Anamın əziz xatirəsinə.

Dedicated to the loving memory of my beloved mother Agida Mammadova (1951-2011).

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Potential for spillover of protozoan parasites from domesticated honeybees and reared bumblebees towards wild bumblebees

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Potentieel voor overdracht van protozoaire ziektes van gedomesticeerde honingbijen en gekweekte hommels naar wilde hommels.

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Cover photo:
On top: *Bombus terrestris* and *Apis mellifera* on *Hylotelephium spectabile.* On the next raws from left to right: *Bombus dahlbomii*, *Bombus terrestris*, *Bombus pascuorum* and *Apis mellifera* on *Cirsium vulgare.*

Contents

Scope and objectives of the study

Pollination is a valuable service, mainly provided by bees, within the ecosystem. Bumblebees, a divers genus within the clade *Anthophila* (bees), are major pollinators of many crops and wild plants in the world. There is considerable evidence that many bumblebee species have shown dramatic declines in recent years. Awareness of danger for declines of pollinators is increasing (Potts et al., 2010; Grunewald 2010). These declines may be ascribed to a range of factors, including the intensification of agriculture, introduction of exotic species, and changes in climatic conditions (Williams 1985; Osborne and Corbet 1994; Goulson 2003; Carvell *et al.* 2006; Rasmont *et al.* 2006). In several studies, range reduction and decline in relative abundance of *Bombus* species have been reported to be associated with pathogens (Colla and Packer, 2008; Grixti *et al*., 2009; Cameron *et al*., 2011).

Trading practices of managed bees, domesticated honeybees or reared bumblebees harbor the intrinsic risk to spread infectious diseases to wildlife. In short, managed bees may act as a parasite reservoir for originally sympatric populations or allopatric populations of wild bees (Meeus *et al*., 2011). Small or declining populations are particularly challenged when the reservoir host repeatedly spills over parasites, leading toward multiple disease outbreaks that, in the worst case, might drive already vulnerable or unmanaged populations to extinction. Therefore in order to effectively conserve wildlife, we need to understand the role of managed bees in parasite spillover, which is the key focus of this dissertation.

Chapter 1 describes the importance of bumblebees (*Bombus* sp.) as key pollinators in natural and agricultural environments. Special interest goes to the plight of bumblebee populations and the role parasites can play in this. Specifically, protozoan parasites and their interaction with the host will be discussed within a multi-host reality. This chapter ends with the current knowledge on parasite spillover and its implication for bumblebee conservation.

Chapter 2 aims to identify the prevalence of parasites in managed bees. Both domesticated honeybees (the western honeybee or European honeybee, *Apis mellifera*) and commercially mass-reared bumblebees (the buff-tailed bumblebee or large earth bumblebee, *Bombus terrestris*) will be screened as these bees undergo anthropogenic transports and thereby harbor the potential to disrupt natural host parasite associations. In addition, the intrinsic ability of a

parasite to perform horizontal transmission will be studied as being a second important factor in parasite spillover.

Chapter 3 consists of two parts. The first part depicts the development of a molecular tool for haplotyping *Apicystis bombi* that is a Neogregarine parasite of bumblebees and which was identified in chapter 2 as an important parasite. In the second part of chapter 3, this molecular tool will then be used to study the *A. bombi* population dynamics within Argentina. The national parks of Patagonia in Argentina are an ideal location to study parasite spillover in relation with the decline of the biggest bumblebee in the world and the only bumblebee native in southern South America, namely the giant bumblebee or *Bombus dahlbomii*. Indeed important drivers of bumblebee decline as agricultural intensification and loss of habitat, are not present in these national parks. In short, the aim of this part was to haplotype different *A. bombi* specimens from different pollinators (bumblebees and honeybees) and locations (Europe, South and Central America) in order to identify the founder population of the *A. bombi* in Argentina and so to verify if pathogen spillover could have occurred.

In **chapter 4**, the protozoan prevalence was investigated within different pollination networks and more specifically the role of the presence of domesticated honeybees within these networks. This study was done in different locations in Flanders (Belgium) with use of the common bumblebee, the common carder bee or *Bombus pascuorum*. A prevalence study focusing on *A. bombi*, that is a shared parasite between honeybees and bumblebees, was performed to identify if honeybees could act as a reservoir population for this parasite.

The final **chapter 5** presents the general conclusions of this thesis. The focus will be on the results of the experiments with mass-reared bumblebees and with domesticated honeybees and their natural host parasites assemblies in wild bee communities. The risks for spillover of parasites by managed bees will be evaluated based on the obtained results. Subsequently, the main areas which need more investigation will be identified to prevent the spread of parasites which harms pollinator communities. The need for new legislative changes in managed bee transportation, empirical studies on pathogenicity and identification of the potential origin of the multi-host parasite *A. bombi* will be discussed.

Chapter I: Introduction

1. Wild bees and their role in preserving biodiversity of nature

A very broad range of wild and managed insect species are responsible for pollination. There are about 20,000 different bee species worldwide (Ascher *et al*., 2014). It is this biodiversity of bees which performs pollination of agricultural crops. Insects pollinate about 80% of all plant species in the world, including most fruits (Calderone *et al*., 2012), many vegetables and some biofuel crops. Bees that completely depend on pollen and nectar of flowers, form the major clade of pollinating insects. For example, most of the tree species of tropical forests are insect-pollinated (Bawa *et al*., 1990; Kato *et al*., 2008). Pollinators contribute to the diversity of wild plant species, habitat and wildlife in general. Therefore, the loss of bees results in a dramatic decline of natural flora. Thus preservation of bees is a key in the general wild life and habitat conservation.

A well-known 'managed' pollinator is the honeybee since this species is often considered as the most important crop pollinator due to its high numbers. However, most crops are better pollinated by wild bees. Indeed a meta-analysis confirmed that the presence of wild bees outperforms honeybees, resulting in higher yields of bee-pollinated crops (Garibaldi *et al*., 2013). Accordingly, fruit, vegetable or seed production of 87 of the leading global food crops is depending on pollinators (Klein *et al*. 2007).

2. Bumblebees and their pollination service

Bumblebees are not only ecologically important because they are prevalent wild pollinators within native plant communities throughout temperate ecosystems, but they also have a high economic value. Indeed bumblebees have become a valuable commercial pollinator in intensive farming (Rasmont *et al*., 2008). Currently, their pollination services in agriculture is worth billions of euros annually (Winter *et al*., 2006; Goulson, 2003).

Bumblebees (*Bombus* sp.) represent a group of about 250 species across the world, performing an important pollination service (Williams *et al*., 2009). Often they are generalist pollinators within pollination networks of flowering plants. Simulation revealed that removal of individual generalist pollinators from pollination networks resulted in a steep decline in plant species diversity (Waser *et al*., 1996; Memmott *et al*., 2004).

Aside from their ecological importance bumblebees are also major pollinators of many crops. For this reason, a Flemish pioneer (veterinary Roland De Jonghe) started the indoor-rearing of buff-tailed bumblebees in 1988. These reared pollinators were an instant success in the pollination of greenhouse tomatoes in the Netherlands and Belgium (Velthuis *et al*., 2006). Subsequently, this practice expanded to other valuable greenhouse crops. An approximate estimation of the turnover in bumblebee rearing industry is around €55 million. The crop value of bumblebee-pollinated tomatoes is €12,000 in Europe alone (Velthuis *et al*., 2006). Currently, open field pollination is a new market for bumblebee breeders. To date, this market is still expanding due to the current losses of domesticated honeybees (Potts *et al*., 2010).

Although initially different companies started only with the Palearctic buff-tailed bumblebee (*Bombus terrestris*), now different species are reared and exported for pollination worldwide. Hence, *B. terrestris* became an invasive species in different parts of the world (see Figure1.1. and 1.3. for details). Therefore breeding companies often use native bumblebee species. In North America native *Bombus occidentalis* and *Bombus impatiens* are widely used species for pollination services (Whittington & Winston 2004). In East Asia (Japan, South Korea), *Bombus ignitus* is well cultivated whereas in China both *B. ignitus* and *Bombus lucorum* are used as a native commercial pollinator (Mah *et al*., 2001; An *et al*., 2007).

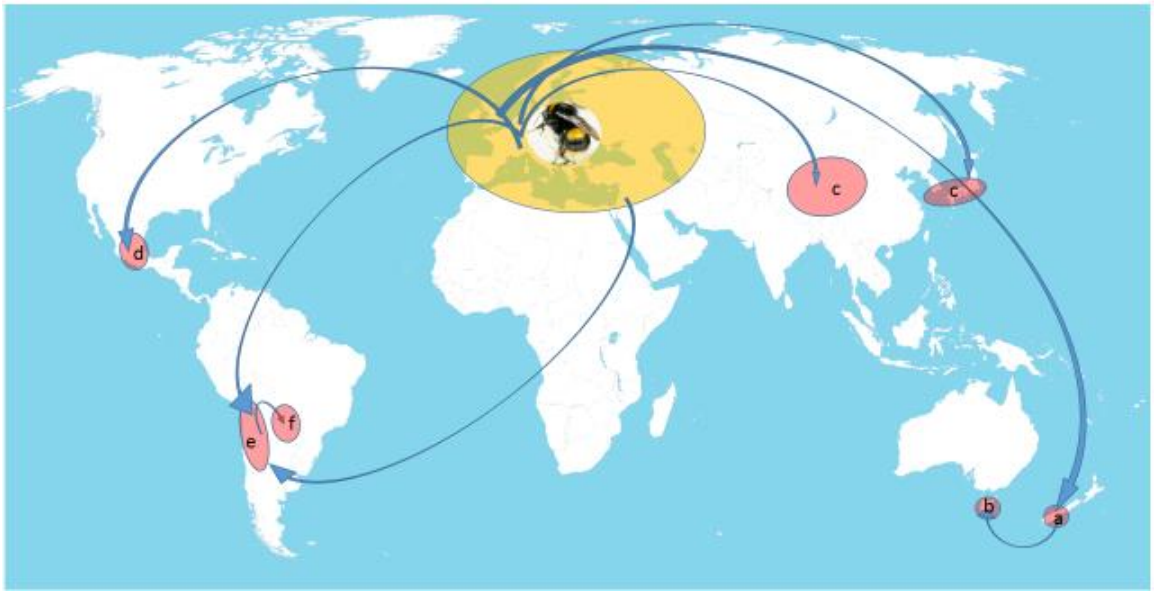


Figure 1.1. Worldwide anthropogenic routes of *Bombus terrestris.* The yellow color indicates for natural habitat of *B. terrestris.* Red colored sites are places where *B. terrestris* was artificially introduced. For detailed border information see Figure 1.3.
a) To New Zealand in 1885 and 1906, there was the intentional introduction of bumblebee queens collected in England (Hopkins, 1914).
b) First discovery in Tasmania on 19 February 1992 (Schmid-Hempel *et al*., 2007). .
c) Since 1991, *B. terrestris* has been imported and has become an essential agricultural pollinator in Asia (Dafni & Shmida, 1996)
d) It was allowed into Mexico in 1995 and 1996 without the knowledge of the U.S. or Canadian regulatory agencies (Flanders *et al*., 2003).
e) The importation to Chile was authorized in 1998 (Ruz, 2002).
f) In early March 2006, it was first recorded in the northwestern area of Argentina's Patagonia region (Torretaa *et al*., 2006) and still it is expanding.

Members of the *Solanaceae* such as tomatoes benefit from pollination of bumblebees (Vergara *et al*., 2012; Dogterom *et al*., 1998.). They are also excellent pollinators of the members of *Ericaceae* such as cranberries and blueberries (Da Silveira *et al*., 2011; Zajmi *et al*., 2011). Bumblebees also contribute to the production of winter oilseed rape *(Brassica napus L.)* (Stanley *et al*., 2013.). Similarly, bumblebees have been reported as good pollinators for alfalfa (*Medicago sativa*), apple orchards (*Pyrus/Malus*), watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*) (Cecen *et al*., 2008; Thomson *et al*., 2001; Stanghellini *et al*., 1997, 1998) (Table 1.1.)

Table 1.1. *Bombus* spp. used for pollination services worldwide (Vergara *et al*., 2012; Dogterom *et al*., 1998; Da Silveira *et al*., 2011; Zajmi *et al*., 2011; Cecen *et al*., 2008; Thomson *et al*., 2001; Stanghellini *et al*., 1997, 1998; Williams *et al*., 2012)

Species	Distribution	Pollination service
Bombus terrestris	Europe, West Asia, East Asia, Coastal Africa, South America.	Greenhouse members of the *Solanacea* such as tomatoes, sweet pepper, eggplant; strawberry, blueberry, red & black currant, blackberry, cranberry, gooseberry, raspberry, apple, pear, plum, cherry, apricot, peach, kiwi, almond and other crops
Bombus ruderatus	Europe, South America (New Zealand, Chili, Argentina)	Red clover
Bombus impatiens	North America (USA, Canada, Mexico)	Greenhouse tomatoes, eggplants, alfalfa and clover; blueberry
Bombus occidentalis	North America (USA, Canada, Mexico)	Greenhouse tomatoes, apple tree
Bombus ignitus	East Asia (China, South Korea, Japan)	tomatoes, eggplants, alfalfa and clover
Bombus lucorum	East Asia (China, South Korea, Japan)	provides pollination for crops in winter greenhouse, strawberry
Bombus hypocrita	East Asia (China, Japan)	Greenhouse tomatoes
Bombus patagiatus	East Asia (China, Japan)	Greenhouse tomatoes

2.1. Bumblebee biology

Life cycle

Different *Bombus* spp. have common features in some aspects of their biology and main life cycle, but they are very different in others. The annual life cycle starts with a spring queen emerging from overwinter hibernation (Figure 1.2.). After feeding on flowers and drinking nectar to gain energy, she develops her ovaries and starts to search for the appropriate nesting place. Having found a suitable place, the queen adjusts nest material to make a small chamber. Then she goes out to collect nectar and pollen which she brings back to the nest in pollen baskets on her hind legs. She moulds pollen into a mass that forms the base of the egg clump. She uses pollen and wax (extruded of between the plates from her abdomen) to cluster the pot shaped structures. After achieving a convenient amount of the egg clump, she constructs honey pots for nectar storage. The queen keeps the eggs warm by shivering her muscles and sitting on her wax nest. A brooding queen can keep her body temperature at about 30-35°C and maintain her egg clump at 25°C despite low outside temperatures. After 4-6 days, the grub-like larvae emerge from the eggs. The

emerged larvae are fed on pollen and nectar collected by the queen. Species differ in the way they feed pollen based on the manner of the feeding process.

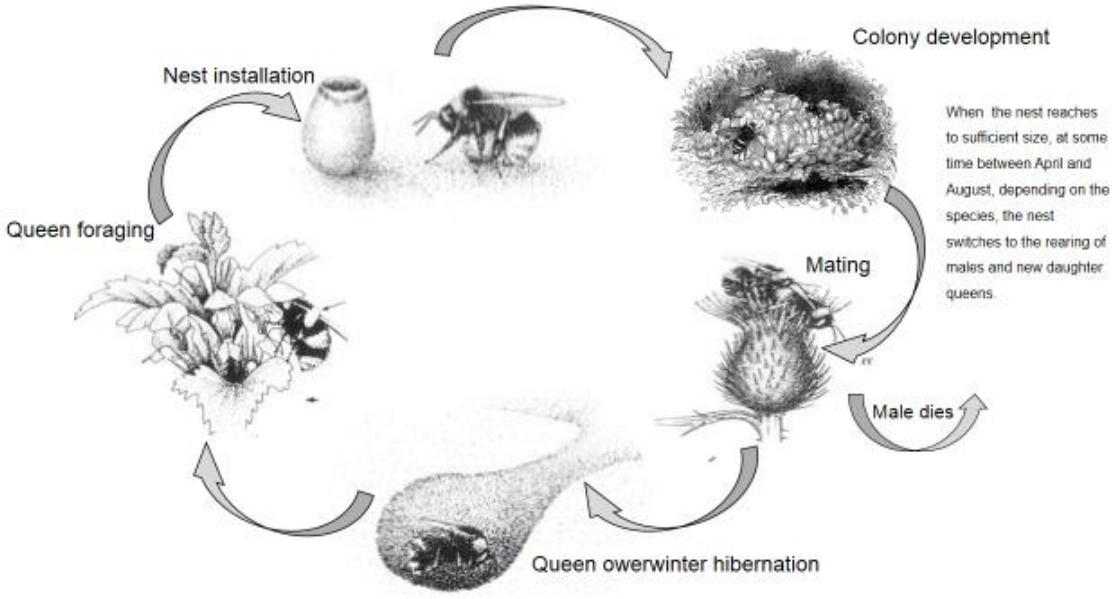


Figure 1.2. Annual life cycle of a bumblebee colony (Goulson, 2010)

"Pocket makers" (such as *Bombus pascuorum, Bombus hortorum*) construct waxen pockets near the base of the larval chamber or brood clump. Into these, the queen deposits the diet for the new emerged larvae by regurgitating a nectar/pollen mixture through a temporary hole in the wax envelope. The larvae feed from the resulting mass (Oliver *et al*., 2011).

In contrast, foragers of "pollen storers" (such as *B. terrestris, Bombus ruderatus, B. lucorum*) deposit the collected pollen in storage pots, and from there, the queen/housekeeping bees bring the pollen to the brood cells and feed it directly to the larvae.

The larvae progress through a series of larval moults in their cell. Later, each larva spins a delicate cocoon chamber wherein the larva develops. The larval development usually

consists of 4 instars. After 10-20 days a much tougher, neater, cylindrical cocoon is formed, wherin the larva pupates. The queen takes care of the nest by scraping the pollen/wax mixture from the pupal cocoons. She reuses it to construct new chambers in which she lays her next batch of eggs. After about two weeks, infertile adult workers, also called callow workers, emerge from the pupae. The exoskeleton and wings of callow workers are still relatively soft and lightly pigmented. The worker acquires her full colors and fluffy appearance after a period of 1-2 days. Wings harden within a period of about 2 days. The emerged workers soon take care of the nest and once their numbers are high enough, then they take over the foraging duties of the queen. From now onwards the queen remains within the nest and devotes herself to egg-laying and brood care. Workers of the colony may vary widely in size and number probably because of differences in received amount of the food or location of larva stadia within the nest (Oliver *et al*., 2011).

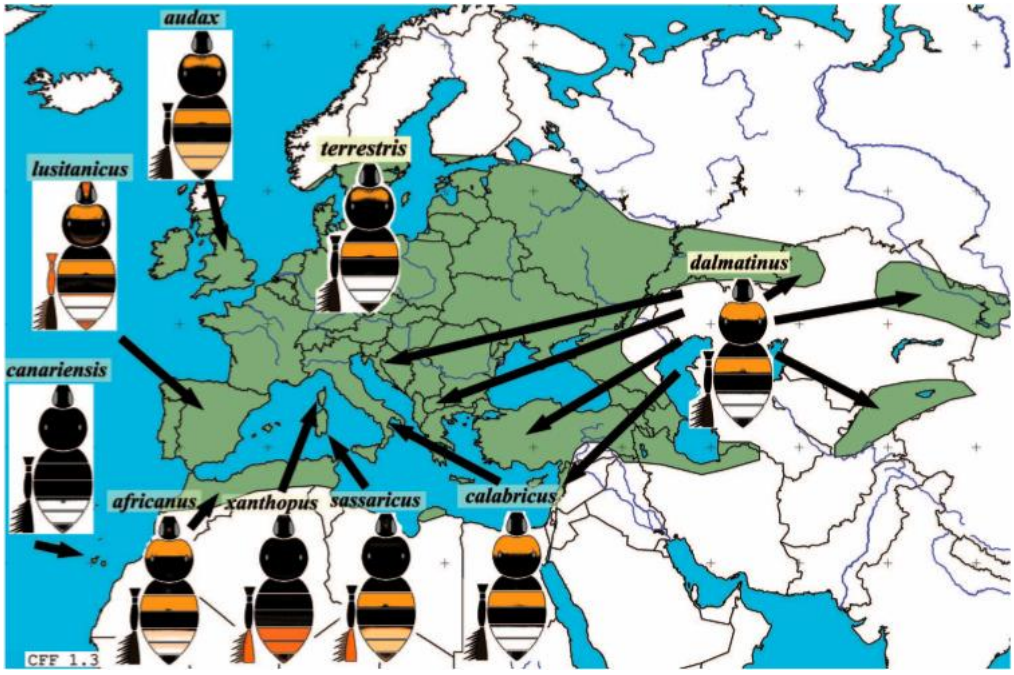
Explained above is the first phase of the colony life cycle, which is called the pre-competition phase (Honk & Hogeweg, 1981). In a second phase, the queen switches to the production of sexuals. She starts to lay fertile off-spring males (haploid) and fertile daughter (diploid) queens in order to allow reproduction of the colony. What exactly triggers this change to sexual production is not well understood. Adequate food stores, chemical cues, nest temperature stability and bee density in the nest are probably all factors. In bees, unfertilized eggs give rise to males and fertilized eggs produce females (queens and workers). After this switch point, the nest enters in the 'competition phase'. Indeed, workers compete with the queen and with each other for male parenthood, as infertile workers can lay unfertilized eggs which develop into haploid bees, being males (Honk & Hogeweg, 1981).

The new males leave the nest for mating. They only forage for themselves. While the new queens fly outside for mating as well but unlike males they often return to the nest at night but they do not normally contribute to provision the colony. She builds up her fat body, and as an additional food store she fills her honey stomach with thick nectar. She then seek a site wherein to dig a burrow for hibernation. Seasonal timing of events in the colony cycle varies with species geographical area and with climate conditions in a particular year (Goulson, 2003).

2.2. *Bombus terrestris*

Bombus terrestris (terrestris – from the Latin "terra" means "earthly") (Figure 1.4.a.) is a heavily built, hairy bee with broad black and golden yellow bands (Oliver *et al*., 2011). In most species, the queen and the worker have the same white colored tail, although in Great Britain, *B. terrestris* (*audax*) is named after the queen's buff-colored 'tail'. Hence, their English name is buff-tailed bumblebee. Indeed there are nine different subspecies of *B. terrestris,* inhabiting the West-Palaearctic region, present within Europe (Figure 1.3.) (Rasmont *et al*., 2008). Especially, *B. terrestris terrestris* and *B. terrestris dalmatinus* have the ability to produce large colonies and to adapt quite well to artificial conditions. Therefore, they were the first species being reared indoors and commercially sold in different world continents. It is a common generalist, pollinating more than 300 flower species in France and Belgium (Rasmont *et al*., 2008). Since its first domestication in 1988, it is widely used as a standard pollinator for tomato greenhouses in Europe. The commercial success of *B. terrestris* caused it to become an invasive species in the world (Figure 1.1.). However, their first artificial extension happened before the commercialization of bumblebee breeding, namely in 1885 when the British subspecies *B. terrestris audax* was successfully introduced from England into New Zeeland to improve the pollination of the forage plant *Trifolium pratense* (Hopkins, 1914)

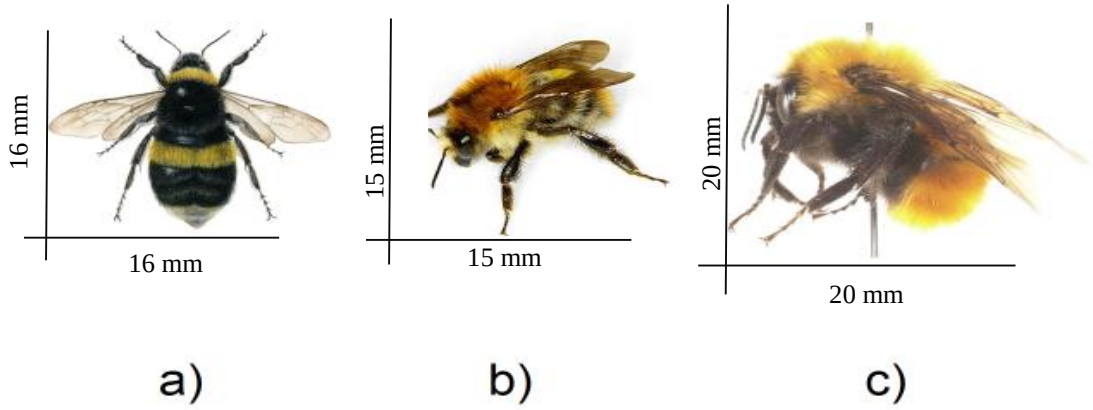
Figure1.3. Natural distribution area of *Bombus terrestris* subspecies (Rasmont *et al.*, 2008)



2.3. *Bombus pascuorum*

Bombus pascuorum is common and has a distinctively ginger colored thorax (Figure 1.4.b.) (Oliver *et al*., 2011). Thereby, it is easily distinguishable from other species in Belgium. Only *B. hypnorum* has the same thorax, but opposed to *B. pascuorum*, it has a characteristic white tail. It can be mismatched with other species, having similar color patterns within the widely distributed West-Palaearctic zone. The hairs of the abdomen of *B. pascuorum* are lighter in color than those of the thorax, and there are also a few black hairs. In a very sunny summer the hairs of older bees can become faded and then appear beige in color. *B. pascuorum* is a long-tongued bumblebee and so potentially a valuable pollinators of deep flowered plants and crops. Workers of *B. pascuorum* are polylectic, but tend to visit flowers of the *Fabaceae*, *Scrophulariacae*, *Lamiaceae* and red-flowered *Asteraceae. B. pascuorum* is known as a carder bee, more specifically the common carder bee, because of its habit of combing material as collected around the nest (carding) to create a covering for the cells containing the larvae. Colonies vary in size and can contain up to about 100 workers (Oliver *et al*., 2011; Goulson, 2003).

Figure 1.4. Photos of a) European buff-tailed *Bombus terrestris;* b) brown-banded carder bee *Bombus pascuorum*, and c) South American orange *Bombus dahlbomii*.



2.4. *Bombus dahlbomii*

Bombus dahlbomii (Figure 1.4.c.) is the largest bumblebee natively distributed in Chile and Argentina (Abrahamovich *et al*., 2004). Of the 250 species of bumblebees, a total of 24 species has a natural habitat in South America (Schmid-Hempel *et al*., 2014). *B. dahlbomii* has a long tongue and tends to feed on deep flowers. It is an important pollinator for local ecosystems in the temperate forests of South America (Murúa *et al*., 2011; Morales *et al*., 2005). It has been suggested that the bumblebee *B. dahlbomii* is under threat by the arrival of the exotic European *B. terrestris* and *B. ruderatus* (Morales *et al*., 2013). This will be discussed in chapter 3.

3. Plight of bumblebee populations.

3.1. Current status of pollinator declines

Pollination is a valuable ecosystem service mainly provided by bees. But there are growing concerns about the declining of both domesticated and wild pollinators worldwide. Declines in the health and populations of bees and other pollinators threaten natural pollination worldwide (Steffan-Dewenter *et al*., 2005). Along with the mounting evidence of the loss of pollinators, the scientific and public awareness increases recognizing the dangers associated with it (Potts *et al*., 2010; Grunewald, 2010).

There are reliable data about the decline of bumblebees, particularly in regions such as Western Europe and North America (Goulson 2008; Grixti *et al*., 2009; Cameron *et al*., 2011; Carvalheiro *et al.*, 2013). Especially in Europe, the decline of bumblebees has been well documented over the past 60 years (Goulson *et al*., 2008). For other wild pollinators, the data is more scare. A good example is the decline of wild pollinator services in Great Britain and the Netherlands, as has been documented by Biesmeijer *et al*. (2006). These authors suggested that specialized species tend to decline more than generalist, fast developing, and more mobile species. The domesticated European honeybee (*Apis mellifera*) also endures losses, and studies showed that the population abundance and its ecological importance in plant reproduction have decreased in different areas of the world (Ellis, 2010, 2012). The recently discovered "Colony Collapse Disorder" (CCD) devastates colonies, leaving hives with a complete lack of bees. CCD is the name given to the mysterious decline of honeybee populations and that began in 2006 (Neumann *et al*., 2010). Aside from CCD, Potts *et al*. (2010) reported consistent declines in colony numbers in central Europe between 1985 and 2005.

It looks that most genera of bees endure losses, although some drivers of losses are surely shared, different drivers act differently among different bee species. If we look at the situations for *Bombus* sp. in detail, we can report a worldwide and drastic decline of many bumblebee species, while some remain abundant (Rasmont, 1995; Westrich, 1996; Goulson, 2003; Sarospataki *et al*., 2005; Goulson *et al*., 2006; Fitzpatrick *et al*., 2007). For example, Since the 1950s in the UK, 3 of the 25 native species have gone extinct, with

another 8 suffering major range contractions (Goulson *et al*., 2008; Williams, 1982; Williams *et al*., 2009).

3.2. Causes of pollinator declines

The causes for the decline of bumblebee diversity are not fully understood, but have been attributed to forage and habitat loss, intensification of agriculture (Williams, 1985; Osborne & Corbet, 1994; Goulson 2003; Carvell *et al*., 2006; Rasmont *et al*., 2006; Mayer *et al*., 2012; Roulston *et al.*, 2011), pesticide use (Williams 1985; Thompson & Hunt 1999; Rasmont *et al*., 2006) or the impact of invasive congeners (Meeus *et al*., 2011).

A contribution factor, making bumblebees more vulnerable toward the above mentioned changing environmental conditions, can be low genetic diversity and inbreeding. Although not proven that this is a general mechanism, Maebe *et al*. (2012) showed that before the drastic decline of *Bombus veteranus* in Belgium (1895-1923), this species already showed inbreeds. Later on, namely after the 1950 and concurrent with agricultural intensification and habitat loss, this once widely distributed species got lost, and to date it is only found in one specific region in Wallonia (Torgny, the province of Luxemburg). It is speculated that the low genetic diversity of *B. veteranus* played an indirect role in future decline of this species (Maebe *et al*., 2013).

A clear causal link between the actual decline of bumblebees and the occurring stressors is difficult to make. Although the above mentioned stressors all have been proven to impact bumblebee health, no single driver has been assigned as the smoking gun. Different stressors influence pollinator populations and interact with each other. Furthermore the presence, impact and interactions of certain stressors on bumblebee populations are location dependent. This being said, habitat or forage loss and the use of agrochemicals are recognized as important drivers of bee losses, especially in agricultural landscapes.

Goulson *et al.* (2008) depicted the importance of the loss of uncultured land, in the modern agriculture after 1950, as an importance driver of forage loss for bumblebees. Second, the use of neonicotinoids and their impact on bee populations have drawn the attention. These insecticides can be found in the main food sources of bees; i.e. from 1 to 23 ppb in nectar

and from 1 to 66 ppb in pollen (Goulson, 2013). Different studies have been performed using field realistic concentrations, showing a reduced fecundity of the queen and reduced bumblebee learning, foraging and homing ability of the workers (Whitehorn *et al*., 2012; Gill *et al*., 2012; Goulson, 2013; Feltham *et al*., 2014).

With the commercialization of bumblebee breeding, anthropogenic movement of bumblebee species happened within and outside of their natural range. These transports can result in a competition with native species. Not only resource and nest competitions can take place, but sometimes the invading species can have a secret weapon. Indeed if reared bumblebee colonies harbor parasites, they may act as parasite reservoirs from which the parasites can spillover to wild bee populations. Different types of parasite spillover can be defined, each with a different risk associated with it, as reviewed by Meeus *et al*. (2011). In short, spillover to sympatric populations of their own species or other species is the least severe, while spillover towards allopatric populations of their own species, and other species, harbors a higher intrinsic risk to cause diversity loss within native bee populations (Meeus *et al*., 2011).

3.3. Spillover from managed bees to wild life.

Parasite outbreaks often occur when anthropogenic change brings wild life into increased contacts with domesticated animals. Transmission of infectious parasites from reservoir populations (usually domestic or commercial) to sympatric wild life populations is known as a "spillover" (Daszak *et al*., 2000). Spillover can cause the emergence of diseases in wild life populations and this in turn can result in a rapid decline in the new host populations. Spillover has a particular threat to endangered species, because the presence of infected reservoir hosts can lower the parasite's threshold density, leading to local (population) extinction.

Are these kinds of spillover events actually realistic? For some sources yes. A good example is reported in 2006 by Colla *et al*. (2006) that commercially reared bumblebees have a higher prevalence of various parasites than their wild counterparts. Since then it should be remarked that a lot has changed and improved. However, several intestinal protozoa like *Crithidia bombi*, *Nosema bombi* and *Apicystis bombi* have still been reported

recently in some reared colonies (Murray *et al*., 2013; Graystock *et al*., 2013). The presence of protozoan is not surprising, as commercial rearing facilities often provide ideal situations for increases in abundance of parasites. Provision of hosts with enough amounts of food and best environmental conditions cause commercial host colonies more likely to survive and reproduce despite high parasite loads (Meeus *et al*., 2011; Brown *et al*., 2000). In North America where European bees have never been introduced, it was hypothesized that the reared native American species *Bombus occidentalis* could harbor European protozoa when it was initially reared in Europe (Cordes *et al*., 2012; Colla *et al*., 2006; Winter *et al*., 2006; Goulson *et al*., 2008).

There are circumstantial evidences that back up the claims about spillover as have been stated above, but none of them are indisputable (Altizer *et al*., 2003; Meeus *et al*., 2011; Arbetman *et al*., 2012). For the North American situation, Cameron *et al*. (2011) reported that higher parasite prevalence and reduced genetic diversity are considered to be realistic predictors of the alarming patterns of the decline of bumblebee species. However, a correlation between spillover and decline is not yet presented. For more details on the decline of *B. dahlbomii* in South America and its relation with parasite spillover, we refer to Chapter 3 in this thesis. The potential spread of parasites from reared to wild bumblebees is investigated by Otterstatter *et al*. (2008) and Colla *et al*. (2006). They monitored wild bumblebee populations near greenhouses for evidence of parasite spillover and found that spillover has allowed *C. bombi* to invade several wild bumblebee species near greenhouses. Although the experimental setup was too small to draw definite conclusion, they were among the pioneers to study these kind of spillovers. In a more extensive study by Murray *et al*. (2013), the highest prevalence of *Crithidia* was observed within 2 km of greenhouses and the probability of infection declined in a host sex- and parasite-specific manner up to 10 km. 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). Therefore, we recommend a prohibition of transport of bumblebees outside their allopatric range.

Moreover, not only bumblebee transport is identified as driver of parasite spillover. Fűrst *et al*. (2014) suggested an emerging parasite problem in wild pollinators that may be driven by *Apis mellifera*. They found that the prevalence of deformed wing virus (DWV) and the

exotic parasite *Nosema ceranae* in honeybees and bumblebees is linked, because honeybees have a higher DWV prevalence and sympatric bumblebees and honeybees are infected by the same DWV strains. So, these authors concluded that *Apis* honeybees are the likely source of at least one major emerging infectious disease in wild pollinators. Spillover event can also be facilitated by domesticated honeybees, and this risk will be discussed in Chapter 4.

3.4. Threat of spillover

Emergent parasites are one of the major threats to biodiversity, and the transmission of novel species or strains of parasites to native species can be particularly damaging. (Daszak *et al*., 2000). The epidemiology and virulence of the parasite in the new host play an important role on the effect of parasite spillover. Parasite spillover seems most likely to occur when the reservoir host and potential novel host are phylogenetically close (Perlman & Jaenike, 2003), and epidemics or emergent diseases are most likely when the novel host population has little life-history, or behavioral or immunological defense against the parasite (e.g., Rosenkranz *et al*., 2010). The first report about parasite spillover due to the commercial trade in bumblebees was by Goka *et al*. (2001). A comparison of 555 bp sequences of the mitochondrial DNA of the tracheal mite *Locustacarus buchneri*, suggested that native reared colonies of *B. ignitus* possessed the same haplotypes as a European haplotype which was found in imported *B. terrestris* samples. In Ireland, Murray *et al*. (2013) found that an infection of the intestinal protozoa parasites *Nosema bombi* and *Crithidia* can be found at higher levels in conspecifics up to 2 km from greenhouses where commercially reared *B. terrestris* hives were used. These authors found that the prevalence of *Crithidia* within adjacent populations of *B. terrestris* was significantly higher within a distance of 2 km from greenhouses compared to a distance of 10 km. Arbetman *et al*. (2012) suggested that the absence of another protozoan as *Apicystis bombi* in the native bumblebee species and in honeybees collected north of current distribution of *B. terrestris* in Argentina, concurred with the hypothesis of *A. bombi* co-introduction with reared *B. terrestris*. Studies of Goka *et al*. (2006) found that the exchange of haplotypes between European and Japanese bumblebees occurred and spillover was suggested as the cause.

4. Overview of bee parasites

4.1. Protozoa

An expanding interest in parasitic protozoa of bees has spawned many recent studies about host conservation (Arbetman *et al*., 2012; Goulson *et al*., 2012; Plischuk *et al*., 2011; Meeus *et al*., 2010). The name protozoa means "first animals". Protozoa are single-celled eukaryotes that commonly show characteristics usually associated with animals, most notably mobility and heterotrophy (Tanada & Kaya, 1993). They are classified in several phyla. More than 50,000 species have been described so far, with most of which are free-living organisms. Protozoa are found in almost every possible habitat. From described species of Protozoa about 500 are parasites of insects (Tanada & Kaya, 1993; Sleigh, 2003). Protozoans are grouped based on their shape and mobility.

Gregarines have been placed in the phylum *Apicomplexa* and represent a group of protozoans that lack cilia. The phylum *Apicomplexa* possesses an apical complex structure that appears as a conical structure at the tapered end (or the apical end) of the cell and that contains micronemes, polar rings and conoid to help the apicomplexan when invading an animal cell (Tanada and Kaya 1993). The gregarines infect invertebrate hosts. They are divided into two groups, the *eugregarines* and *neogregarines*. The major difference between these groups is that eugregarines do not have a vegetative reproduction in the host, while the neogregarines do (Kreier & Baker, 1987).

Neogregarines undergo multiple divisions after entering the host cells. These divisions are called schizogony or merogony. The resulting "merozoites" spread the infection to other tissues in the host. Ultimately, another division also happens before undergoing sexual reproduction (Canning, 1964). Neogregarines are transmitted via contaminated food or by cannibalism of infected hosts. Some of the better known genera are *Mattesia* in beetles (Lord *et al*., 2007) and moths (Valigurová *et al*., 2006) and *Apicystis bombi* in *Apidae* family, that is one of the parasites damaging bumblebees as will be discussed later.

Another group of protozoans infecting bumblebees, belongs to the genus *Crithidia*. They are flagellate parasites that exclusively parasitize arthropods, mainly insects. They have a thin, firm pellicle (outer covering) or a coating of a jelly-like substance. Most

entomogenous flagellates belong to the family *Trypanosomatidae***.** They belong to the class of kinetoplastea. These parasites are named after one of their most unusual features, namely mitochondrial DNA known as kinetoplast DNA (kDNA). Unlike all other DNA in nature, kDNA comprises a giant network of interlocked DNA rings. It replicates independently lying near the base of the flagellum in certain parasitic protozoans. Transmission between hosts usually happens by ingestion of faeces, blood and plant sap or by cannibalism. Infections in some species can persist through the larval stages into the adult stage of the host (Tanada & Kaya, 1993). The best known trypanosome flagellate, namely *Crithidia bombi*, has a single host cycle, occurring only in insects (Schlüns *et al*., 2010). *C. bombi* is a widespread, chronic gut pathogen of bumblebees that might reduce the fitness of bumblebee queens drastically (Brown *et al*., 2003). Transmission occurs between colonies via shared flower sources (Durrer *et al*., 1994). As reported, the bumblebees get infected by ingestion of infectious faeces, and in turn the whole colonies can get infected via the sharing of flowers and contact with infected animals within the nest (Durrer *et al*., 1994; Otterstatter *et al*., 2007).

4.2. Microsporidia

There is another bumblebee-infecting unicellular eukaryotic organism, namely *Microsporidia*. It was initially considered with the protozoa, but to date it is classified with the fungi. The microsporidia are obligate intracellular parasites with a wide range of hosts. There are many microsporidian parasite genera known to infect insects (Wittner *et al*., 1999; Schmid-Hempel *et al*., 1998; Higes *et al*., 2006). The best-known microsporidia genus is *Nosema. Nosema apis* and *Nosema ceranae* are known to parasitize honeybees (Higes *et al*., 2007), while *Nosema bombi* is described as a bumblebee-specific parasite (Cordes *et al*., 2012). Recently, it was discovered that also *N. ceranae* is able to infect bumblebees. If this dual infection has always been the case or is also a consequence of the emergence of *N. ceranae* in European honeybees needs to be studied further (Plischuk *et al*., 2009; Graystock *et al*., 2013). For *N. bombi,* a clear and different pathology has been described in *B. terrestris* and *B. lucorum* (Rutrecht *et al*., 2008). It is a systemic disease in the buff-tailed bumblebee, causing a significant negative impact on the colony

development and indoor-rearing. The effects on *B. lucorum* were less pronounced, potentially because the shorter life cycle did not result in high enough infective amounts of the parasite (Schmid-Hempel *et al*., 1998; Rutrecht *et al*., 2009). *N. bombi* has also been detected in other *Bombus* spp., probably damaging many others hosts (Whittington *et al*., 2003).

The transmission modes of *Nosema* sp. are believed to mainly rely on horizontal transmission, as they produce dormant long-lived spores. Within bumblebee colonies, it is transmitted via infected workers, contaminating shared food sources such as pollen or nectar. Besides, there is some evidence that it may also be transmitted vertically (Rutrecht *et al*., 2008).

4.3. Spiroplasmas

Spiroplasmas are small, helical, motile eubacteria and descendants of gram positive bacteria and lack a cell wall (Regassa & Gasparich, 2006). Spiroplasmas exploit numerous habitats, but are most often found in association with insects. The transmission appears to occur by fecal contamination from infected insect hosts on flower surfaces (Clark, 1982; Raju *et al*., 1981). *Spiroplasma apis* and *Spiroplasma melliferum* are known as parasites of honeybees. *S. apis* (Mouches *et al*., 1983) has been abundantly detected in honeybees in Southwestern France from hives showing symptoms of May disease (Mouches *et al*., 1982). *S. melliferum* from serogroup I2 (Clark *et al*., 1985) has similar symptoms when fed but it is less intense (Clark, 1977; Mouches *et al*., 1982) and at colony level no productivity losses have been observed (Clark, 1977). In the North American bumblebees *Bombus impatiens* and *Bombus pennsylvanicus*, the respective tissues of hemolymph and intestine were found to be infected with serogroup I2 spiroplasmas (Clark *et al*., 1985). Recently, Meeus *et al*. (2012) reported about the presence of *S. apis* and *S. melliferum* in *Bombus pratorum* and in *Bombus pascuorum*, respectively.

5. *Apicystis bombi*

5.1. Introduction

The neogregarine *Apicystis bombi* was considered a low prevalence parasite of *Bombus* sp., and before our work there were only few report about it. However, there is mounting evidence to date that this parasite is cosmopolitan or became cosmopolitan by anthropogenic transports, making this parasite the target of this dissertation. *A. bombi*, also formerly known as *Mattesia bombi*, was first discovered in Canada in 1974 and then reclassified in 1988 as *A. bombi*. It has been recorded in more than 20 *Bombus* spp. (Lipa *et al*., 1992; Macfarlane *et al*., 1995), including commercially breeding species (Murray *et al*., 2013; Graystock *et al*., 2013) and honeybee hives (Ravoet *et al*., 2013) worldwide.

Taxonomy:

Kingdom: Eucaryota

Phylum: Protozoa

Phylum: Apicomplexa

Class: Conoidasa

Subclass: Gregarinasina

Order: Neogregarinorida

Family: Ophryocystidae

Genus: *Apicystis*

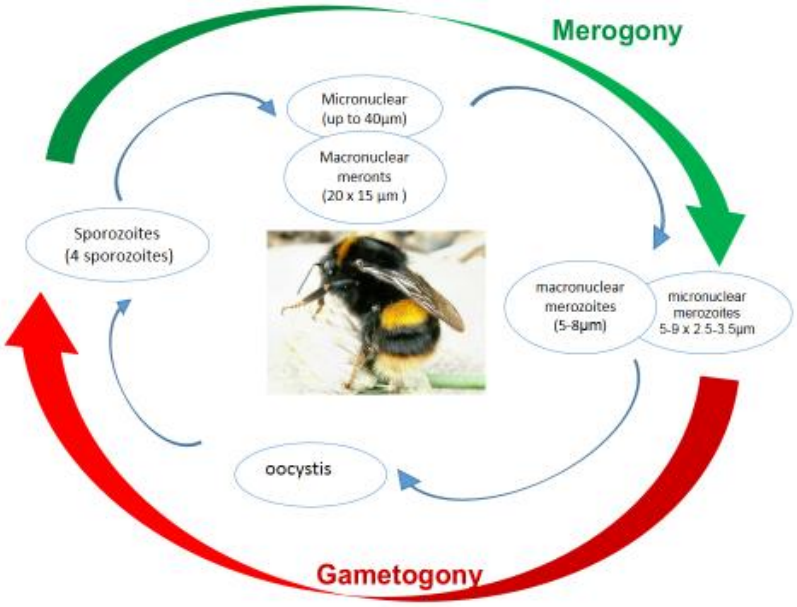
Species: *Apicystis bombi*

5.2. Life cycle

The active stage of gregarine parasite is called a trophozoites, and they may be found within host cells or in the body cavity, body fluids or interstitial spaces between cells. While trophozoites are ideally suited for the parasitic mode of existence, they are not very resistant to external environmental conditions and do not survive long outside of their hosts. For this reason, they develop oocysts, that are thick-walled spores that are able to survive outside the host.

The life cycle of *A. bombi* (Figure 1.5.) starts from sporozoites, that emerge in the intestine from ingested oocysts and penetrate through the midgut wall into the body cavity and infect the fat body cells in which they feed. They grow and undergo merogony (also called schizogony), meaning that their organelles and nucleus multiply. This is is the asexual reproduction of the parasite, resulting in micronuclear meronts of diameters up to 45 µm which contain up to 40 small nuclei, each measuring 1.3 µm. From these the multiple micronuclear merozoites (a motile infective stage) are produced. These are oval or elongated and measure 5-9 x 2.5-3.5 µm. For *A. bombi*, also macronuclear meronts, measuring 20 x 15 µm, have been observed. These give rise to ovoidal or pyriform macronuclear merozoits (gamonts) of 5-8 µm in diameter (Lipa *et al*., 1996). The micro- and macronuclear merozoites enter the sporogony, a sexual and asexual reproduction, in which 4 sporozoites are formed within one oocyst and that is then ready to infect a new host.

Figure 1.5. Life cycle of the protozoan *Apicystis bombi*



5.3. Pathology

A. bombi is thought to have a major impact on infected bumblebee queens by destroying the fat body of the insect due to massive proliferation. It has been claimed that infected spring queen are not able to start up a successful colony, although there is no empirical data on pathogenicity (Schmid-Hempel *et al*., 2001; Macfarlane *et al*., 1995; Rutrecht *et al*., 2008).

6. Dynamics of multihost parasites

6.1. Host-parasite interaction

There are different relationships between organisms, called symbiosis. Indeed symbiosis means that organisms live in an association with one another. In a first approach there are at least three types of relationships based on the quality of the relationship for each member of the symbiotic association:

Mutualism: both members of the association benefit. For bumblebees, one classic mutualistic association is the case that flowers require pollen by an insect vector. Many species of flower co-evolved with insects and became so specialized that they need each other to survive (Bronstein, 2001).

Commensalism: there is no apparent harm to one of the member of the association. In a commensal relationship one partner receives some benefit from the other without harming or benefiting the other partner. Certain hoverflies, e.g. *Volucella bombylans*, live together in the same nest with bumblebees where they lay their eggs. The fly eggs hatch and the larvae feed on nest debris, doing no apparent harm to the bumblebees (Leung & Poulun, 2008).

Parasitism: a relationship is which one species benefits at the expense of the other. The mode of existence of a parasite implies that the parasite is capable to cause damage to the host. A parasite is called a pathogen if its damage to the host results in a disease (Leung & Poulun, 2008)**.**

6.2. One host and one parasite

Here we specifically look at the parasitism of single cellular micro-organisms and its multi-cellular host (i.e., the insect). As a first step we describe a simplified version of reality, being a single parasite in a single host system. Even within this simplified context we cannot speak about one fixed virulence, defined as the parasite-induced fitness loss of the host. Indeed different hosts of the same species react differently to the different specimens of the same parasite. It is important to understand this variability within a host-parasite interaction to further understand how complex host-parasite interactions can work and ultimately how these interactions evolve. The variability in host-parasite interactions has profound effects on the ecology and evolutionary implications of the pathogen, but also of the host (Schmid-Hempel *et al*., 1994). This variability occurs because of genetic variability, age structure, or social and behavioral differences among hosts (Schmid-Hempel *et al*., 1994). But also parasites have genetic variation within their population.

There are variations present in nature that determine the infection success and virulence. Specifically, it is the interplay between host resistance alleles with the parasite virulence alleles that determine the development of the infection. The combination of both host and parasite characteristics creates a host-parasite specificity. Host-parasite specificity implies that some individual parasite strains can be more infectious to some host strains than others. Different parasites are distributed differently among the host strains. As an evolutionary consequence the traits that help a parasite to exploit one set of hosts makes it less able to attack other hosts, or conversely when the traits that help a host to resist one set of parasites makes it less able to resist others (Kirchner *et al*., 2000). If we study this single host-parasite system in its ecological context then we see that often multiple genetically different parasitic strain reside in a single host. A mutation within a single parasite clone or a change of antigenic properties of the parasite within a single infection can cause variability of parasites within their individual host (Schmid-Hempel *et al*., 1994). Indeed parasites have a short generation time compared to their host and therefore they can quickly adapt to exploit their host maximally.

What does this mean for bumblebees? Bumblebees are social insects and have structured populations with a number of colony-forming groups of closely related individuals,

creating a variability within populations. In such populations, spatial closeness implies genetic proximity (Schmid-Hempel *et al*., 1994). This means that the genetic variation in parasites is often associated with geographical and spatial variation. The spatial and social structure of a host population, as well as the migration of hosts, are recognized to represent a crucial element affecting the geographical propagation of directly transmitted infectious diseases (Riley, 2007). Within these so called sympatric locations, the host and parasites have evolved a certain association, being benign or virulent, depending on its transmission potential and host resistance.

Knowing this, it is clear that moving infectious hosts from one location to another allopatric location, may seed the disease in locations with other host-parasite dynamics. Therefore allopatric transport of bees is not only worrisome to introduce new parasites, but can also disrupt existing host-parasite interactions.

6.3. Multihost parasites

A challenge in the study of parasites behavior, ecology and evolutionary biology during the last decade has been to expand the theoretical "one host, one parasite" system and describe the host parasite interaction in a more realistic manner. Therefore, we need to introduce a terminology like, "multi-host parasite" and "multi-parasite hosts".

Multi-host parasites: single parasite species that are exploiting several concurrent host species, for either their whole life cycle or a given stage within it, at both the individual and population levels (Rigaud *et al*., 2010).

Multi-parasite hosts: single host species that are 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 (Rigaud *et al*., 2010).

Despite of the rapid growth of epidemiological studies on multihost parasites and multi-parasites host lifecycles, the evolutionary consequences remain largely overlooked because classical models of virulence evolution focus on simpler, single-host systems (Frank *et al*., 1996). But actually it is this heterogeneity in different biological interactions within the ecosystem that drives the evolution of both host and parasite traits (Schmid-Hempel *et al*.,

1994), influencing the evolution of transmission patterns, parasite virulence and host exploitation (Gandon 2004). This is also true for *Bombus* spp. and in turn their parasites provide a model system for studies on multi-host parasites. Indeed bumblebees together with honeybees live in multi-species assemblages across the majority of the genus range (Williams, 2009). Several parasites are known to occur in *Bombus* spp. and other bees and they play key ecological roles in their population dynamics (Schmid-Hempel, 1998; Tay *et al*., 2005) (Table 1.2.).

Table 1.2. Some pervasive protozoan and *Spiroplasma* parasites of honeybee and bumblebee

	Honeybee	Bumblebees: *Bombus terrestris*[1], *Bombus dahlbomii*[1], *Bombus pascuorum*[2], *Bombus lucorum*[3], *Bombus flavifrons*[3], *Bombus lapidarius*[3], *Bombus terricola*[3], *Bombus pratorum* [4],*Bombus ruderatus*[5]
Protozoa		
Apicystis bombi	X	X[1;2;3;5]
Crithidia bombi	X	X[1; 2]
Crithidia mellificae	X	-
Crithidia expoekii	X	X [1; 2; 3;4]
Microsporidia		
Nosema apis	X	-
Nosema bombi	-	X[1; 2; 3;4;5]
Nosema ceranae	X	X[1; 2; 3;4;5]
Spiroplasma		
Spiroplasma apis	X	X[4]
Spiroplasma melliferum	X	X[2]

6.3.1. Virulence of multihost parasites

It is getting evident that one cannot simply extrapolate the virulence of one parasite in one host to another related host (Woolhouse *et al*., 2001). Depending on the virulence level and transmissibility within and between host species, the parasite can be more or less virulent in a second host compared to the first host. In a first example, when a particular host species does not contribute towards the parasite fitness, then there is no selective constrain on the

parasite virulence in that host. This example will be less relevant in hosts with comparable physiology, behavior and habitat, as both hosts will be able to contribute to parasite fitness. A second example, in which one host is more resistant, this may result that the parasite can evolve a high reproduction and transmission; while in the less tolerant host, this high reproduction will be expressed in a lower host fitness. So, different levels of exploitation in different hosts will yield different level of virulence, transmission and recovery (Gandon, 2004). Thus multi-host parasites can have reservoir hosts where they persist, inflicting damage on a more context dependent manner, while when less tolerant hosts are infected a clear pathology is exposed. The epidemiological and evolutionary process which characterizes the behavior of the parasites in an alternative host, remains unclear and needs to be studied further as it has a crucial importance, especially in the context of emerging diseases (Woolhouse *et al*., 2001).

7. Emerging infectious diseases and the implication for wild bumblebee populations

Emerging infectious diseases (EIDs) pose a risk to human welfare, both directly and indirectly, by affecting managed livestock and wildlife that provide valuable resources and ecosystem services, such as the pollination of crops. The recent dramatic decline in honeybee populations as well as wild bee populations is therefore of significant concern, especially since the driving forces are largely unexplained to date. The speculations about parasite spillover from managed pollinators like honeybees or reared colonies of bumblebees as an important factor for global decline of wild pollinators are increasing (Meeus *et al*., 2011; Evison *et al*., 2012). Well known examples of EIDs are viruses, fungi and mites in honeybees. Some of these honeybee diseases have recently been detected in a variety of non-*Apis* pollinators, including bumblebees (*Bombus sp*.) (Ravoet *et al*., 2014; Murray *et al*., 2013). To study this, both epidemiological and molecular studies are needed in different hosts. Not only parasites determination on species level is needed, but also differences within the parasitic species need to be determined. Indeed if we look at intra-species level, one can question if there is transmission of parasite strains between different host specimens. Here molecular tools are specifically needed to be developed in order to describe population dynamics of bumblebee parasites (see Chapter 2).

Chapter II: Risk assessment of spillover: prevalence and transmission

Parts of this chapter have been published in:

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In this chapter we aim to identify which managed bees are potential reservoirs of parasites. Managed bees undergo anthropogenic transports and thereby harbor the potential to disrupt natural host parasite associations. In the first part, we describe the parasite prevalence in domesticated honeybee hives in Belgium and test parasite infection status of reared bumblebee colonies in Belgium.

In the second part, we screen mass-reared bumblebee colonies placed in different regions of Belgium to assess infection susceptibility of reared colonies and thereby assess the horizontal transmission ability of protozoan and microsporidian parasites. The horizontal transmission of a parasite is an important requisite for parasite spillover.

Part 1. Monitoring managed bees

1. Introduction

Over the last decade global decline of pollination services have been reported (Williams, 1982; Biesmeijer *et al*., 2006; Goulson *et al*., 2008; Cameron *et al*. 2011). The cause of this decline is multifactorial, including parasites, pesticides, nutrition and limited genetic diversity (as reviewed in Potts *et al*., 2010; Vanbergen and the Insect Pollinators Initiative, 2013). Parasites are almost certainly a key players in causing the observed elevated colony losses in wild life (Ruiz-GonzaLez *et al*., 2006; Durrer *et al*., 1994). Understanding the spread and emergence of parasites is crucial in order to effectively preserve wild life (Daszak *et al*., 2000). Parasite spillover from managed pollinators like honeybees, or reared colonies of bumblebees to the environment could play an important role in the decline of wild populations. Especially in America, spillover is discussed as a driver of the more recent decline of wild bumblebees (Cameron *et al*., 2011; Arbetman *et al*., 2012). Recent research has suggested that alien bee species are readily integrated into native plant-pollinator networks, and so they can act as a source of parasites emergence (Arbetman *et al*., 2012; Meeus, *et al*., 2011).

International trade in domesticated bees and bee products has increased considerably over the past few decades and it is expected to continue to increase since technology makes

transport easier and lowers national barriers to trade (Matheson *et al*., 2000). This enables infectious diseases to spread rapidly and to encounter novel hosts (Mutinelli *et al*., 2011; Meeus *et al*., 2011; Graystock *et al*., 2013).

Within the Belgian context two potentially important sources of bee parasites are the domesticated honeybees and the reared bumblebees. Indeed the high presence of parasites known to be associated with wild bumblebee in domesticated honeybees could indicate that domestic bees act as a reservoir of parasite spillover among its congeners. A first evaluation of the risk of parasite spillover from honeybees encompasses an assessment of the prevalence these parasites. The research group of Prof. Dirk de Graaf (Ghent University) within the context of the PhD of Jorgen Ravoet was performing a prevalence study of honeybee parasite within Flanders. This presented a unique opportunity to study some commonly overlooked parasites within the honeybee epidemiology that are potentially very important for sympatric bumblebee populations. The cDNA preparation for an epidemiological study of the typical honeybee parasites was therefore expanded to monitor also parasites like Neogregarines and Spiroplasmas.

The neogregarine family harbors an important bumblebee parasite, i.e. *Apicystis bombi*, which is considered to have an erratic prevalence within *Bombus* spp., and it is expected to have a high virulence (see Chapter 1 - 4.1.). In Europe it has been reported in honeybees, but very sporadically. Observations in Europe by Lipa *et al*. (1992) indicated the presence of *A. bombi* in one *Apis mellifera* worker out of 34 collected ones. Therefore it was mainly described as a bumblebee parasite but not as a honeybee parasite, because no epidemiological studies included this parasite in their screenings until recently. Indeed, Plischuk *et al*. (2011) reported in 2011 the presence of *A. bombi* in honeybee hives in South-America (Argentina). Therefore, this parasite represents an interesting case to study its prevalence in honeybees.

The *Spiroplasma* are small, helical, motile eubacteria, descendants of gram positive bacteria lacking a cell wall (Regassa & Gasparich, 2006). The bacteria, *Spiroplasma apis* and *Spiroplasma melliferum*, are known as honeybee parasites already for a long time (Clark *et al*., 1985; Mouches *et al*., 1983), and also in Asian honeybees (Ahn *et al*., 2012).

However, they seem to be uncommon in honeybees. More recently, Meeus *et al*. (2012) detected *Spiroplasma* in wild *Bombus* spp., implicating that these bacteria can perform a horizontal transmission among conspecifics. Therefore, a large epidemiological study in honeybees is justified.

A second important potential source of parasites are reared bumblebees as discussed in chapter 1 (see 3.3.). For example, Murray *et al*. (2012) studied in 2012 in Ireland the parasite spillover from reared *Bombus terrestris* hives and quantified the prevalence of four parasites. Graystock *et al*. (2013) reported the presence of parasites in 77% of the commercially produced bumblebee colonies from three main producers, which were imported presumably being free of parasites. To evaluate the presence in Belgian breeding facilities we also carried out a parasite screening in reared bumblebee colonies supplied by Biobest (Westerlo). We have chosen to screen for the 3 main parasites reported in bumblebees, i.e. *Apicystis bombi, Crithidia bombi,* and *Nosema bombi.*

2. Material and methods

2.1 Monitoring of honeybee hives

Sampling was done under the framework of a parasite prevalence study in honeybees in Belgium (De Smet *et al*., 2012; Ravoet *et al*., 2013). In July 2011, around 30 bees were randomly sampled at the hive entrance of 363 colonies.

2.2. Monitoring of reared bumblebee colonies

Early stage

Forty eight bumblebee colonies were obtained from Biobest (Westerlo, Belgium). Upon arrival from each colony, we randomly collected 10 workers with a total of 53 ± 19 workers and one queen.

Late stage (Stress simulation)

We checked infection status of 10 reared colonies in a later stage of their development. We also included stress conditions. Stress simulation consisted of cold stress, which was the

containment of the colonies in a non-temperate controlled basement for 7 weeks. Additionally, we induced cold stress for 5 consecutive days with temperatures varying between 2°C and 6°C for 8h per day. The colonies also endured food stress as the last 4 weeks they received insufficient amount of pollen. For the analyses, 18 bumblebees workers were randomly collected from each colony (n = 10) where after they were put under the stress simulation.

RNA extraction.

RNA extraction on pooled honeybee samples was done as described by Ravoet *et al*. (2013). Ten bees per hive were pooled for the extraction.

RNA extraction was done on the pooled samples of reared *B. terrestris* workers as supplied by Biobest. The protocol for RNA extraction from pooled bumblebees is based on the protocol of RNA extraction of honeybees as described by Ravoet *et al.* (2013). The early stage detection of 10 workers consisted of two separated pools of 5 workers, the late stage detection consisted of 3 separated pools of 6 workers. The pooling of 10 and 18 workers was related to the size of the colony and chosen so to have enough specimens to be able to detect an infection being present in the colony, meaning a minimum infection prevalence of 14 and 25%, respectively, with a type I error rate of < 0.05 (Cameron & Baldock, 1998).

Each pool was crushed and homogenized in 4 ml Qiazol® Lysis Reagent. Wild *B. pascuorum* bumblebees were processed individually and crushed in 700 µl Qiazol® Lysis Reagent. Zirconia (0.1 mm) and steel beads (1 mm) were used for homogenization. The tubes were centrifuged at 12000 x g full speed for 3 minutes. A total 1 ml of homogenized material from pooled samples and 500 µl of homogenized material from individual *B. pascuorum* samples was collected. All samples were further processed according manufacturer specifications (RNA extraction kit, QIAGEN) to obtain a final 50 µl of total RNA in RNA-free water. Extracted RNA samples were stored at -80°C prior to molecular detection of parasites.

PCR Analysis

For parasite detection, we used a PCR-based screening method on RNA samples. Five microlitres RNA (variable concentration) were retro-transcribed using random hexamer primers with the Revert AidTM First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer's instructions. All PCR reaction mixtures contained 2 µM of each primer; 1.5 mM $MgCl_2$; 0.2 mM dNTP; 1.25 U Hotstart Taq DNA polymerase (Qiagen) and 1 µl cDNA product. The primers used are shown in Table 2.1. Temperature

Table 2.1. **Primer pairs used for parasite screening**

Target parasites	Primer pairs	Amplicon size
Apicystis bombi	NeoF: CCAGCATGGAATAACATGTAAGG NeoR: GACAGCTTCCAATCTCTAGTCG	260 bp
Apidae species	ApidaeF: AGATGGGGGCATTCGT ApidaeR: ATCTGATCGCCTTCGAACCT	130 bp
Spiroplasma melliferum	Ms-160F: TTGC AAAAGCTGTTTTAGATGC Ms-160R: TGACCAGAAATGTTTGCTGAA	160 bp
Spiroplasma apis	As-636F: CGGGAGAATTTGTCCTATCG As-636R: CCCACTTTAACAATCGGGATG	636 bp

The NeoF/NeoR primer set detects the 18S rDNA of neogregarines (Meeus *et al*., 2010).
The ApidaeF/ApidaeR primer set detects 18S rDNA of *Apidae* species (Meeus *et al*., 2010).
The Ms-160F/Ms-160R species specific primer set detects *S. melliferum* (Meeus *et al*., 2012).
The As-636F/As-636R species specific primer set detects *S. apis* (Meeus *et al*., 2012).

cycles for neogregarines were as described by Meeus *et al*. (2010). Fifteen positive samples (out of 136) for neogregarines were analyzed with *A. bombi*-specific primers and sequenced as well.

Spiroplasmas were detected as described by Meeus *et al*. (2012), based on the 16S ribosomal RNA sequence. Universal *Spiroplasma* primers were used, as sequence specific primers gave cross-reactivity with *Nosema* infection. All positive amplicons were sequenced.

The minimum infection prevalence being able to be detected with the specific sample size within a certain population, was calculated by the FreeCalc2 software (Cameron & Baldock, 1998). This software was designed to calculate freedom from disease. We used the

modified hypergeometrix exact formula to calculate samples sizes needed to detect a certain prevalence with a 95% certainty.

3. Results

3.1. Parasite prevalence in honeybee hives

An overview of the prevalence of the investigated parasites in honeybee hives is given in Table 2.2.

We found molecular evidence that the neogregarine *A. bombi*, primarily known as a bumblebee parasite (Lipa & Triggiani, 1996) was present in 40.8% (148/363) of the screened samples. The 15 sequenced amplicons showed 100% identity with a partial small subunit ribosomal RNA sequence of *A. bombi* (Genbank: FN546182). As all 15 sequences belonged to *A. bombi*, we can conclude that if other neogregarine species would be present their prevalence would be maximum 18% within the parasite population. This would be a maximum prevalence of 7.8% in the honeybee population.

Table 2.2. Parasite prevalence in Belgian honeybee hives

Parasite	Family	Overall prevalence
Apicystis bombi	Ophryocystidae	40.8% (148/363)
Spiroplasma apis	Spiroplasmataceae	0.3% (1/363)
Spiroplasma melliferum	Spiroplasmataceae	4.4% (16/363)

The spiroplasmas *S. apis* and *S. melliferum* were found only in 0.3% (1/363) and 4.4% (16/363) of the tested samples, respectively. One sequence was 100% identical to the *S. apis* strain ATCC 33834 (Genbank: GU993267); while all others matched to *S. melliferum* IPMB4A (Genbank: JQ347516) (4 sequences with 100% identity and 12 sequences with only a single nucleotide substitution).

3.2. Screening of reared bumblebee colonies

No parasites were detected, indicating no or low levels of parasites were present in the reared colonies as provided for the tests.

We calculated the minimum infection level which we can detect with 95% certainty. We screened 10 randomly collected bumblebee workers from each colony (n = 48), and all were negative. The colonies contained on average 53 ± 19 (SD) workers. Thus a minimum infection prevalence of 25% with type I error rate of < 0.05 could be detected.

The screening results on 18 randomly collected workers of stressed colonies of late stage experiment also were negative. These colonies contained 85 ± 41 (SD) workers. Consequently, a minimum infection prevalence of 14% with type I error rate of < 0.05 could be detected.

4. Discussion

This research revealed multiple parasites in the honeybee hives known to infect bumblebees. It is possible, although not proven, that these parasites can spillover toward bumblebees. Indeed honeybees and bumblebees have an overlapping flower network, and shared flower use is an important transmission route of parasites (Durrer & Schmid-Hempel, 1995). Our results do not prove honeybees are a reservoir of parasites for sympatric bee species, but they clearly indicate tha honeybees could be.

We looked more in details for each of the screened parasites. The neogregarine *A. bombi*, primarily known as a bumblebee parasite (Lipa & Triggiani, 1996), is present in 40.8% (148/363) of the screened honeybee hives. This percentage is striking, and was beyond our expectations as this parasite has been barely reported in honeybees in Europe. It still needs to be determined if this is because of a lack of knowledge to detect this parasite or because of its emerging status. But it is evident that it needs to be determined if honeybees infected with *A. bombi* could indeed be acting as a source for spillover toward wild pollinators. However, this protozoan parasite has already been blamed for the decline of native

bumblebee species in Argentina where it is speculated that the presence in Argentina is possibly induced by spillover from invasive *Bombus terrestris* (Arbetman *et al*., 2012); however it should be remarked here that it is still necessary to identify the source of this parasite. In chapter 3, more details are given on the spillover of *A. bombi*.

The bacteria, *S. apis* and *S. melliferum,* are known as honeybee parasites and his already for a long time (Clark *et al*., 1985; Mouches *et al*., 1983). In our screening both bacteria had a low prevalence. The reason for this could be the sampling period. A recent sampling following colonies in a seasonal manner showed the highest prevalence of this bacteria around May (Zheng *et al*., 2014).

If we look at the reared bumblebee colonies we could not detect any parasites. In relation with our sampling effort this means that the prevalence of any parasite is below 25%. Hence the infection prevalence is low as compared to honeybees. Other studies did find parasites in reared bees with a prevalence higher than 25% (Murray *et al*., 2013; Graystock *et al*., 2013). A main difference between our screening and theirs is that these other authors always checked bumblebee colonies after transport. It could be possible that low infection levels within a colony remained undetected as in our study, while the infection level increases after the stress of transport reaching larger numbers of infected hosts and therefore increased parasite density and the probability of parasite detection. Indeed the prevalence and intensity of parasite infections in animals can increase during shipping from the production facilities to the end-user, a phenomenon that is well known in vertebrates and sometimes termed as ‘shipping fever’ (Barham *et al*., 2002). We believe this could be one possible explanation why these colonies had heavy parasite infections upon receipt after transport.

Therefore we induced stress conditions on to 10 reared bumblebee colonies. However, after our stress simulation condition on the reared colonies of *B. terrestris* still no parasites could be detected in a sampling pool of 18 specimens per colony. So we believe that we can make the firm conclusion that the negative diagnostic results of the bumblebee colonies indeed represent that the colonies are parasite free. Improvement on quality assurance control in breeding facilities may be a reason for this. However, our sampling was only a single batch

detection, meaning one large sampling effort at one specific time point at one specific vendor. Therefore more samples and spread time points are needed to get more insight in the prevalence of parasites in bumblebee rearing facilities and their potential role as reservoir species. But these results, not detecting parasites, indicate already that parasite exclusion within closed systems is possible.

Part 2. Protozoan and microsporidian parasites of bumblebees have the ability to perform horizontal transmission

5. Introduction

Many studies have already tried to retrieve evidences of spillover events from reared bumblebee colonies towards wild pollinators. It is evident that the ability of a parasite to spillover into the wild, depends on its ability to perform horizontal transmission. Studies suggest that the trypanosome *Crithidia bombi* has a good potential for horizontal transmission, while the potential of the microsporidian *Nosema bombi* is much lower (Durrer *et al*., 1994; Meeus *et al*. 2011). Also the neogregarine *Apicystis bombi* can infect bumblebees (Graystock *et al*., 2013) but its transmission modes are unknown.

Here we assess whether three important parasites of bumblebees, i.e. *A. bombi, C. bombi*, and *N. bombi* have the intrinsic capacity for horizontal transmission in a natural environment. We measure the infection susceptibility of reared parasite-free bumblebee colonies placed in different environments.

6. Material methods

6.1. Experimental setup

In total 24 reared *Bombus terrestris* colonies were placed in 4 locations (Ghent, Roeselare, Waarschoot and Horebeke) in Eastern and Western Flanders in Belgium. In each location 6 colonies were placed at 2 different study sites (4 locations x 2 study sites x 3 colonies = 24 colonies). The average distance between the regions was 32.8 km ± 11.6 km (SD) and thus considered as independent regions.

In each study site of the different locations we placed 3 colonies close together, i.e. distance between colonies ranged from 10 m to 25 m. A next study site again contained 3 colonies within the same location separated by 1.5 km distance. The radial distance of 1.5 km was chosen so the reared bumblebees had a minimal foraging overlap (Osborne *et al*., 2008)

within a location with similar landscape metrics. All colonies were placed at their study sites on the 10-th of April 2013 and had *ad libitum* of sugar water for 3 days to adjust to their environment. Thereafter sugar water was removed and the colonies remained at their location until the 21-th of May 2013. Thus in total the colonies were 48 day or approximately 7 weeks outside.

At each location, from the wild we collected 10 foraging *Bombus pascuorum* workers, this species was chosen as it is the most prevalent bumblebee at the study sites, and does not have any cryptic color morphs.

6.2. Parasite free bumblebee colonies

Sampling

We used the colonies that were screened in Part 1 of this chapter, therefore they are considered to be free of parasites. As the colonies placed in the laboratory under stress conditions did not develop any parasites infection, we speculate that all, or at least a majority of the developed infections of the colonies placed outside, come from the environment.

Estimation of parasite prevalence in the different study sites

At the end of the experiment bumblebees were counted in each colony and 18 workers per colony randomly collected for future analysis. Colonies consisted of 101 (±74 SD) bees in average.

Sampling of *B. pascuorum* workers occurred at the end of June. All field-caught and reared bees were immediately stored in individual microcentrifuge tubes and then transferred to a -80°C freezer within 8 h for later identification.

RNA extraction and PCR

For RNA extraction we used 3 separate pools of 6 bumblebee workers (in total 18) from the reared colonies placed out in the environment. RNA extraction was done on individual samples of 10 wild *B. pascuorum* workers collected from each locations. In order to

determine parasite prevalence at each chosen location we used the same PCR based screening method on RNA as described in Part1 of this chapter.

7. Results

We sampled *B. pascuorum* workers to assess the prevalence of the parasites in the environment. At each of the 4 locations we had two sampling study sites (2x10 specimens). The mean of the parasite prevalence at each location was calculated.

Table 2.3. Parasite prevalence (%) in different regions of Belgium
a) Parasite prevalence (%) in *Bombus pascuorum*

Regions	*Apicystis bombi*	*Crithidia bombi*	*Nosema bombi*
Gent	60	5	0
Roeselare	25	75	5
Waarschoot	50	60	5
Hoorebeke	70	50	10
Average	51	47	5
SD	19	30	4

b) Parasite prevalence (%) in *Bombus terrestris*

Regions	*Apicystis bombi*	*Crithidia bombi*	*Nosema bombi*
Gent	33	83	33
Roeselare	50	100	50
Waarschoot	33	100	16
Hoorebeke	66	100	0
Average	45	95	21
SD	16	9	25

The prevalence of neogregarine *A. bombi* (51%; ±19 %SD) and the trypanosomatid *C. bombi* (47%; ± 30%SD) in wild *B. pascuorum* workers were comparable, while *N. bombi* infection was very sporadic (5%; ±4 %SD). See Table 2.3a for details. If we compare this with the parasite prevalence in the reared colony, (here we have 6 colonies per location) we see that mainly *C. bombi* was able to infect colonies with elevated level (95%; ± 9

%SD) followed by *A. bombi* (45%; ± 16 %SD) and *N. bombi* (21%; ±25 %SD) (Table 2.3.b).

Prevalence in wild and reared colonies cannot be compared directly. The higher infection level of the reared colonies does not mean that these bees have a higher parasite prevalence than their natural congeners. As for colony detection we pooled 18 bees, while the wild bees are individual foragers.

We found a prevalence of the protozoan *A. bombi* at elevated levels both in reared and wild bumblebee species. The analysis showed only a non-significant weak correlation of 0.18 (Pearson; $P = 0.81$). Although we could not detect a correlation between the prevalence of *A. bombi* within the environment and within reared colonies we still assume that reared colonies got infected by parasites of the environment. This mainly based on the high prevalence of parasites after being placed outside, while the colony with stress in a lab environment stayed free of parasites.

Correlation studies for *C. bombi* and *N. bombi* were not performed, as *C. bombi* infected all colonies and for *N. bombi* the prevalence in the wild was too low to draw real conclusions.

8. Discussion

All three parasites, i.e. *Apicystis, Crithidia* and *Nosema*, which were not observed in the reared colonies when placed in the laboratory under stress conditions (see Part 1 of this chapter) have been detected in the reared colonies placed within different environments. We are not able to infer if the infection level in these reared bumblebees is different from wild *B. terrestris,* because diagnostic PCR only gives an indication on presence or absence of the parasite and is no quantitative measurement. It seems that all three parasites have the ability to perform horizontal transmission. The ability of a parasite to infect the parasite-free colonies depends on at least two properties of the parasite. One, its ability to perform horizontal transmission. Two, its prevalence in the environment.

C. bombi and *A. bombi* have a comparable mean prevalence in the environment. But *C. bombi* infects almost all reared colonies, while *A. bombi* infects 45%. This indicates that *C. bombi* has a better ability to perform horizontal transmission and therefore was able to infect more colonies. It is known that *C. bombi* has the ability to perform horizontal transmission. Where *C. bombi* transmission is thought to be rapid and extensive as new hosts are quickly infected through the faeces in the colony (Otterstatter and Thomson, 2008) and through shared use of flowers by foragers (Durrer & Schmid-Hempel 1995).) Also *N. bombi* was found in different colonies, and therefore able to perform horizontal transmission. Thus all three parasites reached detectable levels within 7 weeks, indicative for their ability to perform horizontal transmission. Because the ability to perform horizontal transmission is a requisite for spillover (Daszak *et al*., 2000), we conclude that the three parasites harbor the intrinsic risk to spillover to sympatric species.

The infection fact of each of these, initially parasite-free colonies, imposes an important question about their infection potential compared to wild specimens. If they are initially free of parasites and have an equal ability to be infected with new parasites, then reared bumblebees rather act as a diluting factor for parasites prevalence in the environment as hypothesized by Whitehorn (2011). Opposed to this, if reared bees readily pick up parasites from the environment, being more vulnerable to real-world conditions because of intensive breeding in a closed environment, then they could act as a parasite reservoir. This is an important factor to consider when efforts are made towards parasite-free bumblebees. Indeed reared bumblebees should be immune competent to fight of parasites, which they will encounter when placed in the field for pollination purpose.

Chapter III: Molecular haplotyping to identify intra-specific variations of *Apicystis bombi* and to study parasite spillover

Part 2 of this chapter has been published in:

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In this chapter we first develop primers to haplotype *Apicystis bombi* (Part 1) in order to study population dynamics of this parasite in Argentina and untangle if parasite spillover has occurred in this region of the world (Part 2)

Part 1. Development of molecular tools for the screening of protozoan parasites in bees

1. Introduction

The occurrence of parasite spillover in wild invertebrates is largely unknown and would almost certainly go unnoticed (Goulson 2008). It is hypothesized that spillover, could be occurring either from commercially-reared bumblebees or domesticated honeybees (Fürst *et al*., 2014; Meeus *et al*., 2011). Today many bumblebees population are already critically endangered (Fitzpatrick *et al*., 2007), therefore spillover events of parasites could drive these population toward extinction (Haydon *et al*., 2006).

In order to investigate if spillover toward native bumblebees has occurred, or is currently happening we need to develop molecular tools to characterize the bumblebee parasites and describe the parasite population dynamics. Knowing the existence of spillover source(s) should lead to appropriate governmental actions to effectively preserve native population. The basic assumption to identify the source of a parasite introduction is that the founder individuals responsible for an invasion will be genetically more similar to individuals of a source population (Dlugosch *et al*., 2008).

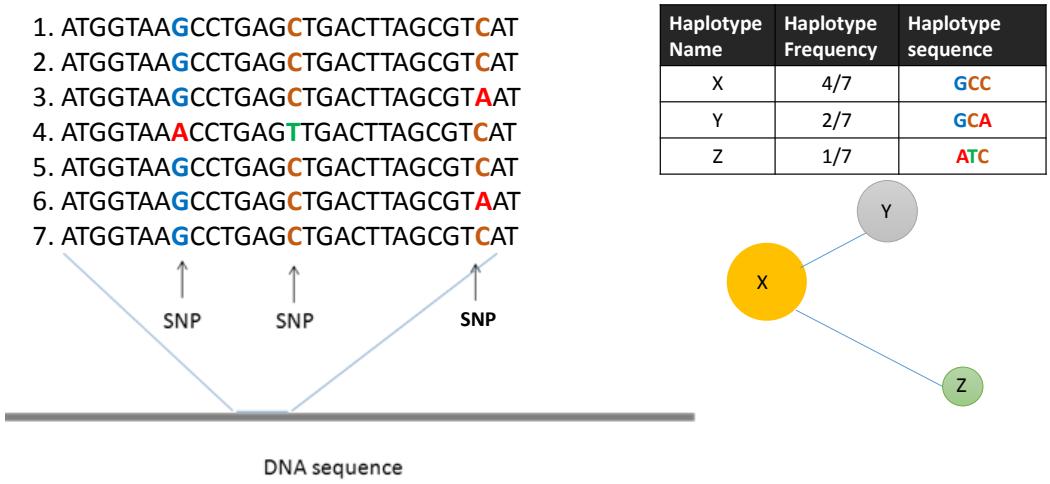
Genetic variation can arise through base substitutions (single nucleotide substitutions), insertion or deletion of DNA sequences (indels), inversion of DNA segments and the rearrangement of DNA segments (Russel 2010). The most detailed markers use single nucleotide polymorphisms (SNPs). A set of specific SNP alleles at particular SNP loci that are close together in one small region of a chromosome can be defined as a haplotype. In

order to build up haplotype maps of genetic differences between individuals small regions of DNA are analyzed.

In Figure 3.1. an example of haplotyping is given. In the example seven specimens are sequenced, revealing 3 haplotypes. Looking at the network map we see that haplotype X and Y are similar with each other, while the haplotype Z, found in one specimen is more distantly related.

In order to start a population genetic survey one must choose the appropriate genetic

Figure 3.1. Graphic presentation of haplotyping procedure: SNP- single nucleotide polymorphisms.

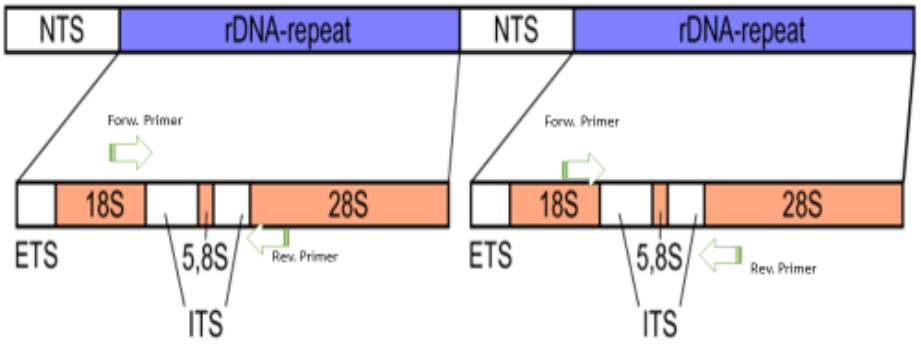


markers. Selecting good markers is essential for haplotyping (Baverstock & Moritz 1996; Brower & DeSalle 1994). An important property of a marker is the mutation rate of the genomic region of the marker, this defines if the marker is sensitive to detect different haplogroups and lineages (Rozhanskii *et al.* 2011). Different molecular markers have different selective constrains, thereby show different mutation and recombination rates. Genetic markers with low selective constrains are needed to study variation within a same species. In general highly conserved regions of DNA are used as a taxonomic marker for assessing phylogeny in many organisms. Faster evolving markers that represent higher resolution on small temporal and spatial scales are used to study specific genetic

relationships within the same species and are used to detect newly introduced unknown populations (Le Roux *et al*., 2009).

The nuclear ribosomal DNA (rDNA) genes have been extensively used for definition of phylogenetic lineages among closely related genera or species (Souto *et al*., 1996; Powers *et al*., 1997; Liu *et al*., 2013). But also to study difference within the same species (Voigt *et al* 1999). This because these genes contain a combination of conserved and variable regions allowing species identification and haplotyping. rDNA are found as parts of repeat units that are arranged in tandem arrays, located at the chromosomal sites known as nuclear organizing regions. Each repeat unit consists of a transcribed region (containing the genes that code the 18S, 5.8S and 26S rRNA and the external transcribed spacers i.e. ETS1 and ETS2) and a non-transcribed spacer (NTS) region. In the transcribed region, internal transcribed spacers (ITS) are found on either side of 5.8S rRNA gene and are described as ITS1 and ITS2. For a schematic representation of the structure see Figure 3.2. The ITS regions are often used for haplotyping studies (Tang *et al*., 1996; Brad *et al*., 2002). The length and sequences of the ITS regions of rDNA repeats have a high mutation rate in different species over different taxa (Baldwin *et al*., 1992; 1995 Chen *et al*., 2010).

Figure 3.2. The eukaryotic ribosomal RNA genes. NTS - non-transcribed spacer, ETS - external transcribed spacers, ITS - internal transcribed spacers



In this chapter we focus on the bumblebee parasite *Apicystis bombi*, which was recently molecularly identified by sequencing of the 18S rDNA (Meeus *et al*., 2010). Sequencing of this gene allows molecular identification of the species, however intra-species variability or the existence of different haplotypes cannot be detected.

It was our goal to design primers to be able to perform haplotyping studies on *A. bombi*. This will allow to assess the genetic structure of the parasites population, hereby inferring the origin of a certain specimen in a certain geographic region and if it is native or recently spilled over from the source/founder populations. But very few sequence information for *A. bombi* or even of related parasite were available on GenBank in order to design primers to pick up suitable genetic markers. There were only 6 sequences that belong to the order of the *Neogregarinorida* posted on GenBank. The class *Gregarina* collects a total of 2796 sequences but most are 18S sequences and 95% belong to only one species, i.e. *Ascogregarina taiwanensis*. Therefore the selection of multiple markers was troublesome. We finally chose the rDNA as a suitable marker.

2. Material and methods

DNA Extraction

A. bombi positive samples came from a screening of *Bombus terrestris* species which invaded Patagonia (Argentina – January 2009 and 2010).

The adult bee (*B. terrestris*) was cut laterally and put in a sterile 1.5 mL or 2 mL microcentrıfuge tube. Bees were homogenized by bead beating with 0.3 g Zirconia/Silica beads 0.1 mm (BioSpec Products, Bartlesville, USA) and 2.5 mm glass beads (BioSpec Products) in a Precellys 24 (Bertin, Montigny-le-Bretonneux, France) and 350 µl Buffer CTL from the Ezna Insect® DNA Isolation Kit (Omega Bio-Tek, Inc) was added. Manufacturer's instructions were followed.

Polymerase Chain Reaction (PCR)

PCR reactions were done in volumes of 25 µl containing 1 µl sample DNA (variable concentration). 1 x Reaction-Buffer, 0.5 µl of dNTPs of 0.2 mM each, 1.25µl of each primer of 10 µM, and 0.5 U of Taq DNA Polymerase.

All PCR-amplifications were done as follows: a first denaturing step of 5 min at 94ºC was followed by 30 cycles of 30 s at 94ºC, primer annealing for 30 s at 56ºC and 1 min extension at 72ºC. The last cycle was followed by 10 min at 72°C. Quality of PCR products was checked on a 1.5 % agarose gel.

PCR product purification

PCR products were purified by ExoSAP (Exonuclease I- Shrimp Alkaline) method (Hanke and Wink 1994; Werle *et al*. 1994).

Cloning

The chosen piece of DNA was cloned using the Sticky-End Cloning Protocol (pJET1.2/blunt Cloning Vector, Fermentas UAB, subsidiary of Thermo Fisher Scientific Inc. V. Graiciuno 8 LT-02241, Vilnius, Lithuania). The plasmid was purified with the E.Z.N.A. Plasmid Mini Kit (Omega Bio-Tek) from 10 bacterial colonies containing the target region.

Sequencing

Cleaned PCR products were directly sequenced by LGC Genomics GmbH. All sequences were edited and aligned using the software BioEdit version 7.1.9 (Hall, T.A. 1999).

3. Results

The ITS region was chosen as a primary candidate marker for haplotyping *A. bombi*. Therefore we needed to sequence the variable regions (ITS1 and ITS2) located between 18S and 28S. In order to design a forward primer we could use the 18S sequence available for *A. bombi* (Meeus *et al*., 2010). The following two forward primers were selected (18SFa-TTACGTCCCTGCCCTTTGTA; 18SFb-CGTGATGGGGATAGACGATT).
For the reverse primer we extracted 28S DNA sequences of related parasites from GenBank. Table 3.1 gives an overview of the primers which were designed. Different combinations were used comparing an infected bumblebee with a non-infected bumblebee.

18SFa/28SR1100a resulted in a band of above 2000 bp, in agreement with the expected length being a PCR fragment of at least 1700 bp in an *A. bombi* infected sample, while no

Table 3.1. Designed primer sets to amplify the ITS or 28S region in *Apicystis bombi.*

Name and sequence of forward primer	Name and sequence of reverse primer	target
18SFa* TTACGTCCCTGCCCTTTGTA	28SR1100a* TCGGAGGGAACCAGCTACTA	ITS
18SFb* CGTGATGGGGATAGACGATT	28SR1300a* CATCGCCAGTTCTGCTTACC	ITS
28SF1100a* TAGGGGCGAAAGACTAATCG	28SR2200* CGAGGCATTTGGCTACCTTA	28S

See fig. 3.4 for the specific location of the primer sequences

product was observed in the non-infected bumblebee, indicating we amplify the ITS region of the parasite (Figure 3.3).

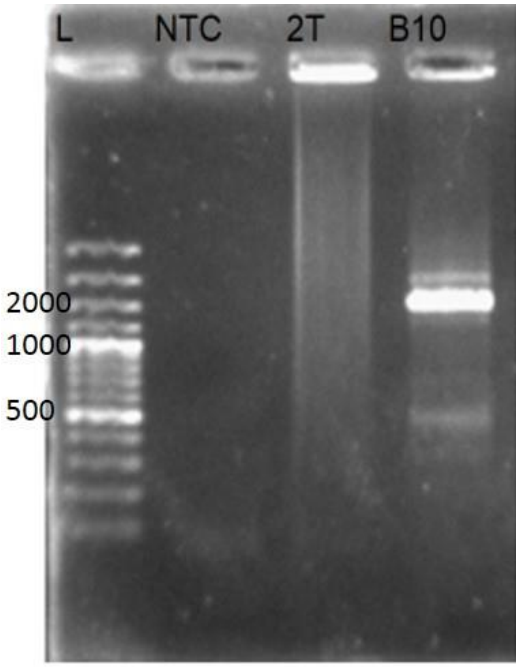


Figure 3.3. The presence of an amplicon above 2000bp in the *Apicystis bombi* infected sample (B10) represents the ITS region of the parasite, amplified by the primer set 18SFa/28SR1100a. L- DNA Ladder, NTC (No template control), 2T-non-infected bumblebee.

We used direct sequencing to verify if we indeed picked up the DNA of the parasite, meaning the PCR product was amplified without any cloning procedure. This procedure resulted in only a short sequence readout of 322 nucleotides (see below Fasta file a) B10-18Sfa). Actually only the first 227bp gave a clear electrogram, while later on a lot of ambiguous base calling was present, ultimately leading toward an unreadable sequence.

The first 119 nucleotides show 100% sequence overlap with the last nucleotides of the 18S of the *A. bombi* isolate NPaw1 subunit ribosomal RNA gene, partial sequence (HQ619890.1) (E value: 1x10-24) and thus represent the 18S of *A. bombi.* The unreadable electrogram actually represents the ITS regions, as there are probably multiple ITS sequences with indels. Cloning of the ITS fragment should generate a single PCR product and therefore a full readout.

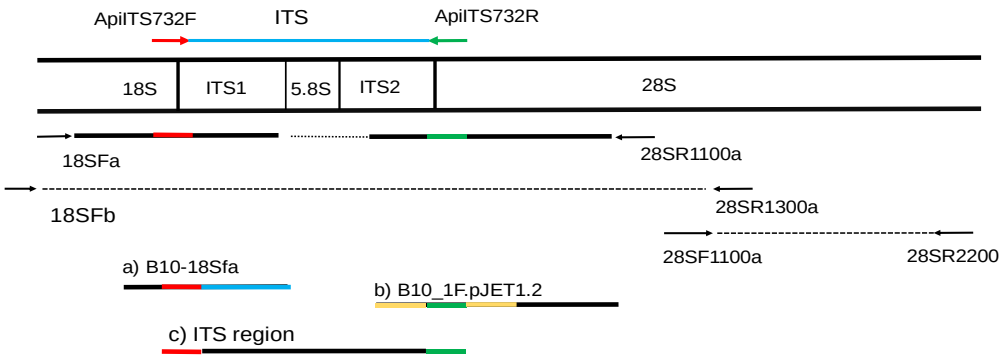


Figure 3.4. A schematic representation of primer development from conserved rDNA regions of *A. bombi.* The primers used further on to amplify the ITS region (ApiITS732F forward (red colored) and reverse ApiITS732R (green colored)) were retrived from the sequences a) B10-18S and b) B10_FpJet. These sequences were identified after cloning the amplicon generated by the primers 18SFa/28SR1100a.

Therefore the PCR product of the 18SFa/28SR1100a primer pairs was ligated into the pJET1.2 cloning vector (CloneJet PCR cloning kit, Fermentas). The plasmid was sequenced with the pJet1.2F primer (CGACTCACTATAGGGAGAGCGGC) (CloneJet PCR Cloning Kit, Fermentas) to pick up a relatively conserved regions of the rDNA including the variable ITS regions in between them. Sequencing of the cloned fragment revealed the start of the 28S (See below Fasta file: c) B10_1F.pJET1.2 28Spart – yellow colored. This procedure lead to the identifications of the sequences surrounding the highly variable ITS regions. These newly sequenced regions were used to design new primers which were able to amplify the ITS region of rDNA of *A. Bombi*. This resulted in a fragment of 730 bp. The forward primers ApiITS732F (TGGAAACAAGTCATTTTTGGA) was chosen as it was close to the ITS1 region (See below Fasta file a) B10-18Sfa - red colored sequence), while the reverse primer ApiITS732R (AGTAACGGCGAGTGAACAGG) was chosen as it was close to the ITS2 region (See below Fasta file c) B10_1F.pJET1.2 28Spart - green colored sequence). Therefore the amplicon has a minimal length to enhance cloning efficiency and a maximal variability in order to screen for inter species variability. In the Figure 3.4. the location of rDNA and primers development for ITS genes were presented.

Sequence information from rDNA of *Apicystis bombi* in FASTA format.

a) B10-18Sfa – Directly sequenced PCR product of 18SFa/28SR1100a primer pairs. Part of 18s region of rDNA.

> B10-18Sfa
GCTCCGGTGAACAATACGGACAATGAATAACT**TGGAAACAAGTCATTTTTGGA**AATATTGTAAACCAA
AGCATCTGAAGAATGAGAAAGTCGTAACACGGTATCTGTAGGTGAACCTGCAGATGGATCATTCATGTT
TTAAAAAATCTATAATTGAATACGTTTTTTATTTAATTTATTAAATAAAAAATGTAGAACAATATAAAGT
TTGTTCAAATATATTGATATATTGACAACATTTTTAATAATGTTGTAAAAAAAAAAAAA

b) HQ619890.1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2; and 28S ribosomal RNA gene, partial sequence

> HQ619890
CGTAACACGGTATCTGTAGGTGAACCTGCAGATGGATCATTCATGTTTTAAAAAATCTATAATT
GAATACGTTTTTTATTTAATTTATTAAATAAAAAATGTGGAACAATATAAAGTTTGTTCAAATA
TATTGATATATTGACAACATTTTCAATAATGTTGTAAAAAAAAAAAAATTGAGAAATATCAAA
TAGTTTTTTGATGTTTTTCAATTTTTTTTTTTTTTNGTAATATATTTAAATGTTATATTTATGACT
ATGTAAATCATTTAATTAAAATTAAAAAAAAANTTTTTAGCGATGGATGTCTTGGGTCTTGTTT
CGATGAAGAACGCAGCAAATTGCGATAAGCAGTATGACTTGCAAATTTCAAAGAATCATTAGA
TTTTTGAACGCGAAAGGTGCCTTTTTGCTTTAAGCAGTAAGGCATGTTCAGTTCAGCGCTCTTA
TTTATTGAATTAATGTGTTTTCACATCTATTCGCTATTAAATGCTATAATTCTTATTATGGCATT
TTGACTATGATACAAAATTGATTATAAGTAATAATATATATAATCAAATGTTTCTAATTTCATT
ATGAGCCTGAGCTTGAACAAGGTTACCCGCTGAATTTAAGCATATTAATAAGCGGAGGAAAAG
AAACTAACTAGGATTTCCTT**AGTAACGGCGAGTGAACAGG**AAAAAGCTCGAGTTGAAAATC
AACATTATATGTTGAATTGTAGTCTCGTGAAACGTTACCAGTGG

c) B10_1F.pJET1.2 28S part – cloned 28S region of rDNA

>B10_1F.pJET1.2
GGAAAAGAAACTAACTAGGATT~CCTT**AGTAACGGCGAGTGAACAGG**AAAAAGCTCGAGTTGAAAATC
AACATTATATGTTGAATTGTAGTCTCGTGAAACGTTACCAGTGGTTGTAATGGGATAAGTCTCTTGAAATA
GGGTGCCAAAGAGGGTGAAAGTCCCATATTTGCTCATTCAACAACTGCGTGCGGTACGTTTTCGTAGAGTC
GCGTTCATTGCGATTGGAGCGCTAAATGGGTGATAAATTTCATCTAAAGCTAAATATAAACAAGACACCG
ATAGTAAACAAGTACCGTGAGGGAAAGATGAAAAGAACTCTGAAAAGAGAGTTAAAAGGAACTGAAATC
GCTAAGAAAGAAGTGGAGGGCTTGTGTTGTTTTTTAATGAAATTTGATTCATATTGTTTCAAAATTTGATTA
AAAAAACTCTGAAATGTTTGTTTCATGGTAATTATGTATTGACAATATAATTTGAAAAAATTATGTCTTTAT
ATTAATTACTGCGAAATAAATTAAAAAAAAACATTATATTTATAAAAGATATAATGTTAAATTTCGGAAAT
ACATATACTCTCCGTCCCGTCTTGAAACACGGACCAAGGAGTCTATCTTATGTGCAAGTACTTTGGTGTGA
AACCAACGTGCGAAATTAAAGTAATTGTTAGTAAAGTAATTGCCCTAACGTCCGGCCATTAAGCTGAGAT
ATAGCATATAGGATAGGACCCGAAAGATGGTGAACTATGCCTGAGTAGGGTGAAGCCAGAGGAAACTCT
GGTGGAAGCTCGAAGCGATACTGACGTGCAAATCGTTCGTCAAACTTGGGTATAGGGGCGAAAGACTAAT
CGAACCATCTAGTAGCTGGTTCCCTCCGAATC

Part 2. Genetic variability of the neogregarine *Apicystis bombi*, an etiological agent of an emergent bumblebee disease.

1. Introduction

Many bumblebees (*Bombus* spp.) populations are undergoing major losses. While range decline for some species has been moderate, others are vanishing rapidly (Cameron *et al*., 2011; Potts *et al*., 2010). Although the trend of decline is evident worldwide, the responsible drivers are diverse (for review see Goulson *et al*. 2008), with each driver potentially interacting with other drivers and acting differently across geographic locations (Brown *et al*., 2009). This complexity makes it difficult to clarify the response to a single driver.

The relatively unspoiled temperate forests of southern Argentina and Chile are the natural habitat of *Bombus dahlbomii*, the largest bumblebee species in the world (Abrahamovich *et al*., 2004). Populations of *B. dahlbomii* appear to be in a steep decline, which has coincided with the recent establishment of the non-native European bumblebee, *Bombus ruderatus*, and more recently of *Bombus terrestris* (Morales *et al*., 2013; Ruz 2002). Already in 2001, before the massive invasion of *B. terrestris* into Argentina, there was concern on *B. dahlbomii* abundance and its relation with the introduction of *Bombus ruderatus* (Morales and Aizen, 2006). In 2014 a survey on bumblebee distribution in Patagonia comparing 2004 with 2010-2012 showed a different spread of *B. ruderatus* compared with *B. terrestris* (Schmid-Hempel *et al.*, 2014). The authors remarked that "wherever *B. terrestris* spreads, the native *B. dahlbomii* disappears although the reasons remain unclear". *B. ruderatus* was intentionally introduced into southern Chile in 1982–1983 from a New Zealand population, which was also an introduced population originating from the UK (Arretz *et al*., 1986), and has subsequently migrated from Chile into Argentina (Abrahamovich *et al*., 2004). *B. terrestris* became established in Chile after the introduction of commercially produced colonies from Belgium and Israel for crop pollination around 1998 (Ruz 2002). It has been hypothesized that the decline of *B. dahlbomii* may be partly due to parasite spillover from introduced bees (Arbetman *et al*., 2013), and in particular that the introduced bumblebee *B. terrestris*, may be the carrier of novel parasites into the environment. Commercially produced bumblebee colonies have

been shown by many studies to carry a wide range of microbial parasites (Colla *et al*., 2006; Meeus *et al*., 2011; Graystock *et al*., 2013; Murray *et al*., 2013), and such an introduction of new parasites could induce emergent infectious diseases with dramatic consequences for native populations (Graystock *et al*., 2013). *Apicystis bombi*, a neogregarine parasite (see Chapter 1.), has been found to be present in Argentina infesting native and non-native bumblebees, as well as the honeybee *Apis mellifera* (Arbetman *et al*., 213; Plischuk *et al*., 2009; 2011), and thus it may be an important parasite driving emerging infectious disease, and potentially involved in the decline of *B. dahlbomii* in this region. However, pathogenicity of *A. bombi* in *B. dahlbomii* needs to be determined in order to infer the risk associated with its spillover. Although empirical data on the pathology of the parasite are limited, the fat body of infected bumblebees is destroyed due to the proliferation of the parasite (Schmid-Hempel 2001), and its presence correlates with high mortality in infected spring queens, preventing them from establishing colonies (Macfarlane *et al*., 1995; Rutrecht *et al*., 2008). Consequently, upon entering novel host populations, *A. bombi* may have the potential to act as an emergent infectious disease agent. However, to date it is unknown whether the *A. bombi* present in populations of European bumblebees and honeybees established in Argentina, was acquired in situ or if it was co-introduced from Europe with them. A previous study using microscopy found *A. bombi* in the invasive *B. terrestris* in northwest Patagonia, but not in native bumblebees from regions which are currently free of *B. terrestris* (Plischuk *et al*., 2009). Similarly, using molecular techniques, *A. bombi*-infected honeybees were only observed in regions invaded by *B. terrestris*, while the parasite was not detected in honeybees from *B. terrestris*-free regions (Plischuk *et al*., 2009). However, *B. terrestris*-free regions were also geographically and climatologically different from the regions where *A. bombi* was found and, in the absence of epidemiological knowledge, these observations cannot by themselves definitively prove that *A. bombi* infections resulted from the introduction of *B. terrestris*. More recently, Arbetman *et al*. (2013) found that 14 out 30 *B. terrestris*, five out nine *B. ruderatus* and one out of nine *B. dahlbomii* specimens collected in northwest Patagonia after invasion by *B. terrestris*, were infected with the neogregarine *A. bombi* (Arbetman *et al*., 2013). Conversely, the parasite could not be detected in any of the 30 of *B. ruderatus* and 52 *B. dahlbomii* museum specimens, collected before the invasion of *B. terrestris* (Arbetman *et*

al., 2013). However, this too is not definitive, because detection limits in the ethanol-stored samples (Arbetman *et al*., 2013) and thus the original source of this parasite in South America is still controversial. Finally, recent molecular screening of honeybee hives (n = 363) in Europe (Belgium) found 40.8% to be positive for *A. bombi* (Ravoet *et al*., 2013) while in Japan two of 69 examined hives (2.9%) were positive (Morimoto *et al*., 2013). However, whether the *A. bombi* discovered in honeybees are of the same strain as those found in *Bombus* species, and whether interspecific transmission is possible, remains unknown. In order to study the interspecific variability of *A. bombi* present in non-native bees that are established in Argentina, i.e. *B. terrestris, B. ruderatus* and *A. mellifera*, we sequenced the highly variable internal transcribed spacer 1 (ITS1) and ITS2 regions as genetic markers. We focused on two questions: (i) do honeybees and bumblebees share the same haplotypes with *A. bombi*, and (ii) do parasite strain haplotypes found in Argentina and Europe exhibit geographical structure? Answering these questions will provide insight into the transmission dynamics and native range of *A. bombi*.

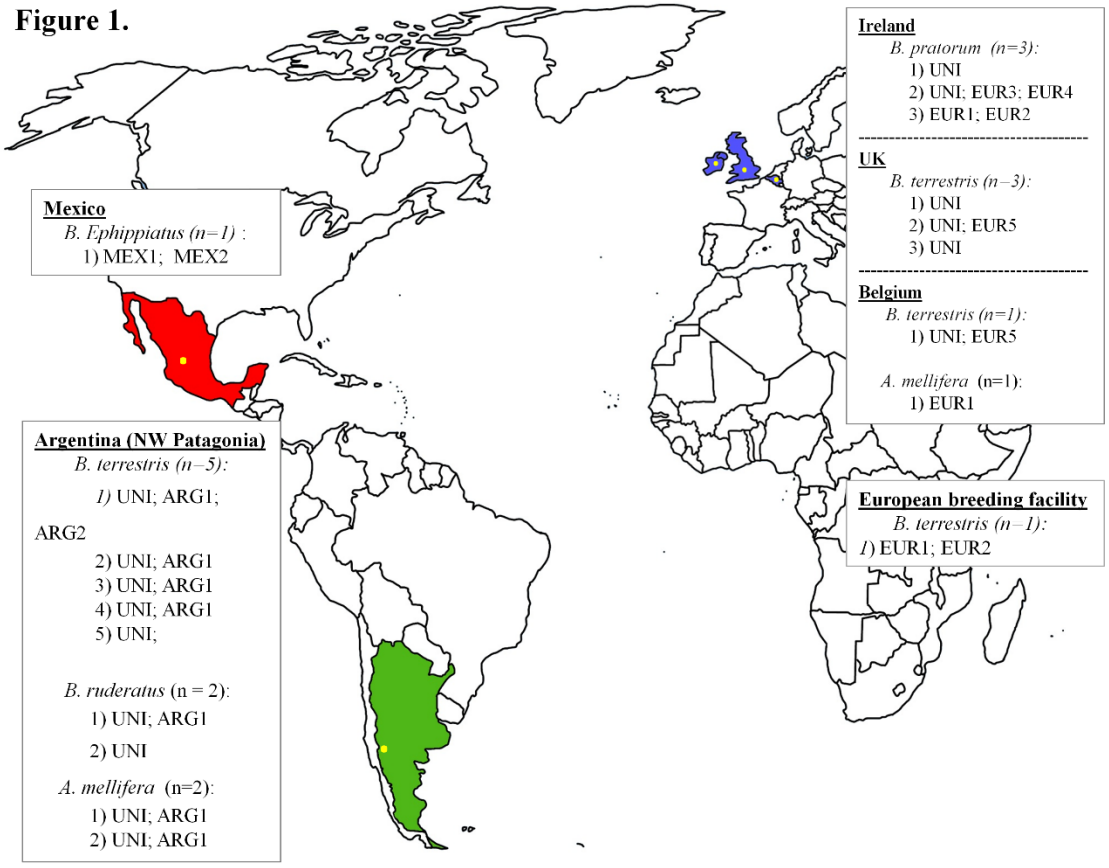
2. Materials and Methods

2.1. Origin of Infected *Apicystis bombi* species

To determine the haplotypes of *A. bombi* present in Argentina, we collected nine *A. bombi*-positive specimens: *B. terrestris* (n = 5), *B. ruderatus* (n = 2) and *A. mellifera* (n = 2). All samples were collected in northwest Patagonia (Arbetman *et al*., 2013; Plischuk *et al*. 2011). The European sampling consisted of eight *A. bombi*-infected specimens: *Bombus pratorum* from Ireland (n = 3); (Rutrecht *et al*., 2008), *B. terrestris* from Belgium (n = 1) and the UK (n = 3), and *A. mellifera* from Belgium (n = 1). Two extra specimens were analyzed: one *B. terrestris* from a commercially produced colony in Europe (Murray *et al*., 2013), and one *B. ephippiatus* native from Mexico (Figure 3.5.). All samples from Argentina were ethanol-stored (Arbetman *et al*., 2013; Plischuk *et al*., 2011), and the other samples were stored at -20°C before extraction. All samples were extracted with EZNAH

Insect DNA kit (Omega Bio-Tek; Norcross, GA) (Meeus *et al*., 2010) with the exception of the samples from the UK which were extracted with a Chelex extraction protocol.

Figure 3.5. Bee sampling infected with *Apicystis bombi* in different geographical areas. Overview of bumblebee (*Bombus pratorum, Bombus terrestris, Bombus ephippiatis* and *Bombus ruderatus*) and honeybee (*Apis mellifera*) samples included in the study, including the numbers of bees from each species at each location. All bees were infected with *Apicystis bombi* and the haplotypes found in each bee are given (UNI, EUR1-5, ARG1, ARG2, MEX1 and MEX2).
doi:10.1371/journal.pone.0081475.g001



2.2. Ethics statement

No national permissions were required to collect samples from the public lands in the locations of Belgium and the UK. Specific permission granted for sampling by the National Botanic Gardens, Ireland, and The Royal Parks, UK. Permits were not required in Argentina for the collection of exotic invertebrates (*B. terrestris*, and *A. mellifera*) from public lands. Permission to collect samples on his private land was provided by Luis Rovera in Argentina. Permission to collect bumblebees (*B. terrestris* and *B. ruderatus*) from Argentine National Parks were obtained from Administracion de Parques Nacionales (Argentina), while permission to export them from Argentina were granted by Secretaria de Desarrollo Sustentable y Medio Ambiente. The field studies did not involve endangered or protected species.

2.3. Haplotyping of *Apicystis bombi*

In order to determine the haplotypes of *A. bombi*, we sequenced both ITS1 and ITS2, which are both highly variable regions of the rRNA. Species-specific primers were designed by Primer3 program (Rozen & Skaletsky 2000): ApiITS732F 5`-TGGAAACAAGTCATTTTTGGAA-3` and ApiITS732R 5`-CCTGTTCACTCGCCGTTACT-3`, amplifying an approximately 730 bp long fragment of *A. bombi* within a bumblebee DNA extract. The amplicon contained ITS1, 5.8S rDNA, and ITS2. PCR reactions were done in 25 ml-reaction volumes, containing 0.2 mM dNTPs, 0.5 mM primers, 16PCR buffer, 1.5 mM MgCl2, 1.25 units of Taq polymerase (Taq DNA Polymerase, recombinant/Invitrogen) and 1 ml DNA template. Amplification was performed using one cycle of 94uC for 3 min, followed by 30 cycles of 95uC for 30 s, 55uC for 30 s, and 72uC for 60 s, and a final elongation step of 3 minutes.

PCR products were cloned using the Sticky-End Cloning Protocol (pJET1.2/blunt Cloning Vector, Fermentas UAB, subsidiary of Thermo Fisher Scientific Inc. V. Graiciuno 8 LT-02241, Vilnius, Lithuania). The plasmid was purified with the E.Z.N.A. Plasmid Mini Kit (Omega Bio-Tek) from 10 bacterial colonies containing the ITS regions, and sequenced (LGC Genomics GmbH (Germany, Berlin).

In order to analyze the different haplotypes, the sequences were aligned using the BioEdit sequence alignment editor (Hall 1999). Not all mutations can be regarded as a different haplotype. It is known that PCR artifacts can occur, with a PCR error rate of e = 0.55561023 (1.8561025 miss incorporations per cycle for 30 cycles) and the probability that a single mutation is caused by PCR error for an amplicon with size 730 bp is P = 0.063. This calculation is based on the binomial probability mass function reported by (Cummings *et al*., 2010). To be conservative, we decided to exclude all mutations which occurred only once in the total dataset. Of the remaining mutations, all haplotypes were confirmed at least three independent times. This procedure resulted in eight different haplotypes in our total data set (n = 18). The exclusion of possible PCR errors could lead toward an under-estimate of the true diversity present. Hence PCR products harboring 3 unique mutations (P = 0.0008) were subsequently re-included in the dataset. In this way, we incorporated two additional haplotypes, both found within the single Mexican sample. The ITS1 contained three mononucleotide repeats, one of 10 nucleotides in length and two of more than 10 nucleotides. It is known that these regions are, because of polymerase slippage, sensitive to mutation errors (Clarke *et al*., 2001). All sequences deposited at GenBank contain the consensus amount of nucleotides plus an N to indicate the unknown amount of extra nucleotides.

2.4. Genetic variation and structuring of *Apicystis bombi*

The pairwise mutational differences between haplotypes was calculated. In order to reveal population structure, the genetic variation within versus among populations was determined using an analysis of molecular variance (AMOVA; Arlequin v3.1). The software compares Fct (difference among groups), Fsc (difference among collections within groups), and Fst (differences among all collections) (Excoffier *et al.*, 2005). The data was grouped in different ways as indicated in Table 3.2. by a, b, and c. In section (a) the groups represent the regions Argentina; Europe and Mexico and the collections are the specimens within these regions, in section (b) the groups represent the different hosts, being *A. mellifera*, *B. terrestris* and *B. ruderatus*, while the collections are the different specimens of these hosts and finally section (c) represents the data only taking the regions Europe and

Argentina into account. Differences in the average within-population sequence divergence of haplotypes of Europe and Argentina were tested using a Mann-Whitney U test. To reconstruct the phylogenetic tree and estimate evolutionary relationships of the haplotypes, the Neighbor-Joining method was used (Saitou *et al*., 1987). Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). Reduced Median (RM) networks were drawn to visualize the evolutionary relationship between different haplotypes using NETWORK 4.6.1 (Bandelt *et al*., 1995). The Median Joining (MJ) algorithm resulted in the same topology.

3. Results

3.1. *Bombus* spp. and *Apis mellifera* are infected with the same species of *Apicystis*

We collected neogregarine infected bees (specimens) from three different locations: Argentina, Europe and Mexico. After sequencing analysis of a part of the 18S region we confirmed that all hosts were infected with *A. bombi*. All were identical to previous samples of *A. bombi* found in *B. terrestris*, *B. ruderatus*, *B. dahlbomii* (Arbetman *et al*., 2013) and *A. mellifera* (Plischuk *et al*., 2011) in Argentina, but also to *A. bombi* found in *B. pratorum* in Europe (Meeus *et al*., 2010). One mutation in the 18S was observed when comparing it with the 18S fragment found in the native Mexican bumblebee *B. ephippiatus* (KC951279). With the use of *A. bombi* specific primers, located in the 18S rRNA and 26S rRNA, we were able to sequence ITS1 and ITS2 (GenBank KF322207–KF322216). From each specimen (n = 18) we sequenced 10 different clones; in total seven bees contained one unique haplotype, nine contained two haplotypes and only two bees contained 3 haplotypes. Most specimens (16 out of 18) therefore contained only one or two different haplotypes, indicating that there is little or no intragenomic variability present in *A. bombi*. When analyzing the molecular variance of the complete data set, 34% of the variability in the data was explained by variation within one specimen (Table 3.2.a). To study *A. bombi* transmission among different host species, we investigated the Argentine subsample (n = 9). Here three different species were sampled in close proximity to each other (samples were at most 25 km apart). We determined whether haplotypes were distributed randomly among these three different species (*B. terrestris*, n = 5; *B. ruderatus*, n = 2; and *A.*

mellifera, n = 2). The two most common haplotypes were present in all three host species (Figure 3.5., 3.6.). The most common haplotype (UNI) was detected in all nine Argentine bees, while the other prevalent haplotype (ARG1) was also found in seven of these specimens (Figure 3.5., 3.6.).

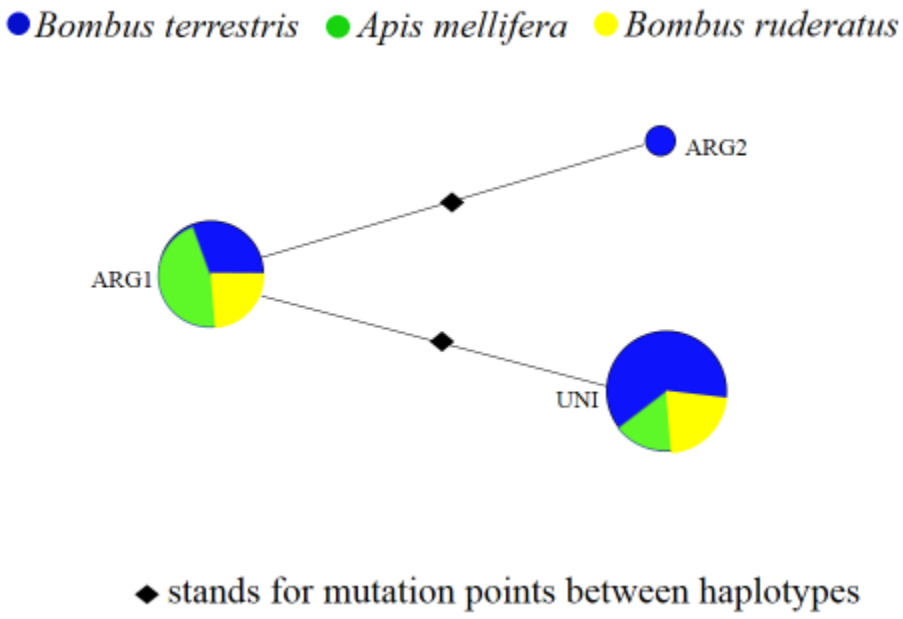


Figure 3.6. The network of *Apicystis bombi* haplotypes (ARG1, ARG2 and UNI) of different bee hosts in Argentina. The relative abundance of a haplotype in the three introduced bee species in Argentina is represented by the size of the circles. The colors represent the different hosts; i.e. *Bombus terrestris* (blue), *Bombus ruderatus* (yellow) or *Apis mellifera* (green), showing the proportion of each haplotype over the different hosts. The black squares represent unobserved single-nucleotide substitutions. doi:10.1371/journal.pone.0081475.g002

Only 17.3% of the total genetic diversity of *A. bombi* was explained by differences among host species. It should be noted though, that this percentage is a consequence of the higher sampling of *B. terrestris* resulting in a unique haplotype (ARG2) for this species, and the majority of the genetic diversity was found within host species: 5.5% among specimens of one host species and 77.2% within a specimen (Table 3.2.b).

Table 3.2.: Analysis of molecular variance (AMOVA) showing the genetic variation distribution of *A. bombi* from (a) all specimens (grouped by location), (b) specimens from Argentina (grouped by host), and (c) specimens from Argentina and Europe (grouped by location).

Source of variation	DF	Sum of squares	Variance components	Percentage of variance	*P*	
a)						
Among regions (Argentina; Europe and Mexico)	2	13.8	0.15	31.8	0	
Within each region						
Among specimens within each region	15	23.4	0.16	33.8	0	
Within specimens	138	21.8	0.16	34.4	0	
b)						
Among hosts	2	2.6	0.04	77.2	0.06	0.17 Fct
Within each host						
Among specimens within each host	6	1.9	0.01	5.5	0.13	0.07 Fsc
Within specimens	2	2.6	0.04	17.3	0.06	0.17 Fst
c)						
Among regions (Argentina and Europe)	1	5.8	0.06	15.2	0.0004	0.15 Fct
Within each region						
Among specimens within each region	15	23.4	0.16	42.9	0	0.51 Fsc
Within specimens	137	20.8	0.15	41.9	0	0.58 Fst

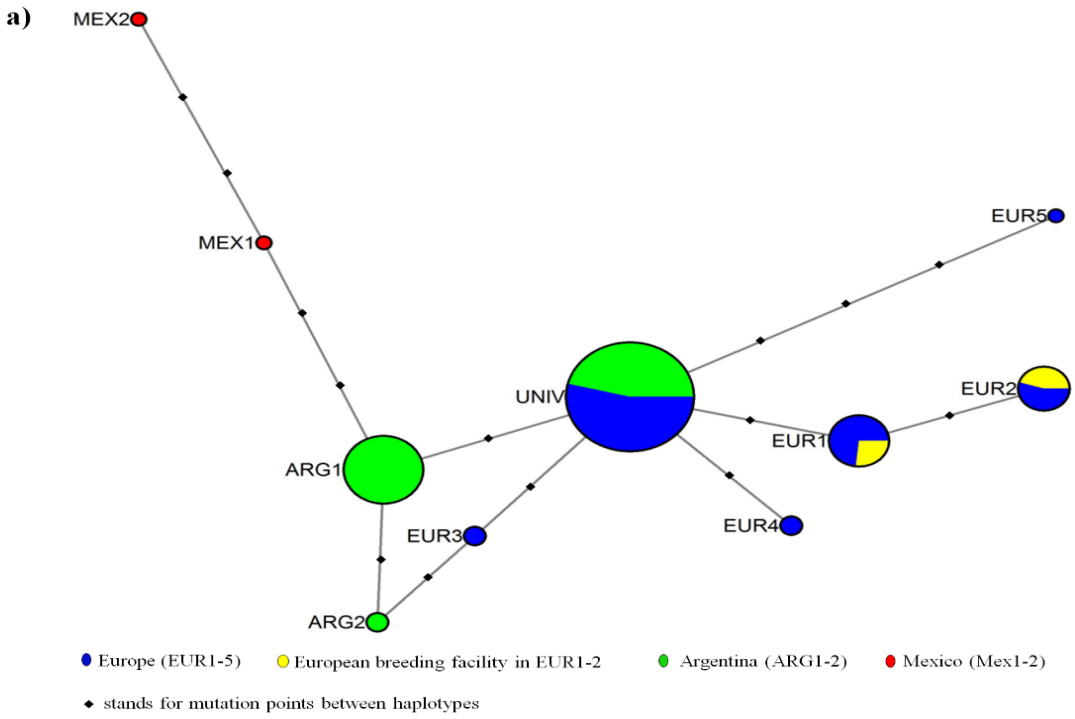
P is the probability of having a more extreme variance component and F-statistic than the observed values by chance alone. F-statistics are a measure for genetic variation with Fct, Fsc and Fst assessing different hierarchical levels of subdivision. The level of subdivision is explained in the first column of the table. doi:10.1371/journal.pone.0081475.t001

3. 2. Argentine and European *Apicystis bombi* share the same origin

We classified *A. bombi* haplotypes according to their geographic location, and also included an infected *B. terrestris* sample from a European commercially produced bumblebee colony (see Murray *et al*., 2013). We found three haplotypes in Argentina (ARG1, ARG2 and UNI, in nine hosts), six haplotypes in Europe (EUR1–EUR5 and UNI, in eight hosts), two haplotypes in Mexico (MEX1 and MEX2, in a single host), and two haplotypes in a commercially produced colony (EUR1 and EUR2). Haplotypes EUR1, EUR2, EUR3, EUR4 and EUR5 were only found in European samples, ARG1 and ARG2 were only found in Argentine samples, and the haplotypes MEX1 and MEX2 were only found in Mexico (Figure 3.7.a). The universal haplotype UNI was detected in all Argentine samples, and in 75% of the European samples. The other prevalent haplotype ARG1 occurred in 78% of the samples in Argentina while it remained undetected in our sampling in Europe. Furthermore, as shown in Figure 3.7.b, the haplotypes found in Europe and Argentina formed a single phylogenetic clade. The genetic variation among geographic regions (Argentina and Europe) only explained 15.2% of the total genetic variation, with most of the variation found among and within specimens of each region (42.9% and 41.9%, respectively) (Table 3.2.c). The genetic structure between Europe and Argentina was therefore small and there was no significant phylogenetic structure across these regions (Figure 3.7.b). The two haplotypes found within the commercially produced bumblebee colony also fell within this European/Argentine cluster.

3. 3. Genetic diversity of *Apicystis bombi* in Argentina and Europe

The mean sequence divergence of *A. bombi* among specimens from Argentina (mean 6 s.d = 0.760.6, n = 9) was significantly smaller than the mean sequence divergence among European specimens (1.561.3, n = 8; Mann Whitney U test: z = 24.98; P = 0.001). Indeed, only three haplotypes were found among Argentine specimens separated by one or two mutations, while the European haplotypes were more diverse (Figure 3.7.). This could indicate that *A. bombi* from Europe was the founder population and those retrieved in Argentina the sink population. However, our sample amount was too low and sampling location distance too variable to draw real conclusions.



Evolutionary relationship between *Apicystis bombi*

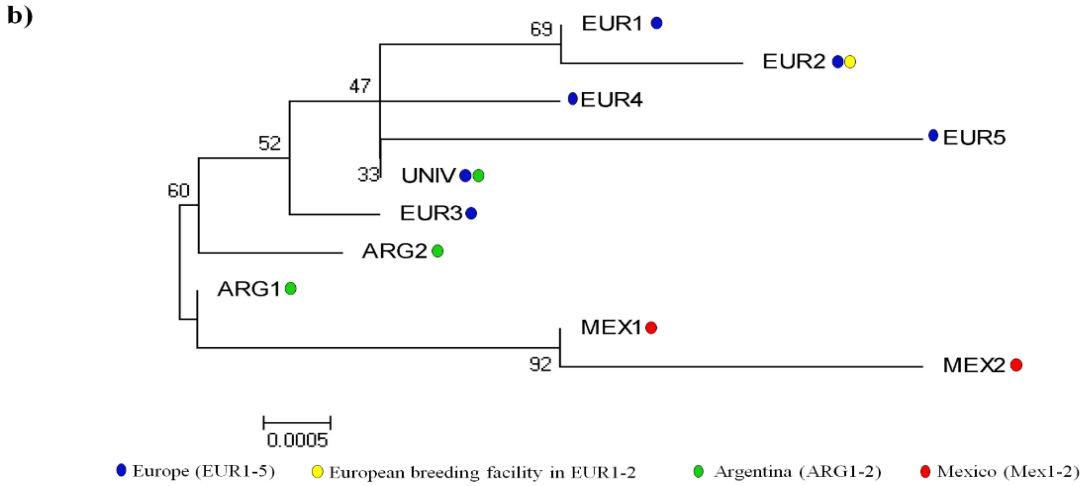


Figure 3.7. Network and evolutionary relationships of *Apicystis bombi* haplotypes. (a) The network depicts the number of mutations between the different haplotypes, and the name of each haplotype informs about the location, the UNI haplotype was found in Europe and Argentina. The circle sizes are proportional to the numbers of bees infected with a certain parasite haplotype and the colors indicate the location of the host. Bees collected in Europe (blue), Argentina (green), Mexico (red), and a commercially produced bumblebee colony in Europe (yellow). The black squares represent unobserved single-nucleotide substitutions. (b) The geographic location in which each parasite haplotype was detected is indicated with colored spots: Europe (blue), Argentina (green), Mexico (red), and a commercially produced bumblebee colony in Europe (yellow). The optimal tree with the sum of branch length = 0.0176 is shown. The percentages of replicate trees in which the associated haplotypes clustered together in the bootstrap test (1000 replicates) are shown next to the branches. doi:10.1371/journal.pone.0081475.g003

4. Discussion

4.1. The ITS region can be used to haplotype *Apicystis bombi*

In this study we used ITS1 and ITS2 as a molecular marker to study genetic variability of *A. bombi*. Although these markers have been widely used, particularly because of their high level of sequence variation, there are caveats regarding their use (Harris *et al*. 2010). Parasites have multiple rDNA loci which makes intragenomic diversity possible, as for example reported for the bumblebee parasite *Nosema bombi* (O'Mahony *et al*., 2007). Because of concerted evolution, intragenomic diversity is generally low (Harris *et al*., 2010). However, different paralogs are possible, making ITS markers uninformative if intragenomic diversity is higher than the intergenomic diversity within a population. In our data, 34% of the variability was within individuals. This percentage may reflect intragenomic diversity or infection with multiple strains. Although we cannot differentiate between these two explanations, we speculate that there is little intragenomic diversity for *A. bombi* parasites for two reasons. First, 16 out of 18 specimens contained either one (7out of 16 specimens) or two (9 out of 16 specimens) haplotypes (across 10 clones). Second, it is often reported that multiple parasite strains infect a single host (Read *et al*., 2001), a phenomenon reported for other bumblebee parasites like *Crithidia bombi* (Ruiz-Gonzalez *et al*., 2012; Schmid-Hempel *et al*., 1999).

A final remark is the limited sampling size and thereby the possibility to miss existing genetic variation. We analyzed 18 positive specimens, already revealing 10 different haplotypes. Moreover we could also detect two more distant related haplotypes from Mexico, indicating that possible intraspecific variation within this limited amount of samples can be detected.

4.2. Honeybees and bumblebees share *Apicystis bombi* haplotypes

Our results indicate that honeybees and the two European bumblebee species, *B. terrestris* and *B. ruderatus*, sampled from the same area in Argentina, were all infected by the same haplotypes of *A. bombi*. This parasite was originally described as a bumblebee parasite, implicated in increased mortality rates in infected queens (*B. pratorum*; (Rutrecht *et al*., 2008)). These authors found that the parasite reemerges in bumblebee workers late in the season, suggesting that some queens survive infection and transmit the parasite to the

progeny and/or alternate reservoirs exist for the parasite (Rutrecht *et al*., 2008). Recent studies suggest that *A. mellifera* could represent an alternative reservoir and/or vector of *A. bombi* (Meeus *et al*., 2011; Ravoet *et al*., 2013; Morimoto *et al*., 2013). Although we have demonstrated that the same parasite species and haplotypes within the species is present in both taxa, we cannot conclude whether *A. bombi* found in honeybees can indeed re-infect bumblebees. Furthermore, it still needs to be determined whether the *A. bombi* found in Argentina represented genuine infections of *A. mellifera*. The fact that honeybee and bumblebee populations are largely sympatric throughout the geographic range of *Bombus* spp., which is a first requirement for parasites to jump between host species (Woolhouse *et al*., 2005), makes the presence of shared parasites probable. Indeed, several honeybee parasites are now known to infect bumblebees (Meeus *et al*., 2011; Evison *et al*., 2012; Genersch *et al*., 2006; Graystock *et al*., 2013; Meeus *et al*., 2012; Singh *et al*., 2010), showing that species jumps between bumblebees and honeybees do not necessarily result in a dead-end host. Furthermore, recent work has confirmed that *A. bombi* from bumblebees can readily infect honeybees (Meeus *et al*., 2011). Although knowledge about multihost parasites is 'scarce', the high rate of interspecies transmission plays a central role in the evolution of virulence in host-parasite networks (Rigaud *et al*., 2010).

4.3. The same *Apicystis bombi* haplotypes are shared between Argentina and Europe

Our fundamental question was whether *A. bombi* detected in Argentina is indigenous or from introduced bees originating from Europe. If *A. bombi* were indigenous in two geographically separated continents, two clearly differentiated clusters should be seen, where one contains parasites of Argentina and the other of Europe. Two separate clusters would also imply that the introduced European bees became infected with *A. bombi* already present in Argentina. Instead we found that the European parasites clustered together with those from Argentina. However, we note that the detection of only one cluster could be the consequence of insufficient sampling in Argentina and/or Europe. Higher sample numbers could reveal separating clusters. However, the homogeneity in the data between Europe and South America strongly suggests that parasites identified in the analyzed samples share the same origin.

We detected low geographic structuring between Europe and Argentina, with only 15.2% of the genetic variation being explained by location. Indeed both locations share the most frequent haplotype (UNI). The haplotypes detected in Argentina (ARG1 and ARG2) are one and two point mutations different from UNI, respectively (Figure 3.7.a). Therefore the number of point mutations between the ARG samples and the UNI is comparable with the amount found in the haplotypes (EUR1, EUR2, EUR3 and EUR4) which are exclusively found in Europe. Thus the differences detected between samples from Europe and Argentina are of the same magnitude as those found within European samples. Geographical structuring in parasites between land masses separated by an ocean has been observed in other taxa; a good example is *Atractolytocestus huronensis* (*Cestoda: Caryophyllidea*), an invasive parasite of common carp introduced from North America to Europe, which exhibits at least 8 mutations or 1.2% sequence diversity in ITS2 of the two geographic locations (Bazsalovicsova *et al*., 2011). We recognize here that one has to be careful when comparing mutational rates of the ITS region of different species. Ideally, reference samples of *A. bombi* from isolated locations without nonnative bumblebees or honeybees would be required to quantify the natural genetic structuring of *A. bombi* within its natural habitats. With the intense and worldwide transport of honeybees and bumblebees such samples are very difficult to obtain. However, it is evident from our data that the weak structuring of *A. bombi* between European and Argentine bumblebees does not reflect the historical Palearctic, Nearctic, and Neotropic separation of the Old World Bombus ancestor (Hines 2008). Rather, it supports a common and more recent origin, which is consistent with the hypothesis that introduced European bees (be it *Apis* and/or *Bombus*) carried

4.4. *Apicystis bombi* into Argentina.

The *A. bombi* haplotypes found in *B. ephippiatus*, native to Mexico, are more distantly related, demonstrating that the ITS region of *A. bombi* is variable enough to identify differentiation at these spatial scales where present. These data also represent the first record of *A. bombi* in this country. *A. bombi* was originally described as *Mattesia bombi* (Liu *et al*., 1974; Lipa *et al*.,1996) and has been found in Ontario (Canada) (Liu *et al*., 1974), suggesting its presence in North America before bumblebee transport started. However, the single sample from Mexico is insufficient to draw any conclusion regarding genetic differentiation within Mexico. The native *B. ephippiatus* is found in a region where

non-native commercially produced bumblebees, *Bombus impatiens*, have become established (Torres-Ruiz *et al*., 2012; Vergara *et al*., 2012). It is uncertain whether the *A. bombi* haplotypes in the Mexican bee represent indigenous, North American haplotypes, or haplotypes spilling over into Mexico from other locations. The Mexico sample does further emphasize the need for a worldwide prevalence and haplotyping study in which regions with intense bumblebee and/or honeybee importation are compared with regions without importation.

4.5. Geographic transmission routes of *Apicystis bombi* and perspectives

Our molecular haplotyping strongly suggests that the origin of the *A. bombi* currently found in non-native Argentine bees is the same as those found in Europe. What mechanisms could explain this? A first possibility is that *A. bombi* originated from Europe and was subsequently introduced into Argentina either by *A. mellifera*, *B. terrestris* or *B. ruderatus*. We have one infected bumblebee sample originating from a commercially-produced bumblebee colony, and its *A. bombi* haplotypes fall within the European/Argentine cluster. This again indicates there is a shared origin of *A. bombi*, but remains uninformative about the original native region of *A. bombi*. Furthermore, the original native locality of commercially produced bumblebees is not always clear, and thus also of the parasites they carry. The two subspecies most commonly used in European breeding factories are *B. t. terrestris* and *B. t. dalmatinus*, with the former originating from a variety of European countries and the latter from Greece and Turkey (Velthuis *et al*., 2006). In addition, Israelian facilities also supplied bumblebees to Chile. Together with the fact that *A. bombi* has been detected in Turkey (Cankaya *et al*., 2006), this makes the Mediterranean region a good candidate to retrieve the original location of the ARG1, ARG2 haplotypes.

A second possibility is that *A. mellifera* became infected with *A. bombi* from a South American host and global honeybee queen transport subsequently homogenized the genetic variation of this parasite. Although this seems a less probable explanation, it is a possibility that the current results cannot exclude. At a minimum, however, *A. bombi* can be considered as an emergent disease in Argentina, in either the native or non-native bees depending on the direction of transfer, with the implication that it may have also rapidly

increased in prevalence in other regions where non-native honeybees and bumblebees have been introduced. It has been recognized that emergent diseases of wild populations pose a substantial threat to the conservation of global biodiversity (Graystock *et al*., 2013; Daszak *et al*., 2000). With bee diversity already being threatened by multiple factors (Potts *et al*., 2010), monitoring of this emergent infectious disease is crucial. Here we present molecular tools to study intra- and interspecies diversity to untangle the spread of this parasite. In order to detect the exact origin and transmission dynamics of this parasite, multiple locations now need to be sampled worldwide, especially the Mediterranean region, preferably including regions with no honeybee and/or bumblebee imports. In parallel, pathological studies are also urgently needed to assess the virulence of the parasite across native and non-native hosts.

Chapter IV: Domesticated honeybees are a reservoir of *Apicystis bombi*

1. Introduction

The mutualistic relationship between plants and pollinators has lead towards a tremendous biodiversity (Bascompte and Jordano 2007.). Herein, bees, have evolved a full reliance on flowering-plant, as all their life stages are completely dependent on nectar and pollen (Michener 2007.). Up to 80% of the plant species are dependent on insect pollination for fruit or seed set, a service particularly provided by a vast variety of wild bees (Gallai *et al*., 2009; Garibaldi *et al*., 2013). This ecological essential service also results in a purely economic value of 9.5% of the total economic value of crops that are directly used for human food (Gallai *et al*., 2009; Potts *et al*., 2010). The honeybee is well known for its commercial pollination potential, increasing yield in 96% of animal-pollinated crops (Potts *et al*., 2010; Klein *et al*., 2007). But honeybees are outperformed by the pollination service of wild bees, as wild insect visitation enhanced fruit set twice as much (Garibaldi *et al*., 2013). Current practices to rescue natural pollination losses by honeybee pollination has therefore better, although less straight forward alternatives, being the restoration of the native pollinator communities. Furthermore massive domestication of honeybees, being efficient foragers, will surely have its impact on forage availability for native sympatric bees, probably aggravating the current threatened status of the bee pollinator community. Indeed forage loss is regarded as one of the primary drivers in current declines of bumblebees, a well-studied genus of bees (Goulson *et al*., 2008). Bumblebees in the neighborhood of honeybee-rich environments tend to be smaller, here the size of the bumblebee is a proxy for the fitness of bumblebees and colony success in that environment (Goulson *et al*., 2009). The severity of this inter species competition depends not only on the abundance of honeybees per area but also on the availability of shared floral sources (Leonhardt *et al.*, 2012). Aside from this direct competition for food between wild pollinators and domesticated honeybees there can also be an impact on host parasite interaction. Honeybees could act as a reservoirs of parasites. Indeed, it has been reported that honeybees and wild pollinators host the same parasites (Ravoet *et al*., 2014). For instance, Kashmir bee virus and Israeli acute paralysis viruses originally reported in honeybees reduce colony startup success in bumblebee micro-colonies (Meeus *et al*., 2014). *Nosema ceranae*, known to harm honeybee hives (Higes *et al*., 2013), is also found in bumblebees (Fűrst *et al*., 2014; Graystock *et al*., 2013). *Apicystis bombi* a neogregarine

parasites infecting the fat body tissue of bumblebees was also found in honeybees (Plishchuk *et al*., 2011; Ravoet *et al*., 2013).

Here in this study we will assess if presence of domesticated honeybees interferes with the natural host parasite prevalence in a wild bee population. We will specifically look at protozoan and microsporidian parasites in *Bombus pascuorum* one the most important pollinator in Belgium and the most dominant bumblebee in the Palaearctic Region (Rasmont and Iserbyt 2010-2013).

2. Material & Methods

2.1. Choice of honeybee-rich and honeybee-poor study sites

We looked at parasite prevalence in 5 locations, each containing 2 paired study sites (5x2). Each location harbored a honeybee-rich study site (HRS) and a honeybee-poor study site (HPS). The choice of a HRS was made based on the distribution of apiaries in the neighborhood of Ghent (Belgium). In Belgium each beekeeper is obliged to register at the Federal Agency for the Safety of the Food Chain. This distribution was used to generate a map of study sites presumed to be rich with apiaries. At each HRS we contacted the local

Table 4.1.: **Number of honeybee hives per location. (Numbers in brackets represents honeybee hives per km^2)**

Location	Honeybee-rich study site	Honeybee-poor study site
Gent	21 (11.9)	0
Roeselare	19 (10.8)	0
Zingem	3 (1.7)	0
Horebeke	8 (4.2)	0
Waarschoot	7 (4.0)	0
Mean ± SE	11.6 ± 3.1 (6.6 ± 1.8)	0 ± 0

beekeepers to further screen for any potential unregistered apiaries and updates about amount of hives. The experiment was performed in May 2013, a year with high winter mortality of honeybee hives in Belgium (Chauzat *et al*., 2014). The updated map was used to pinpoint a new paired study site at a distance of 1.5 km with a minimum number of

apiaries in the neighborhood. If we calculate the number of bee hives surrounding the centre of each study site with a 750 m radius the HRS contain a mean of 6.6 ± 1.8 SD honeybee hives per km^2 compared to 0 ± 0 for the HPS (see table 4.1). The mean number of honeybee hives per km^2 in Belgium is 3.6 (Chauzat *et al*., 2013).

2.2. Paired design of two study sites within one location and statistics

The 2 study sites within one location are separated by 1.5 km. This distance does not exclude honeybees from the HRS to enter the HPS, but we hypothesize that the abundance will sharply drop because of dilutions effects. The distance had two rationales. First, in each location we wanted to measure parasite prevalence in wild *B. pascuorum*, therefore the distance between locations needed to be large enough to ensure that the sampling populations were different. 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 2003). We cannot exclude that some specimens, with nests between the study sites, are foraging in the two sampling places, but they will be scarce. Second, the 2 study sites within one location were chosen to have the same landscape metrics, achievable by keeping the distance between them as small as possible. Figure 4.1. gives an overview of each study site, showing the same degree of urbanization, agriculture landscapes, and forests within each location.

For statistical analyzes of the mean abundance of each genera, and its related F score and *P* value Univariate General Linear Models (GLM) were performed using HRS and HPS as a fixed factor, while the locations were used as a random factor.

Figure 4.1. Overview of study sites per location. Yellow highlighted zones surrounded by red circle indicates honeybee-rich sites (HRS); Honeybee-poor sites (HPS) were surrounded by white circle. White Line depicts 1.5 km distance between study sites.

City site locations: a) Gent; b) Roeselare;

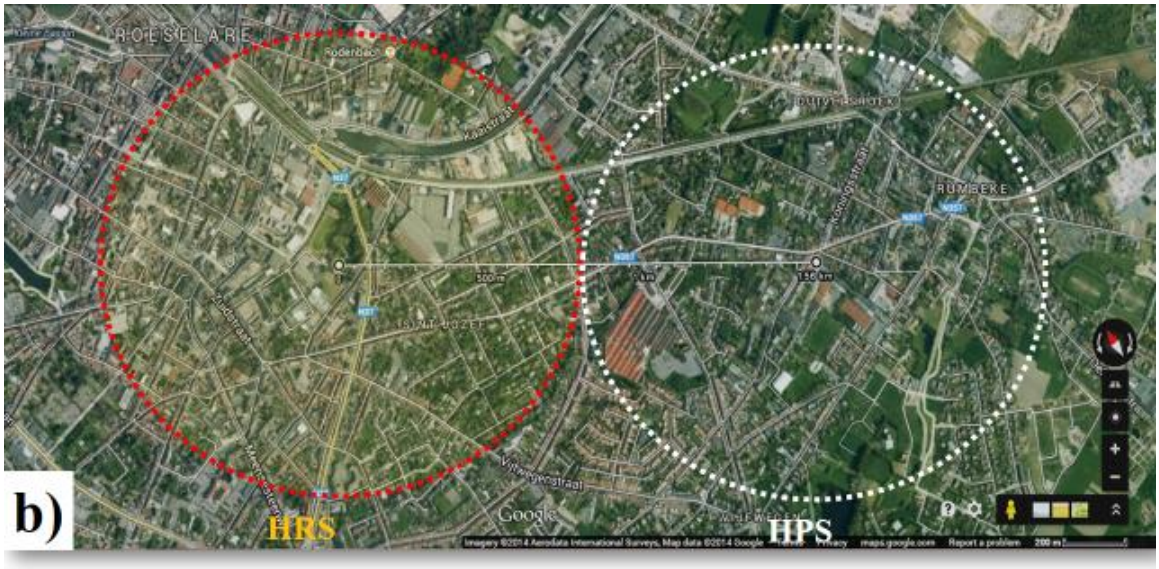
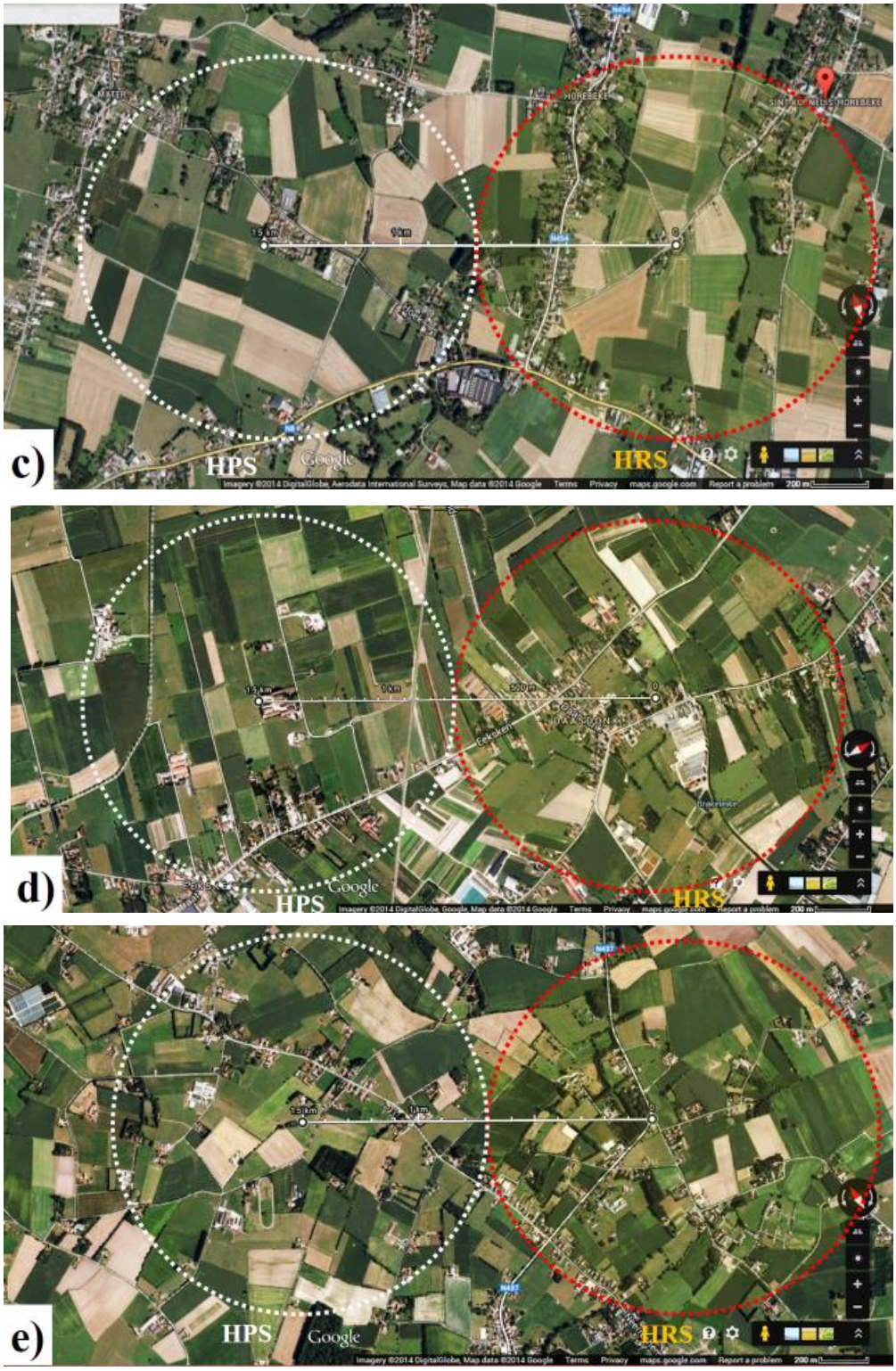


Figure 4.1. Countryside locations: c) Horebeke; d) Waarschoot; e) Zingem



2.3. Measurement of bee community

In each study site we placed 9 (3x3) pan traps in triplicates; each triplicate contains the following 3 colors: white, yellow and blue (Figure 4.2.). The distance between the pan traps within one triplicate ranged from 3 until 5 meters and between triplicates from 10 until 20 meters. Each triplicate of pan traps was placed at a certain height, ranging from 0 to 0.5 meter, depending on the dominated flowers vegetation present. The pan traps were filled with 400 mL of water and a drop of detergent. The total survey time was 48 days but pan traps were checked at intervals of 2 or 3 days. The pan traps were refilled if needed. The collected specimens were temporarily stored in 70% ethanol until pinned for identification (Westphal *et al.*, 2008). The placement of the pan traps must not only give us a verification that the HRS are indeed richer in honeybees, but will also allow us to quantify the pollinator composition at each study site.

Figure 4.2. Placement of pan traps at study sites.

2.4. Parasite detection

We used a PCR based screening method on RNA samples for detection of protozoan and microsporidian parasites (Table 4.2.). Wild *B. pascuorum* bumblebees were processed individually and crushed in 700 µl Qiazol® Lysis Reagent. Samples are homogenized in

special bead-beating machine with steel beads (1mm) and zirconia beads (0.1 mm) in special shock resistance tubes. The tubes were centrifuged at 17,000 g for 3 min. until pellet occurred and 1 ml of homogenized material was collected. All samples were further processed according manufacturer's specifications (RNA extraction kit, QIAGEN) to obtain a final 50µl of total RNA in RNA-free water. Extracted RNA samples were stored at −80°C prior to molecular detection of parasites. Finally parasites were detected by PCR as described in the section 2.2. (Monitoring of reared bumblebee colonies subsection *PCR analysis* in the Part 1 of chapter II.)

Table 4.2. **Primer pairs used for parasite detection**

Target parasites	Primer pairs
Apicystis bombi	NeoF: CCAGCATGGAATAACATGTAAGG NeoR: GACAGCTTCCAATCTCTAGTCG
Crithidia bombi	SEF: CTTTTG GTCGGTGGAGTGAT SER: GGACGTAATCGGCACAGTTT
Nosema bombi	Q NoU F1: GGAGTGGATTGTGCGGCTTA NoB R: ATTCTCGAATCAGGATTCTCTCAGAA
Nosema ceranae	Q NoU F1: GGAGTGGATTGTGCGGCTTA NoC R ACCACTATTATCATTCTCAAACAAAAAACC
Nosema apis	Q NoU F1: GGAGTGGATTGTGCGGCTTA NoA R: CCTCAGATCATATCCTCGCAGAAC

3. Results

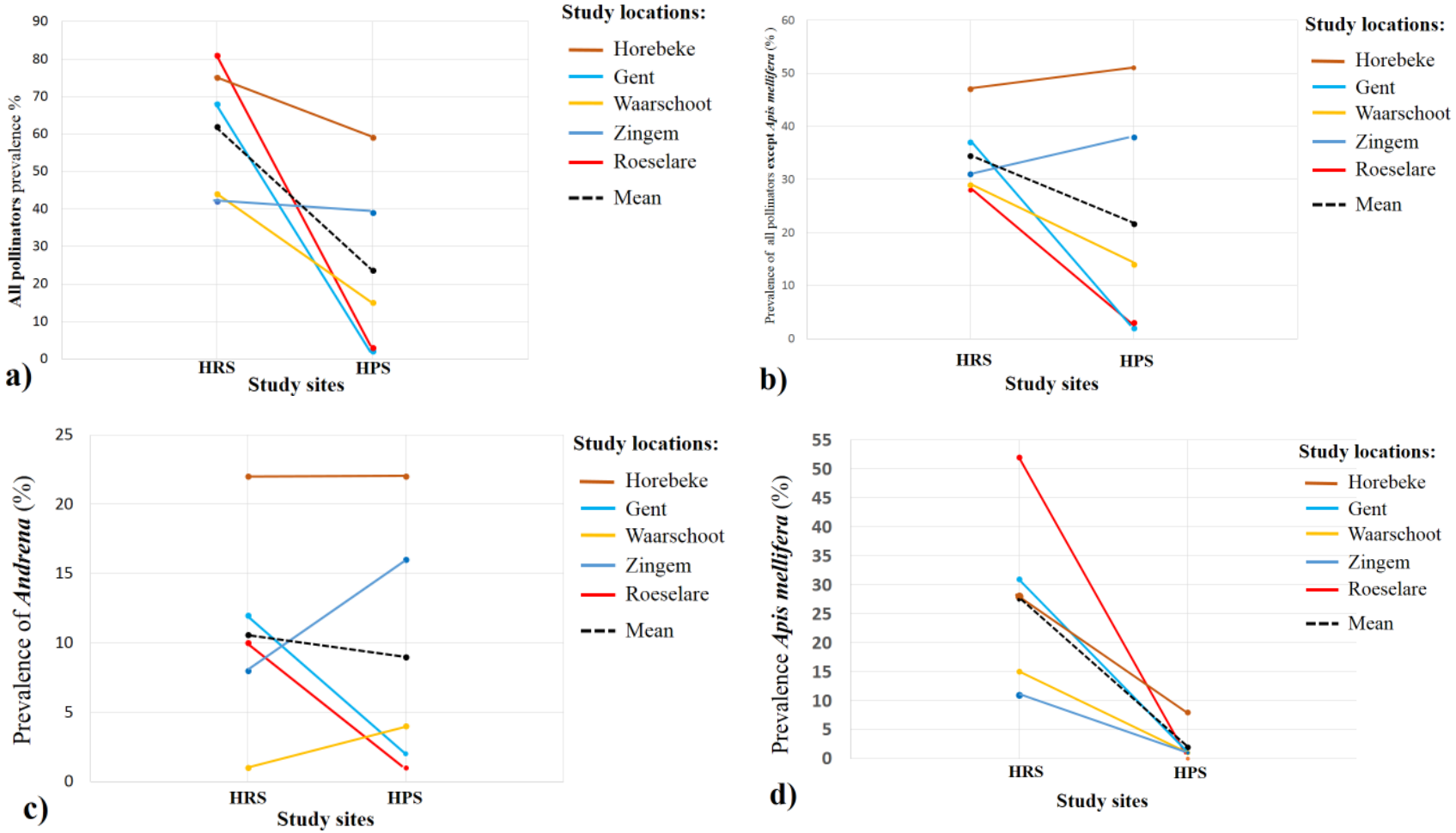
3.1. Pollinator community in honeybee-rich study (HRS) site and honeybee-poor study (HPS) site.

In each study site of each location we assessed the pollinator community (2x5). This showed that all genera, except the *Apis*, of pollinators stayed the same within locations. (See Table 4.3). We can conclude the honeybee-rich (HRS) and honeybee-poor (HPS) study sites within a same location are indeed paired and harbor the same wild bees foraging in it. While the variable factor, *Apis mellifera*, we wanted to create in the setup is present. Indeed in each location more honeybees (*A. mellifera*) are present. The increase in presence of honeybees also resulted in a significant increase of the total amount of pollinators, while the total abundance of other pollinating bees were stable in the two study sites within each locations (Figure 4.3).

Table 4.3. Overall mean relative abundance (%) and standard error (SE) of the most abundant genera found at honeybee-rich study sites (HRS) and honeybee-poor (HPS) study sites in all location.

Genus	Mean abundance and SE		Univariate General Linear Models	
	HRS	HPS	F	*P*
All genera	**62.0 ± 8.0**	**23.6 ±11.1**	**6.891**	**0.034**
Without *Apis mellifera*	34.6 ±3.4	21.6 ±9.8	1.553	0.253
Apis mellifera	**27.4 ±7.2**	**2.0 ±1.5**	**13.880**	**0.007**
Andrena	10.6 ±3.4	8.8 ±4,3	0.096	0.765
Bombus	12.0 ±2.3	6.60 ±3,1	2.032	0.197
Lasioglossum	2.6 ±1.1	2.4 ±1.9	0.007	0.934
Nomada	4.8 ±2.9	0.8 ±0.5	1.595	0.247
Osmia	3.2 ±0.7	2.0 ±1.8	0.355	0.570
Coelioxys	0.2 ±0.2	0.0 ±0.0	1.167	0.316
Dasypoda	0.4 ±0.4	0.2 ±0.2	0.318	0.590
Halictus	0.2 ±0.2	0.0 ±0.0	2.435	0.163
Hylaeus	0.0 ±0.0	0.2 ±0.2	1.167	0.316
Megachile	0.2 ±0.2	0.0 ±0.0	0.933	0.366
Melecta	0.0 ±0.0	0.2 ±0.2	0.875	0.381
Sphecodes	0.2 ±0.2	0.4 ±0.4	0.184	0.681

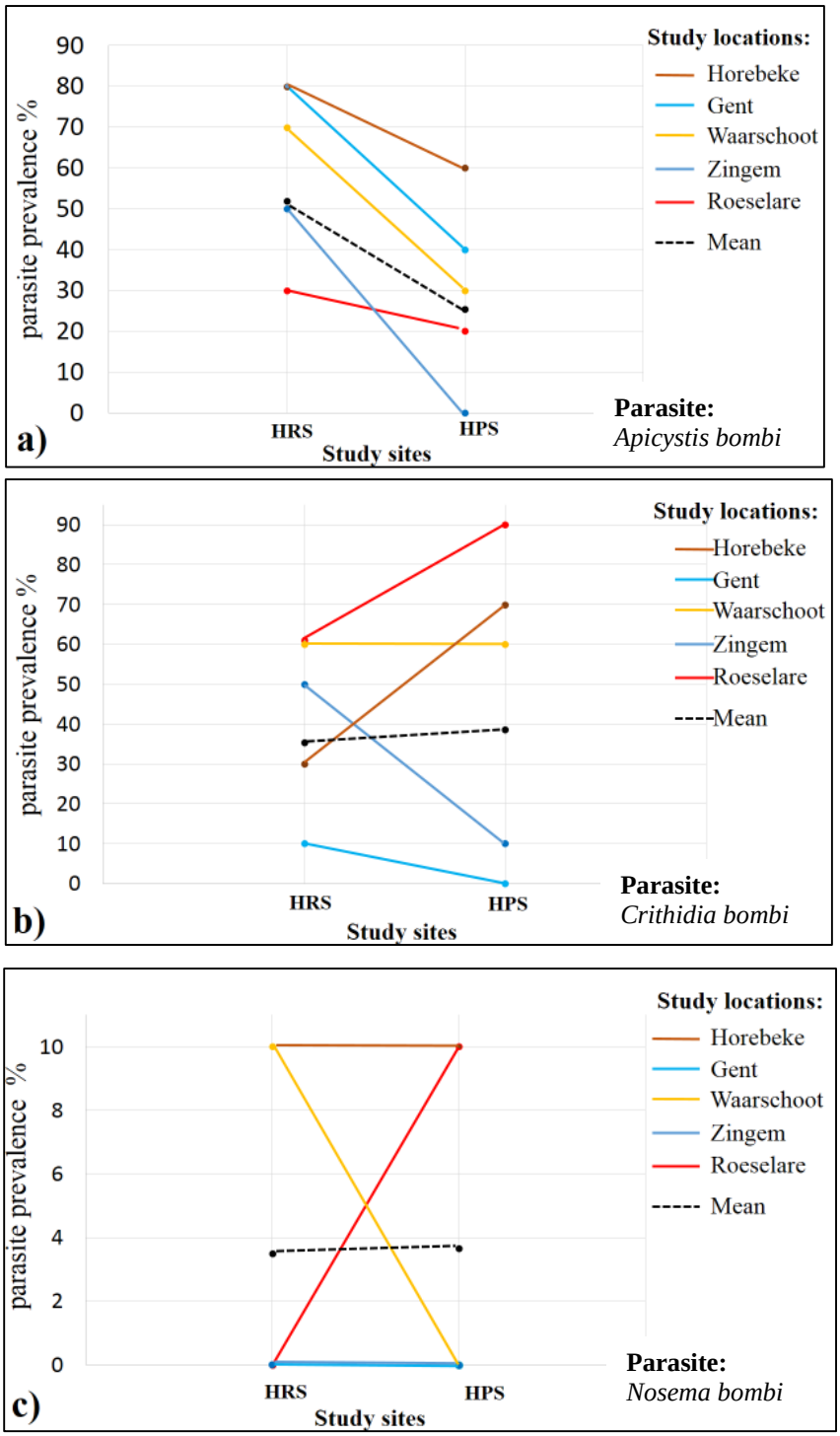
Figure 4.3. Comparative diagram of prevalence of bee genera in honeybee-rich (HRS) and honeybee-poor (HPS) study sites. a) all pollinators; b) all pollinators except *Apis mellifera*; c) *Andrena*; d) *Apis mellifera*



3.2. Parasite prevalence in honeybee-rich (HRS) and honeybee-poor (HPR) studies

The mean prevalence of both *N. bombi* and *C. bombi* did not alter between the two study sites within locations (Fig 4.3. a,b,c). For *A. bombi* we saw a drop in prevalence in each HPS of the 5 different locations (GLM; $F_{\text{Study site}}$ (1,90) = 14.52; P = 0.02); also the prevalence of *A. bombi* between these different locations was different (GLM; F_{Location} (4,90) = 7.52; P = 0.04). But there was no interaction between study site and location (GLM; $F_{\text{Study site x Location}}$ (4,90) = 0.66; P = 0.62). Indeed the effect is clear in all study sites. The prevalence of *A. bombi* drops in the same direction. As explained above, we regard both study sites within a location as paired with each other; therefore we can also perform a non-parametric paired statistical test. Also here a significant drop of *A. bombi* prevalence in the honeybee-poor study sites can be proven (Wilcoxon signed rank test; P = 0.04).

Figure 4.4. Comparative presentation of parasite prevalence in honeybee-rich (HRS) and honeybee-poor (HPR) study sites. a) *Apicystis bombi*; b) *Crithidia bombi*; c) *Nosema bombi*



4. Discussion

We followed the prevalence of *A. bombi* in wild *B. pascuorum* in 5 different locations. Previous epidemiological screening within these locations revealed a high prevalence (i.e. 40.8%) of this parasite in honeybee hives (see Part 1 of Chapter 2). The other two parasites, *Crithidia bombi* and *Nosema bombi*, are reported as typical bumblebee parasites (Otti *et al*., 2007; Brown *et al*., 2003), although a recent publication also found *Crithidia bombi* in Asian honeybees (Li *et al*., 2012). Only *A. bombi* showed a differential abundance in our paired design, being in 5 honeybee-rich study sites (HRS) compared with 5 honeybee-poor study sites (HRS). The prevalence of *A. bombi* actually was doubled in study sites where honeybees were abundant. Therefore our results show that the presence of honeybees can disturb the parasite prevalence. Here honeybees are acting as a reservoir of spillover of *A. bombi*. Our results do not mean that *A. bombi* has a unidirectional transmission from honeybees toward wild bumblebees. It is surely possible that presence of *A. bombi* in honeybees comes from sympatric populations of wild bees including bumblebees. But from the perspective of wild *B. pascuorum*, domesticated honeybees are acting as a reservoir population from which *A. bombi* spills over. It remains theoretically possible that the increase of *A. bombi* was an indirect effect. Indeed the dominant presence of honeybees could have led to drop in food availability, therefore weaker bumblebee colonies, being more immune incompetent (Goulson *et al*., 2009). We argue that this is not the case. Because a same tendency could then also be present for parasites that are not observed in honeybees, which is not. Furthermore Leonhardt & Blüthgen (2002) described that *Bombus* species rely on those plant species that provide pollen of high quality, thus although their foraging pattern overlaps also avoidance can be recorded.

The risk associated with the higher prevalence of *A. bombi* in *B. pascuorum* in HRS depends on the actual pathology of this parasite in bumblebees. This is actually unknown, although it is presumed to be virulent and an initial screening showed that ingested spores were able to reduce the lifespan of bumblebees (pers. comm. Peter Graystock). Thus although further research is needed on the exact impact of this parasite on bumblebee population, and without knowing its exact epidemiological status in different honeybees in Europe or worldwide, we can conclude that domesticated honeybees can negatively impact

other pollinators in their environment, by disruption natural host-parasite associations. The density of domesticated honeybees is important, the mean density of 6.6 ± 1.8 bee hives per km^2 on our experimental sites is realistic for regions where apiaries are present. For Belgium a global mean of 3.6 bee hives per km^2 was reported. In some European countries the mean number per km^2 of bee hives, i.e. Greece 11.4, Hungary 10.7 and Slovenia 7.7 exceeds the density we observed close to the apiaries in our experimental design (Chauzat *et al*., 2013). Therefore we consider the setup of the experiment represent a realistic stress.

Honeybee domestication can thus disrupt host-parasite relations of sympatric bees. Spillover of pathogens from domesticated animals toward their wild counterparts is a major contributor of emergent infectious diseases (EIDs) and therefore carries the potential to induce a lot of damage (Daszak *et al*., 2000; Haydon *et al*., 2006). Especially in threatened populations, and many bumblebee species are endangered (IUCN 2014), could EIDs lead toward local extinction (Woodroffe *et al*., 1999; Mccallum *et al*., 1995). The classical example of an EID after spillover is the viral disease rinderpest, which passed in the 1890s from imported Asian cattle (*Bos indicus*) to the African buffalo (*Syncerus caffer*) and resulted in the loss of 90% of Kenya's wild African buffalo population (Mack, 1970).

Another concern is the local transportation of honeybees between regions where *A. bombi* is present. Our results show that domesticated honeybees can indeed influence the local prevalence of this parasite and thereby increase the parasite pressure in the wild population. In undisturbed host parasite associations a parasite will not drive a species toward extinction (Woodroffe 1999; Mccallum and Dobson 1995). But it is uncertain what happens if natural host parasite association are disturbed. Indeed the collapse of a population and thereby reduced transmission is an important trade-off of parasite virulence (Rigaud *et al*., 2010). It remains to be investigated how the continuous presence of one domesticated species, of which hives are restocked after collapse, interferes with natural mechanisms like local adaptation, virulence evolution and parasite prevalence.

Chapter V: General conclusions and future perspectives

In this dissertation we describe host parasite assemblies in pollinator networks. Aside from the complexity of multihost parasites and multi-parasite hosts, we made a distinction between managed bees and the wild pollinators. The key challenge of this study is to identify the influence of managed bees on natural host parasite interactions. A future question could than be what are the effects of spillover events on the diversity and abundance of endangered pollinators.

At the start of this PhD research there were already some pioneering experiments on the concept of spillover of parasites from reared bumblebees toward wild bumblebees. Especially with a focus on North America and Japan (Colla *et al*., 2006; Ottenstatter *et al*., 2007; and Goka *et al*., 2001, 2006). Although a trend of spillover could be seen, the above cited manuscripts have some power issues on the amount of greenhouse sites sampled or numbers of samples taken before *B. terrestris* invasion. Later on, Murray *et al*. (2013) demonstrated that parasite spillover is conceptually true, by which we mean it can happen. However, it should be emphasized that the implication of the act of spillover from managed bumblebees has not yet been investigated thoroughly. Our study mainly focused the protozoan parasite *Apicystis bombi*. We differentiate ourselves as we do not only focus on reared bumblebees but also on the potential spillover from domesticated honey bee hives. In summary, we study the impact of managed bees on natural host parasite interactions.

1. Reared bumblebees

1.1. Spillover of *Apicystis bombi*

In Argentina an interesting case to study the effects of parasite spillover presented itself. Here the steep decline of *Bombus dahlbomii* was concurrent with the introduction of exotic bumblebee species (Morales *et al*., 2013; Schmid-Hempel *et al*., 2014). A screening done by Arbetman *et al*. (2012) and Plischuk *et al*. (2009; 2011) showed a high prevalence of *A. bombi* in bees sampled in Argentina. Therefore we chose this parasite as focus species, and developed molecular tools to study its population dynamics.

Using ITS1 and ITS2 regions of rDNA to assess the parasite's intraspecific genetic variation in bees from Argentina and Europe, we found a largely unstructured parasite population, with only 15% of the genetic variation being explained by geographic location.

Although our data did not provide information on the direction of transfer, the absence of genetic structure across space and host species suggests that *A. bombi* may be acting as an emergent infectious disease across bee taxa and continents. In summary, the data suggests that *A. bombi* from Argentina and Europe share a common, relatively recent origin. Hence a worldwide study, untangling the possible transport routes of *A. bombi* in relation with known bumblebee routes (see Figure1.1.) is needed. We will discuss about this in more detail in the following section 3.2. as a future perspective.

1.2. Up to parasite free bumblebees

Our monitoring clearly indicated low parasite prevalence in reared bumblebee colonies compared to honeybee hives. Whereas, other screenings did reveal parasites in reared bumblebees coming from different vendors (Murray *et al*., 2013; Graystock *et al*., 2013). The main difference between their and our screening was the transportation factor. In their studies they always checked bumblebee colonies after transport. "Shipping fever", a well-known phenomenon in vertebrates could explain this difference. "Shipping fever" is known from Bovine respiratory disease (BRD) and is the most common and costliest problem encountered in stocker or feedlot calves. During the shipping period organisms may be exposed to many infectious agents. Stress impacts the immune system of animals by making them more vulnerable to certain parasites. As a result, it is possible for animals to develop severe diseases like bronchopneumonia and even die from "shipping fever". (Barham *et al*., 2002). Our experiment, with putting colonies under more severe stress conditions than transportation also did not result in parasite detection and can thus not prove that "Shipping fever" is the cause of this difference.

A single, large batch of bumblebees being free of parasites does not mean that the total breeding facility is clean, but it is a clear indication that high sanitary and quality control of the breeding stock could result in parasite-free bumblebees. Indeed, unlike domesticated honeybees, bumblebees are produced in closed and controlled climate conditions, which creates opportunities for a parasite-free environment. Combinations between optimal parasite detection and exclusion of the influx of new parasites should lead toward parasite-free reared bumblebees. Here diagnostics test with high sensitivity are needed, but also with the throughput to handle large sample amounts to ensure the sampling is statistically relevant. Molecular detection tools meet these criteria. Although the protozoan (Meeus *et*

al., 2010) and microsporidian (Klee *et al*., 2006) bumblebee parasites can be screened with molecular detection techniques, care must be taken on another remaining weak point in the rearing systems. This concern is the feeding source of bumblebees. Since bumblebees are fed with honeybee-collected pollen, a known source for *Nosema* (Higes *et al*., 2008), and other pathogens (Singh *et al*., 2010), influx of pathogens remains possible after the initial breeding stock was declared free of disease. It is evident that pathogen sterilization techniques could greatly reduce infections risks associated with feeding on pollen and thereby enabling a pathogen-free rearing environment. Recently gamma-radiation of honeybee collected pollen has been suggested to reduce this risk (Meeus *et al*., 2014). It was implied that pollen radiation would not only reduce viral incidences in rearing facilities, but also of other pathogens. For example, for *Nosema apis* dosages of minimally 2kGy could kill the parasite, while 10 kGy was able to inactivate the etiological agents *Paenibacillus larvae* and *Aschophaera apis* for American foulbrood and chalkbrood disease, respectively (Melathopoulos *et al*., 2004; Williams *et al*., 2013). At least in this basic mode of operation, there are a range of future experiments that could be done in the matter of elimination of protozoan and other parasites from honeybee collected pollens, and thereby creating the opportunity to have parasite-free bumblebees.

Aside from the fact that a reared bumblebee should be free of parasite it is also important to use native bumblebee species for pollination purposes. The use of native bumblebees does not eliminate the risk associated with spillover, especially if imported honeybee-collected pollen is used. But displacement of native species by invasion of exotic species and its competition is prevented. The breeding with local species has been implemented if the region has "pollen storers", like *B. impatiens* in North America and *B. ignitus* in Japan (Velthuis *et al*., 2006). In the case of South America, only having "pocket makers" bumblebees, the breeding of native species has proven to be difficult. However, efforts have been made and recently both *B. huntii* and *B. atratus* can be bred by the commercial breeding company Biobest to ensure native pollinators for the South American market.

1.3. *Apicystis bombi* performs horizontal transmission

We monitored parasite-free reared colonies within natural environments containing *A. bombi* infected pollinators. We observed horizontal transmission of this parasite, with

already 45%; ± 16 SD (11/24) of the colonies infected after 7 weeks. Although we concluded that its transmission potential of *A. bombi* (45%; ± 16 %SD) is lower of that of *C. bombi* (95%; ± 9 %SD), the amount of positive nests was unexpectedly high. In natural environments the summer queens, those that mate and go into hibernation, are often seen with fat bodies loaded with *A. bombi*. A study on the colony start-up of spring queens compared with uninfected ones is urgently needed (see section 3.3. within this chapter), to estimate the damage this spillover could be doing.

2. Honeybees

2.1. Honeybee hives host the protozoan *Apicystis bombi*

Before studying the possibility of parasite spillover from domesticated honeybees toward wild pollinators, one needs to know which parasites reside in domesticated honeybees. Epidemiological studies on honeybees are omnipresent, but often follow a typical set of the most famous pathogens. In collaboration with the research group of Prof. de Graaf (University of Gent, WE10), an epidemiological screening in Flanders was extended with candidate parasites potentially implicated in spillover events between different genera of pollinators.

The results showed an unexpected high prevalence (40.8% - 148/363) of *A. bombi*, primarily known as a bumblebee parasite (Lipa and Triggiani 1996), in honeybee hives. Indeed, this parasite has been barely reported in honeybees in Europe. Actually only in one paper describing the species, they mentioned about findings of *A. bombi* in one honeybee specimen. What does this high prevalence that we detected mean? Is *A. bombi* one of those forgotten parasites which studies missed to diagnose? Are the detection techniques improving, or are we just searching better? But actually the key question is: is this presence influencing the prevalence of *A. bombi* within wild pollinator communities?

2.2. Domesticated honeybees disrupt parasite prevalence and act as a reservoir of *Apicystis bombi*.

The final part of this thesis investigated the interference of domesticated honeybees with natural host parasites assemblies in wild bee communities. The paired design in 5 different locations, each containing a honeybee-rich and honeybee-poor environment revealed a drop in prevalence of *A. bombi* in each paired honeybee-poor environment. The presence of honeybees is thus disrupting the parasite prevalence. Our results is in agreement with studies of Fűrst *et al*. (2014), demonstrating that domesticated honeybees can be a reservoir of parasites shared with wild congeners. However the exact mechanism by which these inter species transmission occur are not studied yet. Transmission by shared flower use seems to be a plausible explanation as both species are general foragers, however different flower choices within a specific environment have been observed (Leonhardt and Blüthgen, 2002).

As also mentioned for *B. dahlbomii*, the origin of *A. bombi* would have great implication on the risk associated with the spillover events which we detect. But evidently sympatric spillover are less severe than allopatric spillovers (Meeus *et al*., 2011). Thus, did honeybee transport make *A. bombi* a cosmopolitan parasite (see section 3.2)? And what kind of pathology (see section 3.3) study is needed to make proper risk assessment associated with transports of bees carrying this parasite.

3. Future perspectives

3.1. Legislation

Aside from the scientific progress being made about understanding the risks associated with spillovers, and how to get rearing facilities free of parasites, also legislation will need to follow. Currently bumblebee transportation mainly falls into European Union legislation regulated by Commission Regulation (EC) No 206/2010 and Commission Decision 2003/881/EC. The main focus of the legislation is honeybee diseases. None of the potentially virulent bumblebee parasites are covered under this legislation. It would be useful if to expand this list with bumblebee pathogens, otherwise veterinary screenings are likely to be ineffective in controlling the spread of important bumblebee parasites. Furthermore, also for the honeybee, a new list including some emerging disease harmful to wild pollinator should be included. As for now the legislation is mainly meant to protect honeybees, and should be redesigned to ensure no spread of parasites harming pollinator communities because of transport of bees and bee products.

3.2. The origin and transportation routes of *Apicystis bombi*

As identified above it is crucial to determine if *A. bombi* was a cosmopolitan parasite or if it became one because of bee transport. The challenge is to identify the potential origin of *A. bombi* by combining molecular biology studies with distribution routes of managed bees. Therefore additional studies are needed to investigate the haplotype distribution and abundance of *A. bombi* in different host species. It would reveal new out-groups or comparable haplotype clusters demonstrating the most potential origins of the parasite. This kind of haplotyping would require a worldwide screening with an intelligent sampling design following the known transport routes of managed host species. Routes of honeybees and bumblebees need to be separated, including control location with no transport of one or both managed bees. Reared bumblebee colonies are imported by over 50 countries across the globe (Velthuis and van Doorn, 2006) from known breeding facilities. Also global transportation of the western European *A. mellifera* have been documented (Moritz *et al*., 2005; De la Rua *et al*., 2009). Therefore synergy between haplotype distribution results and intensive documentation of bee transport can tackle the questions associated with *A. bombi* spillover. *A. bombi* could thereby form a precedent on anthropogenic

transports of hosts carrying parasites and how they could alter the natural host-parasite association.

3.3. Pathology of *Apicystis bombi*

It has been postulated, but not supported with empirical data, that the *A. bombi* inhibits colony founding, increases workers' mortality, shortens the life span of the queens and causes mortality in early spring queens (Rutrecht and Brown 2008; Schmid-Hempel P 2001). Thus first empirical data on this is needed. But also tolerance studies of parasites comparing different pollinators. We speculate that the bumblebee life cycle is less tolerant to parasite exposure compared to honeybee colonies. For a honeybee colony it is important to prevail the queen to become infected and die, a partial loss of its workforce or foraging abilities because of parasite burden can be compensated by the great number of workers in a single hive. For bumblebees the queen passes through an extremely stressful colony start up on her own, facing the fluctuant weather conditions of early spring (Goulson 2003). One can imagine that even a reduced foraging capacity in these conditions can push successful colony startup into failure. Therefore further empirical studies on pathology of *A. bombi* needs to be undertaken with care to how the pathology of the parasite presents in natural conditions apposed the laboratory conditions.

Thus, the next step is to extend the work from prevalence studies to empirical pathogenicity experiment together with molecular biology studies describing parasites haplotypes along known transport routes of managed bees and natural distribution of host pollinators.

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Summary

Domesticated honeybees and commercially mass-reared bumblebees are important pollinators of agricultural crops. Currently, these managed bees are transported globally. However, a worldwide trade of pathogen-free managed bees is crucial. Parasite spillover from a managed bee reservoir to wild life can cause the emergence of diseases in wild pollinator populations and this in turn may result in a rapid decline of the new host populations.

This thesis consists of three main parts. First, an evaluation of the potential of two managed bee species to spillover parasites. Second, a case study on the spillover of the parasite *Apicystis bombi* in Argentinean bumblebees. Third, a study on the influences of domesticated honeybees on parasite prevalence in wild populations of *Bombus pascuorum*.

In the first part, managed bees were investigated to identify the potential reservoirs of parasites. The monitoring revealed that domesticated honeybee hives host multiple parasites known to infect bumblebees. Among the detected parasites the prevalence of the neogregarine *Apicystis bombi* was 40.8% (148/363) which was much higher than expected since this parasite has been barely reported in honeybees in Europe. In reared bumblebee colonies we could not detect any parasites. Our screening was done on two period stages of colonies. A first monitoring was conducted on early stage colonies (n = 48) upon their arrival from a mass-producing facility (Biobest). Subsequently, we screened a later stage of the colonies where we had induced a stress simulation condition (n = 10). Both screenings were negative. In relation with our sampling effort, this means that the prevalence of neogregarines and spiroplasmas is below 25%. These results imply that parasite elimination within a closed breeding facility is possible.

We also studied the infection susceptibility of mass-reared parasite-free colonies in different environments. The screening for three important parasites of bumblebees, i.e. *Apicystis bombi, Crithidia bombi* and *Nosema bombi,* demonstrated that these parasites have the intrinsic capacity for horizontal transmission in a natural environment. Indeed, these three parasites were detected in the mass-reared bumblebee colonies after 7 weeks of contact with the environment, while the colonies were parasite-free before they were placed outside. Mainly *C. bombi* was able to infect colonies (95%; ±9% SD) followed by *A. bombi*

(45%; ± 16% SD) and *N. bombi* (21%; ±25% SD). This result imposes an important question about the infection potential of reared colonies and their impact to wild specimens. Do reared bumblebees act as a diluting factor for parasites prevalence or can they act as a parasite reservoir when they have a higher infection potential compared to wild bees and readily pick up parasites from the environment?

In the second part of this thesis a molecular tool was developed to study the population structure of *A. bombi*. It was demonstrated that the Internal Transcribed Spacer (ITS) region of ribosomal DNA of *A. bombi* can effectively be used for haplotyping.

Using ITS1 and ITS2, a population genetic study was performed on *A. bombi* to assess the parasite's intraspecific genetic variation in bees from the natural parks of Patagonia in Argentina and different countries in Europe in order to identify a potential parasite spillover route. The most abundant haplotype in Argentina (found in all 9 specimens of non-native species) was identical to the most abundant haplotype in Europe (found in 6 out of 8 specimens). The absence of a genetic structure across space and host species suggested that *A. bombi* may be acting as an emergent infectious disease across bee taxa and continents. Although the data obtained did not provide information on the direction of transfer, it can be assumed that sufficient variability does exist in the ITS region to identify continent-level genetic structure in the parasite.

In the third and final part of this PhD work, the prevalence of protozoan and microsporidian parasites was evaluated in wild populations of *Bombus pascuorum*. We investigated the role of domesticated honeybee hives with natural host parasite assemblies on wild pollinators in 5 different locations in Flanders (Belgium). Each location consisted of honeybee-poor and honeybee-rich study sites. Our paired design in 5 different locations demonstrated a differential drop in prevalence of *A. bombi* in each paired honeybee-poor environment. This clearly indicates that domesticated honeybees may interfere with host-parasite interactions in a wild bee pollinator. The results obtained suggested that from the perspective of *B. pascuorum*, domesticated honeybees are acting as a spillover reservoir for *A. bombi.*

In conclusion, the assessment of the impact of different managed bees on the natural host pathogen association illustrates the necessity of a parasite-free status of bees. Anthropogenic transportation of hosts carrying parasites can add pathogenic stress on wild life populations. Apparently, control of protozoan parasites within closed environments can lead toward a large batch of bumblebee colonies being free of parasites. We speculate that with the existing molecular diagnostic tools and sterilization techniques indoor reared bumblebees can be freed from parasites. At present, the transportation of managed bees is regulated by Commission Regulation (EC) No 206/2010 which is primarily intended to protect honeybee populations. In order to achieve an effective veterinary screening in controlling the spread of parasites, the cover of this legislation should be expanded to other potentially virulent parasites, and the focus should be enlarged to the total pollinator community. Finally, additional research is needed to identify the origin population of *A. bombi* based on the distribution routes of managed bees. This in order to establish if this parasite could be causing an emergent infectious disease in European pollinators or in other world continents.

Samenvatting

Gedomesticeerde honingbijen en intensief gekweekte hommels zijn belangrijke bestuivers van landbouwgewassen. Momenteel worden deze bijen (*Apidae*) wereldwijd vertransporteerd. Bij deze wereldwijde handel zijn pathogeen-vrije bijen cruciaal. Door overdracht van parasieten van de gekweekte bijen naar de inheemse populaties kunnen parasieten in de wilde hommelpopulatie ontstaan, en dit kan op zijn beurt leiden tot een snelle achteruitgang van deze nieuwe gastheerpopulaties.

Dit proefschrift bestaat uit drie grote delen. Ten eerste, een evaluatie van het potentieel tot overdracht van parasieten bij twee gekweekte bijensoorten. Ten tweede, een case studie over de overdracht van *Apicystis bombi* in Argentijnse hommels. Ten derde, een studie naar de invloed van gedomesticeerde honingbijen op de prevalentie van parasieten in de natuurlijke populaties van de hommel *Bombus pascuorum*.

In het eerste deel onderzochten we gekweekte bijen om zo potentiële reservoirs van parasieten te identificeren. Bij de controle van deze gedomesticeerde honingbijnesten werden meerdere parasieten gevonden waarvan bekend is dat deze hommels kunnen infecteren. Van deze parasieten was de prevalentie van de neogregarine *Apicystis bombi* met 40.8% (148/363) onverwacht hoog aangezien deze parasiet nauwelijks gemeld wordt bij honingbijen in Europa. In gekweekte hommelkolonies konden we echter geen parasieten detecteren. Onze screening werd op twee stadia van de kolonies uitgevoerd. Een eerste controle werd uitgevoerd op kolonies in een vroeg stadium (n = 48), namelijk bij hun aankomst uit een commercieel bedrijf (Biobest). Tevens werd ook een later stadium van deze kolonies gescreend, waarbij we ook een biotische stress simulatie conditie uitvoerden (n = 10). Beide screenings waren negatief. Rekening houdend met de door ons uitgevoerde staalname betekent dit dat de prevalentie van neogregarines en spiroplasmas minder is dan 25%. Deze resultaten impliceren dat de verwijdering van parasieten in een gesloten kweekbedrijf mogelijk is.

We bestudeerden ook de infectiegevoeligheid van gekweekte parasiet-vrije kolonies in verschillende omgevingen. De screening op drie belangrijke hommelparasieten, namelijk *A. bombi*, *C. bombi*, en *N. bombi* toonde aan dat deze parasieten de intrinsieke capaciteit hebben voor horizontale overdracht in een natuurlijke omgeving. Inderdaad, deze drie parasieten werden ontdekt in gekweekte hommel kolonies na 7 weken contact met de

omgeving, terwijl ze parasiet-vrij waren voordat ze buiten werden geplaatst. Vooral *C. bombi* kon kolonies (95%; ± 9 SD) infecteren, gevolgd door *A. bombi* (45%; ± 16SD) en *N. bombi* (21%; ± 25 SD).

Uit dit resultaat stelde zich de belangrijke vraag over het infectiepotentieel van gekweekte kolonies en hun impact op wilde bijen. Kunnen gekweekte hommels fungeren als een verdunningsfactor voor parasietprevalentie of fungeren zij eerder als een reservoir van parasieten wanneer ze een hogere infectie hebben dan wilde bijen en gemakkelijker parasieten uit de omgeving kunnen oppikken?

In het tweede deel van dit proefschrift hebben we een moleculaire tool ontwikkeld om de populatiestructuur van *A. bombi* te bestuderen. Hierbij hebben we aangetoond dat de 'Internal Transcribed Spacer (ITS)' regio van het ribosomaal DNA van *A. bombi* effectief kan worden gebruikt voor haplotypering. Wij hebben populatie-genetische studies in *A. bombi* uitgevoerd met behulp van ITS1 en ITS2. Dit om de intraspecifieke genetische variatie van deze parasiet in bijen uit Argentinië en Europa te onderzoeken en op deze manier een mogelijke route van parasietoverdracht te kunnen identificeren. Het meest voorkomende haplotype in Argentinië (teruggevonden in alle 9 stalen van niet-inheemse soorten) was identiek aan het meest voorkomende haplotype in Europa (in 6 van de 8 exemplaren). De afwezigheid van een genetische structuur over zowel de locatie als de gastheer soorten suggereert dat *A. bombi* een opkomende infectieuze ziekte voor diverse bijen taxa en continenten kan zijn. Hoewel onze gegevens geen informatie verstrekken over de richting van deze overdracht, gaan we ervan uit dat er voldoende variatie bestaat in de ITS regio om op niveau van continenten toch een genetische structuur in de parasiet te identificeren. Zoals de stalen van de inheemse hommel *Bombus ephippiatus* uit Mexico, die genetisch verder verwijderd waren van de Argentijnse en Europese stalen.

In het laatste deel van dit doctoraatsproefschrift volgden we de prevalentie van protozoa en microsporidia parasieten op in natuurlijke populaties van *B. pascuorum*. We onderzochten de rol van gedomesticeerde honingbijnesten met hun specifieke natuurlijke parasietgemeenschap, op wilde pollinatoren in 5 verschillende locaties. Elke locatie bestond uit zowel een honingbij-arme als een honingbij-rijke studiesite. Onze gepaarde proefopzet in 5 verschillende locaties toonde een differentiële daling aan van de prevalentie

van *A. bombi* in elke honingbij-arme omgeving, waaruit duidelijk blijkt dat gedomesticeerde honingbijen kunnen interfereren met de gastheer-parasiet interacties in een wilde bestuiver. Onze resultaten suggereren dat vanuit het perspectief van *B. pascuorum*, gedomesticeerde honingbijen kunnen beschouwd worden als een reservoir voor *A. bombi*.

Het onderzoek naar de impact van verschillende gekweekte bijen op natuurlijke gastheer-pathogeen associaties illustreert de noodzaak van de parasiet-vrije status van bijen. Antropogeen transport van gastheren die parasieten dragen, kan de pathogene spanning op wilde populaties doen toenemen. De controle van protozoaire parasieten in gesloten omgevingen kan blijkbaar leiden tot een groot aantal parasiet-vrije hommelkolonies. We stellen daarom dat hommels binnen een massakweek vrij van parasieten kunnen worden gekweekt dankzij de bestaande moleculaire diagnostische instrumenten en de sterilisatietechnieken voor de behandeling van het honingbij-verzameld stuifmeel dat mogelijk kan besmet zijnmet parasieten. Op dit moment valt het transport van gekweekte bijen onder de Commissionaire Verordening (EG) nr 206/2010 die in de eerste plaats bedoeld was om de honingbijpopulaties te beschermen (Commissie Beslissing 2003/881/EG). We menen dat om een efficiënte veterinaire screening te bereiken ter controle van de verspreiding van parasieten, deze wetgeving moet worden uitgebreid tot andere potentieel virulente parasieten, en tevens zou de doelgroep moeten worden uitgebreid naar de totale gemeenschap van bestuivers.

Tot slot als aanvullend onderzoek stellen we voor om de oorsprongpopulatie van *A. bombi* te identificeren op basis van distributieroutes van gekweekte bijen. Dit om na te gaan of deze parasiet een opkomende infectieuze ziekte in de Europese bestuivers of in andere continenten kan veroorzaken.

Curriculum vitae

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1. **Maharramov, J.,** Meeus, I., Maebe, K., Arbetman, M., Morales, C., Graystock, P., Hughes, W.O., Plischuk, S., Lange, C.E., de Graaf, D.C., Zapata, N., de la Rosa, J.J., Murray, T.E., Brown, M.J., Smagghe, G. 2013. Genetic Variability of the Neogregarine *Apicystis bombi*, an Etiological Agent of an Emergent Bumblebee Disease. PLoS ONE 8(12): e81475. doi:10.1371/journal.pone.0081475.

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Posters

1. **Maharramov, J.,** Meeus, I., Vercruysse, V., Smagghe, G. Impact of *Spiroplasma apis* on artificially infected *Bombus terrestris*. 5th European Conference of Apidology. 3-7 September 2012, Halle an der Saale, Germany.

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