Behavioural and social immunity in a eusocial insect, the bumblebee *Bombus terrestris*

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To my family

and to the memory of my uncle,

J.-D. Fouks

“Experience is not what happens to you; it's what you do with what happens to you.”

Aldous Huxley
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**General Introduction**

Evolution is one of the major domains of biology. The first one who have theorised it is Lamarck (Lamarck, 1809) and afterwards Darwin formulated his theory of natural and sexual selection (Darwin, 1859; Darwin, 1871). Darwin theories have been the background of a great part of the modern synthesis of evolution. Darwins theory of natural selection is based on inheritable variation between individuals, which is selected due to better adaptation to the environment and in turn a higher fitness (Darwin, 1859). The environment could be divided in two categories, the abiotic (physical environment) and the biotic environments (living organism environment). The biotic interactions between two species (more precisely two populations of two different species) can lead to reciprocal evolutionary dynamics, called co-evolution (Janzen, 1980). The co-evolution between two species is the result of either antagonistic or mutualistic interactions. Mutualistic interactions lead to mutualism and symbiosis, while antagonistic interactions appear between predator-prey and parasite-host species pairs.

Antagonistic interactions between hosts and parasites (in the broad sense of the term including viruses, bacteria, fungi, protozoans, helminths, arthropods and vertebrates) are a key structuring force in natural populations, driving co-evolution (Thompson and Cunningham, 2002; Harvell, 2004). Dynamics of antagonistic co-evolution has been theorised as a law of extinction by Van Valen (1973); the Red Queen hypothesis, which stipulates that two or more species could evolve reciprocal traits to reduce the selective pressure exercised on each other. In other words, hosts will adapt to reduce the impact of parasites on their fitness, while parasites will adapt to increase their transmission efficiency (fitness) through their hosts, potentially leading to an arms race. This founds expression as negative frequency-dependent selection leading to the fluctuation of host and parasite genotype frequencies over time (Van-Valen, 1973; Hamilton et al., 1990; Lively and Dybdahl, 2000). Theoretical studies gave birth to different models, such as “gene for gene”, “matching allele” and “matching genotype” models, to explain the mechanisms involved in this co-evolutionary processes (Flor, 1942; Agrawal and Lively, 2002; Otto and Nuismer, 2004; Decaestecker et al., 2007). However, it remains quite difficult to gather empirical data of such long-term co-evolution, especially the temporal dynamics of the process (reviewed in Woolhouse et al. 2002, Decaestecker et al., 2007). Except direct evidence of temporal patterns, spatial patterns can also be used to infer such
temporal variations assuming that spatial populations are at different stages of co-evolutionary process (Gandon, 2002). Spatial patterns take on two aspects, either local adaptation or maladaptation (Lajeunesse and Forbes, 2002). Local adaptation is defined as sympatric host-parasite combinations are more compatible than allopatric combinations (an host resists better against sympatric parasites than against allopatric parasites or a parasite has a higher fitness when infecting a sympatric host than an allopatric one), while local maladaptation is the contrary. Anyway both can be interpreted as an ongoing co-evolution between hosts and parasites. Strong evidence for co-evolution is provided by investigating the occurrence of reciprocal variation in host and parasite phenotypes (ideally strongly linked with genotypic components) (reviewed in Woolhouse et al., 2002). Nevertheless, the lack of spatial adaptation or reciprocal variation of host-parasite phenotypes/genotypes is not sufficient to reject the co-evolution hypothesis. Further variables might have blurred the picture of host-parasite co-evolution. The genetic basis of such interactions might be more complex (polygenic trait) than assumed in mathematical models (reviewed in Woolhouse et al., 2002). Environmental factors have also influences on host-parasite interactions, such as phenotypic plasticity (Schulenburg et al., 2009). Moreover, hosts usually are infected by multiple parasite species/strains and parasites infect multiple host species/populations. This could reduce the specific interactions of a host-parasite system (reviewed in Woolhouse et al., 2002).

Hosts have evolved different mechanisms to reduce the parasite burden on their fitness; they can adopt two different strategies either evolved resistance or tolerance against parasites. While resistance prevents or reduces parasite infection, tolerance alleviates the fitness reduction caused by infection. Resistance has an impact on parasite fitness while tolerance does not. It is expected to result in differential host-parasite co-evolutionary dynamics (Boots and Bowers, 1999; Roy and Kirchner, 2000; Miller et al., 2005). Indeed, the fluctuation of host and parasite genotype frequencies over time due to antagonistic co-evolution will appear in case of resistance, but not for tolerance (Miller et al., 2005). Therefore, I will describe only resistance mechanisms adapted to defend hosts against parasite infections. Most studies on resistance mechanisms are mainly focused on the immune system. The immune responses are classically defined as either innate or acquired immunity; the last one being attributed only to vertebrates (Kurtz, 2004). The acquired immunity is the result of specificity and immunological memory after initial exposure to a novel
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parasite (Kurtz, 2004). The innate immunity on the other hand relies on the genetic background of the host to resist against different parasite strains and species, leading to a low-level specificity to a broad range of parasites (Hauton and Smith, 2007). There are two different effectors of the immune response, the humoral and cellular ones. The humoral immune response is the result of constitutive and inducible elements. The two main elements of humoral response are anti-microbial peptides (AMPs) and the prophenoloxidase system. AMPs are regulated and expressed at the transcriptional level by signalling pathways (Toll, Imd, JNK and JAK/STAT pathways) and reduce infection by disrupting the function and proliferation of bacteria and fungi (Epand and Vogel, 1999). The prophenoloxidase system is responsible for melanisation reactions, which creates a physical barrier at a site of wounding or by surrounding parasites thereby avoiding the spread of infections (Cerenius and Söderhäll, 2004). The cellular immune response is mediated by specialised cells, responsible for phagocytosis and encapsulation of parasites. In vertebrates, cellular immune response through lymphocytes B and T allows the adaptive immunity. In order for the immune response to be activated, first the host has to recognise the parasite. This is carried out by pattern recognition receptors (PRRs) that bind specifically to pathogen associated molecular patterns (PAMPs) present on the parasites surface. PAMPs can be used by the host to differentiate the parasite infection giving some degree of specificity (Medzhitov and Janeway, 2000). However, the immune system of insects seems to be more specific than originally thought (Schmid-Hempel, 2005)

Due to the genetic specificity of host-parasite interactions, parasites are likely to infect easier highly genetically related individuals rather than unrelated individuals (Shykoff, Jacqui A. and Schmid-Hempel, Paul, 1991; Liersch and Schmid-Hempel, 1998; Baer and Schmid-Hempel, 1999, 2001; Tarpy, 2003; Hughes and Boomsma, 2004; Tarpy and Seeley, 2006). Furthermore, high density of individuals is beneficial for parasites to increase their transmission, mainly due to the high number of possible reservoirs and the high number of interactions facilitating parasite transmission between individuals (Schmid-Hempel, 1998). Therefore, sociality, despite all its inherent benefits, has drawbacks when facing parasite infection compared to solitary species (Schmid-Hempel, 1998). Indeed, evolution of sociality or group living is mainly due to the benefits of cooperation of genetically related individuals, which thwarts the cost of such cooperative acts (Hamilton, 1964a, b). In addition to the high
genetic relatedness of members of a social group, social animals live in dense groups with high interaction rates between group members (Aron & Passera 2000). This is especially the case for eusocial animals. Eusociality is the highest degree of social structure where the society is divided into different castes, the reproductive caste (called queen caste) and the sterile one (called the worker caste), where there is an overlap of generations and the offspring help their parents to care for their siblings (Wilson 1971). Within the animal kingdom only invertebrates are eusocial, at the exception of the naked mole rats. Most of the eusocial invertebrates are hymenopterans. The sex determination in this family (haplo-diploidy, with females being diploid and males being haploid) is a major factor for their social evolution (Hamilton, 1964b; Trivers and Hare, 1976). The haplo-diploid sex determination system increases the genetic relatedness between related females, which is of 0.75 for full sisters while it is of 0.5 for full sisters in a diplo-diploid sex determination system (Hamilton, 1964b; Trivers and Hare, 1976). Generally, eusocial hymenopterans live in a closed nest founded by a single queen resulting in a high density of genetically highly related individuals. This specificity makes them a prime target to parasites (Schmid-Hempel, 1998). As stated above, eusocial insect colonies provide a rich and stable environment for parasites. Therefore, they have evolved special features in order to reduce the impact of parasites on colonies (Cremer et al., 2007). Indeed, eusocial hymenopterans and social animals in general have developed collective defence mechanisms in order to reduce infection and parasite burden, so called social immunity (Cremer et al., 2007). Social immunity is an important characteristic of social animal immunity, especially in eusocial hymenopterans that possess a reduced number of immune genes compared to solitary species (Evans et al., 2006). Another possible strategy to reduce the parasite burden on eusocial hymenopterans is to increase the genetic diversity within the colony either by multiple mating of the queen (polyandry) or having more than one queen (polygyny) or both (Schmid-Hempel and Crozier, 1999; Hughes and Boomsma, 2006). However, not all eusocial hymenopterans increase genetic diversity within colonies despite the advantages against parasite infections (Schmid-Hempel, 1998). Bumblebees are eusocial hymenopterans whose colonies are founded by a single mated queen (Alford, 1975). Therefore, the whole bumblebee colony is highly prone to be infected once few individuals have been parasitized.

Bumblebees (Bombus spp.) are present mostly in the northern hemisphere and are
important pollinators, feeding on flowers for nectar and pollen (Alford, 1975). The most common bumblebee in Europe is *Bombus terrestris*, the buff-tailed bumblebee. Colonies have an annual life cycle. They are founded by a single queen in early spring. She establishes the first foundations of the nest collecting nectar and pollen; and after the first brood hatched newly emerged workers take over tasks of foraging and nest maintenance. At the midsummer, the colony reaches its highest number of individuals (~200 to 1000 workers) and the queen starts producing sexual individuals (males and gynes). This period is called the competition phase, because workers start to compete with the queen over male production. At the beginning/middle of fall, newly emerged gynes mate with single males and enter hibernation, usually in a cavity in the ground. Then again in spring queens emerge and found new colonies (Alford, 1975). Bumblebees, over this seasonal life, are getting infected by a wide diversity of parasites, ranging from viruses to arthropods and even other bumblebee species (subgenus *Psithyrus*) (Goulson, 2010). Therefore, bumblebees have adapted a battery of mechanisms to defend themselves against parasites. Bumblebees possess, as other eusocial insects, an innate and a social immunity. Bumblebees evolved features at every level of parasite infection such as behavioural alteration of foraging workers infected with conopid flies (Müller and Schmid-Hempel, 1993), consumption of alkaloid nectar reducing *Crithidia* infections, which can be considered as medication (Manson et al., 2010), social activation of the immune system (Richter et al., 2012), efficient utilization of the innate immune system (Erler et al., 2011) and even their social organisation allows for reducing the spread of parasites within the colony (Naug and Camazine, 2002).

One of the most predominant parasites of bumblebees is *Crithidia bombi*, which can infect up to 80% of colonies (Shykoff and Schmid-Hempel, 1991). *Crithidia bombi* is a protozoan, (Trypanosomatidae, Zoomastigophorea) (Lipa and Triggiani, 1988), which infects and performs its life cycle in the mid and hind gut of bumblebees. Little is known about *C. bombi*, but it seems that they are diploid, with both clonal and sexual reproduction (Schmid-Hempel, 2001; Schmid-Hempel et al., 2011; Erler et al., 2012b; Popp et al., 2012) showing a high genetic diversity within natural populations (Schmid-Hempel and Reber Funk, 2004). After 2-3 days post infection infective *C. bombi* cells are released through the faeces of bees (Schmid-Hempel and Schmid-Hempel, 1993). *C. bombi* is transmitted horizontally between bumblebees via direct contact between individuals within the colony (Otterstatter and Thomson, 2003).
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2007) and also via the shared use of flowers (Durrer and Schmid-Hempel, 1994). It can also be transmitted vertically from the queen to her offspring (Imhoof and Schmid-Hempel, 1999). *C. bombi* has low effects on bumblebee colonies under favourable conditions (Brown et al., 2003). However, queens infected by *C. bombi* have a reduced success in colony founding (Brown et al., 2003), colonies have smaller worker populations and produce fewer sexual offspring (Brown et al., 2000). In addition, infected foraging workers have an impaired learning ability for floral cues (Gegear et al., 2006), which reduces the fitness of the colony (Oster, 1976; Ings et al., 2005; Raine and Chittka, 2008). Therefore, bumblebees have developed specific defence mechanisms against this gut parasite. Their immune gene expression is specific to *C. bombi* strains (Riddell et al., 2009). This specificity to resist against *Crithidia* appears to be at the genetic level, since there is natural variation of *Crithidia* resistance occurring between different bee populations allowing for the identification of quantitative trait loci involved in resistance to *Crithidia* (Wilfert et al., 2007). This specificity is reciprocal, since after serial passages of one strain through closely related workers from one colony, this strain has a higher fitness infecting bumblebees of the same colony compared to infections of bees originating from different colonies (Yourth and Schmid-Hempel, 2006). However, there is no strong evidence of local adaptation or reciprocal variation host and parasite phenotypes in natural populations (Imhoof and Schmid-Hempel, 1998b, a; Yourth et al., 2008). This lack of evidence could be due to confounding factors. It has been proven that the gut microbiota plays a key role in bumblebee-*Crithidia* interactions (Koch and Schmid-Hempel, 2012). In addition, *Crithidia bombi* parasitizes a wide range of bumblebee species and individual bees often facing infections by multiple strains of *Crithidia* resulting in drastic changes of the genotypic structure of parasite populations (Salathé and Schmid-Hempel, 2011; Erler et al., 2012b; Popp et al. 2012). Nevertheless, experimental studies (under controlled environmental conditions) show an ongoing co-evolution within this host-parasite system (Schmid-Hempel, 2001; Riddell et al., 2009). Despite a large number of studies focusing on the bumblebee - *C. bombi* host-parasite system, none of them has investigated the most economic defence mechanism against this parasite, which consists of the avoidance of an uptake of *C. bombi*. Avoidance of a parasite saves the costs of parasite damage on the host and for the activation of the immune system. Additionally, in the case of bumblebees, the avoidance of parasites could also
drastically reduce the infection of the entire colony, which could be caused by the high genetic relatedness between nest-mates. Thus, the foraging behaviour of bumblebees was investigated, when facing the choice of either feeding on a contaminated or an uncontaminated flower. We used different types of contamination, either a common pathogen (*Escherichia coli*) or a specifically adapted parasite (*C. bombi*).

Bumblebees are known to use social cues to increase their foraging efficiency, either through the use of scent-marks or direct visual cues from conspecifics (Goulson et al., 2000; Leadbeater and Chittka, 2009, Leadbeater and Chittka, 2009). I set up a second experiment, in which single bees had the choice to forage on two flowers, one contaminated by *C. bombi* and the other not. In one set up, the bees were allowed to use scent-marks left by conspecifics on flowers while in the second set up no other cues than the presence of *C. bombi* on the flower were provided.

Finally, in a third experiment, two molecular diagnostic methods for the quantification of *C. bombi* infections in bumblebees were compared. The quantification of infections has a great importance for several topics (host-parasite interactions (Schmid-Hempel, 2001), ecology (Kremen et al., 2007) and epidemiology (Erler et al., 2012a). Usually microscopic methods are used for this purpose, but when facing large sample sizes, these methods start to become error prone and time consuming. Therefore, a quantitative PCR method and one based on the amount of amplified products of microsatellite markers were compared using different *C. bombi* cell concentrations.
Chapter 1
Recognition and avoidance of contaminated flowers by foraging bumblebees (*Bombus terrestris*)

Bertrand Fouks and H. Michael G. Lattorff

Bumblebee colonies are founded by a single-mated queen. Due to this life history trait, bumblebees are more susceptible to parasites and diseases than polyandrous and/or polygynous social insects. A greater resistance towards parasites is shown when the genetic variability within a colony is increased. The parasite resistance may be divided into different levels regarding the step of the parasite infection (e.g. parasite uptake, parasite intake, parasite’s establishment in the nest, parasite transmission).

We investigate the prophylactic behaviour of bumblebees. Bumblebees were observed during their foraging flights on two artificial flowers; one of these was contaminated by *Crithidia bombi*, a naturally occurring gut parasite of bumblebees (in a control experiment the non-specific pathogen *Escherichia coli* was used).

For *C. bombi*, bumblebees were preferentially observed feeding on the non-contaminated flower. Whereas for *E. coli*, this preference was also observed but at lesser degree than with *C. bombi* contamination, however bumblebees spent more time feeding on the non-contaminated flower.

These results demonstrate the ability of bumblebees to recognise the contamination of food sources. In addition, bumblebees have a stronger preference for the non-contaminated flower when *C. bombi* is present in the other flower than with *E. coli* which might be explained as an adaptive behaviour of bumblebees towards this specific gut parasite. It seems that the more specific the parasite is, the more it reduces the reward of the flower.


**Key words:** activated immune response, *Crithidia bombi*, co-evolution, pollinators, social insect, parasite.
Avoidance of contaminated flowers by bumblebees

Introduction
Among all metazoans, parasites and diseases represent a strong threat reducing the life time and the fitness of an organism (Bonsall, 2004), and also a strong evolutionary force (Salathé et al., 2008). When a parasite is specific to a host, the relation, regarding the evolution, between these two species is linked and may lead to co-evolution. This co-evolution between a parasite and a host results in an arms race (Ebert and Hamilton, 1996; Decaestecker et al., 2007). The host will tend to evolve to reduce the effects of the parasites on themselves. Many levels are involved in resistance to a parasite (Cremer et al., 2007). The first one is the reduction of parasite uptake, allowing individuals to avoid the parasite. The second one is the non-intake of the parasite resulting to a protection against the intrusion of the parasite in the organism. The third one is the reduction of parasite loads inside the host and even the complete elimination of the parasite. The last level is the prevention of transmission of the parasite in order to avoid secondary infection and the infection of the conspecifics.

Eusocial insects provide a rich and stable environment for parasites (Schmid-Hempel, 1998). Indeed, living in a closed nest with a large amount of nest-mates provides a parasite with a lot of individuals to infect in a close and tiny spatial environment. The homeostatic nest conditions may additionally improve parasite survival.

One explanation to the evolution of polyandry in social insects is to reduce the parasite load (Schmid-Hempel, 1998). Indeed, several empirical studies have proved that increasing genetic diversity among nest-mates diminishes the parasite load within the colony (Shykoff, Jacqui A. and Schmid-Hempel, Paul, 1991; Liersch and Schmid-Hempel, 1998; Baer and Schmid-Hempel, 1999, 2001; Tarpy, 2003; Hughes and Boomsma, 2004; Tarpy and Seeley, 2006). Two factors are claimed to be responsible to this. First, the spread of a parasite within a colony is reduced when the worker genotype variability is high; due to the host-parasite genotype-genotype interactions (Otto and Nuismer, 2004). Secondly, the increase of genetic variability within a colony results in an increased likelihood for the presence of individuals resistant to parasites; since different genotypes vary in their resistance to parasites (Baer and Schmid-Hempel, 2003; Hughes and Boomsma, 2004). Monandrous and monogynous species seem so to be more susceptible and defenceless to parasites, when they are established in the nest (Baer and Schmid-Hempel, 2001).
Parasites in social insects appear to be a great concern in ecology since they are responsible for the world wide decline of pollinators; especially in bees (Biesmeijer et al., 2006; Cameron et al., 2011).

In bumblebees, the colony is founded by one single-mated queen (Alford, 1975; Schmid-Hempel and Schmid-Hempel, 2000). This reduces the genetic variation within a colony thereby increasing the risk of the spread of a parasite within the colony. Regarding this, when the parasite is established in one individual, it can spread easily within the colony and affect the entire colony. The most adaptive strategy to resist parasite in bumblebees should be the avoidance of parasite uptake or intake into the colony.

One of the most widespread parasites in bumblebees is *Crithidia bombi*, a trypanosome gut parasite. *C. bombi* may cause a decrease of colony efficiency, a higher mortality of workers and/or a delay on the production of the reproductive caste (Schmid-Hempel, 2001). Transmission of *C. bombi* might occur vertically, but also horizontally by foragers on flowers (Durrer and Schmid-Hempel, 1994). *C. bombi* may be transmitted to other conspecific, even allospecific pollinators, via shared used of flowers (Durrer and Schmid-Hempel, 1994). The presence of this parasite on flowers has been recorded (Durrer and Schmid-Hempel, 1994). The ingestion of this parasite results in a rapid immune response. The immune genes are up regulated 24 hours post infection (Riddell et al., 2009). The same pattern has been shown to occur with non specific parasites (*E. coli*) (Erler et al., 2011). This immune response is known to reduce the learning ability of free flying bumblebees (Alghamdi et al., 2008). *C. bombi* is further known to change the foraging behaviour of bumblebees. When they are infected with *C. bombi*, they spend more time foraging due to a reduce ability to handle the flower (Otterstatter et al., 2005). Bees infected with *C. bombi* reject more flowers and fall more often from the flower (Otterstatter et al., 2005).

To test, whether bumblebees are adapted to resist against a specific parasite and if avoidance behaviour was selected against contaminated flowers; bumblebee colonies were observed during a foraging test. Bumblebees were marked individually and were given a choice between two flowers: one where the pathogen is present in the nectar referred later as “contaminated” and the other where the pathogen is absent from the nectar referred as “non-contaminated”. This experiment was repeated with different pathogens: a common, non-specific pathogen *Escherichia coli* and the specific parasite *Crithidia bombi*. The number of visits, the visit duration and the
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individual feeding on each flower were recorded and compared.

**Material and methods**

*Bombus terrestris*

To test the ability of bumblebees to recognise contaminated flowers, the foraging of bumblebee workers from a commercial colony was observed on artificial flowers under semi-natural conditions within a tent (4 m x 5 m x 2 m) placed outdoors. Four replicates were made for *E.coli* and *C. bombi* experiments with separate colonies. The bumblebee colony was placed on a chair at a distance of two meters from the flowers. The bumblebees were kept in their original colonies and were provided only with pollen *ad libitum*, foraging was for sugar or honey water. The flowers were equidistant from the colony and were placed at 10 cm apart from each other. The artificial flowers were built from a model of the umbel flower from Jordan & Harder 2006 (Jordan and Harder, 2006) and consisted of twelve Eppendorf® tubes (0.6ml) wrapped in blue paper and pinned on a cardboard disc (Ø12cm) by an insect pin. Before the recording, bumblebees were trained to forage on the flowers. During training, the flowers were filled with a mixture of honey and 50% sucrose solution (v/v). The training occurred over 3 to 5 days depending on the frequency of individuals foraging. After training, the observations were started with one of the flowers contaminated by a pathogen. During the experimental period, the flowers were filled with the same mixture as during the training, when no observation was taking place. Bumblebee workers were marked individually using Opalithplättchen (I.D.) glued (ApisPro®) to their thoraces. The individual I.D., the number of visits and the visit duration were recorded for each flower. When individuals lost their marking, they were recorded as unknown individuals and were attributed a different number for each visit. The recording time started when the bumblebee began feeding on the flower and stopped when they departed. When the identification of individual’s marking was impossible (staying on the flower less than 2s), the visit was discarded.

*Escherichia coli*

The first experiment was conducted by infecting one flower with *Escherichia coli*, a non-adapted pathogen. *E. coli* (strain JM109 from Promega®) was cultivated in 30
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ml LB medium as over night culture at 37 °C. After counting with a Fuchs-Rosenthal counting chamber (Roth, Karlsruhe, Germany) according to standard protocols, the cell culture was centrifuged 20 min at 2000 rpm. The LB medium was extracted and the pellet was mixed with a 50% sucrose solution (v/v) in order to get a concentration of \( E. coli \) at \( 10^5 \text{ cells/ml} \). Four commercial bumblebee colonies (Koppert Biological System®) were used containing each 70 to 150 workers. The recording occurred 4 hours per day over a period of 4 days. The flowers were switched every hour.

**Crithidia bombi**

In a second experiment, *Crithidia bombi* was used to infect one of the flowers. *C. bombi* were extracted from wild bumblebees’ guts from Halle (Germany) (No specific permits were required for the extraction of *C. bombi* from wild bumblebees. The sample was on an open area not privately owned and not protected in any way, and concerns only bumblebee workers which are not considered as an endangered or protected animal.). One strain of *C. bombi* cells was cultivated and counted according to the methods developed by Popp & Lattorre 2010 (Popp and Lattorre, 2011). The cell culture of *C. bombi* was centrifuged for 20 min at 2000 rpm. The pure medium was discarded and the pellet was diluted in 50% sucrose solution (v/v) in order to get a concentration of \( 10^4 \text{ cells/ml} \). Four commercial colonies were used (2 from Koppert Biological System® and 2 from Biobest Biological System®) containing each 70 to 150 workers. We used the two commercial sources to test for differences between maintained populations (one population from Central Europe and one from South Europe; possibly different subspecies). The visits were recorded until the total number of visits was 350 for each colony; the flower position was switched 4 times per day in order to account for any side preference of the foraging workers and to get the same number of visits for each flower position per day. For three colonies, the time of recording was 3 days and for the last colony the record was running for a total of 6 days.

**Control**

A control experiment was made to certify the absence of influence of the culture medium on the bumblebee foraging decisions. One commercial colony (Koppert Biological System®) was used for the record and one flower received a mixture of medium and sugar water (concentration: 1.34% according to twice the concentration...
of medium expected in the contaminated sucrose solution of both other experiments). Behavioural recordings were done according to the methods described for the *C. bombi* experiment.

**Statistical analyses**

The avoidance behaviour exhibited by bumblebees was expected to be specific and so should be more frequent when a specific pathogen of bumblebees was present in a flower. Hence the proportion of visits on the uncontaminated flower was compared between the different pathogens. We assigned the value 1 for a visit on the uncontaminated flower and 0 for a visit on the contaminated flower. The proportion of visits on the uncontaminated flower was analysed between the different experiments by a generalized linear mixed effect model with a binomial distribution including as a fixed factor the pathogen type (*E. coli, C. bombi,* and control) and individual and colony I.D., and day of recording as random factors to account for pseudo-replication between days and, between and within colonies.

**E. coli**

The data for feeding duration for each set up were log transformed and analysed with a generalized linear mixed effect model (Bates, 2008; R Team Development Core, 2008) including the individual and colony I.D., and the day of recording as a random factors to account for pseudo-replication between days and, between and within colonies. The contamination of the flower (contaminated or not) and the position (left or right) were included as fixed factors in all models. The distribution of all response variables and their residuals were inspected for symmetry. Factor levels were reduced from the full model by stepwise deletion (model simplification following Crawley 2005 (Crawley, 2005)).

The number of visits was analysed by a generalized linear mixed effect model with a Poisson distribution including as explanatory factors: the contamination, the position; and as random factor: the individual and colony I.D., and the day of recording to account for pseudo-replication between days and, between and within colonies. Factor levels were reduced from the full model by stepwise deletion (model simplification following Crawley 2005 (Crawley, 2005)). Furthermore when a model was better than the null model, another generalized linear mixed effect model was built. In order to test how the proportion of uncontaminated flower visitation changes over days and in regard to the position of the flower, the proportion of visits on the
uncontaminated flower was analysed using a generalized linear mixed effect model with a binomial distribution. The day of recording and the position of the flower were included as fixed factors while the individual and colony I.D., and day of recording as a random factors to account for pseudo-replication between days and, between and within colonies. Factor levels were reduced from the full model by backward stepwise deletion (model simplification following Crawley 2005 (Crawley, 2005)).

\textit{C. bombi}

The same statistical method applied for \textit{E. coli} was used for the visit duration and the preference toward a flower in the \textit{C. bombi} experiment. When testing for the distributions of uncontaminated flower visitation over days and position, a third fixed factor was added to the model: origin of the colony (i.e., company).

In addition, to understand the decision making at an individual level in the \textit{C. bombi} experiment, individuals with different total number of flights were classified in different groups: individuals with less than or equal to 5 flights the naive bees (Riveros and Gronenberg, 2009; Durisko et al., 2011) and individuals with more than or equal to 10 flights the experienced bees. Individuals recorded as unknown were excluded from this analysis.

The naive bees were used to analyse if the individuals were able to recognise and avoid the contaminated flower without experience. So the number of visits between the contaminated and uncontaminated flowers was compared using a Mann-Whitney-U-test.

The experienced bees were further divided in two groups: the rare (10 to 24 flights in total) and the frequent flyers (>25 flights in total). The proportion of visits on the non-contaminated flower was compared between these two groups on each day with a Mann-Whitney U test. In addition, the proportion of visits on the non-contaminated flower for each group was compared between days using a Friedman ANOVA and Kendall coefficient of concordance test.

\textbf{Control}

The same statistical method applied for \textit{E. coli} was used for the control experiment without colony as random factor.
Results

The proportion of visits on the uninfected flower is higher for the *C. bombi* experiment than for the *E. coli* one. For the control experiment, this proportion was lower than for either of the other experiments (Fig 1, GLMM: \( p < 0.001 \)). This highlights an increased preference, or a better ability to avoid the contaminated flower, in the presence of *C. bombi* than *E. coli* (*C. bombi* vs control: \( p < 0.001 \), *C. bombi* vs *E. coli*: \( p < 0.001 \), *E. coli* vs control: \( p < 0.01 \)).

![Figure 1: Proportion of non-contaminated flower visitation between experiments. The bars represent the means between the different colonies and their 95% confidence interval. The foragers were feeding more often on the non-contaminated flower when the other one was contaminated by a pathogen. This proportion increased when the other flower was contaminated with *C. bombi* (GLMM: \( p < 0.001 \); *C. bombi* vs control: \( p < 0.001 \), *C. bombi* vs *E. coli*: \( p < 0.001 \), *E. coli* vs control: \( p < 0.01 \).)](image)

**Escherichia coli**

Bumblebees spent more time feeding on the non-contaminated flowers (Fig. 2a). For the visit duration the best model includes only the contamination as explanatory factor (GLMM: \( p < 0.05 \)). They also exhibited a preference for the non-contaminated...
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Figure 2: Feeding duration, flower preference and proportion of uncontaminated flower visitation for E. coli experiment. A) Feeding duration on both flowers with and without the presence of Escherichia coli (n=1150), B) Visit duration on both flowers with and without the presence of Escherichia coli (n=1150), C) Proportion of non-contaminated flower visitation for E. coli experiment. C (in white) represents the presence of the parasite in the flower and NC (in grey) its absence. For the feeding duration, box plots depict median, interquartile range and non-outlier range; the dots represent the outliers. The bars represent the means between the different colonies and their 95% confidence interval. Foragers feed longer on the uncontaminated flower (GLMM: \( p<0.05 \)), visit it more often (GLMM: \( p<0.01 \)) and are more accurate when the flower is on left position (GLMM: \( p<0.001 \)).

flower. The number of visits observed was higher on the non-contaminated flower than on the contaminated one (best model includes only the contamination as explanatory factor GLMM: \( p<0.01 \), Fig. 2b). The bumblebees visited the non-contaminated flower more often when it was on the left position (best model includes only the position as explanatory factor, GLMM; \( p<0.001 \); Fig. 2c).

**Crithidia bombi**

For the C. bombi contamination, bumblebees spent a similar amount of time foraging on the contaminated as on the non-contaminated one (GLMM: \( p=0.24 \), Fig. 3a), but visit more frequently the non-contaminated flower (best model includes
Avoidance of contaminated flowers by bumblebees

only the contamination as explanatory factor, GLMM: \( p < 0.001 \); Fig. 3b). Moreover the number of visits increases over time and there is a different pattern of visitation between populations. Bumblebees exhibited a stronger preference for the non-contaminated flower. Indeed the best model includes the contamination as an explanatory factor. They also increased the number of visits on the non-contaminated flower over time and there is a different pattern of visitation between populations. Bumblebees exhibited a stronger preference for the non-contaminated flower. Indeed the best model includes the contamination as an explanatory factor. They also increased the number of visits on the non-contaminated flower over time (factor day: \( p < 0.05 \)), for the sympatric population

**Figure 3**: Feeding duration, flower preference and proportion of uncontaminated flower visitation for *C. bombi* experiment. A) Feeding duration on both flowers with and without the presence of *C. bombi* (n=1400), B) Visit duration on both flowers with and without the presence of *C. bombi* (n=1400), C) Proportion of non-contaminated flower visitation over days and between sympatric population (grey dot & continuous line) and allopatric population (black triangle & dashed line) for *C. bombi* experiment. C (in white) represents the presence of the parasite in the flower and NC (in grey) its absence. For the feeding duration, box plots depict median, interquartile range and non-outlier range; the dots represent the outliers. The bars represent the means between the different colonies and their 95% confidence interval. Foragers spend the same time feeding on both flowers (GLMM: \( p = 0.24 \)), visit preferentially the uncontaminated flower (GLMM: \( p < 0.001 \)). The proportion of uncontaminated flower visitation increase over days and for the sympatric population this increase is stronger than for the allopatric population (GLMM: \( p < 0.01 \); factor day: \( p < 0.05 \), interaction between day and population’s origin: \( p < 0.01 \)).
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this increase was stronger (interaction between day and population’s origin: \( p < 0.01 \)). The best model included the day and the interaction between day and the population of origin as explanatory factors (GLMM: \( p < 0.01 \); Fig 3c).

The naive bees are able to avoid the contaminated flowers since they visited more often the non-contaminated flower (M-W-U-test: \( Z = 5.74 \), \( p < 0.001 \)). Among the experienced bees, the frequent flyers have a better cognitive ability or sensory to recognise the contaminated flower than the rare flyers on the first day since they visited the non-contaminated food source more often (M-W-U-test: \( Z = -2.40 \), \( p < 0.05 \), Fig. 4). Although after the first day, the rare flyers increase their number of visits on the non-contaminated flower (Friedman ANOVA: \( \chi^2 = 9.15 \), \( p < 0.01 \); 2nd day: M-W-U-test: \( Z = 0.77 \), \( p = 0.45 \); 3rd day: M-W-U-test: \( Z = 1.49 \), \( p = 0.15 \)). The frequent flyers showed no increase or decrease over time (Friedman ANOVA: \( \chi^2 = 4.26 \), \( p = 0.12 \), Fig. 4).

Control

The medium has no influence on the feeding duration, or the number of visits, since
the null model (without any explanatory factors) was not improved by adding explanatory factors (feeding duration: GLMM: $p = 0.71$; number of feeding events: GLMM, $p = 0.33$).

**Discussion**

Our study assessed the ability of bumblebees to recognise food sources contaminated by an adapted parasite and a non-adapted microorganism under semi-natural conditions. The results highlight the existence of the avoidance behaviour during the foraging of bumblebees, a primitive eusocial insect. In addition, our results show that bumblebee foragers behave differently toward non-contaminated food sources and contaminated ones, with also a difference towards the type of contamination. The *B. terrestris* population originating from the same region of Europe than the *C. bombi* lineage used for the experiment shows a better ability to avoid contaminated flower than the population allopatric with the parasite lineage. This seems to indicate an adaptation not only toward a specific parasite but also to a specific lineage of the parasite; maybe due to the host-parasite genotype-genotype interaction. This is seen at the immune response level where bumblebees show a greater resistance to specific strains of *C. bombi* (Schmid-Hempel and Ebert, 2003). An alternative explanation is a better ability of one population to avoid the contaminated flower compared to the other. It was argued and shown that avoidance behaviour in birds should be specific to a parasite species, but not a parasite strain (Christe et al., 1996; Schmid-Hempel and Ebert, 2003).

Bumblebees spent more time feeding on non-contaminated artificial flowers than on those contaminated by *E. coli* and visit the uncontaminated flower more often (Fig. 1a,b). Many theories on optimal foraging were tested in bumblebees and other pollinators, especially the marginal value theorem developed by Charnov in 1976 (Goulson, 1999; Biernaskie and Gegear, 2007; Lefebvre et al., 2007; Biernaskie et al., 2009; Bar-Shai et al., 2011). The results provided by these different experiments show that bumblebee foraging and patch departure follows a sub-optimal strategy (Goulson, 1999; Biernaskie and Gegear, 2007; Lefebvre et al., 2007; Biernaskie et al., 2009; Bar-Shai et al., 2011). To summarise briefly the strategy exhibited by bumblebees is to stay longer in large patches or patches providing a high reward. Patch departure happens with the decreasing reward of one flower or from the entire
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patch. In our experiment, we can consider one flower composed by 12 inflorescences as a patch. The flowers were filled appreciatively at a similar level and access to the “nectar” was similar between the two flowers. On one hand, this difference in feeding duration between the two flowers could be explained as a preference for the non-contaminated “nectar”, or as most rewarding “nectar”. On the other hand, this difference in feeding duration could also result from the direct presence of the cells or the medium in the sugar water decreasing its energetic value for the bee. This last explanation seems to be contradicted by the Crithidia and control experiment where the presence of the gut parasite and the medium had no effect in the visit duration (Fig. 3a). The effect of position on the proportion of uncontaminated flower visitation could be due to a lateralization of the brain and behaviour in bumblebees (Anfora et al., 2011).

The bees, having the choice between a contaminated food source by a specific gut parasite and a non-contaminated one, visit more often the non-contaminated flower (Fig. 3b). This reveals the clear ability of bumblebees to recognise and avoid sugar water contaminated by Crithidia. In a same context as above (comparing this foraging behaviour with the optimal foraging theorem) this result can be interpreted as flower constancy. Indeed, it was shown that a bee will prefer to visit a flower that she learnt to be rewarding than to spend time visiting other flowers (Waser, 1986; Goulson, 1999; Biernaskie and Gegear, 2007; Lefebvre et al., 2007; Raine, N. E. and Chittka, L., 2007; Biernaskie et al., 2009; Bar-Shai et al., 2011). The presence of C. bombi leads to a perceptive decrease of the reward provided by the sugar water to the bumblebees.

The comparison between the results of the experiments with C. bombi and with E. coli shows a degree of adaptation of bumblebees toward the specific gut parasite C. bombi; since bumblebees avoid food sources contaminated by C. bombi more often than E. coli (Fig 1). Even if they feed longer on the uncontaminated flower when contaminated by E. coli while this pattern is not present with C. bombi (Fig 2a,3a) This result maybe an artefact from the experimental design, as short visits (<2 seconds) may have been the response time to C. bombi (these visits were not recorded). This is correlated with the observation of individuals tasting the nectar without landing on the flower contaminated by C. bombi (personal observation).

C. bombi is a long term and specific parasite of bumblebees resulting in co-evolution between host and parasite (Schmid-Hempel, 2001). According to the red queen
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theory, it should lead to an arms race between a host and his specific parasite (Bell, 1982; Hamilton et al., 1990; Lively et al., 1990). Since the bumblebee colony is composed by full-sibs, a parasite can easily spread between individuals and decrease the fitness of the entire colony (Schmid-Hempel, 2001). Hence the adaptation of avoidance behaviour should be a decisive step with regards to parasitism in bumblebees. This hypothesis is strengthened by our results, since the presence of a non-adapted parasite toward bumblebees decrease the rewarding value of the nectar; moreover the presence of a specific parasite in the nectar leads to the avoidance of the flower. Furthermore, a bumblebee population sympatric with the *C. bombi* lineage showed a better efficiency in avoidance of contaminated flowers than an allopatric population.

Bumblebees use different cues (colours, shapes, odours of the flowers and even social cues) in order to optimise their foraging efficiency (Keasar et al., 1997; Goulson et al., 2000; Goulson et al., 2001; Kunze and Gumbert, 2001; Blarer et al., 2002; Bonsall, 2004; Dornhaus and Chittka, 2005; Worden and Papaj, 2005; Saleh et al., 2007; Renner and Nieh, 2008). These cues allow them to choose the most rewarding flowers through learning. To recognise flowers contaminated with *C. bombi* without feeding on it, bumblebees have to use cues which are perceptible before the ingestion of the contaminated sugar water. At an individual level, the most likely explanation is the presence of the odour produced directly by the parasite, which is the case in ungulates (Fankhauser et al., 2008). A previous study showed that bumblebees avoid flowers containing evidence of past predation events, the cues, used were the sight and the scent of a dead bumblebee (Abbott, 2006). A further possible cue, used to recognise the contaminated sugar water, is the taste of the sugar water. Some workers were observed to extend the proboscis toward the flower and use their tongue to taste the “nectar” without landing before choosing the non-contaminated flower (personal observation).

This learning could also be the result of a colony level learning ability. The recognition of a non-contaminated flower could be provided through social cues. This could be the resultant of the use of cues from the other individuals like a copying behaviour (Goulson et al., 2001; Worden and Papaj, 2005; Saleh et al., 2007; Renner and Nieh, 2008) or the scent marks left on the flower (Goulson et al., 2001; Saleh et al., 2007; Renner and Nieh, 2008). Bumblebees leave a scent mark after visiting a flower (Goulson et al., 2000; Goulson et al., 2001; Saleh et al., 2007; Renner and
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Nieh, 2008). These scent marks can provide different information for a pollinator in regard to its previous experience (Leadbeater and Chittka, 2009). Moreover, nest-mates gain cues through the odour from the successful foragers and honey pots (Dornhaus and Chittka, 2005). Another social cue used by bumblebees for foraging is the copying behaviour; where bumblebees having seen a nest-mate feeding on a specific flower, will subsequently copy their flower choice (Worden and Papaj, 2005). Social learning is supported by our results on *C. bombi* contamination. The proportion of visits on the non-contaminated flower increased over time, while this did not occur with the contaminated flower. In addition, individuals foraging less than 5 times showed a clear preference for the non-contaminated flower without any effect from the position. Since they visit the flowers only a few times, they are not able to learn by themselves (Riveros and Gronenberg, 2009; Durisko et al., 2011). This preference of naïve bees seems to result from the copying behaviour. Naïve bumblebees choose more often flowers occupied by conspecifics (Kawaguchi et al., 2006).

Our result on the individual level shows a difference between rare and frequent flyers cognitive or sensitive abilities (Fig. 4). The frequent flyers choose more often the non-contaminated flower on a first day than rare flyers did. Although, rare flyers are not so sharp on their foraging efficiency, they increased it over days showing learning. Some previous studies have demonstrated that workers from the same colony do not possess the same abilities (Spaethe and Weidenmüller, 2002; Ings et al., 2005; Raine, N. and Chittka, L., 2007; Raine and Chittka, 2008).

Another question comes into mind with regards to these results, why bumblebee population are so heavily contaminated by this specific parasite, if they are able to recognise contaminated flowers? There are many possible explanations. First the transmission of *C. bombi* can be horizontal as vertical so the parasite is also transmitted from the mother colonies to the daughters’ colonies. For the horizontal transmission, the transfer of workers from a colony to another one (Birmingham, 2004; Lopez-Vaamonde et al., 2004) could also play a preponderant role to the spread of the parasite in a population. Regarding the infection of individuals through contaminated flowers some environmental factors can mislead the bees. One could be that the odour (if the odour is the cue used by bumblebees to recognise the contaminated flower) of the flower masks or reduce the ability of bees to detect the parasite; although this is not likely due to their ability to recognise scent marks
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deposited by other bees on the flower (Goulson et al., 2000; Goulson et al., 2001; Saleh et al., 2007; Renner and Nieh, 2008). Another reason could be strong competition for food resources or a reduced availability of the optimal food source, which might force bumblebees to forage on the most rewarding flowers. The most likely explanation for this difference between our experiment and the nature is the small quantity of nectar in a natural flower (~1 to 100 µl) compared to our flower (0.8 ml). With such small nectar quantities in the flower, the amount of *C. bombi* cells is low (compared to our experiment set-up) and should increase the difficulty for a bumblebee to detect their presence.

In conclusion, avoidance behaviour has been selected in bumblebees in order to reduce the uptake of a specific parasite when foraging on flowers. In addition they are sensitive to the presence of a common pathogen in “nectar”. The avoidance of *C. bombi* contaminated food sources appeared through learning at both, the individual and the colony level. This is mediated by the use of different cues: direct cues provided by the contamination (odour, taste, visual) and social cues provided by the other nest-mates (scent-marks, odour from honeypots and foragers, copying behaviour). These results provide a new insight on foraging strategies and resistance to parasites in bumblebees, other pollinators and social insects in general.

![Figure S1 Frequency distribution of number of flights.](image)

**Figure S1 Frequency distribution of number of flights.** The frequency of individuals in regard to their observed number of flights for the *Crithidia bombi* experiment. All replicate colonies are pooled and only the marked individuals are represented.
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Chapter 2

Social scent marks do not improve avoidance of parasites in foraging bumblebees

Bertrand Fouks and H. Michael G. Lattorff

Foraging is a result of innate and acquired mechanisms, and is optimized in order to increase fitness. During foraging an animal faces many threats - such as predation and infection. The uptake of parasites and diseases while foraging is common and an individual should be adapted to detect and avoid such threats, using cues either from the abiotic environment, or the parasite. Social animals possess an additional cue to detect such contaminated food sources: information provided by conspecifics. Bumblebees avoid contaminated flowers, but the cues used by the bees to distinguish contamination remain unknown. We tested under controlled laboratory conditions the use of scent marks derived from other foragers in choosing between a contaminated and uncontaminated flower. As a positive control we tested the bee’s choice towards two flowers, one scented with geraniol and containing a highly rewarding sugar solution and the other not scented and containing a poorer reward. The bees mainly chose the uncontaminated and the rewarding scented flower. Scent marks did not increase the efficiency of the bumblebees in choosing the better flower. The bees from both experiments behaved similarly, showing that the main and most relevant cue used by them to choose the uncontaminated flower is the odour from the parasite itself. The adaptation of bumblebees to avoid flowers contaminated by *Crithidia bombi*, arose from the long term host-parasite interaction between these species. This strong adaptation results in an innate behaviour of bees and a detection and aversion of the odour of contaminated flower nectar.


Key-words: *Bombus terrestris, Crithidia bombi*, host-parasite interactions, social cues, social immunity, social learning.
Introduction

Foraging behaviour and its optimization was and still remains a centre of evolutionary, ecological and neuroscience research. When investigating foraging behaviour in social animals an additional level appears which is composed of the signals, cues and information given by conspecifics in order to choose a resource patch. While foraging, many threats appear such as predators and parasites, leading to a drastic decrease of the fitness of an organism. Thus, organisms should have evolved in order to detect and avoid such threats. In the case of parasitism, the avoidance of parasites is the first barrier against it, which could be less costly than immune responses. Theory incorporates the role of parasites into the optimal foraging models (Lozano, 1991).

In order to detect such threats, an organism can rely on evidence from the environment and also from the parasite itself (Hart, 1990). When living in a society, animals can cooperate to avoid parasites. Indeed, ants and termites avoid directly any contact with parasitic flies, helminths and fungi (reviewed in Cremer et al, 2007). This is called social immunity, since this avoidance depends on the cooperation of a social group. Other levels of social immunity exist, such as hygienic behaviour in honeybees (Wilson-Rich et al., 2008), or allogrooming, where social groups cooperate or behave altruistically to reduce the effect of the parasite on the whole group (Cremer et al., 2007).

Moreover, living in a group facilitates an individual to learn via his conspecifics, known as social learning, which may lead to the evolution of culture in many vertebrate species (Heyes and Galef, 1996). This social learning appears to be of a great importance in honeybees, bumblebees and even in fruitflies and crickets (Chittka and Leadbeater, 2005; Coolen et al., 2005; Kawaguchi et al., 2006; Battesti et al., 2012). The combination of social learning and social immunity has been observed in mammals, e.g. primates (Huffman et al., 2010). However, in invertebrates this has never been studied.

The bumblebee, Bombus terrestris (Linnaeus, 1758), is a model species for investigating foraging mechanisms (Hodges, 1985). Bumblebees use both, innate and learning mechanisms to find resource patches (Plowright et al., 2006), and the social cues allow them to optimize their foraging efficiency (Goulson, 1999). They are able to learn which flowers are the most rewarding with the help of the flower, social cues
Scent marks not used to detect parasite and experience (Kawaguchi et al., 2006; Leadbeater and Chittka, 2009; Hudon and Plowright, 2011; Plowright et al., 2011).

Bumblebees are eusocial insects with an annual life-cycle, whose colonies are founded by a single, once-mated queen in early spring. Their social life and the low genetic diversity within a colony make them a prime target for parasites. Their social organisation provides parasites with a stable and rich environment (Schmid-Hempel, 1998). The low genetic variability within a colony, due to the single mated and unique queen, allows parasites to easily infect every individual within it (Baer and Schmid-Hempel, 1999, 2001). However, their social life also provides them with a different way to fight against a parasite or disease, so called social immunity (Cremer et al., 2007). There are different levels of social immunity from the uptake of the parasite to its transmission to the next generation (Cremer et al., 2007). Social immunity may occur in presence of a parasite (activated response) but also in absence of parasites (prophylactic response) (Cremer et al. 2007; Richter et al. 2012).

Bumblebees are parasitized by *Crithidia bombi*, a well adapted gut parasite of bumblebees (Schmid-Hempel, 2001). This parasite decreases drastically the chance for a future queen to found a new colony, and also the size and the efficiency of new colonies (Brown et al., 2003). This long term relationship leads, according to the red queen theory hypothesis (Bell, 1982), to an arms race. Recently, Fouks and Lattorff (2011) discovered an avoidance behaviour of contaminated flowers, either by a specific parasite (Trypanosoma: *Crithidia bombi*) or by a common micro-organism (Bacteria: *Escherichia coli*), in foraging bumblebees.

The combination of activated social immunity during foraging behaviour exhibited in bumblebees is of importance as parasites might be taken up on shared food patches (Durrer and Schmid-Hempel, 1994). The foraging behaviour of the bees is influenced by parasites (Fouks and Lattorff, 2011) and resulting from that the fitness of flowers might be influenced indirectly.

Here, we investigate the interaction of social information and innate preference in avoiding unrewarding or contaminated flowers. In order to know which cues the bumblebees use for choosing the rewarding (non-contaminated) flower, we record the flower choice of bumblebees during 6 days with two different setups: one where the flowers were cleaned in order to remove scent cues left by conspecifics, and the other where the flowers were not cleaned. In addition, we use a positive control with the same setup without contamination but where the most rewarding flower was scented
with geraniol, to investigate the mechanism used by the bees to distinguish both flowers.

**Materials and methods**

**Bumblebees**

Bumblebees from 3 different colonies were used for the experiment (Koppert). One colony was used for the Geraniol experiment, while two other colonies were used for *C. bombi* experiment in order to avoid any peculiar behaviour from a colony. From each original colony, 2 batches of 25 marked bumblebees (with Opalithplättchen) were housed in a metal cage (14.5cm x 12cm x 2.5cm) containing empty honey pots on a wax frame, and were provided with pollen *ad libitum*. Each bee was trained to fly and feed on an artificial flower for 5 minutes, 3 times a day during a 3 day trial period. The flower consisted of a blue foam paper (Ø 6cm) glued on a piece of wood placed on a plastic cylinder (Ø 2.8cm, 4.5cm), in the center an Eppendorf tube (0.2 mL) was placed. The artificial flower was filled a solution of honey water and washed after each trial with ethanol (50%) (Leadbeater and Chittka, 2009). The foraging trial and experiment occurred in a flight arena (terrarium of 1m x 0.4m x 0.5m, the ground was covered by a green Kraft paper) with the flower placed towards the light source. After these 3 days of training, only the bumblebees who were feeding were kept for the experiment. All the bumblebees were flower naive before the training.

For the experiment, each bee was placed in a flight arena and was given a choice between two artificial flowers (as described above), 10 cm apart from each others and equidistant from the bumblebee entrance. Each group of bees was tested 4 times a day over a period of 6 days. In one flight arena, the flower was washed after every trial with ethanol (50%) in order to allow no cues to help the bees in choosing between the two flowers (referred to as the Individual setup later on), and in the other flight arena the artificial flowers were not washed in order to allow the bees to use the scent marks left on the flower by their conspecifics (referred to as the Group setup later on). The position of flowers was switched regularly between the trials in order to avoid any side bias.

The duration before the bee landed, where she landed, the time period of feeding and switching between flowers after the first landing or after feeding were recorded. When the bee spent more than 3 minutes without landing on a flower, she was put
back to her sub-colony.

**Geraniol experiment**

As a positive control we used a strong odour to indicate the rewarding flower to the bee. We used a sponge to apply a diluted solution of geraniol (>90%, Carl Roth®) (5µL:50ml) on the flower containing the most rewarding “nectar” consisting of sucrose water (50:50, v:v) while the other flower contained a more diluted sucrose solution (30:70, v:v). One colony was used and the sub-colony “Group setup” was composed of 12 individuals, and the “Individual setup” was composed of 11 individuals.

**C. bombi experiment**

The *Crithidia* experiment consisted of one flower with a sucrose solution (50:50, v:v; below referred to as the rewarding flower), and the other flower containing the same sucrose solution (50:50, v:v) including a concentration of 3000 cells/mL of *Crithidia bombi* (strain 076 provided by P. Schmid-Hempel, ETH Zurich) (below referred to as the unrewarding flower). *C. bombi* was cultivated in cell cultures and cell number was quantified according to a standard method (Popp and Lattorff, 2011). In order to avoid any odour or cue from the medium, *C. bombi* cells were washed two times with pure water before preparation of the sucrose solution. Two colonies were used for this experiment, the 2 sub-colonies “Group setup” contained 13 and 12 individuals, and the 2 sub-colonies “Individual setup” contained 14 and 12 individuals.

**Molecular analyses**

After the experiment all bees were snap-frozen. Their guts were removed and crushed in 300µl of aqua dest. DNA was extracted from a 100µl aliquot of the homogenate using the Chelex method (Walsh et al., 1991). DNA was used to genotype samples using a multiplex PCR with the microsatellite primers Cri 4, Cri 4G9, Cri 1.B6 and Cri 2F10 (Schmid-Hempel and Reber Funk, 2004) according to the method described by Erler et al. (2012) (Erler et al., 2012b). Fragment lengths were determined by means of capillary DNA sequencer Megabace 1000 (Amersham Biosciences). The area of the peaks for each microsatellite allele was calculated using the software Fragment Profiler (Amersham Biosciences).

The intensity of the fluorescence signal of the microsatellite alleles (peak height/area in electropherogram) determined by a capillary sequencer (MegaBace 1000, Amersham) is correlated to the intensity of infection (B. Fouks and H.M.G. Lattorff, unpublished). In order to determine the infection intensity we used the peaks of the
microsatellite locus Cri 1.B6, which gives the most reliable estimate (B. Fouks and H.M.G. Lattorff, unpublished). The area of the peaks was compared between the different setups (Group and Individual) using a Mann-Whitney U test. Additionally, a linear regression between the overall proportion of visits on the uncontaminated flower of every bee and the area of the peak was performed.

Allometry analysis

The size of bumblebees is well known to have an effect on their foraging efficiency and learning ability (Spaethe and Weidenmüller, 2002; Spaethe et al., 2007; Chittka and Niven, 2009). In order to rule out any potential bias between the different setups for the C. bombi experiment, the size of the bees was determined by quantifying the length between two junctions of veins on their forewings, as wing length is highly correlated to body size (Muller and Schmid-Hempel, 1992; Müller et al., 1996; Hunt et al., 1998; Klingenberg et al., 2001). Wings were removed, mounted on object slides and digitised. Calculations were done using Image J® software.

Using wing size as a proxy for body size of the bees we tested for the influence of body size comparing the setups (Group and Individual), using a Mann-Whitney U test. We performed a linear regression between the overall proportion of visits on the uncontaminated flower of every bee and their size. Furthermore, we realized a linear regression between the peak’s area of the microsatellite Cri 1.B6 (the intensity of infection of an individual) and the size of the bee.

Statistical analyses

All statistics were realised with the R software (R Team Development Core, 2008).

Behavioural assays

The avoidance behaviour exhibited by bumblebees was expected to increase with the presence of scent marks on flowers and over days as a result of social and associative learning.

The data for feeding duration for each experiment were log transformed and analysed with a generalized linear mixed effect model (GLMM) (Bates, 2008) including the individual I.D. as a random factor to account for pseudo-replication within individuals. The reward/contamination of the flower (rewarding/uncontaminated or unrewarding/contaminated), the position (left or right) and the setup (Group or Individual) were included as fixed factors in the models. For all GLMMs, the distribution of all response variables and their residuals were inspected for symmetry and overdispersion. For model building and simplification (backward stepwise
Scent marks not used to detect parasite deletion), we followed the practical guide developed by Bolker et al. (2008) (Bolker et al., 2009) and Crawley (2005) (Crawley, 2005).

The number of visits was analysed for both experiments (geraniol and C. bombi) by a GLMM with a Poisson distribution including the reward and position as explanatory factors and individual I.D. and day of recording as random factors in order to account for pseudo-replication within individuals.

We assigned the value 1 for a visit on the uncontaminated flower and 0 for a visit on the contaminated flower. The proportion of visits on the rewarding flower was analysed by a GLMM with a binomial distribution including setup (Group and Individual) and position (left or right) and day as fixed factors and individual I.D. as a random factor to account for pseudo-replication within individuals.

For switching between flowers, both after landing and after feeding, we assigned the value 1 when a bee switched from one flower to the other and the 0 when the bee stayed on the first flower. The proportion of switches to the other flower after landing and after feeding were analysed for both experiments (geraniol and C. bombi) by a GLMM with a binomial distribution including as fixed factors: the reward of the flower (rewarding or unrewarding), the setup (Group and Individual), the position (left or right), the day of recording; and individual I.D. as a random factor to account for pseudo-replication.

**Results**

**Behavioural assays**

**Geraniol setup**

As expected, bees fed longer and more often on the most rewarding and geraniol scented flowers (Fig. 1A, GLMM: $P < 0.001$; Fig. 1B, GLMM: $P < 0.001$, Table S1 in supplementary material). Over days the bees show a decreased efficiency feeding on the scented flower: showing a loss of flower constancy, the position of the flower influences the choice of the bees but not significantly (GLMM: the best model is the model containing the position and day as explanatory factors, position: $P = 0.144$, day: $P < 0.05$; see Table S1 in supplementary material). In addition, the bees switch
Scent marks not used to detect parasite

**Figure 1:** Feeding duration, flower preference and switch of flowers after landing and feeding for the geraniol experiment. A) Feeding duration on both flowers with and without the presence geraniol (n = 368), B) Visit frequency on both flowers with and without the presence of geraniol for each individual on the overall trial (n = 368), C) Proportion of switch between flower after the first landing on the non rewarding or rewarding flower (n=18), D) Proportion of switch between flower after the first feeding on the non rewarding or rewarding flower (n=25) For the feeding duration, box plots depict median, interquartile range and non-outlier range; the dots represent the outliers. The bars represent the means between the different colonies and their 95% confidence interval. Foragers feed longer on the most rewarding flower (GLMM: $P < 0.001$), visit preferentially the scented flower (GLMM: $P < 0.001$). The proportion switch is higher when land and feeding first on the less rewarding flower (GLMM: $P < 0.001$, GLMM: $P < 0.001$).
from one flower to the other more often when landing and feeding first on the unrewarding flower (Fig. 1C, GLMM: $P < 0.001$; Fig. 1D, GLMM: $P < 0.001$, see Table S1 in supplementary material). This indicates that bees are more attracted to flowers with the odour of geraniol, and when landing or feeding on the unrewarding flower, potentially due to mistake, they change to the most rewarding flower.

**Crithidia bombi setup**

We found that bumblebees fed longer and more often on the uncontaminated flower than on the one containing the parasite (Fig. 2A, GLMM: $P < 0.001$; Fig. 2B, GLMM: $P < 0.001$, see Table S1 in supplementary material). The bees behave similarly, but less efficiently as in the geraniol experiment. When examining the proportion of workers foraging on the uncontaminated flower according to the setup, it appears that the scent marks do not affect the efficiency of the bees to choose the non-contaminated flower (Fig. 3B). The bees are more efficient when the uncontaminated flower is on the left position (for the bee), and show a non significant difference over days on their efficiency to choose the uncontaminated flower (GLMM: the best model is the model containing the position and day as explanatory factors, position: $P < 0.05$, day: $P = 0.117$; see Table S1 in supplementary material).

For switching to the other flower, the bees react in the same way as for the geraniol experiment but less efficiently, they change from one flower to the other more often after landing or feeding first on the contaminated flower (Fig. 2C, GLMM: $P < 0.001$; Fig. 2D, GLMM: $P < 0.05$; see Table S1 in supplementary material).

**Molecular assays**

First, we confirmed that the infection of the bees is due only to the strain of *C. bombi* applied to the flowers. The multilocus genotypes are identical between the cultivated strain and the infection determined in the bee guts. When comparing the infection intensity between the two setups, it seems that the washing of the flower decreases the degree of infection of the bees (Fig. 4, Mann-Whitney U test: $Z= 2.14$, $p< 0.05$). The ability of the bees to choose the uncontaminated flower did not affect the intensity of infection, showing a transmission of the parasites directly from an individual to the other inside the nest (linear regression: $r^2= 0.018$, $p=0.17$).

**Allometry assays**

No bias between setups was found for the size distribution of the bees (Mann-Whitney U test: $Z= 0.47$, $p= 0.65$). There was also no correlation between the size of a bee and their performance to choose the uncontaminated flower (linear regression:
Scent marks not used to detect parasite

$r^2 = 0.001, p = 0.31$). In addition, the intensity of infection is not correlated with the size of the bee (linear regression: $r^2 = -0.019, p = 0.82$).

**Figure 2:** Feeding duration, flower preference and switch of flowers after landing and feeding for the *C. bombi* experiment. A) Feeding duration on both flowers with and without the presence of the parasite ($n = 810$), B) Visit frequency on both flowers with and without the presence of the parasite for each individual on the overall trial ($n = 810$), C) Proportion of switches between flowers after the first landing on the non-rewarding or rewarding flower ($n = 77$), D) Proportion of switches between flowers after the first feeding on the non-rewarding or rewarding flower ($n = 73$) For the feeding duration, box plots depict median, interquartile range and non-outlier range; the dots represent the outliers. The bars represent the means between the different colonies and their 95% confidence interval. Foragers feed longer on the uncontaminated flower (GLMM: $P < 0.001$), visit preferentially the uncontaminated flower (GLMM: $P < 0.001$). The proportion switch is higher when land and feeding first on the contaminated flower (GLMM: $P < 0.001$, GLMM: $P < 0.05$).
Discussion
As previously shown, worker bees exhibit an avoidance behaviour towards flowers contaminated by *C. bombi* (Fouks and Lattorff, 2011). Bees react to contamination as a decrease of the reward of the “nectar”. Indeed, the same pattern between the Geraniol and *C. bombi* experiments has been observed for the number of visits and their duration (Figs 1A and 2A). Furthermore, they avoid the contaminated flower due to the odour from contamination since they visit the uncontaminated one more often without any other clue differentiating either flower (Fig. 3). They show also no clear learning over days to choose the flower without contamination indicating that the avoidance of the contaminated flower is an innate response. Finally, bees more often change to the rewarding flower when landing on the non-rewarding, contaminated one (Fig. 2D); emphasising the repellent effect of contamination for the bees.

![Figure 3: Proportion of rewarding/uncontaminated flower visitation with and without scent marks for both geraniol and *C. bombi* experiment. A) The proportion of the most rewarding flower visitation between the 2 setups for geraniol experiment (Group: n = 203, Individual: n = 165), B) The proportion of the uncontaminated flower visitation between the 2 setups for *C. bombi* experiment. The bars represent the means between the different colonies and their 95% confidence interval. The use of the scent marks did not significantly improve the efficiency of the bees to feed on the most rewarding flower (Geraniol: the best model does not include the setup as a fixed factor; *C. bombi*: no model was better than the model containing no explanatory factor, Table S1).](image-url)
Scent marks and their significance have been well studied (Goulson et al., 1998; Goulson et al., 2000; Goulson et al., 2001; Saleh and Chittka, 2006; Saleh et al., 2006; Saleh et al., 2007; Witjes and Eltz, 2007; Leadbeater and Chittka, 2009; Witjes and Eltz, 2009; Leadbeater and Chittka, 2011). On the one hand, some studies have shown that scent marks act as repellents for experienced bees, allowing them to choose rewarding flowers more efficiently, as previous visitors might have reduced the available nectar (Goulson et al., 1998; Goulson et al., 2001). On the other hand, some studies report the contrary (Witjes and Eltz, 2007). Finally other studies showed that bees react to scent marks as a function of their previous experience (Saleh and Chittka, 2006; Leadbeater and Chittka, 2009). Recently, it has been shown that naive bees have no preference, neither for flowers already visited nor for the one unvisited (Leadbeater and Chittka, 2011). Scent marks are mainly composed by cuticular hydrocarbons, and they correspond to footprint cues rather than pheromone signals (Goulson et al. 2000; Saleh et al. 2007)(Wilms and Eltz 2008; Witjes and Eltz 2009). These substances are non-volatile and even tiny differences in their quantities are detectable by social insects, which accumulate on the flower after each visit and remain unchanged over a period of 24 hours (D’Ettorre 2008; Saleh et al. 2007; Witjes and Eltz 2009). In our experiment, the scent marks do not increase or decrease the efficiency of the bees to choose the rewarding flower. This could be due to the fact that both flowers were visited. Even so they should have accumulated more on the uncontaminated flower and allowed the bees to choose it more easily. The other possibility is that scent marks are not really useful to facilitate the choice of bees between contaminated or uncontaminated flower, due to the strong cue given by the odour of the parasite (Fig. 3). Some ungulates avoid fields contaminated by faeces containing parasites (Fleurance et al., 2007; Fankhauser et al., 2008). It has also been shown that leaf-cutter ants can discriminate the fungus strain and reject foreign fungus by the odour of the fungus (Ivens et al., 2009). Recently, it has been shown that Drosophila avoids bad smells (Wasserman et al., 2012). The smell might not be directly produced by the parasite but could be an unavoidable interaction of the parasite and the substrate or from the metabolic secretion of the parasite. Indeed, the presence of yeasts inside the nectar of flowers might produce specific odours (Raguso, 2004). Moreover, scent marks are used by the bees through experience and learning; the latter might be impaired by an immune challenge and/or C. bombi infection, as it is
known to decrease learning ability (Gegear et al., 2006; Alghamdi et al., 2008). However, a decrease of learning ability has been observed only when given visual cues: while for the odour cues the immune response does not decrease the learning ability of the bees (Gegear et al., 2006); this corroborates our results with the efficiency of a bee to choose the uncontaminated flower in regard to their infection load. Nonetheless, bees having a supplementary cue upon which to choose the flower feed not significantly more on the uncontaminated flower than the bee with only the odour of the “nectar”, but our sample size is big enough to significantly show this kind of preference (Plowright et al., 2011). Other social cues could have been gathered by the bees in the “individual” setup, such as the odour from the honey pots, or from conspecifics (Dornhaus and Chittka, 2005; Renner and Nieh, 2008; Batte et al., 2012). This would thus be possible if the odour from honey pots and/or conspecifics can be repellent for bees, since only the parasite possesses an odour in our experiment.

In a previous experiment (Fouks and Lattorff, 2011) we found that a bee at a social and individual level seems to learn to forage preferentially on the uncontaminated flower over a period of days. In this previous experiment, entire colonies were placed in the foraging arena; bees were allowed to forage simultaneously on the flowers, and so could rely on their nest-mates to choose the flower. Here we do not find such a significant pattern, but our sample size per day and bee is lower and might not be sufficient to detect a significant learning pattern. It is likely that this learning is strengthened due to social learning via copying behaviour which has been observed in primates who learn by observation to eat medicinal leaves (Huffman et al., 2010), in crickets learning from others to avoid predation (Coolen et al., 2005). Indeed, copying behaviour is a really important cue for naïve bees to choose certain flowers (Leadbeater and Chittka, 2005; Worden and Papaj, 2005; Kawaguchi et al., 2006; Grüter et al., 2010). Furthermore, the infected bees have an impaired learning for visual cues (Gegear et al., 2006; Alghamdi et al., 2008) and reduce their foraging activity after infection due to the immune challenge (Otterstatter et al., 2005). For naïve bees this could lead to rely on conspecifics, which have better learning efficiency and so should feed more often on the uncontaminated flower.

The higher infection intensity in the group of bees foraging on scented flowers is probably due to novel infections directly obtained on the flower. Indeed, it has already been observed that bees transmit *C. bombi* via the flower (Durrer and
Schmid-Hempel, 1994); in our experiment we confirmed that. Since the only difference between the two groups is the washing of the flower which might kill, or remove the parasite, in addition we directly observed the bees defecating on the flower.

![Figure 4: Intensity of infection in regard to the presence or absence of scent marks (n=51). Box plots depict median, interquartile range and non-outlier range; the dots represent the outliers. The washing of the flower decrease the chance of a bee to be reinfected (Mann-Whitney U test: Z=2.14, p< 0.05).](image)

As previously shown, the bees have, in the *C. bombi* experiment, a better ability to recognise the uncontaminated flower when it is on their left side (right side for the observer) (Anfora et al., 2011; Fouks and Lattorff, 2011). The explanation for the side preference stays unclear. Bumblebees have a better ability to learn an odour using their right antenna than their left one (Anfora et al., 2011). They also show preferences in the direction of circling (Kells and Goulson, 2001). This combination of left-right asymmetries could result in the preference to visit a certain position without even choosing the flower. Here, the better ability to visit uncontaminated flowers on the left position could be due to the higher rejection rate combined with higher visitation rate on contaminated flowers on the right position.

Another surprising result is the decreased efficiency of the bee to feed on the geraniol scented flower over time. Even if the reward of the unscented flower was lower, it might still high enough for the bees to select this flower. This might be determined by
the internal sucrose responsiveness threshold of every bee, a feature that is strongly influenced by genetic factors, at least in honeybees (Rueppell et al., 2006). Thus, in the first place bumblebees were attracted strongly by the scented flower, but over time this attractiveness could have decreased realizing that the other flower is also rewarding.

In conclusion, scent marks did not help the bees to choose the rewarding flower. The odour from the contaminated sucrose solution is sufficient for the bees to avoid it, despite a quite high error rate. This is not so surprising given that their ability to distinguish an odour is weak compared to visual cues (Gegear et al., 2006; Milet-Pinheiro et al., 2012).

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Chapter 3

Comparison of two molecular diagnostic tools for the quantification of *Crithidia bombi*, a parasite of bumblebees

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The pollinators decline is of a great importance for ecological reasons; and is partly due to parasitism. Therefore, it is important to detect and quantify parasite infections. Bumblebees are important pollinators and a good model in a context of host-parasite co-evolution with their parasite *Crithidia bombi*. For both studies, it is important to be able to measure such infection. When facing a large sample size, microscopic could be error-prone and time consuming. We tested two different molecular methods to quantify *C. bombi* infection. One is based on the intensity of a PCR product from microsatellite, while the other relies on quantitative PCR. We compared both methods using a dilution series of defined cell numbers. The qPCR method is more precise than the microsatellite method. The microsatellite method performs also well and additionally allows the characterisation of the strain of the parasite, which give more precise information for ecological studies than just detection.


**Key words:** microsatellite, quantitative PCR, host-parasite co-evolution, host-parasite ecology, pollinator, epidemiology, pathology
Introduction

The requirements of diagnostic methods for the detection of parasites/pathogens are dependent on the overall aim of a study. Epidemiological studies tend to focus on presence/absence studies in order to unravel factors contributing to the spread of disease agents (Kleeman et al., 2002; Njiru et al., 2008; Erler et al., 2012a). In contrast, studies driven by evolutionary questions might be interested in the parasite genotypes contributing to infections, in order to determine genotype by genotype interactions, local adaptation and changes in genotype frequencies predicted by models like the Red Queen or the gene for gene theory for host-parasite co-evolution, the last one being used mainly for plants (Flor, 1942; Bell, 1982; Decaestecker et al., 2007). Pathology studies, however, in different contexts (ecology and evolution) need the quantification of the infection rate of a host as the primary data.

Due to technical advances, molecular diagnostic methods are increasingly used due to their cost effectiveness and reliability (Kelley et al., 2006; Leisova et al., 2006; Leblanc-Maridor et al., 2011). Microscopic methods are popular as very little advanced equipment is needed. However, when dealing with large sample size (as in ecological studies (Salathé and Schmid-Hempel, 2011; Goulson et al., 2012) microscopic methods require a long time to process each sample and to measure the infection rates, this might result in increasing error rates over long periods of work (Pattyn et al., 2008; Lim et al., 2010). Molecular methods are more likely to be standardized, allowing for integrative studies and meta-analyses.

During the past years declines of pollinators have been repeatedly reported (Biesmeijer et al., 2006; Ratnieks and Carreck, 2010; Cameron et al., 2011). Factors contributing to pollinator declines have been identified as habitat fragmentation (Murray et al., 2009; Winfree et al., 2009; Potts et al., 2010), pesticide usage (Gill et al. 2012; Stokstad 2012; Whitehorn et al. 2012) and increasing parasite loads (Cox-Foster et al. 2007; vanEngelsdorp et al. 2009; (Cameron et al., 2011). Thus, studies of parasites in insect pollinators are highly demanded, both from an epidemiological as well as an evolutionary point of view.

A major model system in evolutionary ecology of pollinators is the bumblebee (*Bombus* sp., Linnaeus 1758) (Jordano, 1987). Bumblebees are important pollinators for crops and wild plants (Corbet et al. 1991; Lye et al. 2011; Graystock et al. 2013);
Molecular tools for quantification of infection

many of their different biological aspects have been studied (sociality (Bourke and Ratnieks 1999; Lopez-Vaamonde et al. 2004(Huth-Schwarz et al., 2011), cognition (Skorupski and Chittka, 2006; Riveros and Gronenberg, 2009), ecology (Kremen et al., 2007) and host-parasite co-evolution (Schmid-Hempel, 1998)). Bumblebees are eusocial insects with an annual life-cycle, whose colonies are founded by a single-mated queen. Their social life and low genetic diversity within a colony make them a prime target for parasites. Their social organisation provides parasites with both a stable and rich environment (Schmid-Hempel, 1998). The low genetic variability within a colony, due to the single mated and unique queen, allows parasites to easily infect an entire colony (Baer and Schmid-Hempel, 1999, 2001). One of the most widespread parasites in bumblebees is *Crithidia bombi*, a trypanosome gut parasite. *C. bombi* decreases the chance of successful colony foundation by future queens, in addition to the size and efficiency of new colonies (Brown et al., 2003), but represents a minor threat for healthy colonies (Brown et al., 2000). Transmission of *C. bombi* occurs both vertically and horizontally by foragers on flowers (Durrer and Schmid-Hempel, 1994), although foraging bumblebees might be able to avoid contaminated flowers (Fouks and Lattorff, 2011).

Here we adapted and compared two molecular methods to quantify the number of *C. bombi* cells. DNA from different known numbers of *C. bombi* cells in a dilution series were extracted and amplified with either an end-point PCR protocol utilizing microsatellite markers or a qRT-PCR protocol.

**Material and methods**

**Quantification & preparation of *C. bombi* cells**

Two strains of pure *C. bombi* cells (076 and 161 strains provided by P. Schmid-Hempel, ETH Zurich) were cultivated according to the method developed by (Popp and Lattorff, 2011). From each strain, two cell cultures were produced; and for each, the number of cells was counted using a Fuchs-Rosenthal counting chamber in three replicates under a microscope (Olympus®) at 400× magnification. The average of the three replicate counts was used to prepare a dilution series. Nine serial dilutions ranging from 100,000 cells to 10 cells (100,000; 50,000; 10,000; 5,000; 1,000; 500; 100; 50; 10) were prepared in four replicates for each strain in 300 μL of pure medium (Popp and Lattorff, 2011).
Spike control
In addition, a spike control was prepared, where each cell concentration from the previous dilution series of *C. bombi* cells was supplemented with one bumblebee gut, in two to three replicates. This spike control allows accounting for any interaction with the bumblebee gut materials. This spike control was processed in the same way as the dilution series of pure *C. bombi* cells (e.g. DNA extraction, multiplex and quantitative real-time PCR).

DNA extraction
Nuclear DNA of the parasite *C. bombi* was extracted using a modified Chelex protocol (Walsh et al., 1991). A total of 300 µL of each dilution was centrifuged at 3220 g (4000 rpm) for 30 min. The supernatant was discarded and the remaining pellet was homogenized in 100 µL 5%-Chelex solution (Bio-Rad®, Munich, Germany) and 5 µL 1% (10 g/L) proteinase K was added. Samples were processed in a thermocycler using the following program: 1 h at 55°C; 15 min at 99°C; 1 min at 37°C, and a final step for 15 min at 99°C. DNA was stored at –20°C until further processing.

Multiplex PCR microsatellite method
Fluorescence-labelled primers (Cri 4, Cri 1.B6, Cri 2.F10, and Cri 4G9) developed by Schmid-Hempel & Reber Funk (2004) (Schmid-Hempel and Reber Funk, 2004) were used in a single multiplex PCR. A PCR reaction (10 µL) comprised 1 µL template DNA, 5 µL PCR Master Mix (Promega®, Madison, WI), 2.4 µL molecular grade water (J.T. Baker®, Deventer, The Netherlands), 0.2 µL of each forward and reverse primer for every microsatellite locus. The PCR reactions were run in a PE 9700 thermocycler (Perkin Elmer®, Waltham, MA, USA) with the following program: 4 min denaturation at 95°C, then 35 cycles with 1 min, 95°C; 30 sec, 53°C; 30 sec, 72°C, and final elongation at 72°C for 4 min. 35 cycles were used in order to be identical to the microsatellite method of Schmid-Hempel & Reber Funk (2004) (Schmid-Hempel and Reber Funk, 2004). Fragment sizes were analyzed with MegaBACE 1000 Sequencer (GE Healthcare®, Freiburg, Germany) and allele sizes were scored using the software MegaBACE Fragment Profiler v1.2 and inspected and corrected by eye.

Quantitative Real-Time PCR method
The quantitative PCR was conducted with Chromo4™ (Bio-Rad®, Munich, Germany). Each 10 µl quantitative PCR reaction consisted of 1 µl DNA, 0.3 µL of
each primer CriRTF2 and CriRTR2 (Ulrich et al., 2011), 3.4 µl DEPC water and 5 µl SensiMixPlus SYBR & Fluorescein Kit (SYBR-Green) (Bioline®, Luckenwalde, Germany). For each dilution series and replicate, two technical replicates were performed. The following program was used for quantitative PCR reactions: 95°C initial denaturation for 10 min followed by 35 cycles of 15 s at 95°C denaturation, 30 s at 61°C primer annealing and 30 s at 72°C extension step with a subsequent melting curve analysis between 50°C and 98°C, reading the fluorescence at 1°C increments. Opticon Monitor 3 (Bio-Rad®, Munich, Germany) software was used to compute Ct values after baseline subtraction. In case of Ct value differences between replicates larger than 0.5, samples were repeated. All PCR products were checked for correct amplicon sizes by means of the automated multicapillary electrophoresis QIAxcel System with QIAxcel DNA High Resolution Kit (Qiagen®, Hilden, Germany) and melting curve analysis following quantitative PCR. The mean PCR efficiency was 1.86 calculated with LinReg PCR software (Ramakers et al., 2003). The average of the mean Ct values of two corresponding DNA duplicates of the same dilution series corrected with the PCR efficiency of the plate was used for a regression analysis.

**Statistical analyses**

**Multiplex PCR microsatellite method**

The peaks of primers Cri1.B6 and Cri4G9 were chosen for regression analysis. The peak heights from the two other primers were discarded, due to low peak heights and absence in many dilution series. When a strain was heterozygous for a locus the two peak heights were summed. Strain 161 was heterozygous for both loci, while strain 076 was heterozygous for Cri1.B6 and homozygous for Cri4G9. These peak heights were checked for linear regression on the number of cells, which were log-transformed. The variance of the peak heights for each dilution between the two chosen primers was compared using F-tests.

**Quantitative Real-Time PCR method**

Ct values per dilution were averaged over duplicates. The average of the mean Ct values of two corresponding DNA duplicates of the same dilution series was used for covariance analysis. Averaged Ct values were checked for linear regression after log transformation of the number of cells.

**Spike control**

For each method, we compared the regression slope from peak heights or Ct values of spike control data to the one from the dilution series using a t-test.
Comparison of both methods

In order to compare both methods, we transformed the peak height data from the multiplex PCR method and the averaged Ct-value data from RT quantitative PCR using z-scores transformation:

\[ z_i = \frac{x_i - \bar{x}}{s} \]  

(1)

Where \( z_i \) is the z-score, \( x_i \) is the experimental value, \( \bar{x} \) is the mean of all values and \( s \) is the standard deviation.

For the Ct values, we chose the additive inverse z-scores since the slope is negative for the quantitative PCR method and positive for the other method. For each dilution, we compared the variance of z-values between the two method using F-tests. In addition, we compared the regression slopes from the both methods (full model) using a t-test. Moreover, the t-test was applied to compare the best regression slope from the microsatellite methods based on a maximum of 5 000 cells and the previous one from the quantitative real-time PCR (short model). All statistical analyses were made with R (R Team Development Core, 2008).
Results and Discussion

Multiplex PCR method
The relation between peak heights (Cri1.B6 and Cri4G9; Figure 1A & B) and number of cells for both primers follow a highly significant linear regression but with a low correlation factor ($r^2$) (Cri1.B6: $r^2 = 0.45$, $P = 2.46 \times 10^{-10}$; Cri4G9: $r^2 = 0.44$, $P = 6.01 \times 10^{-10}$). We observe a plateau when the number of cells is higher than 5,000 (Figure 2A). This might be due to the nature of this method which is an end-point PCR with a high number of cycles (35 cycles). As mentioned above, the choice of cycle number was made in order to use only one method to both detect and quantify infection. For this reason we recalculate the linear regression and its 95% confidence interval, from 10 to 5,000 cells in order to increase the precision of the calculation and the correlation factor ($r^2$) (Cri1.B6: $r^2 = 0.67$, $P = 1.06 \times 10^{-12}$; Cri4G9: $r^2 = 0.59$, $P = 3.04 \times 10^{-10}$; Figure 1A & B). When comparing the reliability of the primers for the estimation of cell number, the F-test shows only a significant result for 500 cells, where the primer Cri1.B6 has lower variance than Cri4G9 ($F_{7,7} = 0.13$, $P < 0.05$). Moreover, the confidence interval of the linear regression for Cri1.B6 is smaller than the one of Cri4G9. For these reasons, it seems more reliable to infer the infection (number of *Crithidia* cells) of an individual using the peak heights of the primer Cri1.B6. The number of cells should not exceed 5,000. Then the calculation will follow this formula:

$$y = a \cdot \log_{10}(x) + b$$  \hspace{1cm} (2)

where $y$ is peak height of the primer Cri1.B6, $x$ the number of *Crithidia* cells, $a$ the slope of linear regression and $b$ the intercept.

$$\log_{10}(x) = \frac{y + 4206}{3599}$$  \hspace{1cm} (3)

$$x = 10 \cdot \frac{y + 4206}{3599}$$  \hspace{1cm} (4)
Figure 1: Linear regression between peak heights or Ct value and the number of *C. bombi* cells (log-transformed). a) peak heights of microsatellite Cri1.B6, b) peak heights of microsatellite Cri4G9 and c) Ct value from CriRT2. Vertical bars represent the 95% confidence interval; the horizontal bars represent the 95% confidence interval from the *C. bombi* cells counting. The grey triangles represent the data from the spike control. The middle regression line represents the fit of the regression analysis. The upper and lower regression lines represent the 95% confidence interval of the fit for the regression analysis.

Quantitative Real Time-PCR method

The Ct-values were highly correlated to the number of cells (log-transformed) following a linear regression ($r^2 = 0.90$, $P < 2.2 \times 10^{-16}$); Figure 1C), as expected (Ulrich et al., 2011). Ulrich et al. (2011) (Ulrich et al., 2011) were able to detect infection when facing only 3 *Crithidia* cells using 2 µl of DNA with the quantitative PCR method, here we found the same sensibility of the technique at a lower efficiency, we were able to detect an amount of 10 cells with both methods using
only 1 µl of DNA. Moreover, the 95% confidence interval is very small indicating low variance due to methodology. Then the calculation will follow this formula:

\[ y = -3.293 \cdot \log_{10}(x) + 33.811 \]  
(5)

where \( y \) is the Ct-value, \( x \) the number of \textit{Crithidia} cells.

\[ \log_{10}(x) = \frac{y - 33.811}{-3.293} \]  
(6)

\[ x = 10^{\frac{y - 33.811}{-3.293}} \]  
(7)

**Spike control**

The spike control fitted well with the dilution series experiment and the regression slope from both do not differ significantly for each method (Cri1.B6: \( t = -0.16, \text{df} = 55, P = 0.87 \); Cri4G9: \( t = -1.18, \text{df} = 52, P = 0.24 \); CriRT2: \( t = 1.57, \text{df} = 91, P = 0.12 \); Figure 1).

**Comparison of both methods**

The best method and the most reliable one is the quantitative PCR (\( 10^5 \) cells: \( F_{3,9} = 24.53, P < 0.001 \); \( 5 \times 10^4 \) cells: \( F_{8,9} = 15.06, P < 0.001 \); \( 10^4 \) cells: \( F_{7,7} = 11.33, P < 0.01 \); 5 000 cells: \( F_{7,8} = 20.41, P < 0.01 \); 1 000 cells: \( F_{7,8} = 1.20, P = 0.80 \); 500 cells: \( F_{7,7} = 0.27, P = 0.10 \); 100 cells: \( F_{7,7} = 0.13, P < 0.05 \); 50 cells: \( F_{7,7} = 0.17, P < 0.05 \); 10 cells: \( F_{6,6} = 0.46, P = 0.37 \); Figure 2).

![Figure 2: Comparison of microsatellite and quantitative PCR methods. a) Linear regression between z-transformed values from peak heights and additive inverse Ct values and the number of \textit{C. bombi} cells (log-transformed). Bars are 95% confidence interval for z-transformed values (vertical) and cell number (horizontal). The black line represents the fit of the regression analysis from the CriRT2, the grey line represent the fit of the regression analysis from the Cri1.B6 and the grey doted line represents the fit of the regression analysis from the Cri1.B6 based on a maximum of 5 000 cells, b) Variance of the z-transformed values along the the number of \textit{C. bombi} cells (log-transformed) for microsatellite (grey circles) and quantitative PCR (black triangle).](image-url)
However, when facing a low number of *Crithidia* cells, the microsatellite method shows less variance, a lower standard deviation and a model fitting as well the initial cell number as the quantitative PCR (full model: $t = 1.98, \text{df} = 139, P < 0.05$; short model: $t = -0.93, \text{df} = 139, P = 0.35$; Figure 2).

Both methods have been used for experimental purposes and have been published recently (Sadd, 2011; Popp et al., 2012; Fouks and Lattorff, 2013). The host-parasite system, bumblebee - *C. bombi*, has been well investigated for evolutionary purposes (Otterstatter and Thomson, 2007; Wilfert et al., 2007; Ulrich et al., 2011; Fouks and Lattorff, 2013), where it is important to measure the number of *C. bombi* cells. Indeed, the number of *C. bombi* cells is informative for the infection level of a bumblebee and also to measure the fitness of the parasite (Ruiz-González et al., 2012). Furthermore due to their ecological and economical importance, bumblebees and their main parasites have been monitored and also well investigated (Schmid-Hempel and Tognazzo, 2010; Whitehorn et al., 2011; Goulson et al., 2012; Popp et al., 2012). Following the purposes of the experiment the choice of the molecular methods will differ either to have 1) a high precision of the infection rate, then both quantitative PCR and microscopic methods seem suitable or 2) additional information on multiple strains infection and high or low infection rate is required, then the microsatellite method is appropriate. In addition, to relate the presence of different morphological parasite stages ecological or epidemiological studies the recourse of the microscopic methods is necessary (Gorbunov, 1987, 1996; Logan et al., 2005).

For the first time, two molecular methods to determine the infection intensity of bumblebees with *C. bombi* have been tested and compared. These methods are as reliable as microscopic techniques. Moreover, when facing large sample sizes these molecular methods are less time consuming than microscopic techniques. The quantitative PCR method allows high precision, while the microsatellite method allows semi-quantification of infection, not only detection. According to our knowledge it is the first time that a microsatellite method has been developed for the quantification of cell counts.
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General Discussion

To conclude I will first summarise the main findings from the presented experiments implementing new insights from recent studies, then discuss further implications of behavioural defence mechanisms on the bumblebee - *Crithidia* host-parasite system and finally broaden the topic giving a full picture of the co-evolution between bumblebees and *Crithidia*.

Bumblebees have developed a battery of mechanisms to defend themselves against parasites (Schmid-Hempel, 2001; Manson et al., 2010; Fouks and Lattorff, 2011; Richter et al., 2012). These mechanisms range from reducing the parasite uptake to the parasite transmission over a large diversity of processes.

Amongst these processes, bumblebees have adapted their foraging behaviour to reduce the uptake of parasites. Foraging bumblebees recognise and avoid flowers containing parasites (Fouks and Lattorff, 2011, 2013). As in other species, such as ants (Vieira-Neto et al., 2006), ungulates (Fankhauser et al., 2008) and chimpanzees (Samson et al., 2013), the foraging behaviour of bumblebees is influenced by the presence of parasites resulting in an avoidance of certain resource patches. In addition to the avoidance behaviour, bumblebees exhibit a reduction of *C. bombi* load after consumption of alkaloids, which might be contained in the nectar of some flowering species (Manson et al., 2010), such as *Iris pseudocorus*, *Curcubta pepo*, *Rhododendron ponticum* (Baker and Baker, 1975; Adler, 2000). Self-medication is better known in primate species (Huffman, 1998). Thus, such changes in foraging preferences and behaviour due to parasites are confirming the importance for an integration of the parasite component into the optimal foraging theory (Lozano, 1991). The mechanism allowing for an optimisation of foraging behaviour seems to be due to the perception of the parasite presence by bees as a decrease of the flower reward. The bees show the same pattern of foraging behaviour when having the choice between two flowers, one highly rewarding while the other is less rewarding and when having the choice between a non-contaminated and contaminated flower (Fouks and Lattorff, 2013). The avoidance of contaminated flowers seems to be mediated by the odour resulting from the presence of *C. bombi* inside the “nectar” (Fouks and Lattorff, 2013). The recognition of parasites through odour cues has been also reported for nematodes (Zhang et al., 2005; Hasshoff et al., 2007; Pradel et al., 2007; Schulenburg and Ewbank, 2007) and other species (Moore, 2002).
Moreover, this behavioural adaptation represents specificities toward the parasite as bumblebees show a higher efficiency to avoid *C. bombi* (a long-term parasite of bumblebees) contaminated flowers compared to *E. coli* (a common pathogen) contaminated flowers (Fouks and Lattorff, 2011). This reveals the evolution of specific defences against *C. bombi* at a behavioural level, which represents a hint of antagonistic co-evolution between bumblebees and *Crithidia*, at least at the species level. In addition, there is evidence for variation of the ability to avoid contaminated flowers between two bumblebee populations (Fouks and Lattorff, 2011). Populations sympatric with the parasite lineage show a higher avoidance efficiency than an allopatric population. However, in this case these differences between bee populations could not be entirely linked to a specificity of the parasite lineage used. Indeed, it has been reported that bumblebee colonies possess different learning abilities and efficiencies to forage (Ings et al., 2005; Raine and Chittka, 2008). Furthermore, the efficiency to avoid contaminated flowers increases over time at the colony level, but also at the individual level. This could be the result of either individual or social learning. Social learning and the use of public information in the context of foraging in bumblebees has been well documented over the last decades by investigating the use of two different social cues, either conspecific scent-marks (cuticular hydro carbons (CHC)) or direct presence of conspecifics (visual cue). Studies investigating the use of conspecific scent-marks by foraging bumblebees can be summarized in the following way. The use of social cues are dependent of the reward of the resource and thus, also on the experience of the individual bee (Saleh and Chittka, 2006). As for the former case, studies on visual cues also revealed an influence of the individual experience (Leadbeater and Chittka, 2009), but additionally the presence of conspecifics on flowers may act as a local enhancement for foraging bees (Worden and Papaj, 2005; Kawaguchi et al., 2006). Concerning the avoidance of contaminated flowers, it seems that scent-marks do not improve the efficiency of bees to detect and avoid contaminated flowers. However, strong evidence of copying behaviour for flower choice in bumblebees has been published (Worden and Papaj, 2005; Kawaguchi et al., 2006; Leadbeater and Chittka, 2009). Those experiments show that foraging bumblebees use the presence of conspecifics on flowers for choosing certain flowers. This copying behaviour is the resultant of local/stimulus enhancement as the presence of conspecifics on flowers is attractive for foraging bees (Worden and Papaj, 2005; Kawaguchi et al., 2006). In addition,
bees are able to learn through their experience to follow such social cues (Leadbeater and Chittka, 2009), which represents the highest level of social learning (Bonnie and Earley, 2007). Unfortunately, social learning experiments in bumblebees rely on differentiating flower colours rather than odours (Worden and Papaj, 2005; Kawaguchi et al., 2006; Leadbeater and Chittka, 2009). Nevertheless, in this context the local enhancement of naïve bees by means of visual cues is sufficient to increase the efficiency of an entire bee colony to avoid contaminated flowers. This can be seen as the use of public information by foraging bees in order to reduce the uptake of parasites. It represents a collective anti-parasite defence mechanism through the use of stimulus/local enhancement from visual cues and therefore might be a part of social immunity in bumblebees and eusocial insects in general.

Social immunity describes, following Cremer et al. (2007), anti-parasite defences that are the result of collective actions of individuals from the same social entity. A broader definition of social immunity, proposed by Cotter & Kilner (2010), encompasses any individual defence mechanism, which benefits others, being seen as cooperation or altruism, such as parental care or group-living. The broader definition of social immunity allows for the opening of a new scope into the altruism and cooperation evolution paradigm, while the more narrow definition of social immunity allows considering the colony as a “superorganism”, to integrate the parasite pressure into the evolution of social structures and the possibility to investigate convergent evolution between individual and social immunity (Cremer and Sixt, 2009). From either definition, the avoidance behaviour of foraging bumblebees can be categorized as a component of social immunity. Social immunity (according to the definition of Cremer et al 2007) is composed of a huge diversity of mechanisms and covers every step of parasite infection. The first level of defence mechanism against parasites starts at the avoidance of the parasite and the reduction of the parasite intake. Behavioural avoidance of parasites represents a highly economic defence, since it reduces the possible damages caused by the parasite after entering the host and saves the costs for activation of the immune system. Social insects have evolved different behaviours in order to avoid parasite uptake and intake into the colony. For example, ants avoid direct contact with phorid flies (Vieira-Neto et al., 2006); leaf-cutter ants forage in pairs with the major worker caste carrying the leaves back to the nest whereas the minor worker caste are on the leave protecting the major worker against parasite infection (Vieira-Neto et al., 2006), and bees protect the nest entrance against
Discussion

foreigners (Gilliam et al., 1988). Social immunity appears also when parasites establish themselves in the host nest and spread among individuals within the colony. Social insects reduce the parasite spread within a colony using a wide range of behaviours. This is the case for grooming behaviour which can be increased or decreased, depending on the species, if an individual gets infected (Hughes et al., 2002; Bos et al., 2012), social fever in bees (Starks et al., 2000) and hygienic behaviour (Wilson-Rich et al., 2008). Some of these processes can be correlated with immune mechanisms in vertebrates when considering insect colonies as superorganisms (Cremer and Sixt, 2009).

Behavioural defence mechanisms are a common strategy within the animal kingdom to counter parasites (reviewed in de Roode and Lefèvre, 2012 & in Moore, 2002). One of the main parts of behavioural defence mechanisms is the avoidance of parasites, which has been described in detail above. Behaviour is also involved in resistance against parasites in other ways than parasite avoidance, such as self medication (reviewed in Moore, 2002 & in de Roode and Lefèvre, 2012). Indeed, animals’ behaviour has been altered to reduce parasite infection. This is the case for prophylactic and therapeutic medication, where animals have changed their diet and foraging behaviours, either in order to prevent or to reduce infections (reviewed in de Roode et al., 2013); also in mate choice where females will choose their mate depending on its health status (Milinski and Bakker, 1990; Møller, 1990). Moreover, behaviours can also lead to changes in physiological processes reducing or favouring the reduction of parasitic infections, which is the case for behavioural thermoregulation in ectotherms (Kluger et al., 1975). Recently, studies tend to refer of behavioural defence mechanisms as behavioural immunity (de Roode and Lefèvre, 2012). First, immunity takes on different definitions, the broad one including all mechanisms involved in resistance of an organism against parasites mainly employed in evolutionary and ecological studies and the restricted definition which takes into account only physiological mechanisms of immune responses used especially by physiologists and immunologists. When applying the strict definition of immunity, it is easily understandable that behavioural immunity cannot be applicable. Nonetheless, as mentioned above thermoregulation behaviour can be modified in ectotherms in response to parasite infection (Kluger et al., 1975). This change of thermoregulatory behaviour has a direct effect on the immune response and can be seen as an induced fever similar to the homeotherms immune response (Kluger et al., 1975).
In this case it seems proper to use the strict definition of immunity and then call it behavioural immunity. In the case of parasite avoidance and medication, these behaviours do not fall into the strict definition of immunity. Coming back to the social immunity and the concept of the superorganism, analogies in the evolution of individual and social immunity have been reviewed by Cremer & Sixt (2009). In this review the broad definition of immunity has been used. Nevertheless, when taking the strict definition of immunity, analogies between individual and social immunity and their evolution can relate more precisely to the immune system and its evolution. Indeed, social fever (Starks et al., 2000), the reduction of social contacts with infected individuals (Bos et al., 2012), the increase of grooming behaviour to remove parasites (Hughes et al., 2002), which also can lead to some immune memory (Hauton and Smith, 2007), and the structure of colony/social organization can be connected with individual immunity of vertebrates (Naug and Camazine, 2002; Cremer and Sixt, 2009). Therefore, the strict definition of behavioural immunity can also be applied when behavioural defence mechanisms occur in the context of social immunity within the insect colony. Nevertheless, the broad definition of behavioural and social immunity result in linking all the defence mechanisms against parasites, which is not negligible in many aspects of biology such as evolution, epidemiology and ecology; especially to fully understand host-parasite interactions (reviewed in de Roode and Lefèvre, 2012). Therefore when investigating those questions it seems mandatory to use the broad definition of immunity.

Bumblebees, as I have shown with a series of experiments, exhibit a specific resistance against *C. bombi* at a behavioural level. Moreover, at the genetic level three major quantitative trait loci involved in the resistance against *C. bombi* have been found in bumblebees (Wilfert et al., 2007). This specificity against *C. bombi* has also been found at the physiological level with differential gene expression after infection of different *C. bombi* strains (Riddell et al., 2009). A serial passage of *C. bombi* within bumblebee colonies showed an adaptation of the parasite to a specific host colony which results in a decreased ability for this selected parasite strain to infect a “non-familiar” colony (Yourth and Schmid-Hempel, 2006). However, these genotype by genotype interactions might be strongly mediated by the gut microbiome of the bumblebees that is strongly interacting with *C. bombi* during infections (Koch and Schmid-Hempel, 2012). When looking for local adaptation, none or a little
evidence has been found (Imhoof and Schmid-Hempel, 1998b; Lajeunesse and Forbes, 2002). This can be the result of diverse factors such as food-environment outcome (Sadd, 2011), multiple bumblebee hosts for *Crithidia* (Salathé and Schmid-Hempel, 2011; Erler et al., 2012a; Popp et al., 2012), the importance of gut microbiota of bees for interactions with *C. bombi* (Koch and Schmid-Hempel, 2012).

More and more studies point out the importance of symbionts in the resistance against parasites (Haine, 2008; Brownlie and Johnson, 2009). Those studies tend to prove that the specificity of the parasite resistance comes from the interactions between parasite and symbiont strains, rather than parasite strains and host populations (Koch and Schmid-Hempel, 2012; Rouchet and Vorburger, 2012). And finally to complete the picture, it has to be recalled that bumblebees are pollinators and therefore all the effects of parasites on them may have an influence on the flowers’ fitness (Gillespie and Adler, 2012), which should be taken into account for the evolution of host-parasite interactions between pollinators and their parasites.

To conclude, I would like to emphasize the importance to account for all defence mechanisms against parasites when investigating the evolution of host-parasite interactions. Therefore, the choice to choose the broad definition of immunity is primordial to reveal all mechanisms involved in the resistance against parasites such as behavioural immunity, social immunity, ecological immunity and so on. In the bumblebee-*Crithidia* model, we can see that specificities between both species appear at the behavioural, physiological and genetic level. Indeed, the weak evidence of local adaptation between bumblebees and *C. bombi* (Imhoof and Schmid-Hempel, 1998b; Lajeunesse and Forbes, 2002; Yourth et al., 2008) could be due to overlooking the behavioural immunity of bees and other factors (reviewed in Woolhouse et al., 2002; Fouks and Lattorff, 2011; Salathé and Schmid-Hempel, 2011; Koch and Schmid-Hempel, 2012; Erler et al., 2012b; Popp et al., 2012).
Summary

Bees are important pollinators for plants and crops. Recently it has been reported that bee populations is declining. Multiple factors are responsible for this decline. The main factors are the change of agricultural methods mainly with the use of pesticides, the impact of parasites and the interaction of both pesticides and parasites. Bumblebees are eusocial insects, colonies are founded by a single mated queen. Their life history traits make them a prime target for parasites. The defence mechanisms of bumblebees against parasites has been well studied, mainly their immune system. However, little is known about their behavioural immunity. One of the most common and specific parasites of bumblebees is *Crithidia bombi*, a trypanosome infecting bumblebee guts. *C. bombi* reduces the fitness of bumblebee colonies and is transmitted through the shared use of flowers. Therefore, I investigated the foraging behaviour of bees facing contaminated and uncontaminated flowers. Bumblebees showed the ability to recognise and avoid contaminated flowers. They perform better when the flowers are contaminated by *C. bombi* rather than contaminated by a common pathogen (*Escherichia coli*). They also perform better over time, showing a learning process. This learning appears to be mediated through the use of social cues. Bees can use scent-marks deposited on flowers by conspecifics and visual cues (presence of conspecifics on flowers) to help them foraging on flowers. In this case, the scent-marks are not used by foraging bees; they rely on the odour produced by the interaction of *C. bombi* cells with the flower nectar. Nevertheless, the visual cues act as a local/stimulus enhancement for naïve foraging bees, which can explain the learning process at a colony level. I also compared two molecular methods to measure *C. bombi* infection rates in bumblebees. Both methods are reliable and allow a rapid and efficient assessment of *C. bombi* infection rates in bees.

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This work will have never been possible without PD Dr. H. Michael G. Lattorff, who chose me for this PhD project. I would like to deeply thank him for this and naturally for all the help provided; the fruitful discussions on the experiments and in science in general, with always a touch of humour. It has been a great experience to work with him and to know him in both aspects as a scientific and as a person.

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A. Declaration of author contributions


I participated in the design of the experiment, performed the behavioural experiments and analysed the given data and wrote the manuscript. H. Michael G. Lattorff participated in the design of the experiment, in the analyses, writing of the manuscript and provided helpful discussions.


I participated in the design of the experiment, performed the behavioural and genetic experiments and analysed the given data and wrote the manuscript. H. Michael G. Lattorff participated in the design of the experiment, in the analyses, writing of the manuscript and provided helpful discussions.


I participated in the design of the experiment, performed the experiment and analysed the given data and wrote the manuscript. H. Michael G. Lattorff participated in the design of the experiment, in the analyses, writing of the manuscript and provided helpful discussions.
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Education

- **2010-2013**: PhD student in the Molecular Ecology group MLU Halle-Wittenberg (FUGABEE-Project funded by BMBF)
- **2008-2009**: MSc (2nd year) in Insect Sciences (University Francois Rabelais Tours, France)
- **2006-2007**: MSc (1st year) in Ethology (University of Paris 13, France)
- **2002-2006**: BSc in Evolutionary Biology (University of Poitiers, France)
- **2002**: Baccalaureate in Environmental Science with distinction (SAT equivalent) (Jonzac High School, France)

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Work Experiences

- **January 2010 - January 2013:**
  PhD project on **Host-Parasite evolution in eusocial insects** at Molecular Ecology group, MLU Halle-Wittenberg within the FUGABEE-Project funded by BMBF
Appendix

C. Publication list


D. Oral and Poster presentations

- ✔ **Fouks B** & Lattorff HMG. *Avoidance of contaminated flower by bumblebees* (talk). 5th European Conference of Apidology (EurBee), 3rd-7th September 2012 in Halle an der Saale, Germany.

- ✔ Thoisy P, **Fouks B** & Lattorff HMG. *Adaptive evolution of genes with direct ans indirect fitness in social insects* (talk). 5th European Conference of Apidology (EurBee), 3rd-7th September 2012 in Halle an der Saale, Germany.

- ✔ Girault S, **Fouks B** & Lattorff HMG. *Does a pathogen-infected individual blow up the social network in bumblebees?* (talk). 5th European Conference of Apidology (EurBee), 3rd-7th September 2012 in Halle an der Saale, Germany.


Eidesstattliche Erklärung

Halle (Saale), den 27. 10. 2013

Hiermit erkläre ich an Eides statt, dass diese Arbeit von mir bisher weder an der Naturwissenschaftlichen Fakultät I - Biowissenschaften der Martin-Luther-Universität Halle-Wittenberg noch an einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.


Bertrand Fouks

Stand der Veröffentlichungen und Darlegung des eigenen Anteils an den Arbeiten

Design of the project: 50% B.F. 50% H.M.G.L.

Experimental work: 100% B.F.

Data analysis: 80% B.F. 20% H.M.G.L.

Wrote the paper: 70% B.F. 30% H.M.G.L.


Design of the project: 50% B.F. 50% H.M.G.L.

Experimental work: 100% B.F.

Data analysis: 80% B.F. 20% H.M.G.L.

Wrote the paper: 70% M.P. 30% H.M.G.L.

Fouks B. and Lattorff H.M.G. (2013): Comparison of two molecular diagnostic tools for the quantification of *Crithidia bombi*, a parasite of bumblebees. *Entomologia experimentalis et applicata* (accepted)

Design of the project: 50% B.F. 50% H.M.G.L.

Experimental work: 100% B.F.

Data analysis: 80% B.F. 20% H.M.G.L.

Wrote the paper: 70% M.P. 30% H.M.G.L.
