact on wild pollinator assemblies

3.4.1. Measurement of the wild pollinator community

In order to investigate a relation between apiaries and wild bee abundance and diversity we combined pan trapping (Westphal et al. 2008) and transect walks (Wood et al. 2015).

In first, pan trapping (see suppl. dataset S7.3 for detailed description) was achieved in 6 locations and three triplicates of pan traps (3x3) were placed per study site; each triplicate contained the following three colors: white, yellow and blue (Stanley et al. 2013). The distance between the pan traps within one triplicate ranged from 3 until 5 meters and between triplicates from 10 until 20 meter. Each triplicate of pan taps was placed at a certain height, ranging from 0 to 0.8 meter, depending on the dominated flowers vegetation present, and at a distance of 100 ± 50 meter from the centre of the study site.

Second, we did transect walks as this sampling technique gives a better view on pollinator abundance (Westphal et al. 2008). In a total of 10 locations, bees were sampled in 3*50 m transects per sampling period (see suppl. dataset S7.4. for detailed description). Each area was sampled twice between mid May and end of September of 2015 during dry, warm (>15°C) and sunny conditions between 9 and 18.30 hours. Both ADS and ASS study sites were sampled with similar spatial heterogeneity and comparable landscape elements within one location (flowers in gardens).

3.4.2. Statistics

Diversity calculators on pan trapping data are given in supplementary dataset S7.4. For the pan trapping data, we used a GLMM to test the effect of apiaries. Statistics were run in R package lme4 version 1.1-10 (Bates et al. 2015) and we tested against the fixed factor apiary (presence-absence). We also run counts of 'non-Apis', i.e. a summation of *Bombus* and Other wild pollinators counts in the same statistical model with apiary as fixed factor.

4. Results

4.1. Top-down impact: pathogen prevalence

Fparasites the season of 2013 we selected five locations with coupled ADS and ASS site, and similar landscape metrics within each location (see analysis in supplementary data set S7.1, Part 1). After selection of study sites, we counted a mean of 6.6 ± 1.8 honeybee hives per km^2 compared to 0 ± 0 for the ASS over all locations.

Table 7.1. The number of bumblebee nests (n = 3 per study site) infected with a certain parasite or virus; * apiary dense study site; ** apiary sparse study site

Location	Apiary	*Apicystis*	*Crithidia*	*Nosema*	AKI	DWV	BQCV	SBV	Virus	**Total**
Gent	ADS*	1	2	1	0	0	2	1	2	**7**
	ASS**	1	3	1	0	1	1	0	2	**7**
Roeselare	ADS	2	3	2	0	0	0	0	0	**7**
	ASS	1	3	1	0	0	0	0	0	**5**
Horebeke	ADS	3	3	0	0	0	1	0	1	**7**
	ASS	1	3	0	0	0	0	0	0	**4**
Waarschoot	ADS	2	3	0	0	0	1	0	1	**6**
	ASS	0	3	1	0	0	0	0	0	**4**

4.1.1. Pathogen prevalence of bumblebee nests

In total we screened 24 bumblebee nests, i.e. 3 nests per study site. Table 1 gives an overview of infection level after being placed outdoors for 6 weeks (initially all colonies were parasite and virus free following the same screening procedure). *C. bombi* was found in all colonies except one. *A. bombi* was found in fewer colonies, and somewhat more frequently in study sites with apiaries. Virus prevalence was low, SBV and DWV were found in one colony. The most detected virus was BQCV, but here only in 4 out of 15 hives in ADS and in 1 out of 15 hives in ASS. The total count of pathogens showed a positive trend in higher pathogen prevalence in ADS versus ASS, although not significant (P = 0.07; 2-side t-test).

4.1.2. Pathogen prevalence of honeybee hives

Each apiary and honeybee hive was screened individually. All apiaries (n = 30) were screened for BQCV, the DWV complex, SBV and the AKI complex, with a prevalence of 86%, 69%, 62% and 3%, respectively. *A. bombi* was found in nearly all honeybee hives, i.e. 93%.

4.1.3. Parasite and virus prevalence in wild *Bombus* in function of apiaries and *Bombus*

The multivariate analysis of the four viruses and three parasites screened revealed an effect of apiary density (ADS versus ASS) on pathogen prevalence in wild *B. pascuorum* (Res.df = 98, Dev = 30.87; P = 0.001). This effect is shown in figure 7.1.1 after a Bray-curtis visualization. The univariate analysis in figure 7.1.2 showed that both parasites only infecting bumblebees species, *N. bombi* and *C. bombi*, did not alter between the ADS and ASS locations; with low non-significant estimates in table 7.2. We see a marginal and non-significant trend for *C. bombi* in function of *Bombus* counts. For *A. bombi* we detect a consistent drop in prevalence in each ASS compared to ADS, indeed the mean prevalence has a 2-fold decrease from 62% to 30%, mainly explained by the factor apiary.

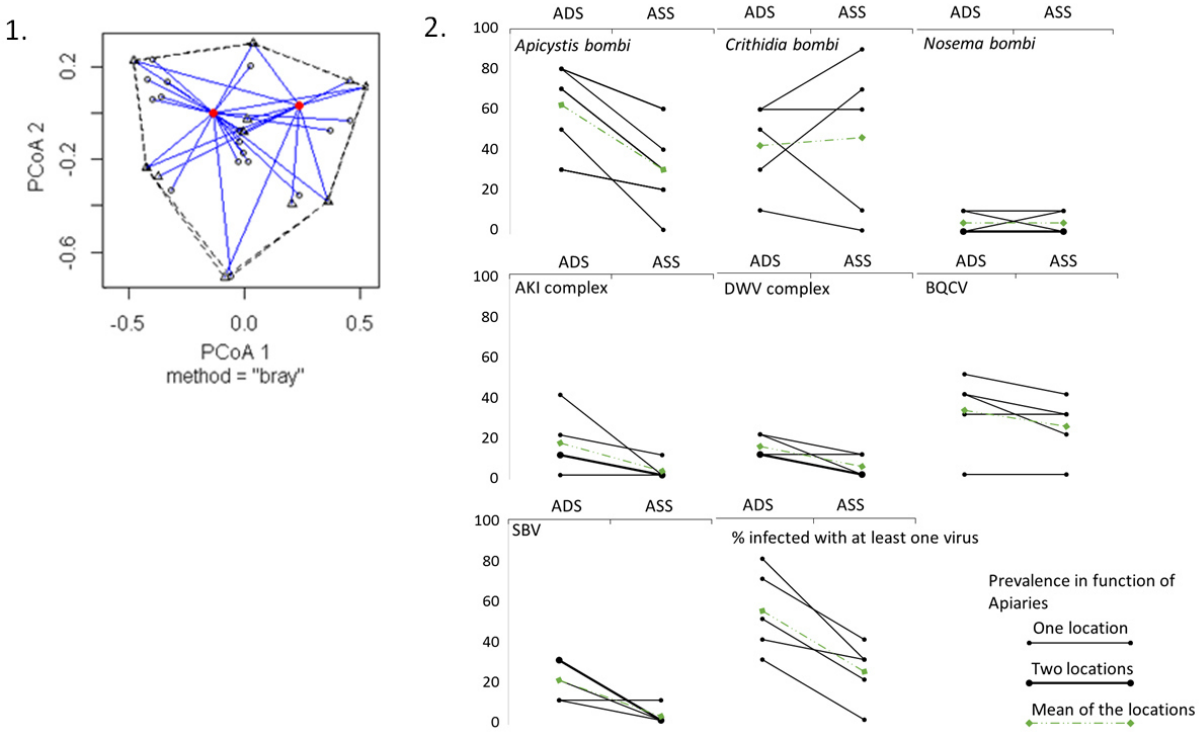


Figure 7.1. Prevalence of the different studied pathogens. **1.** Multivariate stastistics representing the effect of apiaries (location as random); **2.** Univariate statistics: each line connects an apiary dense site (ADS) with apiary sparse site (ASS). The bolded line represents two locations with identical prevalence, the green dashed line is the mean

In ADS, 52% of the bumblebees were infected with at least one virus; while in the ASS only 25% was infected with one virus (Fig. 7.1.1). Again this drop was a systematic drop in all locations, resulting in an overall significant effect of apiary density on virus prevalence and no effect of *Bombus* counts (Table 7.2). If we look at the different viruses individually we see that this effect is largely driven by the

AKI complex and SBV, as both exhibit the same trend towards more viruses in apiary locations. For the DWV complex this trend is less clear, and absent for BQCV (Table 7.2).

Table 7.2. F and p values of the Generalized linear mixed model with presence of apiaries as a categorical main factor and location as random factor; significant values are bolded

parasite / virus	fixed factor	GLMM				AIC	
		Estimate	SE	z value	P	no interaction	with interaction
Apicystis	Apiary	-1.28	0.52	-2.46	**0.01**	129.8	131.8
	Bombus	0.04	0.05	0.87	0.38		
Crithidia	Apiary	0.55	0.55	1.07	0.28	129.1	131.0
	Bombus	0.07	0.05	1.53	0.13		
Nosema	Apiary	0.24	1.10	0.22	0.83	41.3	43.3
	Bombus	0.05	0.09	0.54	0.59		
AKI	Apiary	-2.10	1.12	-1.87	0.06	60.9	66.4
	Bombus	0.07	0.10	0.69	0.49		
DWV	Apiary	-0.93	0.87	-1.08	0.28	63.2	64.7
	Bombus	0.10	0.07	1.44	0.15		
BQCV	Apiary	-0.41	0.52	-0.80	0.43	122.9	124.0
	Bombus	0.00	0.05	0.10	0.92		
SBV	Apiary	-2.35	1.12	-2.10	**0.04**	67.6	66.4
	Bombus	0.03	0.07	0.45	0.45		
Virus	Apiary	-1.39	0.51	-2.71	**0.01**	130.2	130.4
	Bombus	0.00	0.05	0.05	0.96		

4.2. Bottom-up impact: bumblebee nest development

During the season of 2013 and 2015 we selected eight locations (1 overlapping for year effect) with coupled ADS and ASS, and similar landscape metrics within each location (see supplementary data set S7.1, Part 2). After the selection of study sites, we counted a mean of 6.5 ± 6.5 honeybee hives per km^2 compared to 0.2 ± 0.6 for the ASS over all locations.

We employed the potential of *B. terrestris* nests as a bioassay tool to measure competition (see suppl. dataset S7.2 for validation experiments). Figure 7.2 summarizes the data of *B. terrestris* nests (n = 48) placed per triplicate in each of eight coupled ADS and ASS study sites. A significant result was seen after a 6 week development of nests placed at ADS versus ASS, with a lower biomass increase at the ADS sites, confirmed by GLM statistics (estimate = 60.52, SE = 23.19, z-value = 2.609, P = 0.009) following again a best fit by a (x+100) lognormal development model of *B. terrestris* nests.

4.3. General impact on abundance and diversity of wild bees

Finally, during season of 2015 we assessed the general impact of apiaries on proximate pollinaton networks by applying a conventional monitoring of transect walks and pan trapping. In total we selected ten locations with coupled ADS and ASS site, and similar landscape metrics within each location (see suppl. dataset S7.1, Part 3). After selection of study sites, we counted a mean of 8.5 ± 6.4 honeybee hives per km^2 compared to 0.2 ± 0.5 for the ASS over all locations.

4.3.1. Pan trapping data and wild bee diversity indices

Pan trapping was performed in 6 locations over one season (April to July 2015). After meeting criteria of equal sampling within coupled ADS-ASS study sites and drawing rarefaction curves (see suppl. dataset S7.3, part 1), we performed diversity calculators on the total dataset encompassing a total of 1027 wild bees, i.e. 383 and 425 bees in ADS and ASS, respectively, *Apis* counts not included. In order to evaluate diversity differences between ADS and ASS, we performed ecological diversity indices and species richness calculators on both subsets. As described in depth in supplementary dataset S7.3, part 2, we only found Fisher's alpha index (α) differing between ADS-ASS study sites (n = 15; P = 0.011, Paired t-test), independent of *Apis mellifera* counts, as the same effect was found after omitting the *Apis* counts from the dataset (n = 15; P = 0.026, Paired t-test). While Fisher's α is a measure for abundance of uncommon species, these results indicated a lower abundance of uncommon species in close proximity of apiaries (ADS versus ASS), but a general negative effect on wild species diversity was not found.

	Ghent 1		Ghent 2		Ghent 2		Roeselare		Meise		Huizingen		Perk		Berg	
Average Δ biomass	**94,2**	**130,5**	**129,9**	**293,3**	**117,1**	**253,6**	**133,0**	**193,5**	**-54,9**	**28,7**	**-42,5**	**-35,7**	**-39,0**	**-46,1**	**-40,6**	**-17,1**
stdev	90,6	115,7	92,1	81,4	29,5	106,1	142,7	138,1	14,4	120,1	36,8	21,7	16,6	18,3	9,0	9,7
Δ HB vs. 1500m	**+ 36,3**		**+ 163,4**		**+ 136,5**		**+ 60,5**		**+ 83,6**		**+ 6,8**		**- 7,1**		**+ 23,4**	

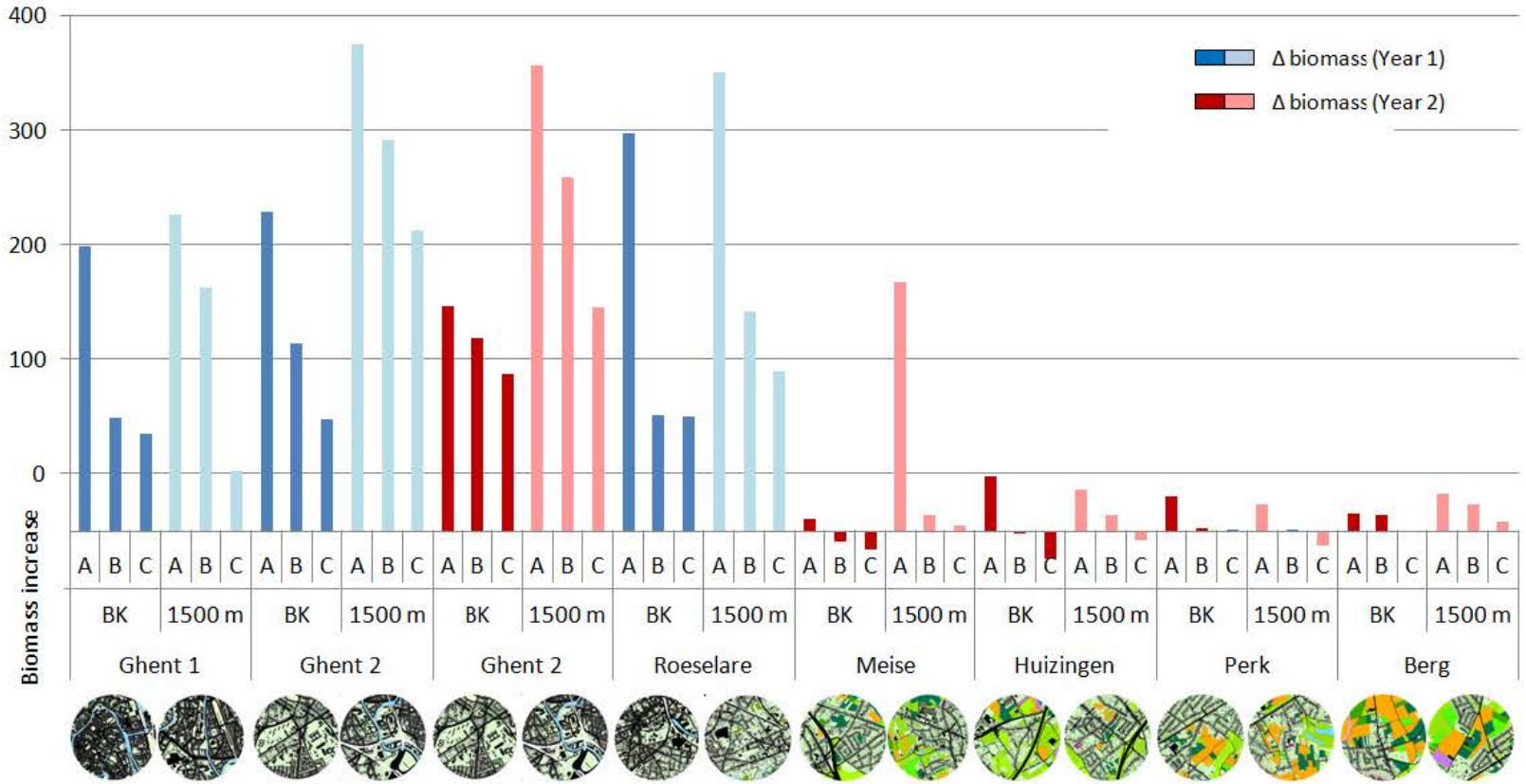


Figure 7.2. Development of *B. terrestris* nests (n = 48) in a spatial matrix of 8 locations during a 2-year survey (2013, blue; 2015, red). Nests are placed in triplicates at each ADS (close to apiary) and ASS (1500 m distance to apiary) and biomass increase is measured after 6 weeks of development

4.3.2. Transect walking data and abundance of wild bees

In total we walked in 80 hours over two periods 6000 m of transects in 20 study sites (10 locations) encompassing urban to semi-rural landscapes to cover different landscapes, as explained in depth in supplementary dataset S7.4. As pan trapping (Table S7.3.1.) indicated that a total of *Apis* and *Bombus* counts represented about half of the dataset (488/1098), and these genera are easiest to observe during transect walks in the field, we classified transect walk counts in three groups, i.e. *Apis*, *Bombus* and other wild bees. This resulted in a total of 2544 bees observed, i.e. 1353 versus 1190 bees in ADS and ASS, respectively, as represented in figure 7.3. Multivariate GLM statistics showed that the number of Apis and Non-*Apis* was significantly different between ADS and ASS sites (multivariate GLM; Res.df = 18, Dev = 22.39, P = 0.004), with the number of *Apis* being significantly lower (Dev = 17.99, P = 0.001) while the number of non-*Apis* (sum of *Bombus* and Other) species increased significantly at ASS sites (Dev = 4.396, P = 0.017). However when considering all counts of bees in ADS and ASS, total of pollinators is higher in close proximity of apiaries, but this effect was only supported by a strong trend (Dev = 23.02, P = 0.058).

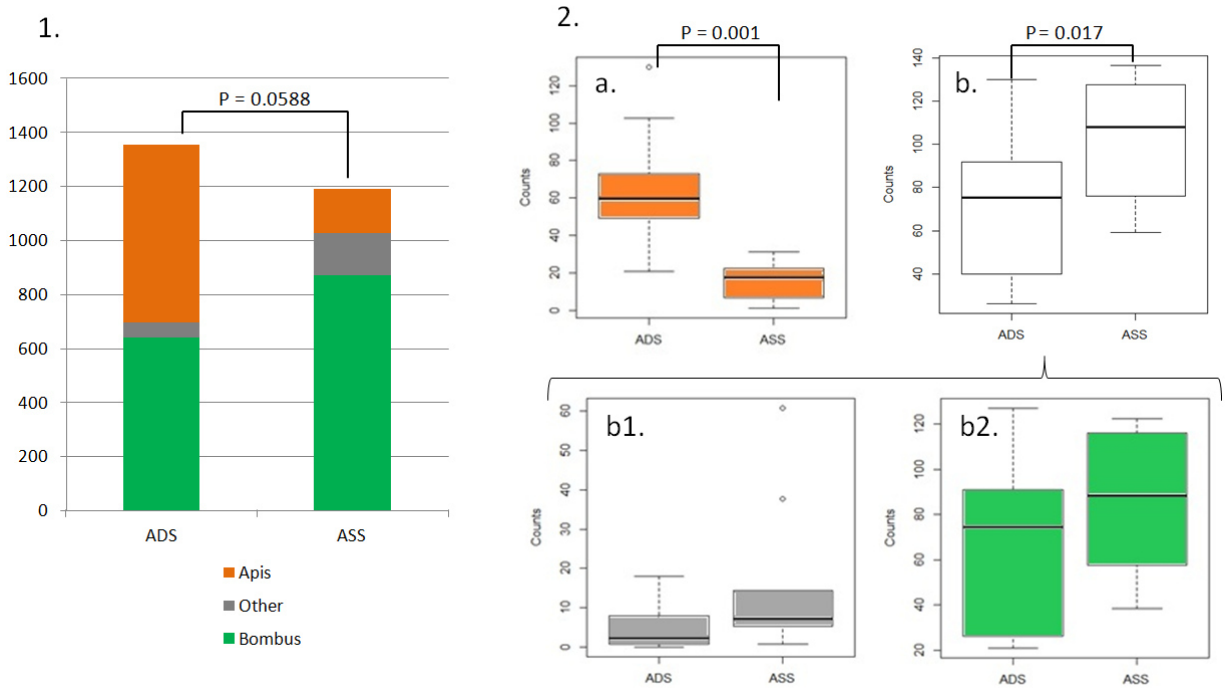


Figure 7.3. Transect walk data in locations each composed of an ADS and ASS (n = 20); **1.** Total counts of *A. mellifera* (Apis), *Bombus* sp. (Bombus) and other wild bee spp. (Other); **2.** Boxplots representing counts and significant P-values between ADS and ASS of (**a.**) *Apis* and (**b.**) non-*Apis* bees, i.e. summation of (b1.) Other and (b2.) *Bombus*

5. Discussion

5.1. Apiary density inversely correlates with wild pollinator abundance independent of landscape types

It has been shown in previous studies that in rewarding flower patches a positive correlation between numbers of *Apis* and *Bombus* exists (Potts et al. 2003, Kohler et al. 2008, Samnegard et al. 2011, Banaszak and Dochkova 2014), which reflects their overlapping foraging pattern. Our results, employing the power of a coupled ADS-ASS study design, show that flower patches (transects) close to apiaries harbored fewer wild bees (suppl. dataset S7.3), and less uncommon solitary bee species (Fisher's alpha index in suppl. dataset S7.3; and Figure 7.3). As our monitoring was performed over different anthropogenic landscapes, ranging from urban areas (31.8 % impervious surfaces) with no agricultural elements present, to semi-rural landscapes (6.2 % impervious surfaces and 34.9 % agriculture), the negative relation on sympatric wild bee abundance is probably not linked to a specific anthropogenic landscape studied.

Beside, the total counts of bees (*Apis* and non-*Apis*) tends to be higher (P = 0.058; Figure 7.3) in study sites dense of apiaries, indicating a stable to positive pollination service function within sites supplemented with honeybee hives, with honeybees as generalist flower visitors. However, this rationale can only be drawn when only focusing on the number of pollinators, while this is somewhat in contrast with recent new insights showing that wild bees are more efficient pollinators and enhance fruit set twice as much despite their lower number (Greenleaf and Kremen 2006b, Holzschuh et al. 2012, Woodcock et al. 2013).

5.2. Apiary density and evidence of competition towards sympatric *Bombus terrestris*

Since an overlap in habitat niches exists (Steffan-Dewenter and Tscharntke 2000, Thomson 2006), competition for floral resources between Apoidea bees could lead to an impaired development of proximate sympatric bumblebees. Indeed, our results showed a significantly lower nest 'biomass increase' when nests of *B. terrestris*, one of the most common bumblebee species in Belgium (Rasmont and Pauly 2010) and Europe (Rasmont and Iserbyt 2010-2016), are developing in proximity of an apiary (ADS versus ASS). Not ignoring the confounding effects designing our experiment in a way that ecological variables do not interfere making conclusions (Stout and Morales 2009), we proved that assessing the nest parameter 'biomass increase', a measure of bumblebee nest fitness (Goulson et al. 2002, Parmentier et al. 2014), together with a coupled study design of ADS-ASS is an effective design to measure competition effects of apiaries on sympatric bumblebee nests. Indeed, we found no confounding impact of pathogens on development of nests over a short period of study (6 weeks) and the possible effect of pesticides (Szabo et al. 2012, Lundin et al. 2015), landscape context, agri-environment schemes (Williams and Osborne 2009) and other anthropogenic

influences (Dupont et al. 2011, Goulson and Hughes 2015) are reduced by the coupled ADS-ASS study site design with equal landscape metrics. Our result is therefore an important extension and confirmation of exploratory work that suggested first evidence for competition of honeybee apiaries towards sympatric wild bumblebees.

Next to this, our results also indicated that when bumblebee nests developed better (at ASS sites) more *Bombus* spp. were counted (Figure 7.3). This suggests that, even when other *Bombus* are more prevalent in the environment, bumblebee nest fitness is better when density of apiaries is lower, indicating for an inverse relationship between apiary density and *Bombus* fitness. In comparison, experimental results of Thomson (2006) in North-America also showed that mean numbers of individual counted *Bombus* foragers within flower patches on a given transect increased with greater distance from introduced *Apis* colonies.

5.3. Apiary density and correlation of *Apis*-associated pathogens within sympatric *Bombus pascuorum*

Since honeybees host the same pathogens also found in other Apoidea bees (Genersch et al. 2006, Singh et al. 2010, Peng et al. 2011, Evison et al. 2012, Graystock et al. 2013a, Levitt et al. 2013, Ravoet et al. 2014), they could act as a reservoir for pathogen spread influencing top-down processes of wild bee ecosystems. Indeed, the prevalence of some pathogens (e.g. DWV and *Nosema ceranae*) in wild bumblebees has been linked with the presence of honeybees, while this result was largely based on spatial overlap at shared flower patches (Fürst et al. 2014). In contrast to previous studies (Fürst et al. 2014, Graystock et al. 2014, McMahon et al. 2015) we here investigated the impact of apiary density on prevalence of associated diseases, including three protozoa and four viruses, on sympatric wild bumblebees. Our results of GLMM (Table 7.2) showed that it was the factor apiary presence which mainly explained pathogen prevalence in wild *B. pascuorum*; while *Bombus* count almost never improved the model. We clearly showed a correlation with *Apicystis bombi*, SBV and general virus prevalence, while *Nosema bombi* and *Crithidia bombi* did not correlate with apiaries. We advocate that shared pathogens, also detected in honeybees showed the highest prevalence in wild *B. pascuorum*. Indeed, the lower prevalence of *N. bombi* and *C. bombi* can be explained as these pathogens have never been detected in honeybees. Thus, the prevalence of shared pathogens, both parasites and viruses, detected in sympatric wild *B. pascuorum* is related to the density of apiaries. This result observed is independent of the landscape type as we tested both in a variety of urban to semi-rural anthropogenic landscapes. We therefore enlarge the effect of honeybees on wild bees based on spatial overlap at shared flower patches in previous studies (Fürst et al. 2014) to a general effect of apiaries independent of shared flower use. As is has been shown that forage trips of honeybees take place not only to rewarding flower patches, but also within one km from the hive

(Eickwort and Ginsberg 1980, Couvillon et al. 2014, Seeley 1995), we speculate that honeybee density in natural environments can be a general predictor of shared pathogen prevalence in wild sympatric bees.

5.4. Mechanisms of pathogen spread towards wild bumblebees

Different mechanisms can explain the relation between apiaries and pathogens prevalence in wild bumblebees. A possible explanation could be that bumblebees have a weaker immunity in proximity of apiaries as it has been reported that a drop in food availability could weaken bumblebee species, making them less immune competent (Moret and Schmid-Hempel 2000). Although we showed a correlation on proximate bumblebee nest fitness (biomass increase), we argue that immune incompetence was not the major effect that played here. Indeed, if this would be the case, *Bombus*-specific parasites like *C. bombi* and *N. bombi* (Brown et al. 2003b, Otti and Schmid-Hempel 2007) would also be higher in apiary dense sites. As this was not the case, a general weakening of *Bombus*' immunity due to apiary density is a less likely explanation.

Honeybees of apiaries could act as active vectors of pathogens. For viruses, being omnipresent in honeybee hives (Fürst et al. 2014, McMahon et al. 2015), a transmission by flowers inoculated by infected honeybees seems possible. Sequencing of the identified viruses to reveal population structuring could enlighten further evidence for interspecies transmission. Indeed, previous studies showed that BQCV, IAPV and DWV retrieved in honeybees and bumblebees cluster together (Singh et al. 2010, Fürst et al. 2014). While our study does not encompass vast areas, making it improbable to detect higher clustering over host than over distance, viral sequencing within different European countries could be interesting as a setup to study inter-species transmissions and host-virus networks.

Although apiary presence is a predictor for pathogen prevalence in wild *B. pascuorum*, this must not mean honeybees are the reservoir for a certain pathogen. It can also mean that honeybees act as mechanical vector. They could merely be spreading an infection hotspot towards more flowers, as experiments in flight cages have identified flowers as hotspots for intra-species transmission of parasites and viruses between honeybees and bumblebees (Graystock *et al.* 2015). We argue that a relation with *A. bombi* could be a consequence of such mechanical vectoring. Although *A. bombi* is being retrieved in honeybees and in our sampling locations of the 2013 study, being East- and West-Flanders in Belgium (suppl. dataset 7.1) 93% of the apiaries (n = 28) tested positive for *A. bombi*, infection status in these hives is often low. Indeed, microscopic analyses in our lab on the fat body of honeybees only rarely reveal *A. bombi* oocysts (data not shown). This is probably explaining why this parasite has barely been detected with a microscope in epidemiological studies of honeybees. Since the description of this bumblebee parasite (Liu et al. 1974, Lipa and Triggiani 1996), it was only

microscopically found in a single honeybee specimen of Finland in 1990 (Lipa and Triggiani 1996) and two decades later it was rediscovered in European honeybees from Northwestern Patagonia (Argentina) (Plischuk and Lange 2009, Arbetman et al. 2013). If honeybees act as mechanical vectors (spreaders) instead, one would expect that the same principle should hold for flowers initially contaminated with *C. bombi*. Yet, this must not be the case, the half-life of *C. bombi* on flowers has been estimated as 77 min (Otterstatter and Thomson 2008). Therefore, taking into account this fast rate of decay, we expect that mainly the initially contaminated flower will contribute to the infection of new bumblebees. Indeed different factors are influencing parasite or virus infection dynamics: the initial inoculum amount on the flower, rate of decay of the particle and infectivity. If a fast decay is expected and high titers are needed to be infective, than a mechanical vectoring is less probable. We therefore encourage more research on transmission pattern of both viruses and parasites within different pollinator assemblies. This will greatly influence how important the initial inoculators and/or the indirect spreaders are.

5.5. Apiaries and top-down effect of diseases on sympatric *Bombus terrestris* nests

In parallel to the wild captured *B. pascuorum*, we also screened workers from *B. terrestris* nest placed in ADS versus ASS. Initial screening did not show any parasite nor virus, while after foraging outdoors we found that mainly the gut parasite *Crithidia* could colonize, and for the viruses mainly BQCV was detected. The latter could indicate that bumblebees are an important host for BQCV. In comparison, McMahon *et al.* (2015) reported for BQCV prevalence a negative correlation with bumblebee count, but a positive with honeybee count (McMahon et al. 2015). When making a summation of total pathogen counts in individual nests per study sites (table 1), a trend was also found in that total counts of pathogen were higher in ADS versus ASS. While a trend was observed in only four locations studied, this result is in line with parallel observations of pathogens detected in wild *B. pascuorum* captured in ADS versus ASS.

5.6. Mitigation

In general, our results indicate a competition towards sympatric bumblebees and results of a higher pathogen prevalence in proximity of apiaries. We observe at least a correlation between the presence of apiaries and prevalence of shared parasites and viruses in sympatric bumblebees. In addition, we here showed an inverse correlation between apiary density and abundance of wild pollinators. We want to emphasize that our selection of sites with many apiaries did not result in unrealistic dense sites. In our setup of the 2013 study on pathogens, the ADS had a mean density of 6.6 ± 1.8 bee hives per km^2, while in Belgium the mean number of honeybee hives per km^2 is 3.6 (Chauzat et al. 2013). In some European countries the mean number of hives per km^2 exceeds the 6.6

density of our setup, i.e. Greece 11.4, Hungary 10.7 and Slovenia 7.7 (Chauzat et al. 2013). We acknowledge that some of the honeybees from the apiary site will forage at the site with few apiaries, but that their abundance will be lower at the sites with few apiaries. Since our experimental setup already shows an impact over a relative short distance, being 1.5 km, we recommend that local transport of domesticated honeybees should be restricted, especially in early spring, the most fragile period of the bumblebees and many solitary bees' life cycle. Second, we recommend less import of honeybee queens and thus the use of local honeybee lineages adjusted to their climatic and vegetation zone, and mating within these zones could lead toward less disturbance of pathogen associations. Currently, some specific breeding races are preferred by beekeepers leading towards import of honeybee queens and transport of honeybees (De la Rua et al. 2009). This practice could potentially introduce new pathogens into a certain location, while the use of local lineages opens new avenues for breeding programs towards honeybees with lower pathogen loads.

Finally it will be important to determine the carrying capacity of different environments in regard with honeybee support and sympatry with wild bees. We recommend a better surveillance so that authorities can track individual honeybee hives to get insight in overlaps of domesticated bees and wild bees.

6. Supplementary datasets

Supplementary dataset S7.1: coupled study sites, their apiary density and their spatial-variation

Spatial variation of landscapes and consequently differences in land cover can impact on bee abundance (Sydenham et al. 2014, Scheper et al. 2015), nest development (Parmentier et al. 2014) and parasite prevalence (Fürst et al. 2014). To obtain equal landscapes when defining an appropriate study design, we searched for locations with one apiary dense site (ADS) and one apiary sparse site (ASS) (in each separate experiment, we counted the number of honeybee hives per km^2) which showed comparable landscape metrics. After selection of study sites, a circle with a radius of 750 m was set as working area for all ADS and ASS study sites. Defining equal landscape metrics within each location, we obtained a coupled ADS-ASS study design and this set up was used as an experimental backbone trough all experiments.

To confirm equal landscape cover, an analysis was done in Geographic Information Systems (ArcGIS v. 10.2.2, ESRI). For each study site we overlaid available ArcGIS land cover maps for Flanders and identified several habitat types in the set study area. We defined following land covers: 1) impervious areas including buildings, streets and other hardened surfaces ("impervious area"); 2) semi-natural habitats encompassing permanent grassland and biodiversity rich, biodiversity valuable land and forests ("semi-natural habitat"); 3) arable land with mainly insect pollinated crops encompassing orchards of fruit, nut-producing trees and legumes ("insect pollinated crops"); 4) arable land mainly encompassing fodder crops ("fodder crops"); 5) grassland encompassing biodiversity poorer grasslands ("arable grassland"); 6) artificial green areas mainly encompassing gardens, public green areas such as parks ("artificial area") and 7) rest fraction encompassing railways tracks, riverbeds, etc. ("rest fraction"). To test multivariate dispersing, such as the landscape cover of study sites, a PERMANOVA analysis is often used (see e.g. (Legendre and Anderson 1999) and (McArdle and Anderson 2001)). Thus, using 7 landscape metrics (as defined supra) we ran the "adonis" call in R package "Vegan", a function for the analysis and partitioning sums of squares using semimetric and metric distance matrices (Oksanen et al. 2016). The technique uses the existing method of redundancy analysis but allows the analysis to be based on Bray-Curtis or other ecologically meaningful measures through the use of principal coordinate analysis (PCoA) (Legendre and Anderson 1999). Next, the differences were visualized by plotting a PCoA using the "betadistr" function (in R package "Vegan"). We tested separately the equality of landscapes within locations in the three datasets of this study, ie. (**part 1**) the 2013 experiment on prevalence of virus and parasites, (**part 2**) the 2013 and 2015 experiment on competition between domesticated honeybees and bumblebees, and (**part 3**) the 2015 experiment on wild bee assemblages in ADS versus ASS study sites.

Part 1- 2013 study: virus and parasite prevalence

It has been shown that there exist local variation of diseases prevalence associated with Apoidea bees (e.g. McMahon et al. 2015) for viruses and (Imhoof and Schmid-Hempel 1998) for protozoa). Therefore we have chosen to cover a spatial variation in our study set up, we selected five locations with different landscape metrics (**Figure S7.1.1a**) each having one coupled ADS and ASS site. As shown in **table S7.1.1**, PERMANOVA statistics showed significant differences of landscape metrics between locations (PERMANOVA; Res. Df = 6; F-score = 62.396; P = 0.01), but not between coupled study sites within locations (PERMANOVA; Res. Df = 6 ; F-score = 0.261; P = 0.58). This result is also visualized in **Figure S7.1.1a/b**. Detailed results of the PCoA analysis including eigenvalues and average distance to medians are given in **Table S7.1.2**.

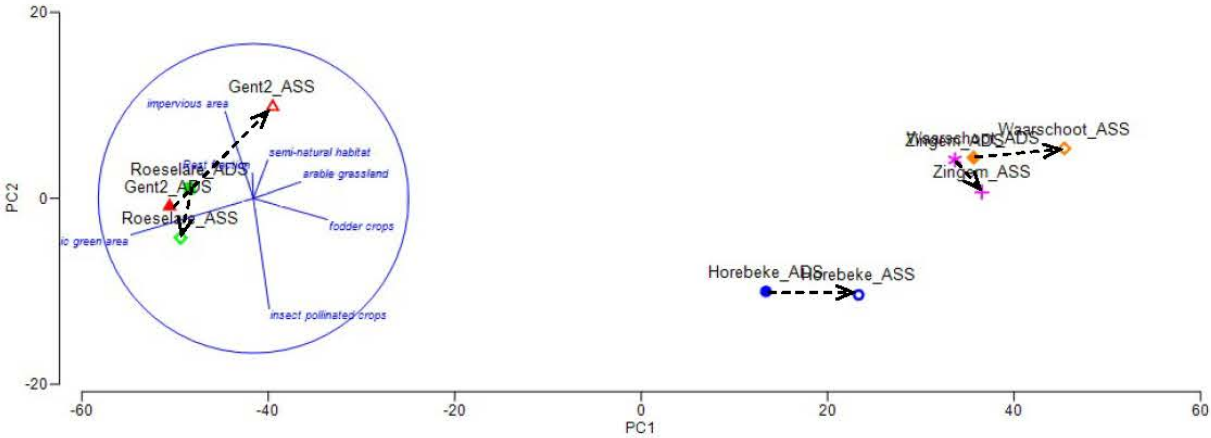
Table S7.1.1. Strata analysis on landscape metrics for the **2013 dataset**: effect of **locations** and coupled **study site** (ADS vs. ASS)

```
Call: Adonis

Strata: loc13
Permutation: free
Number of permutations: 99
Terms added sequentially (first to last)
```

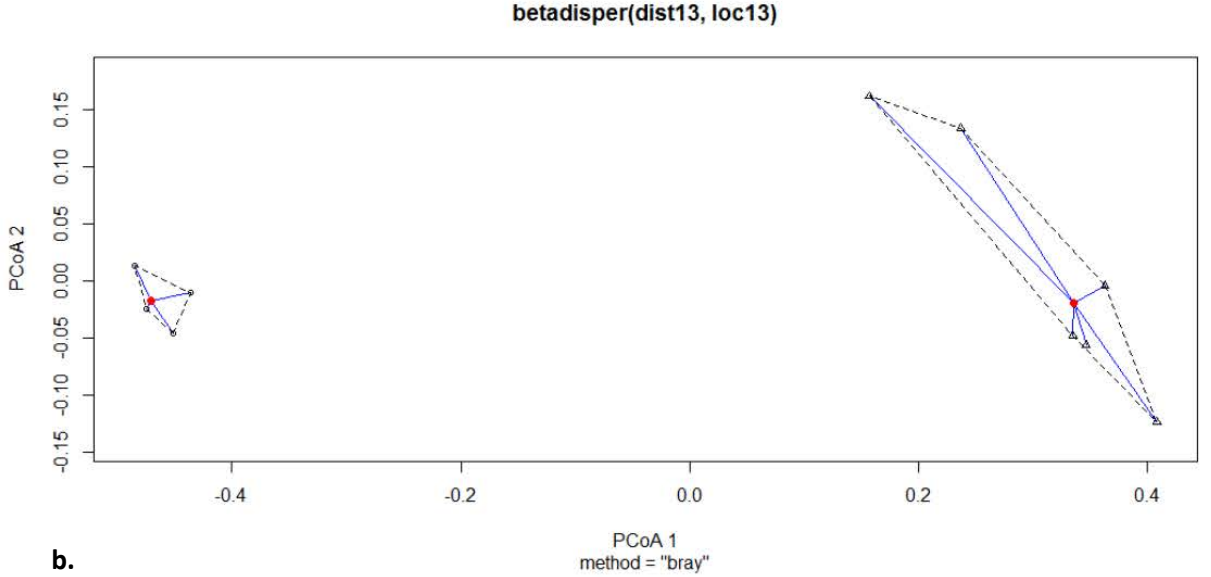
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
loc13	1	1.42325	1.42325	62.396	0.90621	0.01 *
site13	1	0.00596	0.00596	0.261	0.00380	0.58
loc13:site13	1	0.00449	0.00449	0.197	0.00286	0.66
Residuals	6	0.13686	0.02281		0.08714	
Total	9	1.57055			1.00000	

Table S7.1.2. Principal Coordinates Analysis (PCoA) of the **2013 dataset**: values visualizing effect of landscape and coupled study site (ADS and ASS) within locations: Eigenvalues for PCoA axes and distance to median

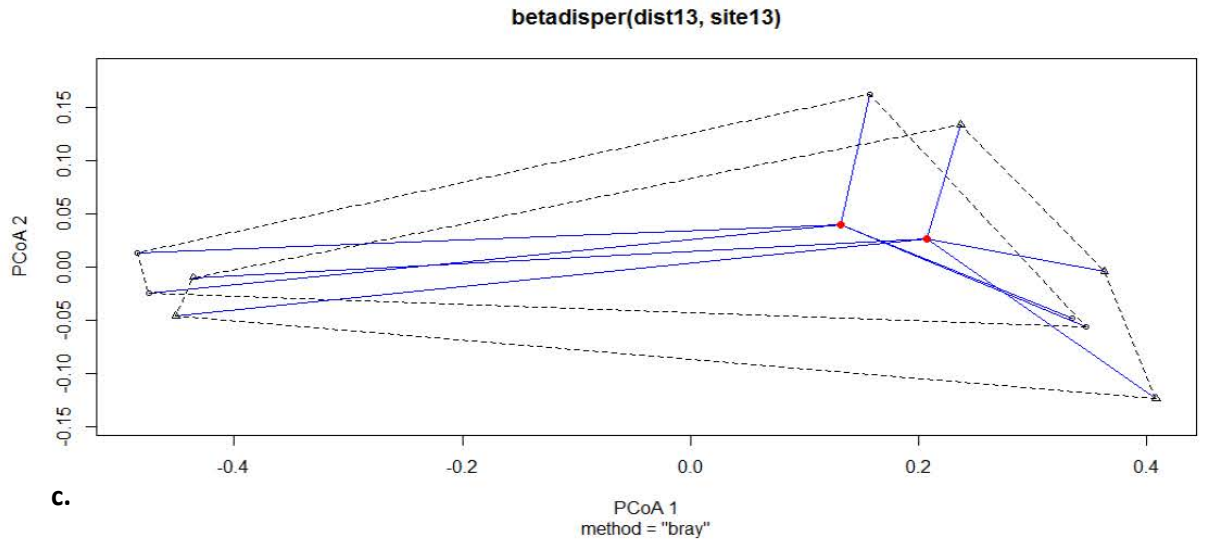
```
Call: betadisper

No. of Positive Eigenvalues: 7
No. of Negative Eigenvalues: 2
Average distance to median (landscape)
Urban  Semi-rural
0.07486 0.12528
Average distance to median (study site)
  ADS    ASS
0.3652 0.3722

Eigenvalues for PCoA axes
 PCoA1   PCoA2   PCoA3   PCoA4   PCoA5   PCoA6   PCoA7   PCoA8
 1.4660  0.0678  0.0274  0.0065  0.0043  0.0027  0.0021 -0.0020
```



a.



b.



c.

Figure S7.1.1. Landscape metrics analysis of all locations and coupled study sites (an arrow from ADS to ASS illustrates coupling within each location) in the 2013 study. **a.** PCoA visualizing the principal landscape covers; **b. and c.** PERMANOVA statistics showing a significant of location (b.) and no effect of study sites (c.)

Part 2- 2013 and 2015 study: competition between honeybees and bumblebee nests

Considering a possible temporal variation in our set-up, especially related to bumblebee hive development (Elbgami et al. 2014), in a second year (2015) we selected extra locations having overlapping study sites with the first year (2013) to account for the year effect in our modeling. In total, we selected seven locations with different landscape metrics (**Figure S7.1.2a**) each having one coupled ADS and ASS study site. As shown in **table S7.1.3**, PERMANOVA statistics showed significant differences of landscape metrics between locations (PERMANOVA; Res. Df = 10; F-score = 12.870; P = 0.02), but not between coupled study sites within locations (PERMANOVA; Res. Df = 10 ; F-score = 0.156; P = 0.87). This result is also visualized in **Figure S7.1.2a/b**. Detailed results of PCoA analysis including eigenvalues and average distance to medians are given in **Table S7.1.4**.

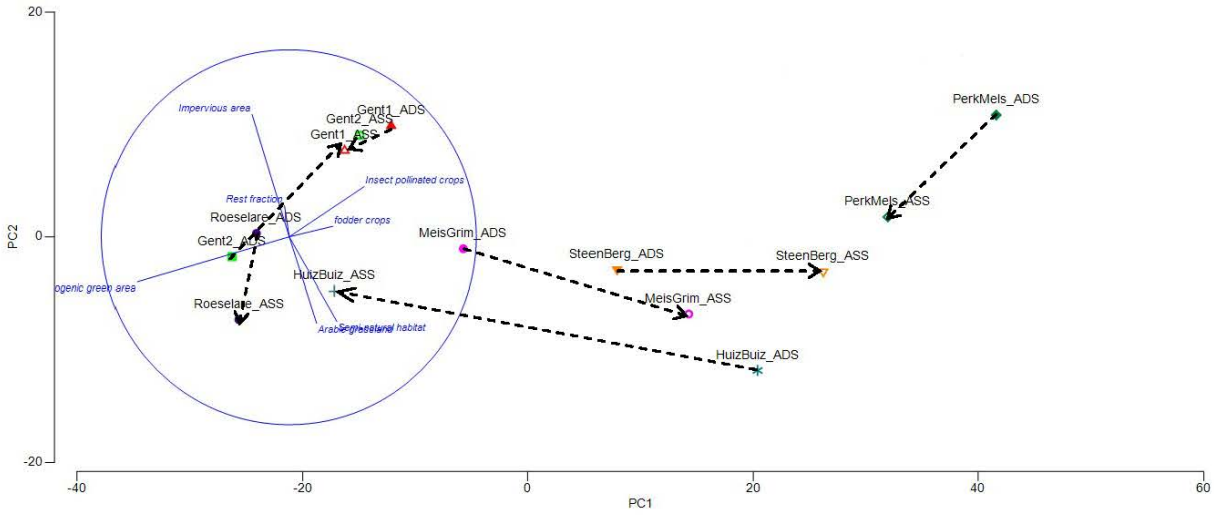
Table S7.1.3. Strata analysis on landscape metrics for the **2013 and 2015 dataset**: effect of **locations** and coupled **study site** (ADS vs. ASS)

```
Call: Adonis

Strata: loc1315
Permutation: free
Number of permutations: 99
Terms added sequentially (first to last)
```

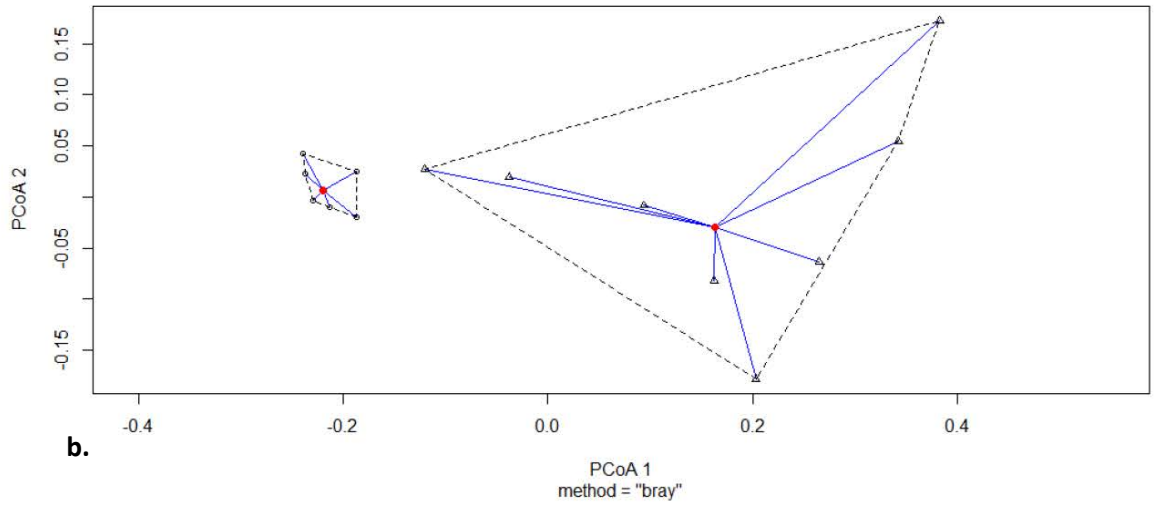
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
loc1315	1	0.48895	0.48895	12.8702	0.56008	0.02 *
site1315	1	0.00593	0.00593	0.1562	0.00680	0.87
loc1315:site1315	1	-0.00179	-0.00179	-0.0472	-0.00205	1.00
Residuals	10	0.37991	0.03799		0.43518	
Total	13	0.87300			1.00000	

Table S7.1.4. Principal Coordinates Analysis (PCoA) of **2013 and 2015 dataset**: values visualizing effect of landscape and coupled study site (ADS and ASS) within locations: Eigenvalues for PCoA axes and distance to median

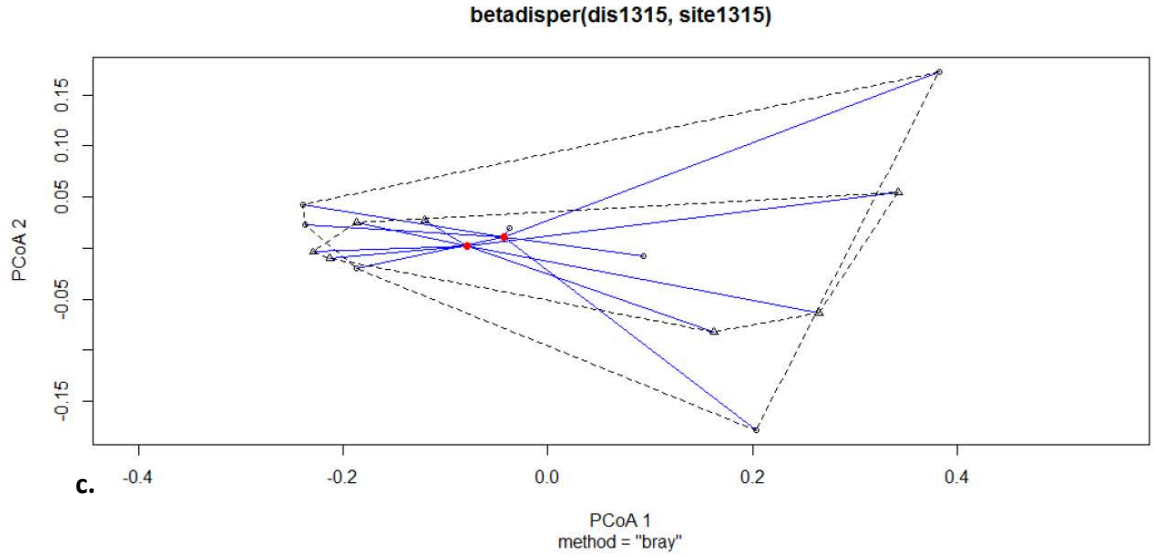
```
Call: betadisper

No. of Positive Eigenvalues: 10
No. of Negative Eigenvalues: 3
Average distance to median (landscape)
Urban  Semi-rural
0.08725 0.19153
Average distance to median (study site)
  ADS    ASS
0.2290 0.2336

Eigenvalues for PCoA axes
 PCoA1  PCoA2  PCoA3  PCoA4  PCoA5  PCoA6  PCoA7  PCoA8
0.7086 0.0797 0.0589 0.0239 0.0085 0.0047 0.0031 0.0009
```



a.



b.



c.

Figure S7.1.2. Landscape metrics analysis of all locations and coupled study sites (an arrow from ADS to ASS illustrates coupling within each location) in the 2013 and 2015 study testing for competition between domesticated honeybees and bumblebee nest. **a.** PCoA visualizing the principal landscape covers; **b. and c.** PERMANOVA statistics for testing the effect of location (b.) and study sites (c.)

Part 3 - 2015 study: effect on abundance and diversity of proximate wild bee assemblages

Considering a possible top-down (diseases and protozoa, 2013 study) and bottom-up impact of domesticated bees (2013 and 2015 study), we finally aimed to test a general effect on proximate wild bee assemblages. Therefore, we again selected six locations (partly overlapping with the 2015 and/or 2013 study) with different landscape metrics (**Figure S7.1.3a**) testing the effect on wild bee abundance and diversity. In the ADS sites, we counted a mean of 8.5 ± 6.4 honeybee hives per km^2 compared to 0.2 ± 0.5 for the ASS over all locations. As shown in **table S7.1.5**, statistics following the adonis call showed significant differences of landscape metrics between locations (PERMANOVA; Res. Df = 8; F-score = 4.365; P = 0.03), but not between coupled study sites within locations (PERMANOVA; Res. Df = 8; F-score = 0.035; P = 0.95). This result is also visualized in **Figure S7.1.3a/b**. Detailed results of PCoA analysis including eigenvalues and average distance to medians are given in **Table S7.1.6**.

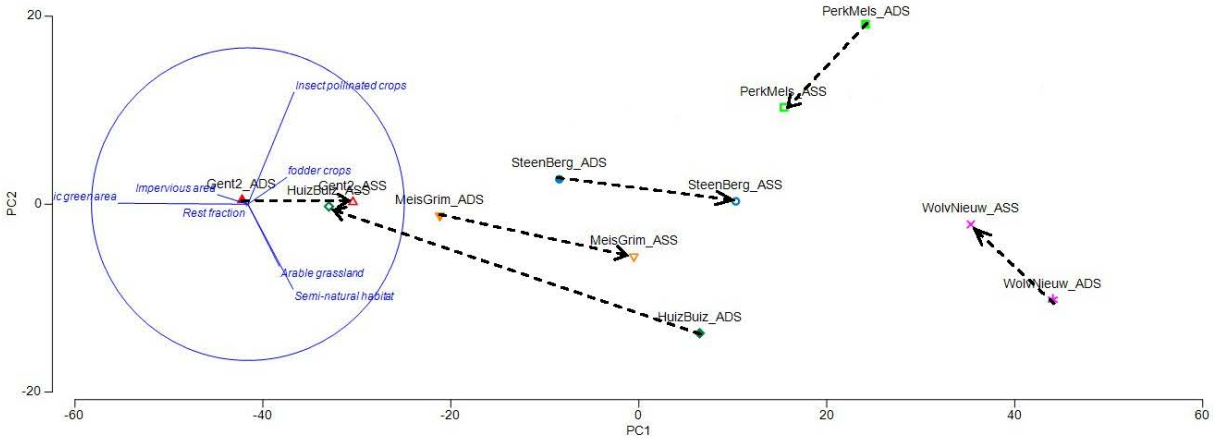
Table S7.1.5. Strata analysis on landscape metrics for the **2015 dataset**: effect of **locations** and coupled **study site** (ADS vs. ASS)

```
Call: Adonis

Strata: loc1315
Permutation: free
Number of permutations: 99
Terms added sequentially (first to last)
```

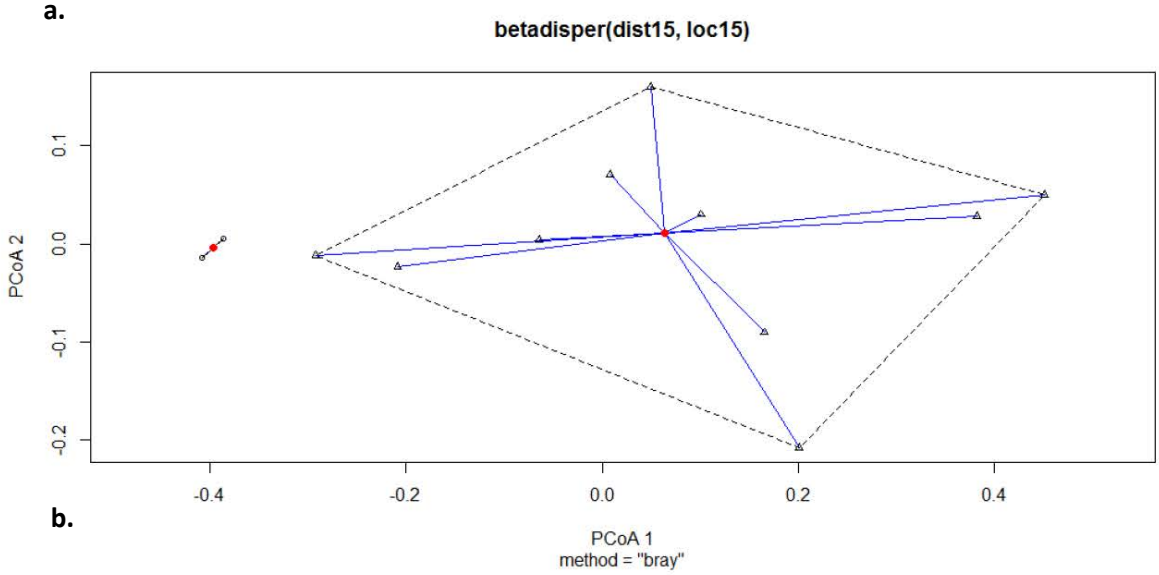
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
loc1315	1	0.37463	0.37463	4.6935	0.36511	0.03 *
site1315	1	0.00279	0.00279	0.0349	0.00271	0.95
loc1315:site1315	1	0.01010	0.01010	0.1266	0.00985	0.92
Residuals	8	0.63856	0.07982		0.62233	
Total	11	1.02608			1.00000	

Table S7.1.6. Principal Coordinates Analysis (PCoA) of the **2015 dataset**: values visualizing effect of landscape and coupled study site (ADS and ASS) within locations: Eigenvalues for PCoA axes and distance to median

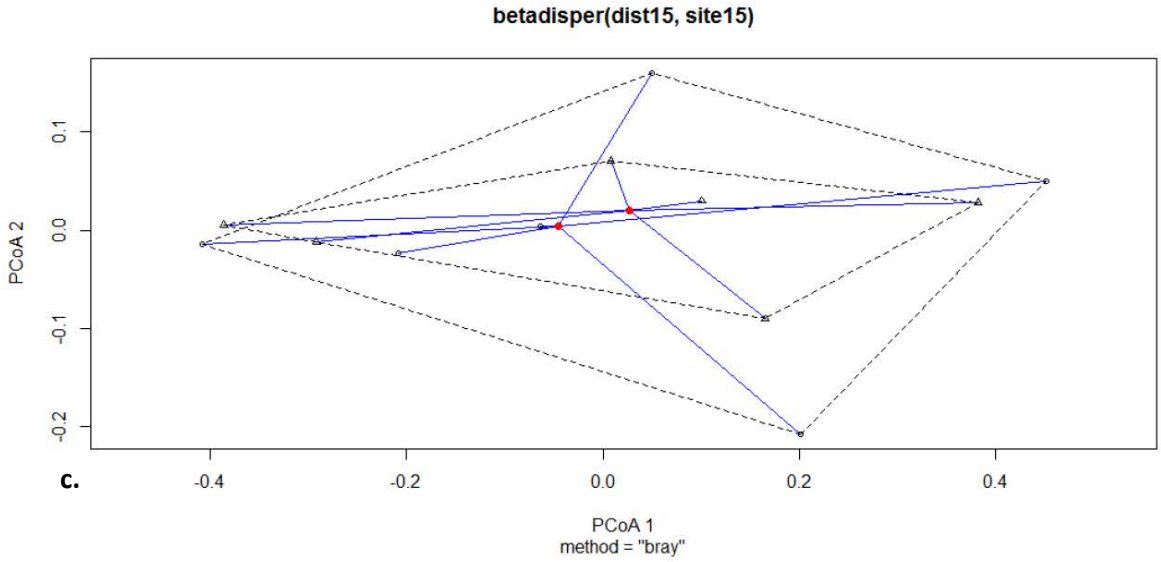
```
Call: betadisper

No. of Positive Eigenvalues: 8
No. of Negative Eigenvalues: 3
Average distance to median (landscape)
Urban  Semi-rural
0.08124 0.23162
Average distance to median (study site)
  ADS    ASS
0.2696 0.2477

Eigenvalues for PCoA axes
PCoA1  PCoA2  PCoA3  PCoA4  PCoA5  PCoA6  PCoA7  PCoA8
0.8781 0.0872 0.0400 0.0225 0.0159 0.0104 0.0037 0.0021
```



a.



b.



c.

Figure S7.1.3. Landscape metrics analysis of all locations and coupled study sites (an arrow from ADS to ASS illustrates coupling within each location) in the 2015 study. **a.** PCA visualizing the principal landscape covers; **b. and c.** PERMANOVA statistics for testing the effect of location (b.) and study sites (c.)

Supplementary dataset S7.2: impact of diseases on development of domesticated bumblebee nests

Commercial nests are produced in a commercial rearing facility for biological pollination in agriculture, here we placed these standardized nests of 45 (± 19.9 SD) workers in different study locations (Figure S7.2.1) and followed their development. Based on earlier experience, the parameter 'biomass increase' has been shown to correlate with environment quality parameters; we therefore focused on this parameter in this study (Goulson et al. 2002, Parmentier et al. 2014). However, it has also been shown that these nests can be infected with pathogens which could impact on bumblebee host fitness (Brown et al. 2003b, Yourth et al. 2008, Schlüns et al. 2010, Koch et al. 2012, Whitehorn et al. 2013). Thus, when using these nests as a bioassay tool, it can be argued that different pathogens including protozoa and viruses could also interfere with bumblebee nest development. We therefore have two controls to overcome a possible pathogen bias when using these nest as a bioassay tool: (1) we only used nests with no pathogens present before they were placed outdoors; (2) we checked if pathogen infection at the end of the experiment could be related with certain nest development parameters.

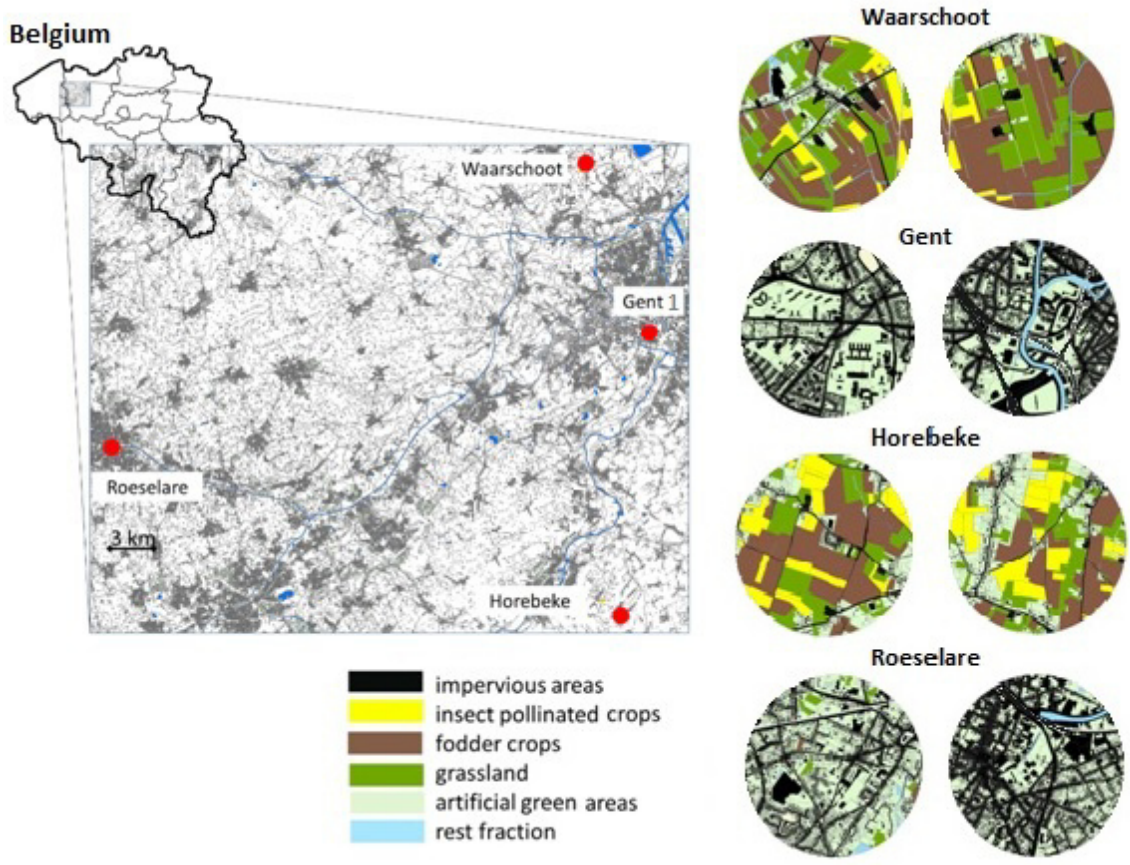


Figure S7.2.1. Locations and study sites selected for this experiment. Both semi-rural (Waarschoot, Horebeke) and urban (Gent, Roeselare) locations were included; in each study site three *B. terrestris* nests were placed randomly within a distance of 60 ± 50 m to the center

Table S7.2.1: Primer and probes for parasite and virus detection

Target	Primer / Probe	Sequence	Size (bp)	Reference
Apicystis bombi	Primers - Neogregarine	F: CCAGCATGGAATAACATGTAAGG R: GACAGCTTCCAATCTCTAGTCG	±260	(Meeus *et al.* 2010)
Crithidia bombi	Primers - different genera within the trypanosomes	F: CTTTTGGTCGGTGGAGTGAT R: GGACGTAATCGGCACAGTTT	±420	(Meeus *et al.* 2010)
Nosema	Primers *Nosema apis,* *Nosema bombi,* *Nosema ceranae*	F universal: GGAGTGGATTGTGCGGCTTA R apis: CCTCAGATCATATCCTCGCAGAAC R bombi: ATTCTCGAATCAGGATTCTCTCAGAA R cera: ACCACTATTATCATTCTCAAACAAAAAACC	 ±80 ±85 ±100	This study
AKI complex	ABPV, KBV and IAPV	RT (ABPV) CAATGTGGTCAATGAGTACGG RT (KBV&IAPV) TCAATGTTGTCAATGAGAACGG MLPA-LPO gggttccctaagggttggaCTCACTTCATCGGCTCGGAGCATGGATGAT MLPA-RPO $^{P-}$ACGCACAGTATTATTCAGTTTTTACAACGCCCtctagattggatcttgctggcac	104	(De Smet *et al.* 2012)
DWV complex	DWV/KV, VDV-1	RT TCACATTGATCCCAATAATCAGA MLPA-LPO gggttccctaagggttggaTGACCGATTCTTTATGCAGCGAGCTCT MLPA-RPO $^{P-}$TACGTGCGAGTCGTACTCCTGTGACAtctagattggatcttgctggcac	95	(De Smet *et al.* 2012)
BQCV	BQCV	RT CGGGCCTCGGATAATTAGA MLPA-LPO gggttccctaagggttggaCTTCATGTTGGAGACCAGGTTTGTTTGCCGACTTACGGAA MLPA-RPO $^{P-}$TGTCGTTAAACTCTAGGCTTTCCGGATGGCTTCTTCATGGtctagattggatcttgctggcac	122	(De Smet *et al.* 2012)
SBV	SBV	TGGACATTTCGGTGTAGTGG MLPA-LPO gggttccctaagggttggaCGTTGATCCAATGGTCAGTGGACTCTTATACCGATTTGTTTAATGGTTGG MLPA-RPO $^{P-}$GTTTCTGGTATGTTTGTTGACAAGAACGTCCACCTTCAGCCATTCAGCtctagattggatcttgctggcac	140	(De Smet *et al.* 2012)

DWV complex; AKI complex: acute bee paralysis virus, Kashmir bee virus and Israeli acute paralysis virus complex; BQCV: black queen cell virus; SBV: sacbrood virus

During spring/summer of 2013 we placed bumblebee nests outdoors and let them foraging freely in different anthropogenic environments (urban and rural landscapes). **Figure S7.2.1** illustrates the selected study sites within Flanders (North-Belgium). We measured parameter biomass increase of the nests and screened for Apoidae-associated parasites (n=3) and viruses (n =4) when the nest were placed outdoors and after 6 weeks foraging. We then tested correlation of total number and individual infections with hive development (biomass increase).

1. Screening of diseases in commercial nests placed outdoors

Sampling and screening procedure

B. terrestris bumblebee nest (n =24) was screened for 3 parasites with broad-range primers, for *Crithidia*, *Apicystis*, *Nosema* and 4 viruses/complexes using stand specific primers, i.e. deformed wing virus (DWV), Sacbrood virus (SBV), Black queen cel virus (BQVC) and viruses of the Acute-Kashmir-Israeli-complex (AKI). All primer set used are given in **table S7.2.1.** Each nest was screened twice: before placing outdoors and at the end of the experiment. At the beginning, nests contained 53 ± 19 workers and we randomly collected 10 workers from each nest to achieve a detection power of 25% with α =0.95 . At the end of the experiment, bumblebees were counted again with a total of 101 ± 74 workers; we then randomly sampled 18 workers to achieve a detection power of 15% with α = 0.95.

The RNA extraction procedure was started by pooling bumblebees (i.e. 2 separate pools of 5 bumblebee workers or 3 separate pools of 6 bumblebee workers)

Results

Before the nests were placed outdoors (n = 24), no single protozoa *Crithidia*, *Apicystis*, *Nosema* or virus (DWV, SBV, BQVC) or viruses complex (AKI-complex) could be detected.

At the end, after about 6 weeks foraging outdoors, we again screened nest samples for *Crithidia*, *Apicystis*, *Nosema*, DWV, SBV, BQVC and the AKI-complex. Results for individual infections per nest are given in **table S7.2.2**. We found no infections for viruses under the AKI complex; therefore the AKI complex was omitted from the analysis.

Table S7.2.2. Infections of protozoa and viruses detected in *B. terrestris* nests after foraging 6 weeks outdoors in rural and urban environments

Location (type)	Study site	DWV	SBV	BQCV	*Apicystis*	*Crithidia*	*Nosema*	Total infections	biomass increase
Ghent (urban)	A	0	0	0	1	1	0	2	113.4
		0	1	1	0	0	1	3	229.1
		0	0	1	0	1	0	2	47.2
	B	1	0	1	0	1	1	4	212.7
		0	0	0	0	1	0	1	291.9
		0	0	1	1	1	0	3	375.4
Horebeke (rural)	A	0	0	0	1	1	0	2	25.2
		0	0	0	1	1	0	2	21.3
		0	0	0	1	1	0	2	3.0
	B	0	0	0	1	1	0	2	5.3
		0	0	0	0	1	0	1	49.7
		0	0	1	0	1	0	2	-7.5
Roeselare (urban)	A	0	0	0	0	1	0	1	49.7
		0	0	0	1	1	1	3	51.5
		0	0	0	1	1	1	3	297.7
	B	0	0	0	0	1	0	1	350.0
		0	0	0	0	1	1	2	141.5
		0	0	1	1	1	0	3	88.9
Waarschoot (rural)	A	0	0	1	1	1	0	3	-1.8
		0	0	0	0	1	0	1	132.5
		0	0	0	1	1	0	2	66.5
	B	0	0	0	0	1	0	1	42.5
		0	0	0	0	1	0	1	-2.5
		0	0	0	0	1	1	2	-17.8

2. Distribution of nest 'biomass increase' data

Following the set-up of this experiment, we measured biomass increase at the end; and these values are given in the last column of **table S7.2.1**. Biomass increase does not follow a normal distribution (Figure 2), which is confirmed after running the Shaprio-Wilk normality test (W = 0.8452; P = 0.00017); call "Shapiro.test" in R package "stats" was used. We performed a (X + 20) transform on the data to obtain positive values before log-transforming the data for biomass increase. After log-transformation the data showed a normal distribution pattern (**Figure S7.2.3**), which was confirmed after a Shapiro.test (W = 0.9413; P = 0.174). Thus, we followed the rationale that bumblebee nest development is best described by a log-normal distribution pattern, an observation which was also recently reported by Crone and Williams (2016).

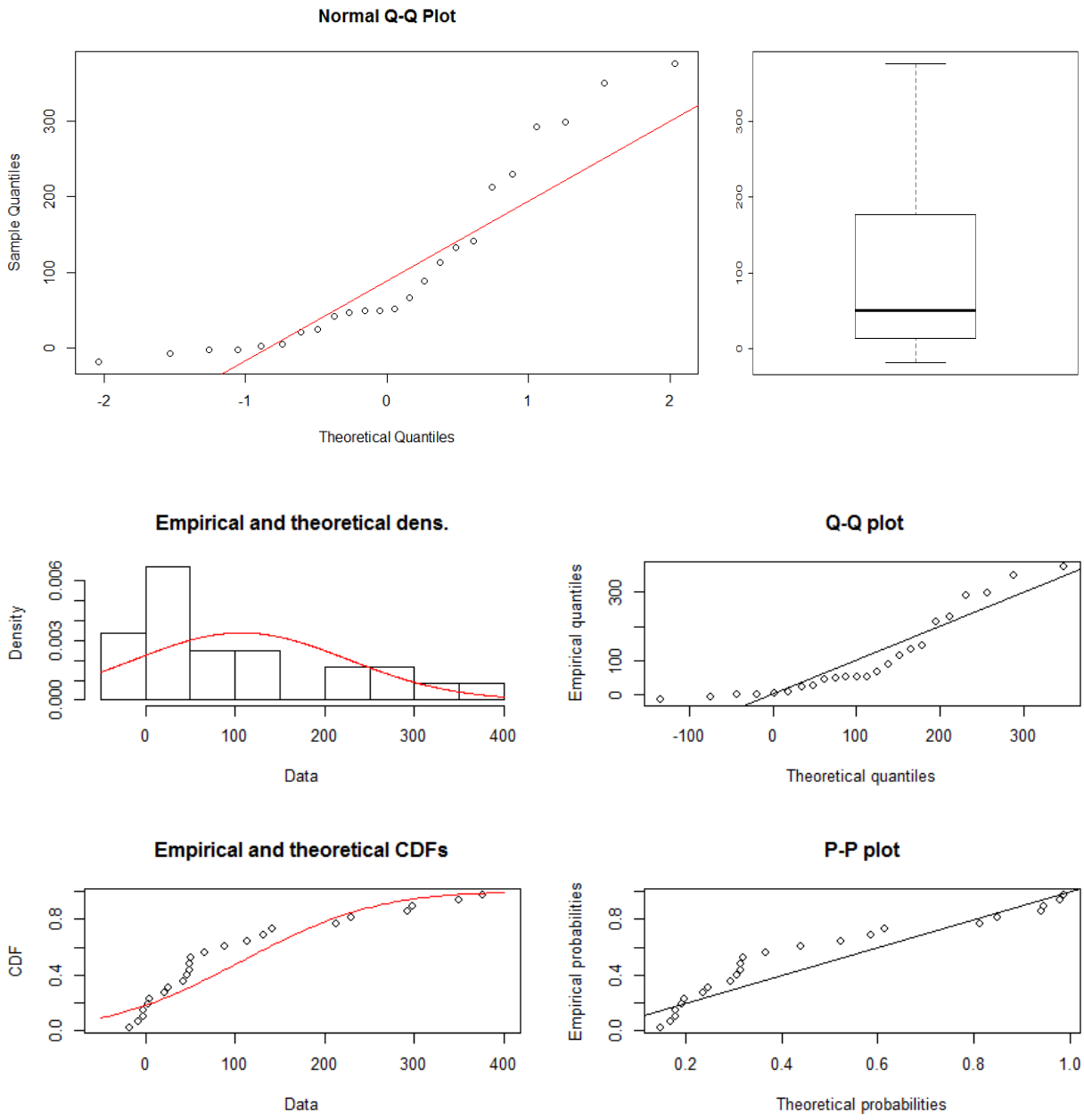


Figure S7.2.2. Basic test statistics after fitting a normal distribution pattern on nest development parameter 'biomass increase'

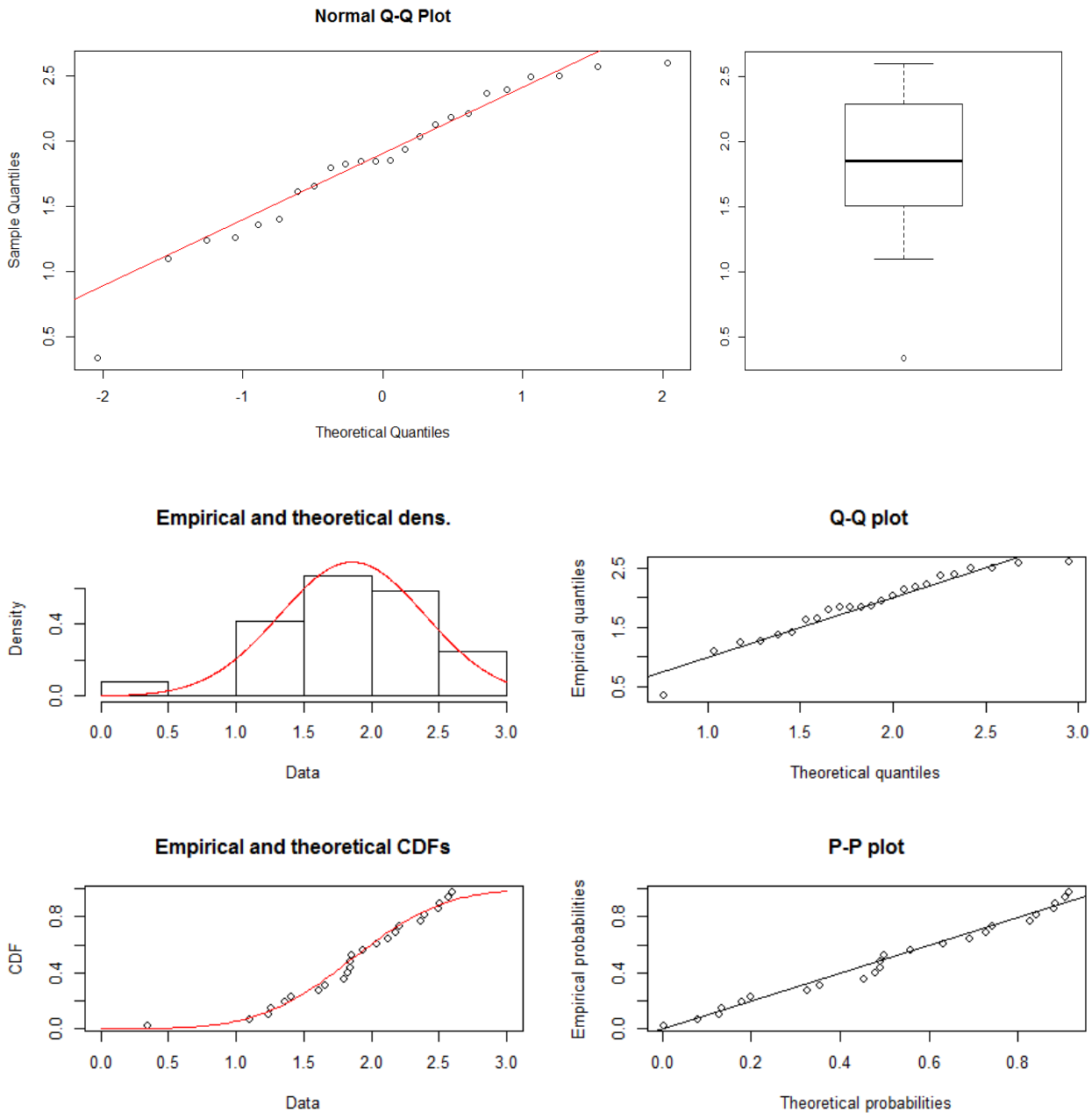


Figure S7.2.3. Basic test statistics after fitting a normal distribution pattern on nest development parameter 'biomass increase' after a log (x + 20) transformation of the data

3. Correlation of pathogen load to nest biomass increase

After running a log (x + 20) transformation on biomass increase data, we performed multivariate statistics in R package "lme4" running a GLMM. In the model we included a "log-transformed biomass increase" as dependant variable and "pathogen load", "landscape type" and their interaction as independent variables, "location" was included as random factor. The results of multivariate and univariate statistics are given hereunder.

Model:

```
> lmer<-
lmer(Path_biomass$biomasslog20~Path_biomass$Tot.path*landscapeF+(1|locationF))
> summary(lmer2)
Linear mixed model fit by REML ['lmerMod']

REML criterion at convergence: 247
Scaled residuals:
     Min       1Q   Median       3Q      Max
-1.39229 -0.53973 -0.04842  0.46717  1.80883

Random effects:
 Groups   Name        Variance Std.Dev.
 locatieF (Intercept) 1075     32.79
 Residual             7879     88.76
Number of obs: 24, groups:  locatieF, 8

Correlation of Fixed Effects:
            (Intr) Pt_$T. lndscF
Pth_bmss$T. -0.931
lndschpFstd -0.763  0.710
Pth_bm$T.:F  0.793 -0.851 -0.911
```

	Estimate	Std..Error	t.value	**p.z**
(Intercept)	96.20210	83.55948	1.1513008	0.2496085
Path_biomass$Tot.path	***-39.90596***	***44.47194***	***-0.8973290***	***0.3695434***
landscape	91.72054	109.55996	0.8371721	0.4024958
Path_biomass$Tot.path:landscape	39.68911	52.24995	0.7596009	0.4474932

Univariate Tests:

	DWV		**SBV**		**BQCV**		**Apicystis**		**Crithidia**	
	Dev	Pr(>Dev)	Dev	Pr(>Dev)	Dev	Pr(>Dev)	Dev	Pr(>Dev)	Dev	Pr(>Dev)
(Intercept)										
Path_biomass$biomass	0.67	0.808	0.884	0.808	0.365	0.808	0.112	0.808	0.047	0.808
landscapeF	0.763	0.675	0.63	0.675	0.993	0.675	0.01	0.955	0.007	0.955
locationF	4.922	0.715	4.841	0.715	6.993	0.523	5.028	0.715	0.368	0.715
landscapeF:locationF	0	0.787	0	0.787	0	0.455	0	0.787	0	0.787

	Nosema	
	Dev	Pr(>Dev)
(Intercept)		
Path_biomass$biomass	0.803	0.808
landscapeF	2.143	0.452
locationF	3.291	0.715
landscapeF:locationF	0	0.787

Multivariate statistics showed that there was no main effect of pathogens on nest development (log biomass increase) (estimate = -39.90; z-value = -0.897; P = 0.4024), and no difference was observed between landscapes (estimate = 91.72; z-value = -0.837; P = 0.4024) or an interaction between pathogens and landscapes (estimate = 39.68; z-value = 0.760; P = 0.447).

Beside, univarate statistics of individual pathogen infections showed the same results (all P values > 0.45). The main result is also shown in figure S7.2.4 as boxplots of biomass increase (log transformed) show no difference when plotted against total number of pathogens.

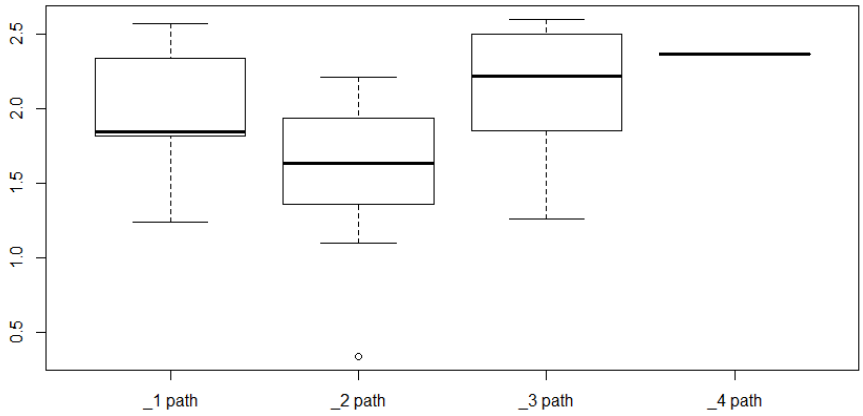


Figure S7.2.4. Boxplots of nest development parameter 'biomass increase' (log transformed) plotted against total number of pathogens (sum of protozoa and viruses) per nest.

4. Conclusion

We investigated if nest 'biomass increase', a parameter to assess environmental quality to sustain bumblebee development, could be influenced by the presence of pathogens. Here pathogens could be a confounding factor as certain pathogens could impair nest development. Therefore we screened nests (n=24) before placing outdoors and showed no single infections of Apoidae-associated protozoa *Crithidia*, *Apicystis*, *Nosema* and viruses DWV, SBV, BQVC, and the AKI-complex.

After 6 weeks in the outdoor environment we detected a scattering of infections of the above mentioned pathogens in the nests. After summation of individual infections per nest, we found single (7/24), double, (10/24), triple (6/24), and quadruple (1/24) infections scattered in samples of nests placed in different locations.

When inferring nest biomass increase, which we showed was best described by a log-normal distribution model following the rationale of Crone et al. (2016), to total pathogen load per hive, we found no effect (estimate = -39.90; z-value = -0.897; P = 0.4024) within different landscapes. Also when testing individual pathogens, no effect was found (univariate statistics: P > 0.45).

We therefore conclude that, after 6 weeks of foraging outdoors, pathogens do not show measurable effects on nest development. The parameter 'biomass increase' can therefore be employed as a measure of environmental metrics such as for recourse availability.

Supplementary dataset S7.3: impact of apiaries on diversity of wild bee assemblages

To measure the effect of apiaries on wild bee species diversity, we selected six locations with similar landscape metrics of coupled study sites ADS (apiary dense site) and ASS (apiary sparce site) within locations (supplementary dataset, visualized in **Figure S7.1.3a/b** and **Tables S7.1.5** and **S7.1.6**). In each study site over an 8 week period (half of May until 10th July) using three triplicates of pan traps (3x3); each triplicate contains the following three colors: white, yellow and blue (Stanley et al. 2013). The distance between the pan traps within one triplicate ranged from 3 until 5 meters and between triplicates from 10 until 20 meter. Each triplicate of pan taps was placed at a certain height, ranging from 0 to 0.8 meter, depending on the dominated flowers vegetation present, and at a distance of 100 ± 50 meter from the centre of the study site. The pan traps were filled with 400 ml of water and a drop of detergent with 37% formaldehyde solution to avoid putrefaction. The total survey time was 8 weeks and pan traps were checked at intervals of 3 ± 2 days. The pan traps were refilled if needed. The collected specimens were temporarily frozen in -20°C fridge until pinned for identification (Lebuhn et al. 2016).The pan traps were filled with 400 ml of water and a drop of detergent with 37% formaldehyde solution to avoid putrefaction.

Table S7.3.1 gives an overview of all specimen collected (n = 1098), per location, study sites ADS (total n =628) and ASS (total n = 470), and individual sample points within study sites. While not taking into account the number of *Apis* bees (i.e. all non-*Apis* taxa), we counted less specimen in ASS (n =383) verus ASS (n = 425) study sites. Total number per species and genus are given meanwhile.

1. Rarefaction curves

In order to evaluate if we collected up to an appropriate depth, rarefaction curves were drawn calling the “rarefaction” function in R package “vegan” (Oksanen et al. 2016). **Figure S7.3.1.a** represents an overview of rarefaction curves of all sample points (n = 36) within 6 locations of Gent, Meise, Perk, Grimbergen, Melsbroek and Wolvertem. We observe that most of the curves are going to an asymptote indicating sufficient sample depth, except for two points (indicated with an arrow). As these two sampling points were from the same ASS study site within location Melsbroek, we omitted this location from further analysis. Thus, we redraw rarefaction curves (n = 30) as represented in **Figure S7.3.1.b.** which now indicated sufficient sampling depth for a subset of five locations. Consequently, we omitted 71 collected specimen from total (n = 1027) for further analysis.

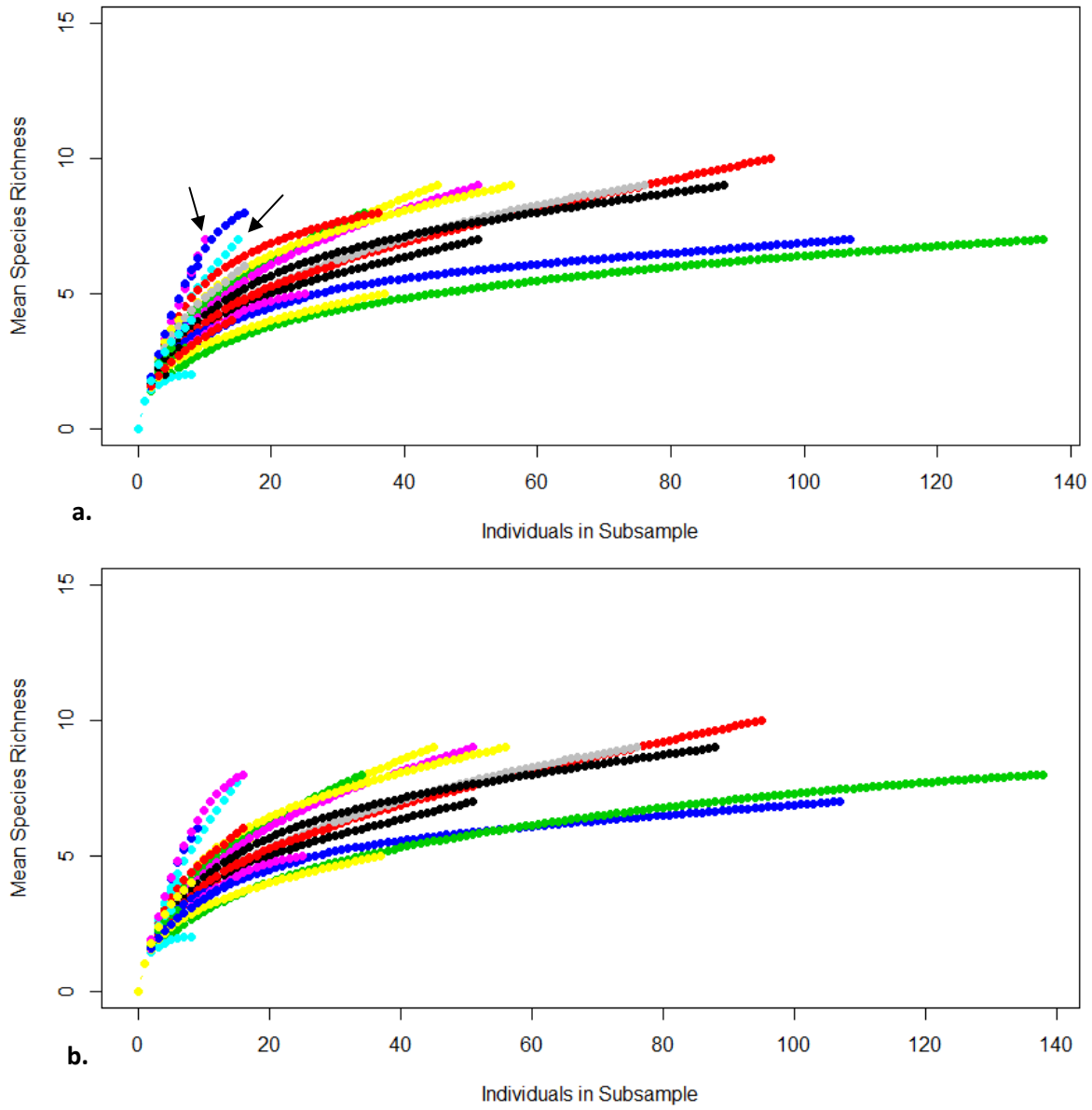


Figure S7.3.1.a. Rarefaction curves of each sample point (n = 36), i.e. in 6 locations (12 coupled study sites) of Gent, Meise, Perk, Grimbergen, Melsbroek and Wolvertem; **b.** Rarefaction curves in subset without location Melsbroek (10 coupled study sites; sample points n = 30)

In order to ensure an equal sampling of wild bees between ADS and ASS, when comparing wild bee assemblages between these coupled study sites, we evaluated rarefaction curves for our data divided into subsets according ADS or ASS sites ((2 x 5 study sites each having 3 pan trapping points). In parallel, we also calculated diversity parameters and run statistics on calculated parameters of both subsets (see 2.), based on average counts per study site.

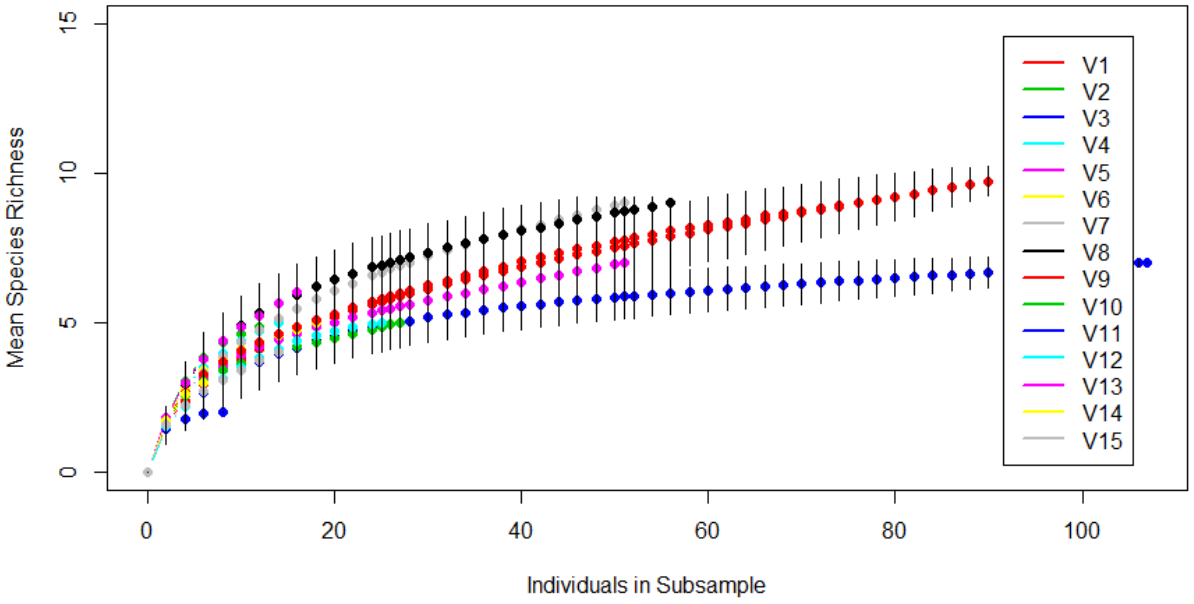
Table S7.3.1. Overview of sampled wild bees up to species level during 2015 survey in different locations and coupled study sites of ADS and ASS

Location / Site / Species	Ghent 2						Meise						Perk						Grimbergen						Melsbroek						Wolvertem						Total
	ADS			ASS			ADS			ASS			ADS			ASS			ADS			ASS			ADS			ASS			ADS			ASS			
	L01	L02	L03	L04	L05	L06	L07	L08	L09	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L27	L28	L29	L30	L31	L32	L33	L34	L35	L36	
Andrena	**16**	**2**	**8**	**5**			**5**	**4**	**1**	**4**		**1**	**1**	**4**	**2**	**9**	**1**	**1**	**1**		**2**	**8**		**2**	**4**		**1**	**1**	**1**	**1**	**1**		**1**		**2**	**3**	**92**
angustior				1																																	1
apicata	1																																				1
bicolor	1																																				1
flavipes	1		1	1										1		6			1			3															14
fulva															1																						1
haemorrhoa	1													1								1					1				1						5
humilis	1								1	2		1			1																						6
minutula	3	2		1																																	6
pandellei	4		1																																		5
praecox	1																																				1
sp.	3		5	2			3	4					1	2		1	1	1			2	2		1	3			1	1	1			1		1		35
sp.2							1			2						1									1										1	1	7
sp.3																1																				2	3
vaga			1				1															2		1													5
Anthocopa																**1**																					**1**
sp.																1																					1
Apis	**57**	**4**	**71**	**1**	**3**	**16**	**1**	**13**	**4**	**3**	**1**	**2**	**26**	**7**	**28**	**4**		**4**	**5**		**2**	**3**			**8**	**2**	**1**		**1**	**1**	**5**	**2**	**9**	**2**	**1**	**3**	**290**
mellifera	57	4	71	1	3	16	1	13	4	3	1	2	26	7	28	4		4	5		2	3			8	2	1		1	1	5	2	9	2	1	3	290
Biastes														**1**																							**1**
troncunum														1																							1
Bombus	**7**	**3**	**8**	**2**	**2**	**17**	**5**	**25**	**6**	**8**	**1**		**3**	**10**	**11**	**12**	**17**	**11**	**13**	**2**		**1**	**2**		**4**	**4**	**3**		**4**	**2**	**5**	**3**	**3**	**2**	**1**	**1**	**198**
hortorum						1											1																					2
hypnorum																1				1									1		2	1		1				7
lapidarius	1					3	1	2						2	1		1	3	3								1			1		1					20
muscorum																	1	1																			2
pascuorum	1	1				2	1		2	1			2	1	1		5	2										1	1		1	1					23
pratorum										1																											1

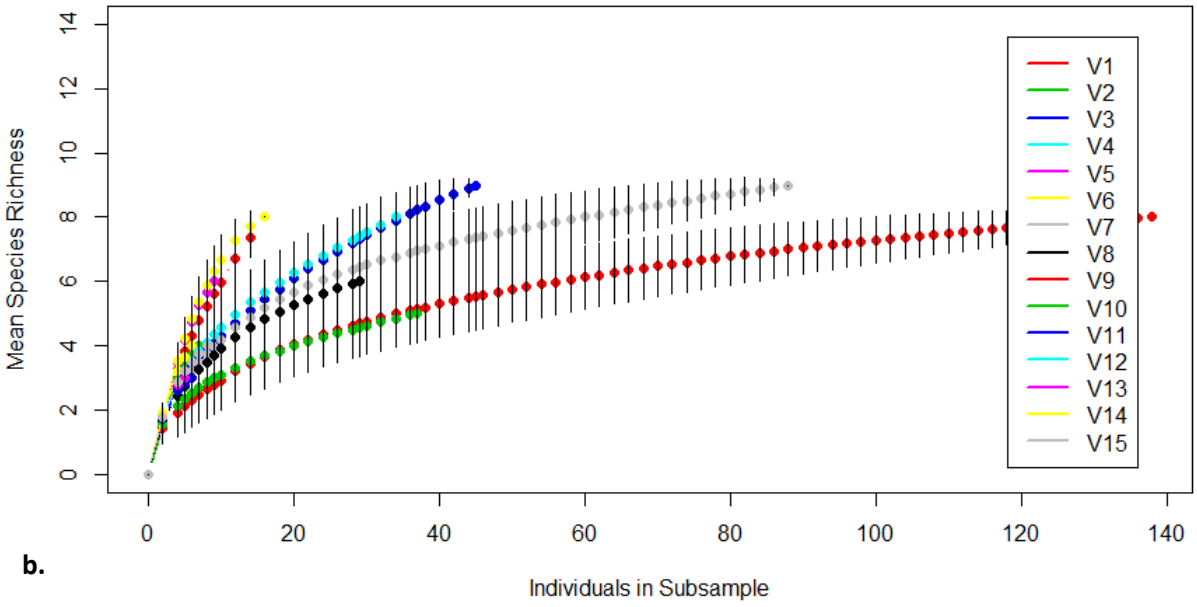
sp.			1			1					1						1																				4
sp.2						1																															1
terrestris	5	2	7	2	2	9	3	23	4	6			1	7	9	11	8	5	10	1		1	2		4	4	2		3		1	1	2	1	1	1	138
Chelestoma	1		1	1		1												1																			5
campanularum			1																																		1
florisomne				1														1																			2
rapunculi	1																																				1
sp.						1																															1
Coelioxys													1																					1			2
inermis													1																								1
sp.																																		1			1
Colletes																													1						2		3
hederae																													1						2		3
Dasypoda						1				1		2	1	8	2	3		103																			121
hirtipes						1				1		2	1	8	2	3		103																			121
Dufourea	3							1			1		5	1	1	1	3		1			1			2			1							2		23
dentiventris	1															1	2																		1		5
minuta																	1																				1
sp.	2							1			1		5	1	1				1			1			2			1							1		17
Halictus	1					1	1	1		1		1		1		1	1				3				8						1				3		24
sp.								1								1																			1		3
tumorolum	1					1	1			1		1		1			1				3				8						1				2		21
Heriades			1	1	1	2		1					3		1		2	2							1				1								16
truncorum			1	1	1	2		1					3		1		2	2							1				1								15
Hylaeus										1				2	1						1				1												6
brevicornis														1																							1
gibbis														1																							1
signatus																									1												1
sp.										1					1						1																3
Lasioglossum	7	4	14	4	1	4	2	6	6	15	2		10	22	27	48	5	14	7	6	17	24	3	2	8		2	2	1	2	3		1		3		272
calceatum	5		2	2	1				2	5	2		5	1	18	6		1	2	2	7	21			2										1		85
laticeps			4	1		1	2			3				6	1	1	2	2							1		2			1			1				28
leucozonium			1			1		5		1			2	1	1	2		2	3			1			1			1	1								23

lucidulum						1										2																					3
malachurum				1						2				1		1		1													1						7
morio		3								2				11	4	8		4			1										1						34
nitidulum			2										1	1		6	1	2	1	4	5	1	2	2	4			1		1							34
semilucens													1			1																					2
sexstrigatum																1																			1		2
sextrigatum	2		2			1			1	2								2																			10
sp.			1						1						2	3	2		1																1		11
villosulum		1	2					1	2				1	1	1	17					4		1														31
zonulum																						1									1						2
Nomada	**1**	**1**		**1**		**1**									**3**	**9**															**1**					**1**	**18**
panzen																															1						1
Sp1.	1	1		1		1									3	1																				1	9
sp.2																6																					6
Sp 3.																2																					2
Osmia	**1**								**1**	**1**	**2**															**1**			**1**			**1**			**2**		**10**
bicornis	1								1	1	1																		1						1		6
caerulescens											1																										1
leaiana																																			1		1
sp.																										1						1					2
Panurgus	**1**		**4**			**2**					**2**												**1**														**10**
banksianus											1																										1
calcaratus	1		4			2					1												1														9
sphecodes													**1**																								**1**
monilicornis													1																								1
Stelis				**1**														**2**						**1**					**1**								**5**
breviuscula				1														2																			3
punctulatissima																								1													1
sp.																													1								1
TOTAL	**95**	**14**	**107**	**16**	**7**	**45**	**14**	**51**	**18**	**34**	**9**	**6**	**51**	**56**	**76**	**88**	**29**	**138**	**27**	**8**	**25**	**37**	**6**	**5**	**36**	**7**	**7**	**4**	**11**	**6**	**16**	**6**	**14**	**5**	**16**	**8**	**1098**
Total site	**216**			**68**			**83**			**49**			**183**			**255**			**60**			**48**			**50**			**21**			**36**			**29**			
Total location	**284**						**132**						**438**						**108**						**71**						**65**						

Figure S7.3.2.a/b represents rarefaction curves of sample points (n = 15) within ADS and ASS study sites. We observe that generally an asymptotic curve is achieved for both subsets, indicating a sufficient depth on sampling points



a.

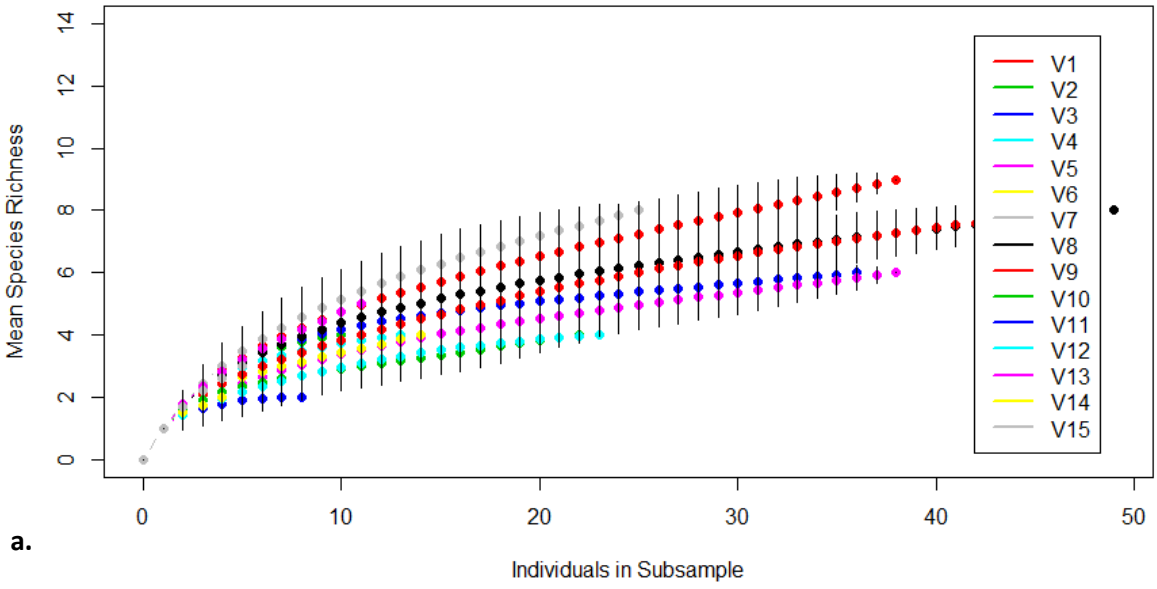


b.

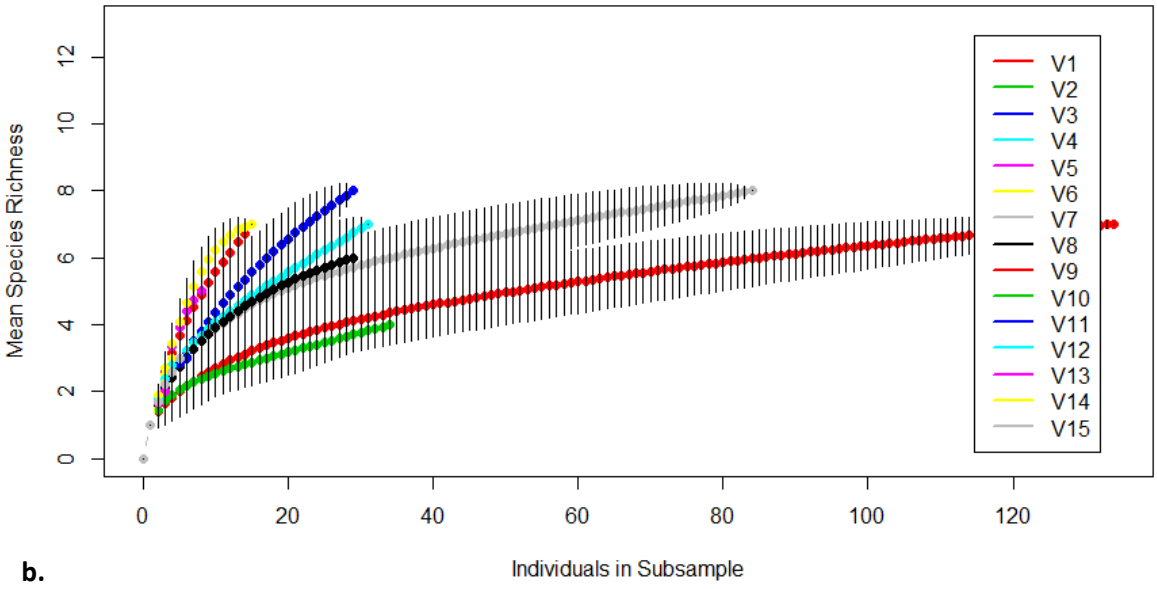
Figure S7.3.2. Rarefaction curves on sample points (n = 15, represented by "V + number" in figure) within 5 locations of Gent, Meise, Perk, Grimbergen and Wolvertem of subsets ADS (**a.**) and ASS (**b.**).

Because under part 2. we will discuss the same sample points without *Apis* counts, we here investigated on sampling depth of reduced datasets encompassing all sampled wild bees except *A. mellifera*.

F**igure S7.2.3.a/b** again represents rarefaction curves of sample points (n = 15) but without *Apis* counts within five locations, divided by subsets of ADS and ASS study sites. We again observe that generally an asymptotic curve is achieved for both subsets, indicating a sufficient depth for reduced datasets.



a.



b.

Figure S7.3.2. on sample points (n = 15, represented by "V + number" in figure) without *A. mellifera* counts within 5 locations of Gent, Meise, Perk, Grimbergen and Wolvertem of subsets ADS (**a.**) and ASS (**b.**)

2. Diversity analysis ADS versus ASS sites

In order to evaluate diversity differences between ADS and ASS, we performed ecological diversity indices and species richness calculators on both subsets: Shannon-Weaver (or Shannon) index (H), Simpson and Inverse Simpson index, Species richness (S), Pielou's evenness (J) and Fisher's alpha index (α). These indexes on the subsets are calculated in R package "vegan" by the "diversity" call, option "shannon", "simpson" and "inv", respectively (Oksanen et al. 2016).

The Shannon index is defined as H = -sum p_i log(b) p_i, where p_i is the proportional abundance of species i and b is the base of the logarithm, mostly the natural logarithm (b=e) is used. Both variants of Simpson's index are based on D = sum p_i^2. Choice "simpson" returns 1-D and "invsimpson" returns 1/D.

Pielou's measure of species evenness, is calculated as $J = H'/\ln(S)$ where H' is Shannon diversity and S is the total number of species in a sample, across all samples in dataset.

Fisher et al. (1943) describes mathematically the relationship between the number of species and the number of individuals in those species. It is widely applied, especially in entomological research (Taylor, 1978). In R package "vegan", "fisher.alpha" estimates the α parameter of Fisher's logarithmic series (Oksanen et al. 2016).

In **table S7.3.2**, the ecological diversity calculators on subsets of ADS and ASS are given and a comparison is made applying a two-side t-test. These results showed that there is no difference observed in number of genera, diversity (calculated by the Shannon and (inverse) Simpson index) and eveness (Pielou's evenness index). In contrary, only a significant difference was observed in the Fisher's alpha index (P = 0.011; paired 2-sample t test; n = 3 per site). According to Hayek and Buzas (1997), this index is a useful index provided the ratio of the total number of individuals to the species number (N/S) exceeds 1.44, which is the case in all our sampling points. Thus, this index visualises the abundance of rare wild bee taxa and indicates a difference observed between ADS and ASS.

However, this observation could be biased due to the direct impact of number of *Apis* bees observed, with a higher number of *Apis* near apiaries compared to at a distance of 1,500m. Therefore, we recalculated the above mentioned calculaters on a subset being the same dataset with the counts of *A. melliera* removed. Results are given in **table S7.3.3**. We achieved the same trend for the diversity calculators, again with only the Fisher's α calculator being significantly different between ADS and ASS (P = 0.026; Paired 2-sample t test; n = 3 per site, total n =15).

We therefore conclude that there is an impact on rare species abundance due to apiary density, while a general impact on wild bee species diversity cannot be concluded. The impact on species abundance will be further discussed in **Supplementary dataset 7.4** by the use of pan trapping data and in main text.

Table S7.3.2 Ecological diversity indices and species richness calculators including all collected bees on subsets (individual sampling points, n =15) of ADS and ASS and comparison by a 2-sized t-test

	Species number		Shannon-Weaver		Simpson		Inverse simpson		Rarefaction		Pielou's evenness		Fisher's α	
	ADS	**ASS**	**ADS**	**ASS**	**ADS**	**ASS**	**ADS**	**ASS**	**ADS**	**ASS**	**ADS**	**ASS**	**ADS**	**ASS**
1	10	8	1,3396	1,8364	0,5992	0,8047	2,4952	5,1200	1,6056	1,8583	0,5818	0,8831	2,8197	6,3672
2	5	4	1,5125	1,2770	0,7653	0,6939	4,2609	3,2667	1,8242	1,8095	0,9397	0,9212	2,7824	3,8784
3	7	9	1,1363	1,5657	0,5298	0,7170	2,1269	3,5340	1,5348	1,7333	0,5839	0,7126	1,6784	3,3830
4	5	8	1,3904	1,5823	0,7143	0,7249	3,5000	3,6352	1,7692	1,7469	0,8639	0,7609	2,7824	3,2982
5	7	6	1,3806	1,7351	0,6736	0,8148	3,0636	5,4000	1,6871	1,9167	0,7095	0,9684	2,1963	7,8669
6	5	4	1,3878	1,3297	0,7222	0,7222	3,6000	3,6000	1,7647	1,8667	0,8623	0,9591	2,2932	5,2446
7	9	9	1,5323	1,4770	0,6836	0,6593	3,1604	2,9356	1,6973	1,6669	0,6974	0,6722	3,1712	2,5105
8	9	6	1,7358	1,2675	0,7704	0,6088	4,3556	2,5562	1,7844	1,6305	0,7900	0,7074	3,0312	2,2973
9	9	8	1,5053	0,9489	0,7136	0,4249	3,4921	1,7389	1,7232	1,4280	0,6851	0,4563	2,6563	1,8494
10	5	5	1,2583	1,0108	0,6639	0,5245	2,9755	2,1029	1,6895	1,5390	0,7818	0,6280	1,8051	1,5583
11	2	3	0,5623	1,0114	0,3750	0,6111	1,6000	2,5714	1,4286	1,7333	0,8113	0,9206	0,8559	2,3877
12	5	3	1,0496	1,0549	0,5088	0,6400	2,0358	2,7778	1,5300	1,8000	0,6521	0,9602	1,8794	3,1666
13	6	3	1,5607	1,0549	0,8225	0,6400	4,1290	2,7778	1,8083	1,8000	0,8710	0,9602	3,4869	3,1666
14	3	8	1,0114	2,0140	0,5714	0,8594	2,5714	7,1111	1,7333	1,9167	0,9206	0,9685	2,3877	6,3672
15	4	4	0,9911	1,2555	0,6939	0,6875	2,1304	3,2000	1,5714	1,7857	0,7150	0,9056	1,8708	3,1836
average	6,067	5,867	1,290	1,361	0,654	0,676	3,033	3,489	1,677	1,749	0,764	0,826	2,380	3,768
STDV	2,374	2,295	0,297	0,330	0,118	0,111	0,863	1,404	0,117	0,136	0,115	0,159	0,685	1,855
Paired t-test	0,816		0,540		0,608		0,294		0,132		0,236		**0,011**	

Table S7.3.3 Ecological diversity indices and species richness calculators not including *A. mellifera* counts on subsets (individual sampling points, n =15) of ADS and ASS and comparison by a 2-sized t-test

	Species number		Shannon-Weaver		Simpson		Inverse simpson		Rarefaction		Pielou's evenness		Fisher's α	
	ADS	ASS	ADS	ASS	ADS	ASS	ADS	ASS	ADS	ASS	ADS	ASS	ADS	ASS
1	9	7	1,6665	1,7095	0,7452	0,7822	3,9239	4,5918	1,7653	1,8380	0,7585	0,8785	3,7252	5,1087
2	4	3	1,2799	1,0397	0,7000	0,6250	3,3333	2,6667	1,7778	1,8330	0,9232	0,9464	2,4710	5,4526
3	6	8	1,4790	1,4196	0,7361	0,6231	3,7895	2,6530	1,7571	1,6450	0,8254	0,6827	2,0560	3,6519
4	4	7	1,2203	1,4081	0,6746	0,6785	3,0727	3,1100	1,7308	1,7010	0,8802	0,7236	1,9742	2,8163
5	6	5	1,0911	1,5596	0,5291	0,7813	2,1235	4,5714	1,5434	1,8930	0,6089	0,9690	2,0042	5,7051
6	4	3	1,1033	1,0397	0,6225	0,6250	2,6486	2,6667	1,6703	1,8330	0,7958	0,9464	1,8708	5,4526
7	8	8	1,7123	1,3536	0,7648	0,6284	4,2517	2,6911	1,7967	1,6360	0,8234	0,6509	4,0683	2,1740
8	8	6	1,5531	1,2675	0,7205	0,6088	3,5782	2,5562	1,7355	1,6310	0,7469	0,7074	2,7145	2,2973
9	8	7	1,3413	0,8421	0,6224	0,3910	2,6483	1,6419	1,6356	1,3940	0,6451	0,4327	2,7413	1,5701
10	4	4	0,9562	0,7937	0,5454	0,4446	2,2000	1,8006	1,5714	1,4580	0,6898	0,5726	1,4307	1,1775
11	2	3	0,5623	1,0114	0,3750	0,6111	1,6000	2,5714	1,4286	1,7330	0,8113	0,9206	0,8559	2,3877
12	4	3	0,8378	1,0549	0,4272	0,6400	1,7459	2,7778	1,4466	1,8000	0,6044	0,9602	1,3993	3,1666
13	5	2	1,3667	0,6365	0,6942	0,4444	3,2703	1,8000	1,7636	1,6670	0,8492	0,9183	3,5381	2,6223
14	2	7	0,5623	1,8989	0,3750	0,8444	1,6000	6,4286	1,5000	1,9050	0,8113	0,9759	1,5918	5,1087
15	3	3	0,9503	0,9503	0,5600	0,5600	2,2727	2,2727	1,7000	1,7000	0,865	0,8649	3,1666	3,1666
average	5,286	5,214	1,195	1,217	0,609	0,623	2,842	3,038	1,652	1,712	0,770	0,806	2,317	3,478
STDV	2,268	2,155	0,368	0,363	0,137	0,130	0,893	1,311	0,130	0,154	0,099	0,175	0,948	1,583
Paired t-test	0,933		0,877		0,784		0,648		0,273		0,503		**0,026**	

Supplementary dataset S7.4: transect walks in ADS and ASS to monitor wild bee abundances

When testing a general effect of apiaries on proximate wild bee assemblages, we also performed transect walks to relate apiary density with wild bee abundances. We selected 10 locations (4 extra in comparison to locations of pan trapping; locations were categorized between urban and semi-rural locations to cover different landscapes and during different time periods from spring until autumn in 2015. We used the sampling protocol defined by Stip (2014) which we slightly modified; transect walking scheme is represented in **figure S7.4.1**.

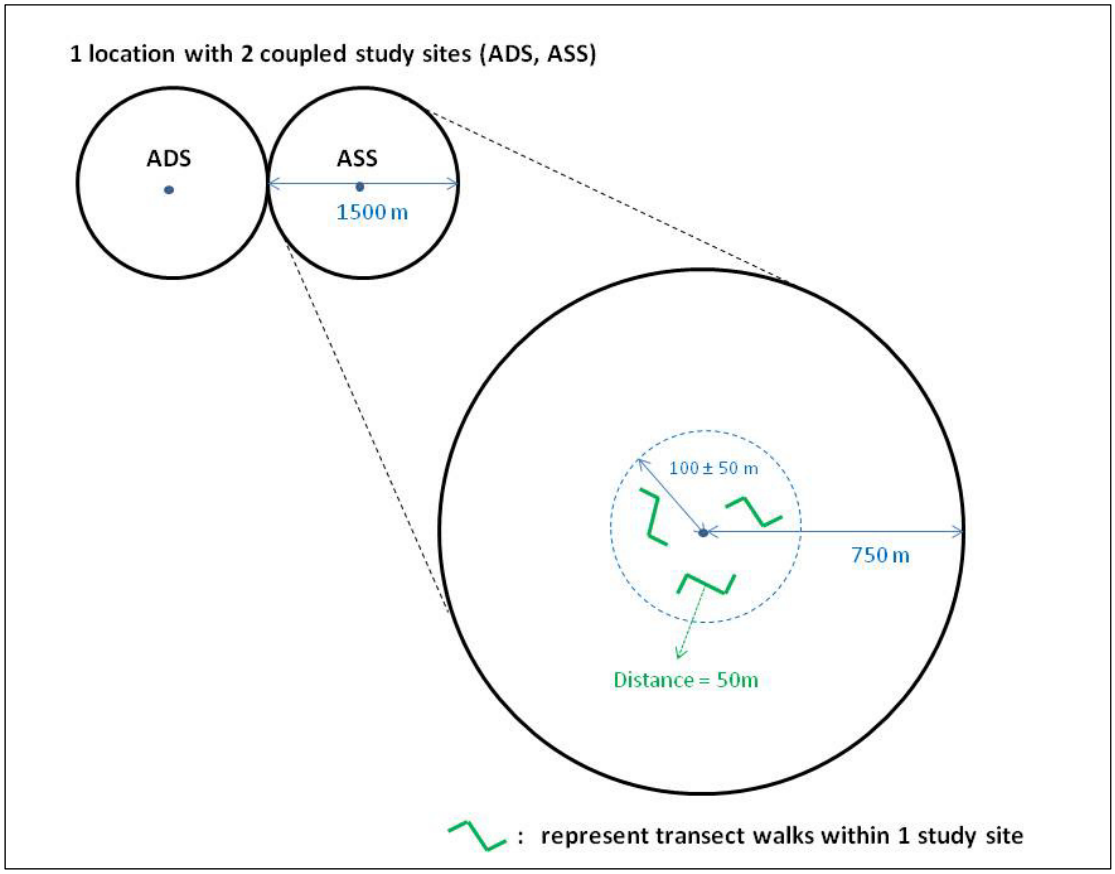


Figure S7.4.1. Representation of transect walk schemes within one location and ADS/ASS study sites

In all twenty study areas (ADS and ASS), bees were sampled in 3*50 m transects to obtain a 150 m transect walk per sampling period. Each area was sampled twice between mid May and end of September during dry, warm (>15°C) and sunny conditions between 9 and 18.30 hours. To make sure that both ADS and ASS study sites were sampled with similar spatial heterogeneity, transects were walked within a distance of 100 ± 50 m from the centre of the study site; each transect environment had comparable landscape elements (being gardens with flowers). The sampling took place on flowers in bloom; with often the same plant species occurring in the different gardens, an overview

of major flowering plants during the transect walks is given in **table S7.4.1**. In each site we monitored on *Lavendula*, which was a very popular garden flower plant for most bee species, followed by *Trifolium*, *Deutzia* and *Salvia*.

In summary, 300 m² of transects (150 m² during two sampling periods) were sampled in each of the twenty ADS and ASS study sites. Total surveying for one location area took one day on average.

In each transect we counted all observed bees for at least twenty minutes (Westphal et al. 2008). Monitoring time per transect was set at 20 ± 5 min. and honeybees and most bumblebees were visually identified to species level in the field. Individuals of *Bombus terrestris, Bombus lucorum Bombus cryptarum* and *Bombus magnus* were aggregated in a *B. terrestris*-group; we did the same for *Bombus pascuorum* and *Bombus humilis* in a *B. pascuorum*-group (Rasmont and Pauly 2010). All other bee species were grouped as represented under class 'other'.

Results

In total we walked in 80 hours 6000 m of transect walks over two periods in 20 study sites encompassing both urban to semi-rural landscapes. This resulted in a total of 2544 wild bees observed. The exact number of *Apis*, *Bombus* and other species counted per location and ADS/ ASS study site is given in **Table S7.4.2**.

Table S7.4.2. observed bees in transect walks in locations (n=10) each having one coupled ADS and ASS study site

Location	Landtype	Site	*Apis*	*Bombus*	Other
PerkMels	Semi-rural	ADS	129.87	81.4	4.8
		ASS	22.2	82.87	6.93
SteenBerg	Semi-rural	ADS	21.0	96.4	1.6
		ASS	6.27	120.47	0.67
Gent2	Urban	ADS	51.73	77.87	0.8
		ASS	18.0	116.0	14.4
HuizBuiz	Semi-rural	ADS	71.0	91.0	1.0
		ASS	31.27	122.27	5.33
MeisGrim	Semi-rural	ADS	59.33	71.27	0.8
		ASS	27.0	94.06	3.13
WolvNieuw	Semi-rural	ADS	102.53	127.0	3.0
		ASS	22.6	112.4	6.2
Leuven	Urban	ADS	49.2	27.0	16.8
		ASS	9.6	52.0	7.2
Merelbeke	Semi-rural	ADS	60.0	22.0	18
		ASS	1.33	57.47	14.07
Melle	Semi-rural	ADS	73.0	21.0	8.0
		ASS	17.6	38.4	37.6
Oudenaarde	Semi-rural	ADS	39.87	26.2	0.0
		ASS	7.0	75.8	60.8

Table S7.4.1. Main flowering plants blossoming where bees were observed in transect walks

Location	Site	*Achilea*	*Buddleja*	*Catalpa*	*Dahlia*	*Daucus*	*Deutzia*	*Echinaceae*	*Fallopia*	*Geranium*	*Helianthus*	*Hypericum*	*Lamium*	*Lavendula*	*Lonicera*	*Majorana*	*Phacelia*	*Rododendron*	*Rosa*	*Salvia*	*Sedum*	*Solidago*	*Tanacetum*	*Taraxacum*	*Thymus*	*Tilia*	*Trifolium*	*Verbena*	TOTAL
PerkMels	ADS											x		x						x			x				x		3
	ASS													x		x				x						x	x		3
SteenBerg	ADS													x				x	x								x	x	2
	ASS		x								x			x				x		x									3
Gent2	ADS						x			x		x		x	x	x							x	x			x		7
	ASS			x			x			x			x	x			x			x							x		6
HuizBuiz	ADS									x				x						x						x	x		3
	ASS				x					x				x													x		2
MeisGrim	ADS	x					x			x				x				x			x			x			x		6
	ASS												x	x			x	x		x				x			x	x	5
WolvNieuw	ADS						x		x					x				x	x										3
	ASS									x	x	x		x						x			x				x		5
Leuven	ADS		x			x								x		x	x		x	x		x			x		x		8
	ASS			x			x				x	x		x					x			x					x	x	6
Merelbeke	ADS		x											x						x		x	x				x		4
	ASS		x				x							x					x			x					x		4
Melle	ADS		x				x				x			x					x		x						x		5
	ASS				x		x			x		x		x									x				x	x	5
Oudenaarde	ADS						x	x		x	x			x							x			x			x		6
	ASS						x	x			x	x		x				x			x			x			x		7
	TOTAL	1	5	2	2	1	10	2	1	8	6	6	2	20	1	3	3	6	6	9	4	4	5	5	1	2	18	4	

Statistics

To understand the impact of apiaries on non-Apis wild bees, we first grouped all '*Bombus*' and 'other' counts in a new parameter 'non-Apis' bee counts and performed statistics to test differences between ADS and ASS sites. Dependant parameters in our model used were 1) binary absence or presence of apiaries (ADS or ASS), 2) counts of Apis, 3) Landtype use and two parameters, 4) 'location' and 5) 'period' to include random spatio-temporal effects.

The model was thus build as follows:

```
> Pantrap_Model1

Global model call: lmer(formula = non_Apis ~ (Apiary_far + Apis) * Landtype +
(1 | Period) + (1 | Location), data = Transectwalks15P1en2R)
```

To visualize all effects, we called the "all.effects" plot function in the same R package "Lme4", which is represented in **figure S7.4.2**.

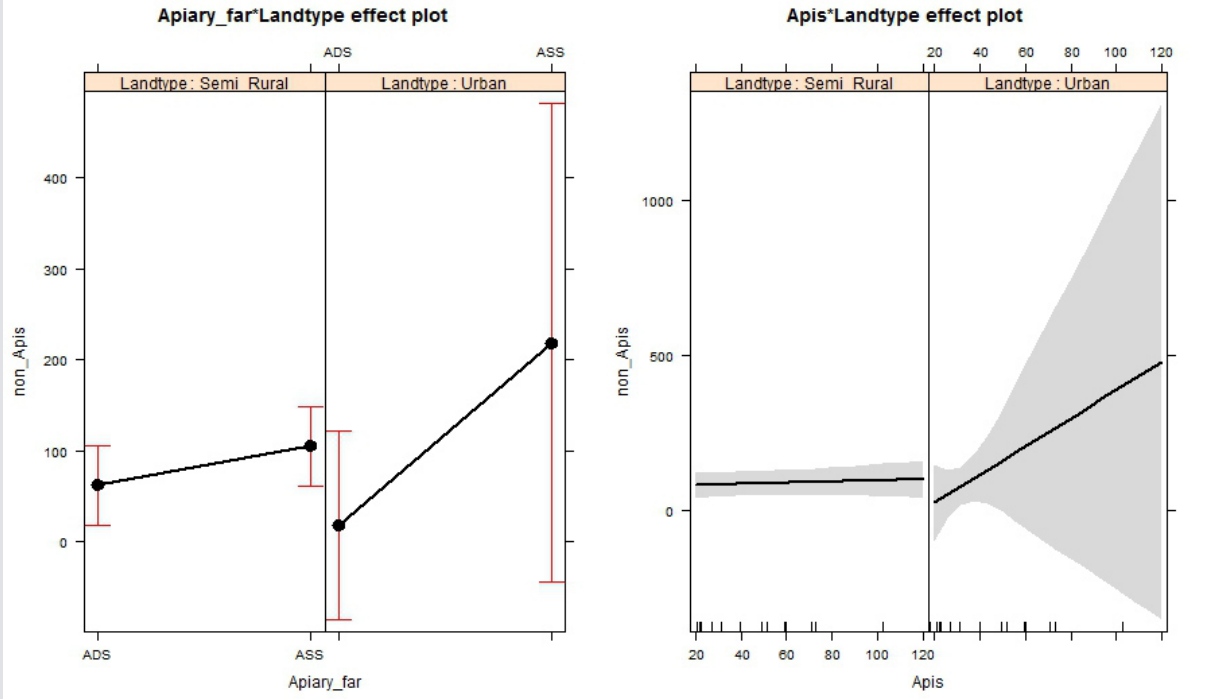


Figure S7.4.2. All effects plots showing effect of factors and interactions in unreduced model 1 for coupled ADS and ASS study sites

To achieve the best model starting from our more complex model, we called the "lmer" function in combination with the "dredge" function in package "MuMIn". The latter function generates all combinations and interactions between different parameters of the intital more complex model and calculates AIC scores. Based on these AIC scores we compared models as best models show a lowest AIC score within a delta AIC of of 4. Results showed that the model (Df =7, AIC = 178.0) only containing the factor ADS-ASS, landscape type, and interaction between best explained the number

of wild bees; however the model without interaction could also be accepted as the AIC score (Df = 6, AIC = 181.4) had only a delta of 3.47 thus below 4.

```
Model selection table
   (Int) Apr_far     Aps Lnd Apr_far:Lnd Aps:Lnd df  logLik  AICc delta weight
14 66.68       +           +           +          7 -77.354 178.0  0.00  0.770
6  66.61       +           +                      6 -81.524 181.5  3.47  0.136
16 58.15       +  0.1261   +           +          8 -77.657 184.4  6.36  0.032
2  65.51       +                                  5 -85.062 184.4  6.37  0.032
32 54.70       +  0.1879   +           +       +  9 -74.810 185.6  7.58  0.017
8  56.71       +  0.1505   +                      7 -81.857 187.0  9.01  0.009
4  56.86       +  0.1349                          6 -85.413 189.3 11.25  0.003
24 57.20       +  0.1342   +                   +  8 -81.250 191.6 13.55  0.001
5  83.40                   +                      5 -89.090 192.5 14.43  0.001
7  97.78         -0.3493   +                      6 -88.174 194.8 16.77  0.000
1  82.25                                          4 -92.826 196.3 18.28  0.000
23 96.38         -0.3171   +                   +  7 -87.328 198.0 19.95  0.000
3  95.52         -0.3377                          5 -91.952 198.2 20.15  0.000
Models ranked by AICc(x)
Random terms (all models):
'1 | Period', '1 | Location'
```

We thus simplified the model omitting the *Apis* count factor. As visualized in figure 2, this factor generates same effects as the binary absence or presence of apiaries (ADS or ASS), but with less variation.

```
> Pantrap_model2

Global model call: lmer(formula = non_Apis ~ Apiary_far * Landtype + (1 | Period) +
    (1 | Location), data = Transectwalks15P1en2R)

  ---                          Estimate Std..Error     t.value     p.z
(Intercept)                  66.6758487   22.33827  2.98482643 0.002837393
Apiary_farASS                33.1250000   11.74072  2.82137740 0.004781791
LandtypeUrban                -5.4425154   18.60940 -0.29246050 0.769934557
Apiary_farASS:LandtypeUrban   0.4416667   26.25305  0.01682344 0.986577467
```

When calling "summary" to obtain test statistics of this reduced model, we obtain a clear significant result for factor Apiary, which is the difference between ADS and ASS sites (Z=33.125, t = 2.82, P = 0.0047) and no significance for landscape type or interaction.

When again visualizing factor effects, calling the "all.effects" plot, which is represented in **figure S7.4.5,** we indeed see no difference between landscape types semi-rural and urban. Therefore, we further simplified the model to obtain the best fitted model only representing the random factor 'Apiary':

```
> Pantrap_Model3
Global model call: lmer(formula = non_Apis ~ Apiary_far + (1 | Period) + (1 |
Location),
    data = Transectwalks15P1en2R)

---
Model selection table
  (Int) Apr_far df  logLik  AICc delta weight
2 65.51       +  5 -85.062 184.4  0.00  0.997
1 82.25          4 -92.826 196.3 11.91  0.003
Models ranked by AICc(x)
Random terms (all models):
'1 | Period', '1 | Location'
    ----
             Estimate Std..Error  t.value          p.z
(Intercept)   65.51215  22.150212 2.957631 0.0031001313
Apiary_farASS 33.21333   9.909184 3.351773 0.0008029589
```

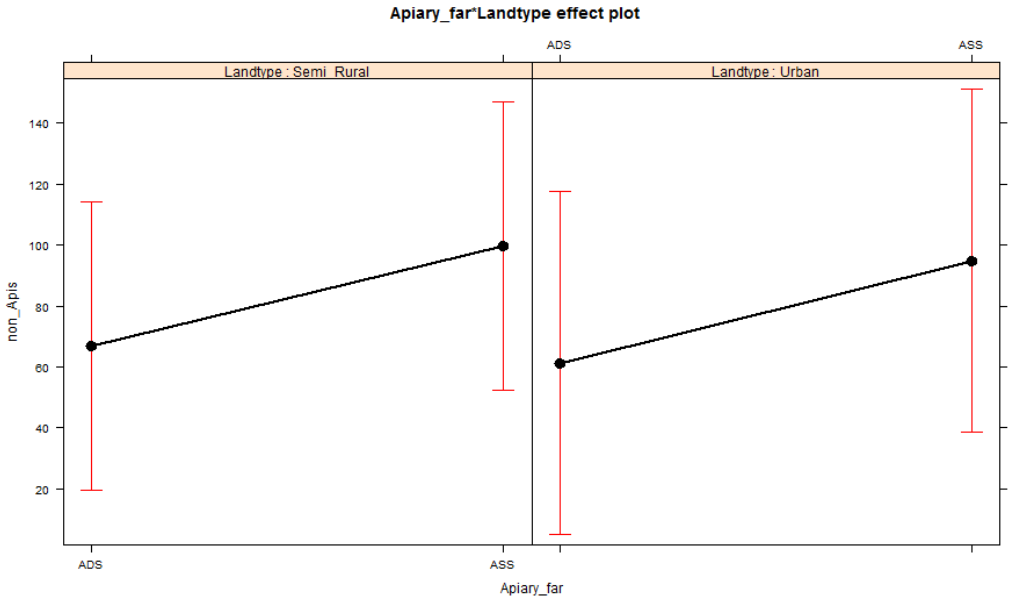


Figure S7.4.5. All effects plots showing effect of factors and interactions in reduced model 2

Finally, we rerun statistics with only apiary as a variable factor and 'location' and transect walk 'period' as random factors. This model showed a clear significance between ADS and ASS coupled study sites (Z=33.21, t = 3.35, P = 0.00080).

Conclusion

We can conclude that there is a significant effect (Z=33.21, t = 3.35, P = 0.00080) of apiaries on abundance of non-Apis wild bees (*Bombus* and other species) in close proximity of apiaries.

Chapter 8: General conclusions and future perspectives

The interaction between domesticated animals and wild species, as a consequence of modern agriculture, threatens important ecosystem services (Moleon et al. 2014). Such interactions also exists between managed and sympatric wild bees and are the backbone of this dissertation. We studied two main managed pollinators: reared bumblebees and domesticated honeybees. Hence, this discussion is divided in two main parts and future perspectives are given meanwhile. In a first part, we focus on the gut microbiota of reared bumblebees of *B. terrestris*, both towards composition and stability. As spillover of gut pathogens has been reported, we wanted to know if the bacterial microbiota of reared bumblebees harboured exotic bacteria and how much they resemble the wild host. Beside, we wanted to investigate if a shift in their gut microbial composition could mediate the emergence of gut pathogens. In a second part, we focused on *A. mellifera* and its interference with sympatric wild bees. We discuss spillover of *A. mellifera* associated parasites and viruses. Beside, competition for resources in their free foraging native range will be discussed. Finally, in regard to a general conservation of pollinators, mitigation measures for domesticated bees are briefly discussed, and in relation to a future use of free foraging managed bees in the natural environment.

1. The gut microbiota of reared bumblebees and its implications outdoors

1.1. A "bottleneck" microbiota in reared versus wild bumblebees

As spillover of gut pathogens between reared and wild bees has been reported (Graystock et al. 2015a, McMahon et al. 2015), we wanted to know the impact of the gut microbiota of managed bumblebees towards wild bees. In order to unravel the potential differences between managed and wild *Bombus* sp., we assessed the gut microbiota towards composition (chapter 2) and stability (chapter 3). We showed that the microbiota of bumblebees reared under controlled conditions indoors (temperature, relative humidity and pathogen-free) have a stable gut microbiota, primarly composed of bacteria belonging to the Acetobacteriaceae (*Bombella intestini*), Neisseriaceae (*Snodgrassella alvi*), Orbaceae (*Gilliamella apicola*), Lactobacillaceae (*Lactobacillus* sp.) and Bifidobacteriaceae (*Bifidobacterium* sp.). We referred to a "bottleneck" microbiota of managed bumblebees, as we showed that these reared hosts harbour a simplified gut microbiota, composed of a subset of the microbiota associated to the wild host.

Here, in chapter 1 and 2 we also categorised the gut microbiota into core and non-core. Following the definition given by Cariveau et al. (2014), microbiota categorized as "core microbiota" include any operational taxonomic units (OTUs) with closest hits to sequences previously found exclusively or primarily in the guts of honey bees and bumble bees (genera *Apis* and *Bombus*); "non-core microbiota" are then bacteria that show a more erratic appearance in the guts of pollinators. In

the first chapters we mainly state that reared bumblebees lost some non-core bacteria, as the gut microbiota of wild captured bumblebees are a combination of core and some non-core gut microbiota following the definitions and descriptions in literature (Koch and Schmid-Hempel 2011a, Cariveau et al. 2014). However the division in "core" and "non-core" bacteria is a work in progress and probably reflects the somewhat early phase in which microbiota research in bees currently is. Indeed at the beginning of this PhD, research on gut microbiota in bees was only starting and thus we followed this dual division in the first chapters.

The question remains: how important are "core" versus "non-core" bacteria? To answer this question, knowing the functionality of both groups, or the functionality of certain bacterial associations could be enlightening. However, as we are only at the beginning of this research, very few information is available to make a link between the exact function(s) and gut microbial species of bumblebees. While then this binary grouping makes sense, however, when looking at general microbial gut composition in bumblebees, still considerable variation is retrieved within different bumblebee species. Indeed, we found that wild captured bumblebees also showed gut profiles with a high to dominant abundance of Enterobacteriaceae and Bacteriodetes, two phyla regarded as non-core. Moreover, this seems not to be a local phenomenon and has also been observed in wild captured North-American bumblebees of *Bombus bimaculatus* and *B. impatiens* (Cariveau et al. 2014). Given this general observation, it seems that at least some bacteria classified as non-core may also be associated and thus functional to the host. Probably, they might also contribute to host well-being while these bacteria are not found exclusively in the hosts (and nests) of bees, but also in the natural environment. Thus, the division in "core" and "non-core" is too simplistic and does not reflect the real situation that different gut bacteria interact with each other forming bacterial associations. For instance, if we make a comparison with the situation in humans, it is known that three bacterial enterotypes exists (i.e dominated by *Rumminococcus*, *Bacteroides* or *Prevotella*) and that these different enterotypes are all functional with their own typical mode of action such as producing vitamins and antimicrobial peptides (Arumugam et al. 2011)

Knowing this, it is perhaps more suited to look at multiple bacterial associations trying to identify bacterial enterotypes in bumblebees, rather than to divide specific bacteria into "core" and "non-core" groups. The use of enterotypes has already been suggested by Li et al. (2015), who reported that the gut microbiota of wild bumblebees clustered in two distinct enterotypes, an observation which was largely consistent over 25 species tested. Here, Enterotype 1 is then mostly composed of 'vertically transmitted bacteria', i.e. *G. apicola*, *S. alvi*, and *Lactobacillus* spp., while Enterotype 2 is a combination of both vertically transmitted Lactobacillus sp. and 'horizontally acquired microbiota' from the environment. This is an interesting rationale, and it might be enlightening to test which are essential bacteria from the hive (uptake by vertical transmission) and

which bacteria from the environment (uptake by vertical transmission) contribute to generate functional and stable enterotypes.

Then, another question arises: is the bottleneck still a form of a natural enterotype? The limited gut microbiota of reared bumblebees could represent the minimal set of (core) bacteria needed, that functions well in a controlled indoor environment. Indeed, as a consequence of indoor rearing, in sterile nest cages (while wild *B. terrestris* are nesting underground) we could explain the observation of a less biodiverse "bottleneck" microbiota. This then reflects the bacteria mainly transmitted by social contact between bees and those being able to survive the hibernation process of the founding queen (Billiet et al. 2016). Within the conditions of the breeding facility, no contact with external stressors and food available *at libitum,* the functionality of such a reduced bottleneck microbiota is sufficient. In contrast, the importance of a more diverse microbiota might prove to be more essential when bumblebees are exposed to unfavourable or variable outdoor conditions. Then an essential interplay exists between vertically and horizontally acquired bacteria, the lumen of the host and the conditions of the environment with many variable factors.

1.2. A gut microbial shift towards Enterobacteriaceae

In chapter 3 we showed that the reared bottleneck microbiota is initially stable for a period (about 6 weeks) but then, when moved to outdoors, especially the newborn workers in the old nests (then about 8 weeks old) show Enterobacteriaceae dominated gut profiles. This is visualized in figure 8.1.a. Here, in chapter 3 we cited again Cariveau et al. (2014) that linked the presence of these "non-core" bacteria to a significant higher presence of *Crithidia,* or vice versa, the higher presence of "core" bacteria to a significant lower presence of *Crithidia.* This phenomena was largely driven by the presence of Enterobacteriaceae and their correlation with the presence of the gut pathogen *C. bombi*. This association can be seen as first argument to link the proliferation of both Enterobacteriaceae and opportunistic gut pathogens when reared bumblebees with a bottleneck microbiota are placed outdoors. However, when having a look in dept, a more nuanced situation must be drawn:

1) The link between lower *C. bombi* prevalence and vertically transmitted (core) gut microbiota of Betaproteobacteria (*Snodgrassella*) is significant (P=0.004), while a significant effect was not found for the proportion of vertically transmitted (core) Gammaproteobacteria (*Gilliamella*) (P = 0.37) (Koch and Schmid-Hempel 2011b);

2) No significant relationship arised for the presence or absence of the intracellular microsporidian parasite *Nosema bombi* (Koch and Schmid-Hempel 2011b, Cariveau et al. 2014);

3) A correlation (P = 0.024) between core gut microbiota and *Crithidia* in wild *Bombus* spp. was made; here Cariveau et al. (2014) defined core bacteria as Alphaproteobacteria (Alpha 2.1), Betaproteobacteria (*Snodgrasella*) and Gammaproteobacteria (*Giliamella* and *Ca. Schmidhempelia*), while the status of Alphaproteobacteria as core can be debated. Indeed, its presence in wild bees is not always consistent: some wild bumblebee species show a lower proportion of these bacteria (e.g. *B. bimaculatus* mean 0.48, *B. impatiens* mean 0.76) (Cariveau et al. 2014); Beside, Alpha 2.1 bacteria are also associated with floral nectar.

Related to the latter, we also found that Alpha 2.1 are lacking in most of the reared gut profiles (*B. terrestris* samples in chapter 2 and 3). At first sight, this bottleneck microbiota may thus show the same protective function compared to wild individuals having the same core microbiota, but this reared insect gut may lose its established properties faster in outdoor condition, probably due to the lack of some bacteria from the natural environment. Indeed, while the abundance of these horizontally transmitted, environmental microbiota could proportionally be low, their impact could be much higher, i.e. they can function as 'keystone species'. Then, following the rationale of functional gut enterotypes (Li et al., 2015; see supra) which can also be composed of horizontally acquired bacteria from the environment, we advocate that the environment also inhabits opportunistic bacteria that are positive for the wild host. Indeed, within the large family of Enterobacteriaceae, multiple functions have been attributed to such opportunistic bacteria. Yet, the final answer regarding these and other environmentally acquired bacteria in wild bumblebees and their exact function remains to be solved as these microbiota can have a dual function (i.e. protective or harmful) to the host. In extension to this, it has been shown recently in humans that a non-optimal gut microbiota (due to inflammation) can boost horizontal gene transfer via plasmids between pathogenic and normal *Escherichia coli* leading to parallel flourishing of both types of *E. coli* (Stecher et al. 2012). Yet, more research is needed to uncover the exact function of different environmentally acquired bacteria, the conditions of their proliferation, and how they are linked with pathogens and the suppression or proliferation thereof.

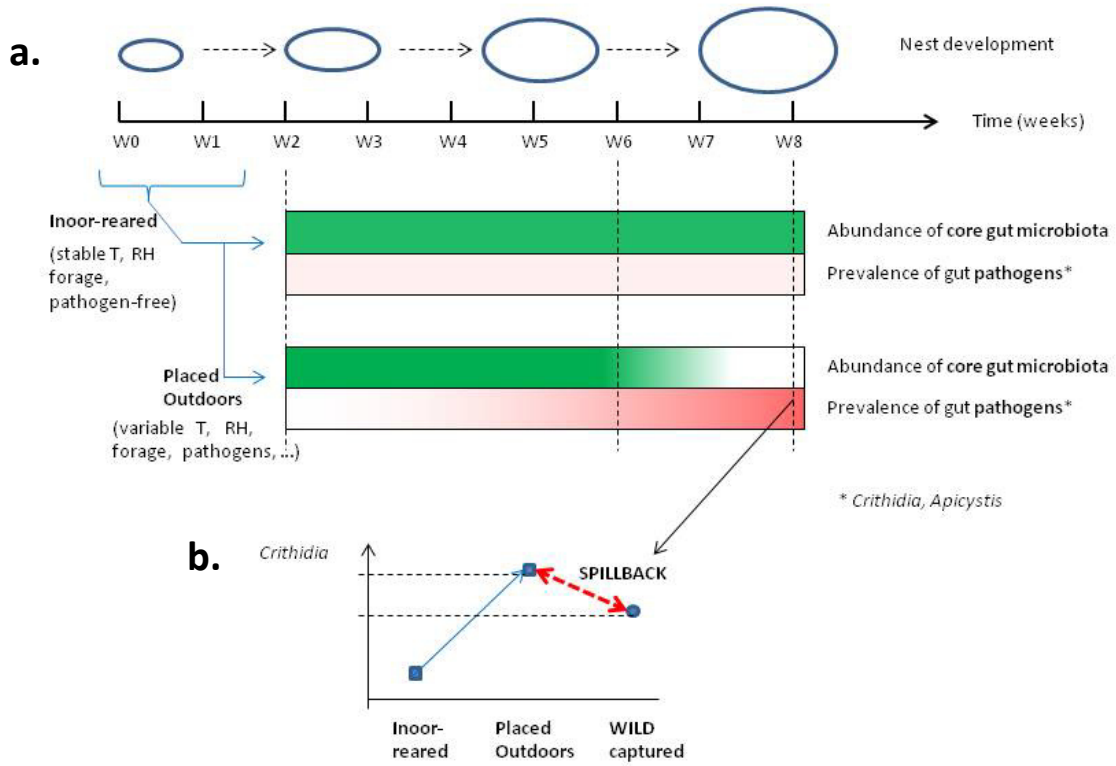


Figure 8.1. Schematic overview of main results from chapters 2 to 4; (**a**) reared bumblebee nests and impact towards gut microbiota and pathogens when developed continuously under stable conditions or placed outdoors under variable natural conditions; (**b**) impact of the latter situation towards pathogens in wild bumblebees: visualisation of pathogen spillback principle

1.3. Reared bumblebees foraging outdoors and "spillback" of gut parasites

It is known that bumblebees with a heavily impaired microbiota are more susceptible to pathogen intrusion than bumblebees with the typical vertically acquired (core) gut microbiota (Koch and Schmid-Hempel 2011b). Here, we showed (chapter 4) that foragers of reared bumblebee nests, from 8 week old nests had a higher prevalence of *Crithidia* compared to wild captured specimen form the same environment, as represented in figure 8.1.a This is an important observation which can have major consequences towards pathogen spread when these nests are being used for pollination purposes for about 8 weeks in an outdoor environment. Indeed, it has been demonstrated that bumblebees from commercially reared nests who escaped from greenhouses (Colla et al. 2006, Dafni et al. 2010, Goka 2010) can lead to higher pathogen prevalence in native wild populations (Murray et al. 2013, Graystock et al. 2014). This phenomenon is often explained by "pathogen spillover", meaning the managed (bumble)bees were infected with pathogens before they are placed in their new environment and from this reservoir they could infect wild species. However, we advocate that another mechanism could have played here, called "pathogen spillback" (Kelly et al. 2009). According to this mechanism, managed (bumble)bees are not the reservoir species before

they were placed in their new environment, but they become infected from bees (wild bees or honeybees) in the environment due to a higher susceptibility for their pathogens; they thus become a reservoir species to infect wild bees at a second order. In relation to our results, we therefore can make the comparison, as demonstrated in Figure 8.1.b. It has been reported that pathogens can be present in managed bumblebees nest infecting wild species (Murray et al. 2013, Graystock et al. 2014); however in chapter 7 we showed that large batches of nests (n = 45) are free of parasites before placing outdoors (week 2), while this result is in sharp contrast with their infection status after placing outdoors with almost all nest infected with *C. bombi* after 8 weeks (18/24 and 9/24 for *B. terrestris* nest samples and wild *B. pascuorum*, respectively). Next, these host can then serve as a new source of pathogens, spreading to sympatric wild bees, which is represented by the striped double arrow in Figure 8.1.b.

According to Kelly et al. (2009), this is an important but disregarded mechanism of pathogen spread when domesticated and wild congeners come into contact with each other. While we could not give definite proof if this concept here, we do give the first indications of this mechanism in the spread of the important trypanosomic gut parasite *Crithidia* in bumblebees.

1.4. Future perspectives on gut microbiota of reared bumblebees

Starting from a bottleneck microbiota we showed that the gut microbiota of reared bumblebees shifted to an Enterobacteriaceae-dominated gut microbiota in newborn bees from the 8 week old nest after being placed outdoors. Our results from individual samples then also indicated a potential of pathogen spillback towards wild congeners, at least for one important gut parasite. This “bottleneck microbiota” - probably lacking some keystone species - could be disrupted and then perhaps provide the ideal environment for certain parasites leading to an intensive replication in these bumblebee hosts. In regard to an ongoing and increased use of native commercial reared nests in open field crops, this points to a further in-depth investigation under different stressors and outdoor conditions (Sydenham et al. 2014, Goulson et al. 2015). Therefore, we give future perspectives both for applied and fundamental research within this topic.

Next, a question remains unsolved combining the results of the first chapters, i.e. if we suppose a link between a bottleneck microbiota and a higher gut pathogen load after several weeks outdoors: is the bottleneck microbiota or the shifted gut microbiota responsible for a higher prevalence of gut pathogens after being placed outdoors? To solve this important question, an interesting avenue is to investigate when (in time) the bottleneck microbiota of reared bumblebees loses its protective function, if there is a link with horizontally transmitted gut microbiota when a major uptake is observed, and thus if a bottleneck microbiota can eventually be linked with immune incompetence in

bees. Therefore, efforts for culturing individual (bumble)bee-associated gut bacteria and gut microbial associations should be made under different conditions to gain more information about their functionality and colonization dynamics in the bumblebee host. In general, the aim should be to create a richer, more diverse "wild-type" microbiota in reared bumblebees which remains stable when nests are used outdoors and functions better in fitness and pathogen protection. Here, the potential of probiotics may also be interesting for future research in regard to boosting the immune competence of bumblebees. Currently, there are already some probiotic formulations on the maket, e.g Micro4bee® is a commercial formulation (including strains of *Lactobacillus* and *Bifidobacteria*) to protect honeybees against pathogens such as American Foulbrood. Next to this, the potential of priming reared bumblebees guts with positive (keystone?) bacteria or other functional groups such as floral yeasts from the environment can be an interesting avenue for future research. Especially when bumblebee nests are increasingly being used for pollination purposes outdoors for longer periods (over 6 weeks), this research should have priority.

In second, more fundamental research could untangle a possible link between pathogen proliferation and the presence/absence of typical commensal and environmentally acquired bacteria such as Enterobacteriaceae. Probably, the discovery of Stecher et al. (2012) that gut inflammation in mammals can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae, leading to parallel flourishing of both, may be an interesting avenue of further research in bumblebees. Gaining insights in the metabolic pathways present in bumblebee gut bacteria (both horizontally and vertically acquired) can form a steady basis to develop a hypothesis on interactions between horizontally and vertically acquired bacteria within the host and under different (environmental) conditions.

2. Impact of apiaries on sympatric bees

In a second part of this dissertation, we looked at the impact of domesticated bees on sympatric wild congeners, and here we focussed on *A. mellifera*, the number one managed insect crop pollinator used worldwide. The main research question was: can apiary density interact with drivers of sympatric wild bee ecology? As competition for resources (Steffan-Dewenter and Tscharntke 2000, Elbgami et al. 2014) and spillover of parasites (Graystock et al. 2015a) and viruses (Fürst et al. 2014) between domesticated and wild pollinators are likely to be important factors in the pollinator crisis, we focused on these key drivers, as represented in figure 8.2.

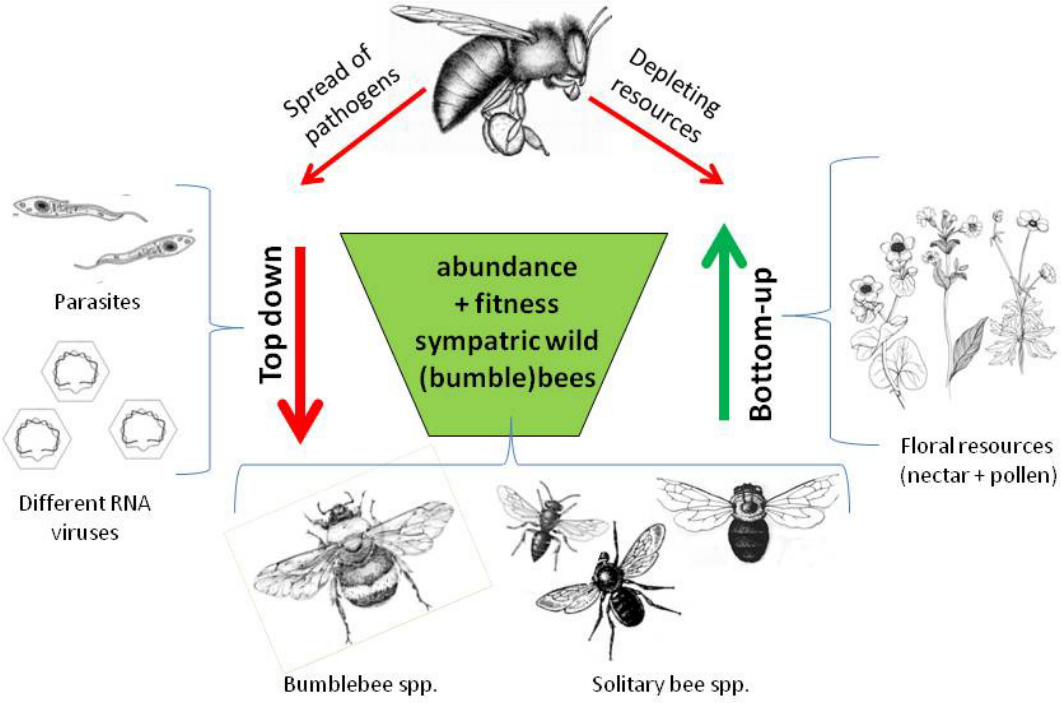


Figure 8.2. Schematic overview of main results from research chapters 5 to 7; Domesticated honeybees from apiaries (represented by a foraging honeybee on top with collected pollen in corbicula) interfere on multiple levels, i.e. bottom-up drivers (parasites, viruses) and top-down drivers (depleting floral resources) of wild (bumble)bees

2.1. Impact of honeybees competing with sympatric wild bees

Using conventional monitoring schemes of pan traps and transect walks, in chapter 7 we showed that apiary density inversely correlates with wild bee abundance. Besides, employing a bioassay of *B. terrestis* nests (chapter 5), we showed that apiary density impacts on bumblebee nest fitness after only 6 weeks (chapter 7). This significant effect was seen independently of pathogen prevalence and spatial landscape variation as we tested in a diverse range of urban to semi-rural anthropogenic locations over two years of study. Thus, in general, we showed a competition for floral resources by apiaries on sympatric wild (bumble)bees. While proxy measurements in literature

already indicated negative impacts of honeybees, other studies showed a positive correlation between number of *Apis* and *Bombus* on flower patch level (Potts et al. 2003, Kohler et al. 2008, Samnegard et al. 2011, Banaszak and Dochkova 2014). Yet, the latter result is not a contradiction with our results. Indeed, it is known that rewarding flower patches attract many different pollinators (Kohler et al. 2008, Hennig and Ghazoul 2012, Dupont et al. 2014, Scheper et al. 2015); hence seeing a positive correlation between *Apis* and *Bombus* (at flower patch level). In our study design, the monitored transects were randomized; including rewarding flower patches indeed attracting a multitude of pollinators, but in apiary dense sites a bigger fraction was composed of honeybees and lower counts of wild bees were recorded. Indeed, when calculating a correlation between *Apis* and *Bombus* counts in a selection of locations with only high *Apis* counts (13/30 study sites; transect walk data chapter 7) we did observe a clear trend ($P = 0.067$; correlation = 0.51; data not shown) retrieving almost the results obtained from previous studies that solely based their monitoring on rewarding flower patches.

These combined results thus indicated that a resource competition between honeybees and sympatric wild pollinators generally exists, and especially at highly rewarding flower patches with overlapping floral resources. This can have some major consequences towards wild pollinators. Probably wild bumblebees and solitary bees shift to forage on other, less rewarding flowering plants or change their temporal activity pattern as a response to increased foraging by honeybees? In comparison, this behaviour has been reported in wild bees when non-native honeybees were introduced, for example in Australia and New-Zealand (Huryn 1997, Goulson 2003b, Paini 2004, Stout and Morales 2009). In this regard, a competitive effect of honeybees would be less effective for specific plant pollinators such as oligolectic bees. In contrast, competition by generalist honeybees would then be maximised for oligolectic bees foraging on generalist flower plants as they would use these surrogates when lacking their specific floral resources. Beside one can argue if there were here food limitation in such regions? Indeed, floral resources can be available, but efforts to extract resources would demand more energy (more flowers to be visited as flowers are less rewarding) resulting in a lower net energy availability for the wild bee host, thus impacting on their fitness.

Next to this, while we did observe a clear effect in two coupled study sites at an inter-distance of 1.5 km, the spatial context of this influence needs to be recognized; i.e. what are the effects of competition on different spatial distances to apiaries, this in relation to rewarding and less rewarding flower patches. Following honeybee foraging trips decoded from waggle dances as described in literature (Couvillon et al. 2014), i.e. dense forage trips within about 1 km from the hive and also to rewarding flower patches within 12 km from the hive, we would expect that within a distance of about 1 km from the hive, there will be a general competition effect on sympatric bees independent of flower patch quality, while further away this effect will mostly be seen at rewarding flower patches; however this hypothesis remains to be tested in the field.

2.2. Impact through pathogen spread

Domesticated honeybees could also impact on pathogen prevalence in wild bees. With disease's being an important top-down drivers of wild animal populations (Schwarz et al. 2013). Both in chapter 6 and 7 we looked at prevalence of wild bee diseases, i.e. parasites, common and uncommon viruses, in relation to apiary presence.

We showed that within sympatric wild *B. pascuorum* the total number of pathogens correlated with apiary density. Yet, for some common diseases, the interaction with apiaries and diseases in wild bees can be expected, as it is known that honeybees share RNA-viruses as well microsporidian parasites with wild pollinators including bumblebees (Fürst et al. 2014, McMahon et al. 2015). Indeed, in chapter 7, we clearly demonstrated the link of common viruses, i.e. DWV, SBV and those under the AKI-complex. In comparison, previous studies also showed that DWV, BQCV and IAPV retrieved in honeybees and bumblebees cluster together (Singh et al. 2010, Fürst et al. 2014). Therfore, we explained that honeybees most likely act as meachanical vectors (spreaders or prime hosts) of these viruses.

Beside, we also showed a correlation between apiary density and *A. bombi*, an *Apis*-specific pathogen, a result which is in agreement with Graystock *et al.* (2013b). However, microscopic analyses in our lab of the fat body of honeybees only rarely reveal *A. bombi* oocysts (data not shown) indicating that apiaries here rather act as a mechanical vector instead of an active vector as we expect for shared viruses. In the case of *A. bombi* it is probably explaining why this parasite has barely been detected with a microscope in epidemiological studies of honeybees, with few cases reported in Finland in 1990 (Lipa and Triggiani 1996) and European honeybees from Northwestern Patagonia (Argentine) (Plischuk and Lange 2009, Arbetman et al. 2013). While we thus indicate apiary presence as predictor for pathogen prevelance in wild *B. pascuorum* species, we argue that this must not mean that honeybees are always the reservoir species for a certain pathogen, and they can also act as an active mechanical vector.

2.2.1. Multi-host interactions involved with honeybees

When integrating the observations of honeybee associated diseases, our results can serve as a good example to demonstrate the multi-host dynamics as defined by Rigaud et al. (2010). Generally, interspecies transmission is playing a central role in the evolution of the pathogen and its virulence to different hosts. Virulence may evolve to be higher or lower in different host species, depending upon transmission and the virulence tradeoff across host species (Woolhouse et al. 2001). Specifically, in a situation where hosts differ in quality, and transmission rates allow, pathogens should evolve towards optimal virulence in their prime host. For instance, we speculate that honeybees are acting as the prime host for RNA viruses transmission, such as for DWV. Then, the

honeybee is the host enabling the best viral fitness (in terms of reproduction). In particular, if the honeybee (prime host) is more resistant, the virus should evolve higher virulence, which will only be expressed in other bees host (Gandon 2004). If the virus has lower optimal virulence (i.e. replication) in this second species (e.g. if the host is less resistant and thus selects for lower virulence), then killing its prime host, will lower its changes to transmit to new bee hosts and thus survival of the virus. Therefore an optimal virulence is ascertained in the prime host; if not another species can take over the role of the prime host.

However, while we here explained for one virus, it is clear that the general principle in host-parasite evolution from a single-host single-parasite system is not applicable anymore, and that virulence in terms of impact on the host can thrive in certain individuals, if transmission dynamics and viral fitness are not impeded in its prime host. It can be speculated that hosts with a comparable life-history, behavioral, or immuno-logical defense against the pathogen will have similar resistance towards the infection. But it is clear that there are big differences among bee species, which could allow that certain parasites are benign in one host, while lethal in others. Yet, it is likely that the honeybee will be the prime host in some cases for pathogens we reported, due to the 'intrinsic properties' of honeybees. Indeed, honeybees live in high numbers together (an average honeybee hive ranges between 30,000 to 60,000 individuals), are generalist pollinators foraging on a vast number of plants, and are omnipresent in most parts of the world, making it an ideal strategy for a pathogen to evolve together with this prime host, then acting an ideal spreader of the pathogen towards other wild congeners.

2.2.2. Viruses associated to multiple Apoidea bees

In regard to the uncommon viruses (chapter 6), we discovered for the first time Bee Macula-like virus (BeeMLV; formerly called *Varroa destructor* Macula-like virus VdMLV (de Miranda et al. 2015)) and Lake Sinai virus (LSV) in multiple wild bumblebee hosts of *Bombus pascuorum*, *Bombus lapidarius* and *Bombus pratorum*. We only found an effect of apiary density towards the spread of BeeMLV, but not in all locations. Thus, it seems that the transmission of these uncommon viruses is rather a local phenomenon, rather than a clear link with honeybees can be drawn. While no significant effect of apiaries was observed for LSV, we reported a new isolate with closest match to a clade isolated from *A. mellifera* as reported by Ravoet et al. (2015) speculating for a possible link with honeybees in the spread of this virus, which we could not prove here. However, multiple reservoirs for LSV are known, as it has also been found in solitary bee species of the genera *Andrena* and *Osmia* (Ravoet et al. 2014). When clustering our LSV isolates together with those availbale in Genbank (about 40 sequences) it becomes clear that the phylogenetic structure of this virus is very complex, and an interesting topic for further research. Recently, a series of new LSV-viruses were described,

sometimes based on partial genomes (eg.: Daughenbaugh et al. (2015)) Probably, the multiple LSV-strains observed represent different viruses which could be linked to a typical Apoidae host. Yet, more information about transmission routes and pathological data of this and other uncommon viruses in different hosts (honeybees, bumblebees and solitary bees) by means of specific infection experiments is needed, as has been achieved for more common viruses such as ABPV, IAPV or SBPV (Meeus et al. 2014, Niu et al. 2016). For LSV, it would be useful to further untangle this complex probably into different viral species or a complex of different viruses with closely related species such as the AKI-complex.

2.3. Mitigation measures and future perspectives related to apiculture and wild bees in general

We demonstrate relations between apiary density and wild bee abundance, bumblebee nest development and pathogen prevalence in *B. pascuorum*. Competition for the same resources and spillover of shared pathogens could be underlying mechanisms explaining the influence of apiaries on proximate wild bee ecology. Although we do not have final results to draw a mechanistic conclusion, it seems that apiary density is an important parameter when considering mitigation actions for the conservation of wild bees.

Our experimental setup already shows an impact over a relatively short distance, being 1.5 km, this could represent an initial area of focus for mitigation measures, while further study awaits if impact over larger distance is seen. However we expect that this will not be the case for food competitions; over 1 km honeybees mainly forage to highly rewarding flower patches (Couvillon et al. 2014). In contrast, pathogen spread can be expected over a longer distance, as previous studies showed a link between honeybee presence and pathogen prevalence in wild bees sampled at random from rewarding flower patches (Fürst et al. 2014, McMahon et al. 2015).

Firstly, our results indicate the need of designing mitigation measures related to forage availability. The placement of honeybee hives in different anthropogenic and natural landscapes should be further investigated, taking into account the density of apiaries in relation to the available forage in the landscape, at least within a short distance of 1 km from the hive. Further research experiments should therefore investigate the relation between apiary density, wild bee abundance, and forage/nesting availability in positive landscape elements for bees (Sydenham et al. 2014), such as remaining forest edges, structure and edge density of agricultural plots, semi-natural or permanent grasslands,... A key point to consider is the presence and quality of flowers in relation to added honeybee abundance. Field research suggest that, when no mass flowering occurs, a maximum of 3 beehives/km² should be advised (van de Spek 2012), while the actual situation largely exceeds this number in many cases. For example, our results indicate an average of 6.6 hives/km² in

honeybee dense sites (in Belgium the mean number of honeybee hives per km^2 is 3.6), while in some European countries the mean number of hives per km^2 is even higher, i.e. Greece 11.4, Hungary 10.7 and Slovenia 7.7 (Chauzat et al. 2013). We therefore encourage more research in relation to different natural and anthropogenic landscapes to set up local forage maps. Such maps should encompass forage quantity of different wild flowers, but also common flowers grown in gardens and identify their floral resource quality (floral characteristics such as nectar availability or inflorescence architecture) within the spatio-temporal variation of a landscape under study. Having such forage maps, policies should identify potential zones for placing apiaries, but also delimit vulnerable zones.

Secondly, in relation to pathogen spread mitigation measures should be undertaken at larger spatial scales. We recommend a better survey of transport of honeybee hives. In this regard, one should address to the function of hive transport and added value of placing hives, i.e. to gain hive products such as honey or wax, or to aid in pollination services. In the case of gaining bee products, placement of hives should be linked with zones of extra available forage, as can be indicated in forage maps explained supra. In the latter case, for example when honeybees are used in the pollination of orchards such as cherry or apple (Breeze et al. 2011), mitigation measures should focus on the restoration of pollination services of wild bees, as latest research shows that wild bees are pollinating crops more effectively (Garibaldi et al. 2013). Beside, going to fixed apiaries can be allowed to tackle negative impacts reported.

Probably, for most vulnerable ecosystems (for example those inhabiting rare wild bee species) or landscapes with reduced forage restriction of honeybee hive transport seems useful. In comparison, restriction of placement of extra honeybee hives in nature reserves is already recommend in literature, e.g. in Israel (Shavit et al. 2009). Currently, international transports of honeybees and bumblebees falls into the European Union legislation by the Commission Regulation EC No 206/2010 and Commission Decision 2003/881/EC. However, this legislation is based on diseases associated with honeybees such as Varroasis and Nosemosis, and should therefore be extended to diseases generally linked with Apoidea bees. Yet, in some cases we recommend that local transport of domesticated honeybees should also be restricted, especially in early spring, the most fragile period of the bumblebees and many solitary bees' life cycle (Peeters et al. 2012). Besides, general measures can be recommended, such as less import of honeybee queens and thus the use of local honeybee lineages adjusted to their climatic and vegetation zone, and mating within these zones could lead toward less disturbance of pathogen associations. Currently, some specific breeding races (e.g "carnica" or "buckfast") are preferred by beekeepers leading toward import of honeybee queens and transport of honeybees (De la Rua et al. 2009). This practice could potentially introduce new pathogens into a certain location and disturb the natural flower-pollinator assemblies and their associated local pathogens (Goulson and Sparrow 2009). Therefore, the use of local

lineages could open new avenues for breeding programs towards local honeybees with lower pathogen loads and in sympatry with other wild pollinators.

Next to this, as latest research showed that wild insects are not only enhancing fruit set (Garibaldi et al. 2013) but are crucial for many wild plants, it could therefore be argued that rescuing the pollination losses by domesticated bees has better, although less straightforward alternatives, being the restoration of the native wild pollinator communities. Therefore, programs enhancing pollination services should be more focused on enhancing local wild pollinators in general. For example: how to intrinsically enhance wild pollinator communities due to smart management practices in unfrieldly environments such as intensified rural landscapes? This also opens new avenues for further research, with a major impact not only for the conservation of ecosystems and their important services but for humanity as a whole.

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Summary

Over the last few decades populations of pollinators are declining severely at international to local scales. This is an emerging threat instigating both ecological and economic concerns and is gaining attention worldwide. Their decline has likely multifactorial causes including spread of (gut) parasites and viruses, anthropogenic changes of the environment, such as urbanisation and monocultures, agrochemicals, introduction of exotic species and interactions between them. In **chapter 1**, the multifactorial problem of bee decline will be summarised, and the current knowledge about the interaction between domesticated and wild animals is given, with a special interest of those between allopatric or sympatric domesticated bees and wild bees. At the end of the chapter, an overview of Apoidae-associated viruses and protozoan parasites is given and is briefly discussed in the light of a multi-host reality.

Counteracting the pollinator crisis has resulted in management of pollination services, making the use of domesticated honeybee hives and artificial reared bumblebee nests a common practice. However, managed pollinators can also act as a stressor to native bee populations in decline, for example through spillover of alien pathogens or competition for food. While such effects of domesticated bees interfering with wild bees have been mostly studied outside their non-native range towards allopatric native bees, studies on sympatric wild congeners hardly exist. Yet, also within their native range, domesticated bees can interfere with their wild counterparts. Therefore, this dissertation focussed on the interaction of domesticated bees (both bumblebees and honeybees) on sympatric native bee fauna.

In a first part of this dissertation, we discuss the impact of the gut microbiota of reared bumblebees of *B. terrestris*, towards composition and stability of their gut microbiota and whether these could be considered as a source of invasive bacteria or mediate in the emergence of gut pathogens towards wild congeners.

As for mammals, insect health is strongly influenced by the composition and activities of resident gut microbiota. Yet, the gut microbiota of reared bumblebees was not investigated to date. In **chapter 2** a comparison is made on the gut microbiota of reared bumblebees of *B. terrestris* to wild congeners. We show that these reared hosts have a simplified gut microbiota, which we call “bottleneck microbiota”, primarily composed of a subset of the core microbiota associated with the wild host, and belonging to the Acetobacteriaceae (*Bombella intestini*), Neisseriaceae (*Snodgrassella alvi*), Orbaceae (*Gilliamella apicola* and *Schmidhempelia bombi*), Lactobacillaceae (*Lactobacillus* spp.) and Bifidobacteriaceae (*Bifidobacterium* sp.). Beside, in **chapter 3**, the plasticity of gut microbiota of reared bumblebees under controlled conditions or conditions in an outdoor environment were investigated. We show that the bottleneck microbiota in reared bumblebees can lead to a shifted Enterobacteriaceae-dominated gut microbiota in newborn workers in an old hive (then about 8

weeks old) at the expense of the core bacteria *Snodgrassella* sp. and *Gilliamella* sp. when these nests are foraging outdoors. This is an important observation which can have major consequences towards pathogen spread when these nest are being used for pollination purposes in the outdoor environment for a longer period (over about 6 weeks). Therefore, in c**hapter 4** we focussed on the impact of reared bumblebees towards proliferation of *Crithidia* and *Nosema,* two important gut pathogens. We placed reared *B. terrestris* nests (2 weeks old) outdoors in different locations and compared prevalence of gut parasites in nest samples (then about 8 weeks old) with wild counterparts in the same environment. We found that especially *C. bombi* had a significant higher prevalence in comparison to wild *B. terrestris* bumblebees. This is an interesting observation as it has been shown that this gut-infecting parasite has an impact on the survival of *B. terrestris* queens over hibernation, colony founding, and the subsequent reproductive fitness of colonies. As reared bumblebees, initially screened to be free from parasites may become infected with parasites from wild bees we add that the prevalence of at least one important disease, *C. bombi* can be higher in comparison to wild congeners. We explain that this may lead to the spread of the pathogen to wild pollinators inferring to a "pathogen spillback" mechanism.

In a second part, we discuss the impact on sympatric wild bees of *A. mellifera*, the prevailing used domesticated pollinator. The main research question was: can apiary density interact with drivers of sympatric wild bee ecology? We discuss spillover of *A. mellifera* associated diseases and competition for resources in their free foraging native range.

In **chapter 5**, we first investigate the potential use of standardized nests of *B. terrestris* as a bioassay tool, determining environment quality to allow bumblebee development. We show that, by assessing their nest development parameters and making a correlation with poor or rich landscape metrics, the parameter 'biomass increase' is a useful measure assessing hive fitness.

In the light of new diseases and their possible link with spillover, **chapter 6** investigates on recently described Apoidea-associated viruses within pollinator networks and in relation to apiary density. We discovered for the first time Bee Macula-like virus (BeeMLV; formerly called *Varroa destructor* Macula-like virus VdMLV and Lake Sinai virus (LSV) in multiple wild bumblebee hosts of *Bombus pascuorum*, *Bombus lapidarius* and *Bombus pratorum*.

While in previous chapter we focused on diseases not yet reported in wild bumblebees, in **chapter 7** we investigate the impact of apiary density and the spillover of established diseases towards sympatric wild bees. In parallel, we also investigate on competition for resources towards sympatric bumblebees and we therefore employed the standard-nest bioassay (chapter 5).

In short, we report on multiple interactions of domesticated honeybees, both top-down and bottom-up drivers, and their impact on the abundance and diversity of sympatric wild bee communities each

in proximity and at a distance of apiaries in a matrix of athnropogenic location in Flanders (North-Belgium). Combining monitoring schemes and analysis of EIDs in Apis and non-Apis bees, and field experiments with bumblebee nests (the standard-nest setup of chapter 5), we demonstrate multiple correlations of apiary density with sympatric non-*Apis* bees both for competition of resources, higher prevalence of honeybee-associated diseases (*Apicystis bombi* and viruses including SBV, and AKI-complex), and lower abundance of wild bees. Our results show that honeybee-dense sites interact simultaneously on multiple drivers of wild bee decline.

The final **chapter 8** presents the general conclusions of this thesis. We synthesise the impact of managed honeybees and bumblebees on wild populations at different levels, starting with associated gut microbiota. In the first part of this dissertation we showed that the gut microbiota of reared bumblebees harbours a subset of the gut microbiota associated to the wild host. Given this "bottleneck microbiota", we further showed that the stability of the gut microbiota of reared nests is stable under controlled conditions, but was shifting after 6 weeks outdoors. While a major uptake of Enterobacteria was then observed, we also showed that these microbiota were not exotic and can also be found in the outdoor environment. However, a major shift in gut microbiota can have its implications which is further discussed in this chapter. In a second part, we mainly investigated on the impact of honeybees towards sympatric wild bees. We showed a significant higher prevalence of honeybee associated viruses and parasites in wild bumblebees collected in close proximity of apiaries. Beside, we also demonstrated a competition effect with wild bumblebees as their nests gained less weight in comparison to nests at a distance of apiaries. Next to the impact on ecological "top-down" (i.e. parasites, viruses) and "bottom-up" (i.e. competition) processes, we showed a general negative effect of apiaries on abundance of wild bee taxa. These important results are further discussed in chapter 8. Possible mitigation actions for the beekeeping sector are given, basically advising the reduction of i) the pathogen load in bee hives, ii) the sector's mobility and iii) implementation of forage density maps, managing the equilibrium between available forage and foragers in different ecosystems.

Samenvatting

In de afgelopen decennia zijn populaties van bestuivers sterk achteruit gegaan, zowel op lokale als internationale schaal. Dit is een opkomende bedreiging voor zowel de ecologische als de economische belangen en komt daarom wereldwijd in de aandacht. Hun daling heeft waarschijnlijk multifactoriële oorzaken, waaronder de verspreiding van (darm)parasieten en virussen, antropogene veranderingen van de omgeving, zoals verstedelijking en monoculturen, landbouwchemicaliën, introductie van exotische soorten en interacties tussen deze stressoren. In **hoofdstuk 1**, wordt het multifactoriële probleem van het verdwijnen van bijen samengevat. Daarnaast wordt de huidige kennis over de interactie tussen gekweekte/gedomesticeerde en wilde dieren gegeven, met een bijzondere aandacht voor deze tussen allopatrische of sympatrische gedomesticeerde bijen en wilde soortgenoten. Aan het einde van dit hoofdstuk wordt een overzicht van Apoidae-geassocieerde virussen en parasieten gegeven en kort besproken in het licht van een realiteit waar meerdere pathogenen en gastheren met elkaar interageren.

Het aanpakken van de bestuiver crisis in de landbouw heeft geleid tot het beheer van de bestuiving diensten, waardoor het gebruik van gedomesticeerde honingbijen en commercieel binnenshuis gekweekte hommelnesten een gangbare praktijk is geworden. Echter, kunnen deze 'gemanagede bestuivers' zelf ook fungeren als een stressor voor de inheemse wilde bijen, bijvoorbeeld door middel van spillover van uitheemse pathogenen of de concurrentie voor voedsel. Dergelijke effecten van gedomesticeerde bijen die interageren met wilde bijen werden meestal bestudeerd in hun niet-natuurlijke verspreidingsgebied ten opzichte van allopatrische inheemse bijen, en studies over sympatrische interacties met wilde soortgenoten bestaan nauwelijks. Toch kunnen ook binnen hun natuurlijk verspreidingsgebied gedomesticeerde bijen interfereren met hun wilde soortgenoten. Daarom richt het onderzoek in dit proefschrift zich op de interactie van gedomesticeerde bijen (zowel hommels als bijen) op de sympatrische inheemse bijenfauna.

In een eerste deel van dit proefschrift bespreken we de impact van de darmflora van deze commercieel binnenshuis gekweekte hommels van *B. terrestris*, meerbepaald over de samenstelling en de stabiliteit van hun darmflora en hoe ze een bron van invasieve bacteriën zouden kunnen zijn of hoe ze ervoor kunnen zorgen dat (vreemde) pathogenen in wilde soortgenoten terecht komen.

Zoals bij zoogdieren, wordt de gezondheid van insecten sterk beïnvloed door de samenstelling en de activiteit van de darmflora. Toch werd de darmflora van de commercieel gekweekte hommels niet onderzocht tot nu toe. In **hoofdstuk 2** wordt daarom een vergelijking gemaakt tussen de darmflora van deze *B. terrestris* hommels in vergelijking met hun wilde soortgenoten. We tonen aan dat deze gekweekte gastheren een vereenvoudigde darmflora hebben, die wij "bottleneck microbiota", noemen en hoofdzakelijk samengesteld zijn uit een deelverzameling van de microbiota geassocieerd met de wilde gastheer. Deze behoort tot de genera Acetobacteriaceae (*Bombella intestini*), Neisseriaceae (*Snodgrassella Alvi*) , Orbaceae (*Gilliamella Apicola* en *Schmidhempelia Bombi*), Lactobacillaceae (*Lactobacillus* spp.) en Bifidobacteriaceae (*Bifidobacterium* sp.). Daarnaast

wordt in **hoofdstuk 3** de plasticiteit van de darmflora van deze gekweekte hommels onder gecontroleerde condities of omstandigheden in de buitenlucht verder onderzocht. We laten zien dat de “bottleneck microbiota” in deze hommels kan leiden tot een sterk verschoven microbiota profiel richting een door Enterobacteriaceae gedomineerde darmflora bij pasgeboren werksters in het oude nest (dan ongeveer 8 weken oud). Dit gaat ten koste van de hommel-geassocieerde bacteriën *Snodgrassella* sp. en *Gilliamella* sp. wanneer deze nesten worden buiten gezet en vrij kunnen foerageren. Dit is een belangrijke constatering, die grote gevolgen kan hebben voor de verspreiding van pathogenen wanneer deze nesten worden gebruikt voor bestuivingdoeleinden in de buitenomgeving voor een langere periode (meer dan ongeveer 6 weken). Daarom richten we ons in **hoofdstuk 4** op de impact van de deze gekweekte hommels richting proliferatie van *Crithidia* en *Nosema*, twee belangrijke darmpathogenen. We plaatsten commercieel gekweekte *B. terrestris* nesten (2 weken oud) buiten op verschillende locaties en vergeleken de prevalentie van darm parasieten in werksters van deze nesten (toen ongeveer 8 weken oud) met wilde soortgenoten gevangen in dezelfde omgeving. We vonden dat vooral *Crithidia bombi* een significant hogere prevalentie had in vergelijking met wild gevangen *B. terrestris* hommels. Dit is een interessante observatie omdat in de literatuur van deze darmparasiet effecten werden beschreven op de overleving van *B. terrestris* koninginnen, hun winterslaap, kolonievorming, en de daaropvolgende reproductieve fitness van hommelkolonies. Als deze gekweekte hommels, in eerste instantie gescreend en vrij van parasieten nog meer besmet raken met deze parasieten dan hun wilde soortgenoten, kunnen deze laatste opnieuw besmet worden (“spillback principe”). Hoewel we dit laatste nog niet konden bewijzen, tonen we hiermee wel aan dat ten minste één belangrijke ziekte, *C. bombi* hiertoe zou kunnen bijdragen en in dit hoofdstuk wordt dit mechanisme verder gekaderd.

In een tweede deel van het proefschrift bespreken we de impact van *Apis mellifera*, de meest gebruikte gedomesticeerde bestuivers wereldwijd, op wilde bijen. De centrale onderzoeksvraag was: kan de dichtheid van bijenstanden een invloed hebben op verschillende onderdelen van sympatrische wilde bijen ecologie? We bespreken hierbij spillover van honingbij-geassocieerde ziektes en hoe ze voedselconcurrentie kunnen veroorzaken in hun natuurlijk verspreidingsgebied.

In **hoofdstuk 5** onderzoeken we eerst de mogelijkheid om gestandaardiseerde gekweekte hommelnesten van *B. terrestris* als een biotoets te gaan gebruiken. Dit doen we door middel van het bepalen van de correlatie tussen nestontwikkeling en kwaliteitparameters van het landschap. We tonen aan dat 'biomassatoename' hierbij een goede parameter is om nest fitness te gaan scoren.

In het licht van nieuwe ziektes en hun mogelijke koppeling met de verspreiding ervan, onderzoeken we in **hoofdstuk 6** enkele recent beschreven Apoidae-virussen binnen bestuiver netwerken en in relatie tot bijenstand dichtheden. We beschrijven ook voor het eerst Bee Macula-like virus (BeeMLV; voorheen VdMLV) en Lake Sinai virus (LSV) in meerdere wilde gastheren van hommels, namelijk *Bombus pascuorum*, *Bombus lapidarius* en *Bombus pratorum*.

Terwijl we in het vorige hoofdstuk ons hebben gericht op nieuwe ziektes, onderzoeken we in **hoofdstuk 7** de gevolgen van bijenstand dichtheden en de verspreiding van gevestigde ziekten op sympatrische wilde bijen. Daarnaast onderzoeken we ook hoe deze honingbijstanden kunnen concurreren met wilde hommels voor voedsel, waarbij we gebruik maken van de biotoets uit hoofdstuk 5. Kortom zullen we meerdere interacties van gedomesticeerde honingbijen bespreken, zowel naar ziektes (Eng: "top-down drivers") als voedselcompetitie (Eng: "bottom-up drivers"), en hun impact op de rijkheid en diversiteit van sympatrische wilde bijengemeenschappen in een matrix van antropogene landschappen in Vlaanderen (Noord-België). Door het combineren van verschillende proefopzetten tonen we meerdere negatieve correlaties van bijenstand dichtheid met sympatrische niet-*Apis* bijen aan, zowel voor de concurrentie van het voedsel, hogere prevalentie van honingbij-geassocieerde ziekten (*Apicystis bombi* en virussen met inbegrip van Sacbrood virus en AKI-complex), en lagere aanwezigheid van wilde bijen. Onze resultaten tonen aan dat op plaatsen met veel honingbijkasten gelijktijdig op meerdere niveaus van wilde bijen negatief wordt ingespeeld.

Het laatste **hoofdstuk 8** geeft tenslotte de algemene conclusies van dit proefschrift. Een synthese wordt gegeven, startend met de darmmicrobiota van hommels. In een eerste deel van dit proefschrift toonden we aan dat gekweekte *B. terrestris* hommels een deelverzameling hebben van deze teruggevonden in wilde soortgenoten, wat we verarmde of "bottleneck" microbiota noemden. We toonden vervolgens aan dat deze bottleneck microbiota stabiel blijft onder gekweekte omstandigheden, maar dat deze compleet veranderen na 6 weken in open veld. Terwijl we een grote opname van Enterobactriaceae aantoonden, zagen we ook dat deze opgenomen darmpathogenen niet exotisch zijn en overeenstemmen met deze in de natuurlijke omgeving. Nochthans kan zo een veranderde microbiota zijn impact hebben en dit wordt verder bediscussieert in dit hoofdstuk. In een tweede deel onderzochten we voornamelijk de impact van honingbijen op wilde bijen. We toonden een significante hogere prevalentie aan van honingbijgeassocieerde virussen en parasieten in wilde hommels verzameld in de onmiddellijke omgeving van imkerijen. Daarnaast toonden we ook een competitief effect aan met wilde hommels gezien hun nesten minder groot werden ten opzichte van nesten verderaf van imkerijen. Naast deze impact op ecologische "neerwaartse" (i.e. parasieten, virussen) en "opwaartse" (i.e. competitie) processen, toonden we ook een algemeen negatief effect aan van imkerijen op het aantal wilde bijen. Deze belangrijke resultaten worden verder bediscussieert in hoofdstuk 8. Tot slot bespreken we mogelijke beheermaatregelen voor de imkerij. In het bijzonder adviseren we 1) een betere en bredere controle op de prevalentie van ziektes in bijenkasten/standen, 2) het beperken/reguleren van de mobiliteit binnen de sector en 3) het opstellen van een soort 'foerageerkaarten' waarbij voedselaanbod en maximale plaatsingsdichtheid van honingbijkasten worden uitgewerkt, om het evenwicht tussen aanwezige vedselplanten en pollinatoren te gaan beheren.

Curriculum vitae

PERSONAL

Laurian Parmentier was born in Ghent, Belgium on August 27th in 1982. He obtained his high school degree in Sciences at H.-Hartcollege, Waregem in 2000. In 2004 he obtained his Master degree in Applied Engineering in Biochemistry, cum laude, at University College Ghent dept. PIH, Kortrijk and he continued his studies to obtain his Master of Science in Bioscience Engineering in 2006 and MaNaMa in Teaching in 2007 at the University of Ghent. During his Master thesis he investigated on applications of arthropods in the aquaculture (enrichment of *Artemia* sp.) and biological agriculture (rearing on food substitutes of *Macrolophus* sp. as biocontrol agent).

After some years working in the private sector as innovation engineer and later as head of an air monitoring department, he returned to the academic world to start his PhD. In April 2012 he began his PhD research in the field of entomology at the Lab of Agrozoology within the Department of Crop Protection at the faculty of Bioscience Engineering under the supervision of Prof. dr. ir. Guy Smagghe and dr. Ivan Meeus. During his PhD research, he investigated multiple parameters of reared bumblebees and domesticated honeybees, including gut microbial stability and prevalence of diseases as well as their impact on ecological drivers of wild bees when interfering in their natural environment. His results were and will be published in international journals.

WORK EXPERIENCE

2012-2016	Researcher at Ghent University Subject: The impact of domesticated honeybees and reared bumblebees on ecological drivers of sympatric wild congeners Place : Laboratory of Agrozoology, Department of Crop protection, Faculty of Bioscience Engineering, Ghent University. Coupure Links, 653, 9000 Ghent.
2009-2012	Head of Air Monitoring department at Eurofins nv. Place: Venecoweg 5, 9810 Nazareth
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SCIENTIFIC OUTPUT

A1 peer reviewed publications

Parmentier L., Meeus I., de Graaf D.C., Smagghe G. 2016. *Varroa destructor* Macula-like, Lake Sinai and other new RNA viruses in wild bumblebee hosts (*Bombus pascuorum*, *Bombus lapidarius* and *Bombus pratorum*). *Journal Of Invertebrate Pathology* 134: 6-11

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