HOW TO BUILD A HEALTHY COLONY:
AGE STRUCTURE AND IMMUNE STRATEGIES IN BUMBLEBEES

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SUMMARY

Longevity is an important aspect in any organism’s life history, from humans to bacteria. Trade-offs with other traits and optimization of life-history strategies against an ecological background (including parasites) will shape longevity patterns across individuals, populations, and species.

In social insects worker lifespan strongly affects colony success. Long-lived workers may be favoured as they allow for large colony size, a general fitness measure in social insect colonies. At the same time, long-lived and therefore old workers should pose an increased risk of parasite transmission within the colony. This is because older workers are more likely to have encountered a parasite, and moreover defence (immune) functions commonly decline with age. To counter this epidemic risk, social insects may have evolved several adaptations related to worker age. Old workers, for example, tend to be foragers and often have limited contact with younger workers engaged in in-nest tasks. Bumblebees do not show such clear age-related changes in behaviour and might be especially vulnerable to infection through old workers in the colony. In this thesis I used the bumblebee, *Bombus terrestris*, and its trypanosomatid gut parasite, *Crithidia bombi*, to test the role of worker age on the resistance to and transmission of this ecologically important parasite.

In the first Chapter I demonstrate that long-lived workers indeed may be a risk for the colony when exposed to parasites. Experimentally altering birth and death rates in infected worker groups showed that a long-lifespan resulted in more infected workers. Because I controlled for group size and differences in worker immunity, increased transmission should be a direct consequence of the parasites’ residence in a long-lived host.

In the second Chapter I tested how worker resistance relates to longevity and colony size, yet another trait that is positively related to parasite spread. I show that colonies with long-lived workers were not only more resistant to experimental infection but long-lived workers were typically also found in larger colonies. Increased individual resistance in colonies with long-lived workers may reflect the high risk of parasite transmission associated with both worker longevity.
and living at high densities. Individual resistance, however, may not necessarily be relevant to decrease parasite transmission at old age, because immune functions (a component of resistance) may decline with age. In the third Chapter I show that immune senescence does not affect functional immunity in aged workers, but resistance to *C. bombi* is maintained. To conclude, a ‘healthy’ reproductive colony may on one hand sustain colony growth by producing long-lived workers, but must compensate for the increased risk of having many old workers by investing in functional immunity to avoid within-colony epidemics.

Based on my findings in this thesis, I suggest a scenario where parasite pressure positively correlated to colony size and worker longevity acts as a selective force for the evolution towards complex social structures. This hypothetical framework highlights the importance of parasitism in determining colony structure and simultaneously sets the stage and calls for further investigations.
ZUSAMMENFASSUNG


In einem ersten Kapitel demonstriere ich, dass langlebige Arbeiter in der Tat ein erhöhtes Infektionsrisiko für die von Parasiten bedrohte Kolonien darstellen können. Ich habe experimentell die Geburts- und Todesraten innerhalb von infizierten Arbeitern variiert und konnte zeigen, dass eine längere Lebensspanne mehr infizierte Arbeiter zur Folge hatte. Nachdem ich für
die Gruppengröße und Unterschiede in Arbeiterimmunität kontrolliert habe, sollte die erhöhte Parasitenverbreitung eine direkte Folge der längeren Verweildauer der Parasiten in den langlebigen Arbeitern sein.


1.1 GENERAL INTRODUCTION

Animals live in groups when the benefits of a social lifestyle outweigh its costs. Social insects, the ants, bees, wasps, and termites, for example, live in some of the largest animal societies and therefore are subject to a wide range of costs and benefits. A single colony can consist of several million individuals (Janzen 1973). Social insect colonies populate virtually all continents of our planet (Suarez et al. 2001), and E.O. Wilson (1971) estimated that $10^{15}$ individual ants live on the earth at any time. This spectacular ecological and evolutionary success of social insects is typically assigned to their social lifestyle (Wilson 1971), which may have increased their efficiency in brood care, foraging, and anti-predator defence. At the same time, a social life style should offer amenable conditions for parasites (Schmid-Hempel 1998). In a world full of parasites, the risk of living in groups is additionally increased when individuals live at high densities (Pie et al. 2004), as density increases the probability of encountering pathogens because individuals are bound to interact more frequently, which favours the transmission to naïve (uninfected) nest mates (Otterstatter & Thomson 2007; Naug 2008). Furthermore, in typical social insect colonies individuals are also closely related to one another (Hölldobler & Wilson 1990), which creates a disproportionally homogenous genotypic environment, facilitating the spread of the parasite through the colony still further (Shykoff & Schmid-Hempel 1991; Liersch & Schmid-Hempel 1998; Hughes & Boomsma 2004). The observation that antibacterial activity appears to increase with the level of sociality (Stow et al. 2007) may reflect this basic dilemma.

Social life and parasite defence

To counter the increased risk of parasites associated with their social lifestyle, social insects, along with other social species, have evolved adaptations that complement the individual immune defence system and can efficiently contain an epidemic at the colony level (Cremer et al. 2007; Wilson-Rich et al. 2009; Parker et al. 2011). These so-called social defences (social because they are generally not available to solitary species), act at all steps of colony infection; from parasite uptake, to parasite invasion, establishment and spread in the nest. To reduce the risk of primary colony infection, for example, workers may simply avoid contact with locations or individuals
rich in parasites (a relatively simple, but effective behaviour that is also observed in solitary species) (Orr et al. 1995; Moore 2002). Furthermore, upon infection, workers can be excluded from the colony. Specialized guards, together with other workers, are known to attack or exclude infected honeybee workers at the entrance of the hive (Drum & Rothenbuhler 1985). Within the colony, the transmission of the parasite and its establishment in the nest may be controlled by various defences mediated at the colony level (Cremer et al. 2007). In this context, a number of sanitary behaviours have been described that might be of particular importance for preventing parasite spread within the colony, including the removal of hazardous material and carcasses (Julian 1999; Hart & Ratnieks 2002), removal and inactivation of infectious spores from either the own body (self –grooming), or from the body of exposed nest mates (allo-grooming) (Schmid-Hempel 1998; Rosengaus et al. 1998; Reber et al. 2011) and the disinfection of brood with poison produced by the ants themselves (Tragust et al. 2013).Anti-bacterial and anti-fungal compounds as in ants (resin) (Chapuisat et al. 2007) and honeybees (mold) (Gilliam et al. 1988) are also imported from outside the colony and have prophylactic effects. A recent study described the use of a very low dose of a parasite to generate the effect of social vaccination (Konrad et al. 2012), up-regulating immune function, and thus better protection of the colony from secondary infections. However, as the activation of the immune system is costly (Moret 2000), social vaccination may be adaptive only under high and persistent parasite pressure. Nevertheless, the extraordinary abundance and complexity of behavioural adaptation are suggestive of the importance of parasites during social evolution.

Age polyethism with a note on worker longevity

‘Age polyethism’ is a hallmark of many social insect colony organizations and refers to the delegation of non-reproductive tasks in relation to age e.g. (Calderone 1995; Seid & Traniello 2006; Mersch et al. 2013). In its purest form, workers perform a specific task at a distinct age in a defined spatial area. More specifically and with respect to diseases, young workers typically perform tasks in disease-privileged areas of the colony, such as assisting the queen or brood in the brood or queen chambers, whereas older workers are more likely to be foragers or engage in other
tasks at the periphery or outside the nest. Such a system may reduce interactions, with old workers being socially more isolated, and as a consequence introduces a spatial and temporal barrier to a spreading disease (Schmid-Hempel & Schmid-Hempel 1993).

Age-related changes in behaviour, among other things, raise the question of worker life span or longevity. In general, long-lived workers should be selected for as they allow for large colony size (Carey 2001). Larger colony size in turn usually correlates with efficiency and higher colony reproductive output (Muller & Schmid-Hempel 1992; Palmer 2004; Lopez-Vaamonde et al. 2009). At the same time, long-lived and therefore, on average, the presence of more older workers in the colony, should impose an increased threat of parasites and parasite transmission to the colony. This is because older workers are not only more likely to have encountered parasites but may also be less protected as the immune system senesces over an animal's life (Doums et al. 2002; Moret & Schmid-Hempel 2009; Stanley 2012). One part of this thesis asks how worker longevity affects parasite transmission and whether age-based polyethism may mitigate this threat by restricting the interactions of older, infection prone, workers.

The organization of bumblebees
Work in this thesis was done with bumblebees and their parasites. Bumblebee colonies are headed by a single queen, typically singly mated (Schmid-Hempel & Schmid-Hempel 2000), and consist of up to ca. one hundred workers. After the colony has been founded by the queen and established with the emergence of the first brood, workers tend to the queen's eggs and engage in non-reproductive tasks such as brood care, foraging, and guarding. Bumblebees have, at best, only partly evolved social defences against parasites. Examples are the (weak) tendency of (trypanosome-) infected workers to leave the nest, a behaviour known also from ants (Korner-Nievergelt 2003), or the observation that workers parasitized by conopid parasitoid flies seek out cold temperatures and thus leave the nest at night so as to suppress parasite development in their body (Müller & Schmid-Hempel 1993). Prolonged survival in this case should add to colony efficiency.
Bumblebees also show no clear-cut division of labour according to age (Cameron 1989; Jandt et al. 2009) but task affinity based on body size is common. In particular, small workers are more likely to perform hive tasks whereas bigger workers forage or guard the nest (Goulson et al. 2002; Yerushalmi et al. 2006). In terms of social defences, bumblebees, therefore, may depend mainly on a functional individual immune system to counter density effects in the face of parasites. Also, immune senescence together with weak age-related adaptations should render bumblebee parasite defences sensitive to a demographic shift towards old workers.

How to build a healthy colony
In this thesis I used the bumblebee Bombus terrestris L. and its trypanosome gut parasite Crithidia bombi (Lipa & Triggiani 1988) to investigate the importance of worker age on the infection and transmission of this ecologically relevant parasite. B. terrestris is a primitive eusocial insect. ‘Primitive’ in the sense that there is no clear-cut task division and reproduction is virtually confined to queens. B. terrestris inhabits temperate zones of the Palaearctic and follows an annual life cycle (with partly bivoltine cycles in warmer areas such as the Mediterranean). After hibernation, mated queens found colonies in spring on their own, which subsequently grow in number of workers until, in late summer, the colony eventually reproduces by producing sexuals (males and gynes, the daughter queens) after which the colony collapses. C. bombi infects per os, is picked up via contaminated flowers (Durrer & Schmid-Hempel 1994) and spreads through the colony by infective cells shed in the faeces of workers. Costs to infection are mainly paid by young queens that – if infected- often fail to found new colonies (in roughly half of the cases), whereas increased mortality in workers was only observed under harsh conditions (i.e. under food restriction). The parasite cannot live outside its host and thus should thus be under selection to infect young queens, which are the only individuals going into and surviving hibernation.

From an evolutionary perspective, ‘healthy’ is a colony state that allows survival and reproduction such as to leave as many daughter queens as possible that themselves found new colonies in the next year. As daughter queen production correlates with colony size (Muller & Schmid-Hempel 1992), 'health' should allow achieving large colony size and few infected
daughter queens. An insight of how worker lifespan in *B. terrestris* colonies may relate to colony health is given in the following chapters. Each chapter will introduce the respective background for the study and discuss the consequences of its findings. The General Discussion attempts to summarize the thesis' results and synthesize them more conceptually.
1.2 THESIS OUTLINE

Chapter 1. General Introduction

Chapter 2. Colony pace: A life-history trait affecting social insect epidemiology.
For a given colony size, the colony may produce many short-lived workers or few long-lived workers. In this Chapter I investigate whether differences in colony pace reflect a life-history strategy against parasite transmission. I infected groups of bumblebees with the infectious gut parasite *Crithidia bombi* and experimentally manipulated birth and death rates to mimic slow and fast pace. I showed that fewer workers were infected in colonies with a higher turnover rate. Few infected workers means less infective cells and a lowered probability of daughter queens being infected. I therefore propose that a high worker turnover rate, hence many short-lived workers can act as a strategy of defence against a spreading disease.

Chapter 3. Worker longevity and its relation to colony resistance against infectious parasites in a social insect
Many short-lived or few long-lived workers may be of different ‘immune quality’. Here, we test a quality/quantity tradeoff with respect to resistance to *C. bombi*. I first monitored the relationship between worker lifespan and the number of workers a colony produced. In a second experimental, I infected workers and measured the standing expression of bumblebee immune genes. I found that immunity in bumblebees indeed is linked to longevity such that individuals from colonies that produced long-lived workers were phenotypically better-protected. Colonies with short-lived workers however were small colonies, which may be maintained by the success of short-lived workers in preventing an epidemic within the nest.

Chapter 4. Worker longevity in a primitively eusocial insect: Immune senescence does not affect worker resistance to an ecologically relevant parasite.
Colonies with long-lived workers inevitably have more old workers at any time. This Chapter investigates the age effect on *C. bombi* resistance over a workers lifespan. I first tested age related resistance, then I compared the resulting transmission potential to behavioural adaptations, such as worker activity and distance to naive workers, which are likely to influence parasite spread. I
found that immune senescence may not necessarily affect resistance to *C. bombi* but that age effects should be relevant for the spread of the parasite and may be mediated by behaviour rather than by infection intensity.

**Chapter 5. General discussion and a hypothetical scenario: How parasite pressure may act as a driving force of colony social organization**

I summarize my thesis findings and suggest a scenario where parasite pressure selects against colony size and worker longevity selects for the evolution of complex social structure.
References


Konner-Nievergelt, P. 2003 July 28. Dynamics and consequences of immune system activation in


Mersch, D. P., Crespi, A. & Keller, L. 2013. Tracking Individuals Shows Spatial Fidelity Is a Key Regulator of Ant Social Organization.


2. COLONY PACE: A LIFE-HISTORY TRAIT AFFECTING SOCIAL INSECT EPIDEMIOLOGY

Abstract

In social insects, the worker turnover rate (its ‘pace’) shows considerable variation between colonies. This has epidemiological consequences for parasites because in ‘fast-paced’ colonies, with short-lived workers, the time of residence in a given host will be reduced. Here we investigate whether differences in colony pace reflect a life-history strategy against parasite transmission. We infected bumblebees (Bombus terrestris) with the infectious gut parasite Crithidia bombi and experimentally manipulated birth and death rates to mimic slow and fast pace. Fewer workers and, importantly, fewer last generation workers were infected in colonies with a higher turnover rate. This suggests increased fitness in “fast-paced colonies, as daughter queens exposed to few infective cells are less likely to be infected and have a higher chance of founding their own colonies. We therefore propose that a high worker turnover rate acts as a strategy of defence against a spreading disease; parasitism therefore likely drives the variation in worker lifespan commonly observed in social insects.

§ A version of this Chapter is in preparation as S. D. Buechel & P. Schmid-Hempel.
**Introduction**

The evolutionary transition from a solitary to a social lifestyle is considered a main reason for the spectacular ecological success of social insects (Wilson 1971; Szathmáry & Smith 1995). At the same time, social living may impose several disadvantages. Infectious parasites, for example, may spread more easily when individuals live together at high densities, are closely related, and interact frequently with one another - conditions that are characteristic for social insect colonies (Shykoff & Schmid-Hempel 1991b; Schmid-Hempel 1998; Naug & Camazine 2002; Pie et al. 2004; Otterstatter & Thomson 2007). It is therefore conceivable that social insects should have evolved a broader and more efficient immune system compared to their solitarily living counterpart. This expectation is not confirmed by the available data, as social insects in fact seem to have fewer immune genes than solitary insect species (Evans et al. 2006). However, social insects may have alternative or complementary ways to defend themselves against diseases and the transmission of pathogens. Among these non-immunological defences are those that are based on altruistic behaviour (e.g. mutual grooming to remove parasite spores), collective decision-making (e.g. increase in nest temperature), and other collective actions that reduce parasite loads or the impact of infection [reviewed in (Cremer et al. 2007)]. This so-called ‘social immunity’ can effectively contain an infection at the colony level and so help to sustain colony growth, survival and reproduction.

One aspect that has not been well covered so far is that social insects may also mitigate fitness losses caused by parasitic infection by adjusting life-history parameters such as growth, reproduction and survival (Moret & Schmid-Hempel 2004) see also review in (Agnew et al. 2000). Life-history theory suggests that individuals should alter the timing and investment in these traits to maximize their lifetime fitness in a given environment (Stearns 1992). The presence or absence of a parasite arguably is a crucial environmental factor contributing to an organism’s optimal pattern of resource allocation (Perrin et al. 1996; Sorci et al. 1996). A life-history strategy that accelerates the host’s schedule of reproduction, for instance, should be favoured when parasites shorten the remaining hosts lifespan and curtail it's reproductive capacity (Agnew et al. 2000;
Evidence for a life-history response to parasitism maintained throughout the evolution of sociality however, remains scarce.

Here we investigate a life-history parameter that is unique to social insects - the turnover of individuals within a colony as given by worker lifespan and birth rate of new workers, defining a colony pace. In most social insects, typically, only the queen reproduces, while the worker caste is sterile (Hölldobler & Wilson 1990). Thus, worker turnover is akin to the turnover of cells in a body, and an individual life takes a different meaning from that in most other organisms. With this approach, the growth and reproductive success of a social insect colony depends on the effort of its workforce, which is a function of the total number of worker present at any one time and is thus given by the worker turnover rate. In bumblebees, only if a critical worker number is attained does the queen reproduce and the colony thus reaches sexual maturity (Muller & Schmid-Hempel 1992).

In the bumblebee, *Bombus terrestris*, some colonies seem to have higher worker turnover rates than others (Regula Schmid-Hempel pers. comm., and pers. obs.). In "fast-paced" colonies workers die younger, but new workers are produced more frequently than in "slow-paced" colonies - even under otherwise similar conditions (e.g. in the laboratory). For an infectious parasite, an increased production of short-lived workers has epidemiological consequences, since the expected time of residence in a given host will be reduced. Hence, transmission events must become more frequent and, according to standard theory, short host lifespan should select for rapid replication in parasites, which is typically associated with higher virulence and greater infectivity.

To test this idea, we manipulated worker turnover rate in experimental colonies of *B. terrestris* to measure its effect on the spread of the infectious parasite, *Crithidia bombi*. Moreover, we predict that a decrease in residence time of infection and more frequent transmissions reduces the genotypic diversity of the parasite by eliminating non-suitable strains (see (Yourth & Schmid-Hempel 2006). A reduction in parasite diversity at time of colony reproduction is important
because high strain diversity increases the likelihood of daughter queens becoming infected (Ulrich et al. 2011). Eventually, at the end of a colony cycle, fast-paced colonies may thus have fewer infected workers with fewer persisting strains, which may result in fewer daughter queens being infected and thus higher colony founding success in the next generation.

Methods

Parasites and insects

The bumblebee (Bombus terrestris) is a eusocial insect with annual lifecycle. In spring, the singly mated queens, after emerging from hibernation, found colonies that grow in numbers of workers until the colony eventually reproduces in late summer; only the mated daughter queens undergo hibernation to start next generation colonies. The trypanosomatid gut parasite, Crithidia bombi, is common in most populations (Shykoff & Schmid-Hempel 1991a; Salathé & Schmid-Hempel 2011; Ruiz-González et al. 2012). It cannot live outside the host (Schmid-Hempel et al. 1999) and hence underlies strong selective pressure to infect daughter queens - the only individuals that survive the winter. But at least in our study area, only 10 -15% of spring queens (daughter queens) are infected with C. bombi. Colonies contract the infection either from other colonies via shared use of flowers or via faeces deposited in the nest. The parasite is genetically highly diverse (Schmid-Hempel & Reber Funk 2004; Salathé & Schmid-Hempel 2011). The time between established infection and subsequent transmission ranges between two days for fast developing genotypes and 10 days for slower strains (Schmid-Hempel & Schmid-Hempel 1993). C. bombi increases worker mortality under stressful conditions and substantially reduces the colony founding success of daughter queens (Brown et al. 2003).

Collection and culturing

B. terrestris queens emerging from hibernation were collected in spring 2010 from two distinct populations in Switzerland (near Aesch BL; and Neunforn TG) and allowed to initiate colonies in the laboratory. As soon as the first workers emerged, colonies were transferred to circular perlite
nests (Pomeroy & Plowright 1980). Seventeen uninfected colonies were reared to reach a sufficient number of workers so as to serve as donor colonies for bees used in this experiment. All bees were kept under standardized laboratory conditions (28± 2 °C, 60% RH) with constant red light illumination and pollen and sugar water provided ad libitum.

The parasite, *C. bombi* was sampled from faeces of naturally infected queens that originated from the same two populations in 2010. Single infective *C. bombi* cells were then isolated with a fluorescence-activated cell-sorter (FACS) and maintained clonally in liquid medium (Salathé et al. 2012). The six strains used in this experiment had distinct multi-locus genotypes at five polymorphic microsatellite loci and could therefore be easily distinguished by genetic markers.

**Infection and experimental manipulation of the worker turnover rate**

Workers from 17 donor colonies were assembled into experimental groups housed in boxes (with two treatments each, n = 34 boxes in total), and exposed to high or low worker turnover rates. The experiment started by placing four naïve (uninfected) workers and six workers each infected with a distinct *C. bombi* strain (six strains in total per box) into a given box. Each experimental group consisted of 10 workers at all times. A sketch of the experimental design is given in Figure 1.

![Figure 1](image)

**Figure 1.** Experimental design of the study. We induced different worker turnover rates in initial groups of 6 infected and 4 naïve workers housed in boxes (left, see legend). To subsequently create a high worker turnover rate, two random workers from the group were exchanged against two naïve worker (circular arrows) every three days. To create low worker turnover, workers were exchanged every 6 days only. The prevalence of infected workers in each group was measured on days 14, 30, and 38 (arrows). Six naïve workers, mimicking workers of the last cohort were added to the respective groups at day 30.
For the infections, and to mimic a natural colony background, four (non-age controlled) workers were selected at random from each donor colony (i.e. a total of 68 workers), starved for two hours and presented with 10 µl inoculum containing 20'000 cells of a single *C. bombi* clone in medium and sugar water (1:1 ratio). Workers that did not ingest the cocktail after 60 min were excluded from the experiment to ensure primary infections and viability of the infectious cells. This procedure was repeated six times for each donor colony (and for the six trains) always infecting four workers with one out of six *C. bombi* clones (i.e. resulting in 24 workers per donor colony; 408 exposed workers in total). Bees were then kept individually to prevent cross infection and eight days later their faeces were visually checked for the presence of *C. bombi* cells, confirming their infection status. Subsequently, two (out of the four) successfully infected workers for each of the six *C. bombi* strains were randomly assigned to one out of two experimental groups (low, high turnover rate) and each experimental group was completed with four naïve workers from the respective donor colony to yield the total of ten workers per experimental group (a colony x turnover combination); each box thus had an infection prevalence of 60% of hosts. Four donor colonies were found to be completely resistant to either one or two strains; the respective experimental groups (both low and high turnover) thus started the experiment with an infection proportion of 50% or 40%.

To create a high turnover rate, every third day, we randomly exchanged two workers from the group against two randomly chosen naïve workers from the same donor colony. For the low turnover rate, two randomly chosen naïve workers from the experimental group were exchanged only every sixth day. This experimental protocol mimicked the “birth” and "death" rate in a *B. terrestris* colony in the sense that workers are "reared" at different rates, get exposed to infections, and are removed from the pool of infection at an arbitrary time due to uncorrelated, external events. The time intervals were chosen based on the observed differences in the average worker lifespan among colonies of *B. terrestris*. In the present case, average worker life spans of the colony with the longest living worker are about twice the average life span of the colony with the shortest living workers (Chapter 3). Furthermore, worker life span in the field is in the order of 4
weeks, which roughly corresponds to the low turnover treatment used here. We exchanged workers from experimental groups following this treatment schedule for a total of 30 days and sampled all workers on day 38. This schedule thus allowed workers to live up to the expected 4-6 weeks, which is typical for natural conditions (Goulson 2003). The proportion of infected workers over time was monitored by visually checking the faeces of all 10 workers in the treatment groups after 14, 30 and 38 days.

On day 30, an additional six naïve workers from the respective donor colonies were added to each treatment group (n=30 for high and low turnover rate). These individually marked workers were introduced to mimic the last generation of workers in the colony (late workers) that are responsible for raising sexuals (daughter queens and males). At the same time, this cohort of workers represents the parasite environment to which the emerging daughter queens are exposed. Late workers were exposed to the treatment groups for eight days, which is about the time young queens spend in the nest before they leave for the mating flight [see (Bourke 1997)]. As colonies produce the last workers together with first males, which leave the colony before eventually queens emerge, workers were no longer exchanged, once the last cohort was added. All workers were sampled on day 38 and their gut tissue was genotyped to determine the number and identity of *C. bombi* strains that successfully infected bees exposed to different worker turnover rates (Figure 1). For groups were all workers provided faeces samples (n=20 for high and low turnover rate) we additionally measured the average concentration of infective cells per treatment group with a haemocytometer (Neubauer improved counting chamber).

**Genotyping of infections**

We dissected out the whole gut of all workers and extracted genomic DNA with a Qiagen DNAeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol for purification of total DNA from animal tissues. *C. bombi* infection was detected by the amplification and visualization (1.5% agarose gel) of a part of the *C. bombi* Cyt b gene with *Crithidia*-specific primers (CB- Cytb2-F and CB-Cytb2-B, described in (Schmid-Hempel & Tognazzo 2010). The *C.
*bombi* strains present in successful infections were thereafter genotyped at the *C. bombi* microsatellite markers Cri4, Cri2F10, Cri4G9, Cri16 and Cri1B6 (Schmid-Hempel & Reber Funk 2004) following the protocol in (Koch & Schmid-Hempel 2011) and the amplification product was run on a MegaBACE sequencer (GE Healthcare, Glattburgg, Switzerland). The microsatellite peaks were scored twice with MegaBACE Fragment Profiler software v1.2 by an observer blind to the treatment groups.

**Analysis and statistics**

Determinants for the number of infected workers over time were analysed using a generalized linear-mixed model (GLMMs) with a binomial error structure and logit-link function. Because workers did not provide a faeces sample every time the infection status was assessed, we used the number of infected/ total number of sampled workers as independent response variable. In the full model we included the experimental groups (high versus low turnover rate) and the three time points at which we assessed the number of infected workers per group (14 days, 30 days, 38 days) as fixed factors while the donor colony of each experimental group and the individual groups were used as random factor. Note that including each individual group as random factor accounts for repeated measurement over time (14 days, 30 days, 38 days). Model selection was done by backwards elimination of non-significant terms (Crawley 1993). If interaction terms are not presented, they did not have a significant effect. For every time point measured, we conducted post hoc comparisons based on paired sampled t-tests on the model residuals and sequential Bonferroni-correction (Holm 1979) to analyse differences in the number of infected workers between groups with a high and low worker turnover rate. We used an analogous model to test the influence of a high worker turnover rate on the prevalence of infected last cohort workers. The model included the experimental groups (high versus low turnover rate) as fixed factor and the donor colony of the experimental groups as single random factor, because the infection status of last cohort workers was assessed only at day 38. Identical model parameters were applied to test the number of persisting *C. bombi* strains in high versus low turnover groups, except that the number of strains after 38 days were used as dependent and the number of strains at treatment
start as independent variable, because not all experimental groups started the experiment with six infected workers and thus six \textit{C.bombi} strains. The distribution of the \textit{C. bombi} strains that successfully infected workers exposed to a high versus low turnover rate was analysed using Fishers exact test and the average infection load of workers in high versus low turnover groups was tested with a Wilcoxon signed rank test for paired samples. All statistical analyses were carried out in IBM SPSS Statistics version 19.0 or R statistical software version 2.15.2 (R Core Team 2012). Model assumptions were met for all linear models presented.

**Results**

A high turnover rate in a group of bumblebee workers significantly reduced the spread of the infectious parasite \textit{C. bombi}, resulting in an average infection prevalence of $\text{Prev} = 0.45$, for workers exposed to a high turnover rate, and $\text{Prev} = 0.6$ for workers exposed to a low turnover rate (GLMMs; $F_{1,98} = 13.517$, $p < 0.0001$). In 14 out of 17 paired experimental groups (high vs. low turnover rate from the same donor colony), fewer workers were infected when exposed to a high turnover rate. In two of the remaining pairs, there were fewer infected worker in the group with the low turnover rate and in one pair workers were equally infected regardless of the turnover rate they were exposed to. The infection was lost after 30 and 38 days in three groups exposed to a high turnover rate, while in two groups with a low turnover rate all workers became infected.

Even though the differences in infection proportion between experimental groups slightly varied over time (Figure 2, GLMMs: $F_{2,98} = 3.329$, $p = 0.04$), the high turnover rate reduced the proportion of infected workers at all times measured (paired t-tests; 14 days: $t_{16} = -2.234$, $p = 0.04$; 30 days: $t_{16} = -3.562$, $p = 0.003$, 38 days: $t_{16} = -2.569$, $p = 0.021$).
Figure 2. The proportion of infected workers in groups of *B. terrestris* workers with high and low turnover rate (n=34), measured 14 days, 30 days and 38 days post-experimental infection at time '0'. Shown are the estimated marginal means (±SE) from a model with turnover rate and time as fixed factors, and colony and individual group as random factors (see text). Note that workers were exchanged for 30 days while the final proportion of infected workers was measured at day 38.

Importantly, a higher worker turnover rate also resulted in fewer last cohort workers contracting the infection (Figure 3, GLMMs: $F_{1,28} = 6.136, p = 0.02$), and this difference in infection prevalence between groups with different worker turnover rates was not due to different numbers of *C. bombi* strains infecting either treatment (Figure 4, GLMMs: $F_{1,28} = 1.258, p > 0.1$). We further did not find that different strains infected workers with a high turnover rate when compared to workers with a low turnover rate (Fishers exact test: n= 12, p > 0.1). Finally, the average infection load in groups with a high turnover rate did not differ from the infection load of workers exposed to a low turnover rate (paired Wilcoxon signed-rank test: $Z = -1.274, n= 20, p > 0.1$).
Figure 3. The proportion of infected last cohort workers after they were exposed for 8 days to worker groups with a high and low turnover rate (GLMMs: $F_{1,28} = 6.136$, $n= 30$, $p = 0.02$). Bars represent the estimated marginal means ($\pm$SE) derived from a model with turnover rate as fixed factor, and colony as random factor (see text).

Figure 4. Percentage of different C. bombi strains (shades of grey, letters A-F) present in groups of B. terrestris workers ($n= 30$) with a high and a low worker turnover rate, 38 days post-experimental infection.

Discussion

In this study we aimed to understand the consequences of different worker turnover rates for a spreading disease. We found that fewer workers and, most importantly, fewer workers that mimicked the last cohort of workers were infected in groups with a higher worker turnover rate. Few infective cells circulating in the colony therefore should result in fewer infected daughter queens at the end of the colony cycle. Contrary to expectations, we did not find that, at the end of the experiment, fewer parasite strains infected workers exposed to a high turnover rate, nor did we find that different strains infected bumblebee workers exposed to different turnover rates. A high turnover rate of workers did also not increase the infection intensity of C. bombi. Our results
therefore suggest that a high worker turnover rate can be a life-history strategy that protects the colony against an infectious parasite.

The main virulence effect of *C. bombi* seems to be in the founding queens. *C. bombi* infection greatly reduces the colony founding success in daughter queens of *B. terrestris* after they emerge from hibernation the following spring (Brown et al. 2003). Furthermore, the number of *C. bombi* strains circulating in the colony is an important factor predicting the infection rate of daughter queens (Ulrich et al. 2011). A high turnover rate in our experiment did not reduce the number of persisting strains but decreased the number of infective cells circulating in the colony. As daughter queens can only be infected with exposure to a high dose (personal communication S. Barrière) a high worker turnover rate should result in fewer infected daughter queens. The observed low infection prevalence among late workers moreover must be due to a short transmission period of workers exposed to a high turnover rate.

In natural colonies, a high worker turnover rate would inevitable coincide with a demographic shift towards younger workers. This may additionally lead to increased defence levels, since the immune system of bumblebees declines with age (Doums et al. 2002; Moret & Schmid-Hempel 2009). Taken together, fast pace might prevent a parasite from spreading through a colony. In line with results from a serial passage experiment (Yourth & Schmid-Hempel 2006), a high turnover rate did not correlate with selection for increased virulence of *C. bombi*, if infection intensity can be taken as a correlate. Note that *C. bombi* was exposed to the high turnover rate for more than 100 generations (Salathé et al. 2012).

Important with regard to worker turnover rate in *Bombus* is that the colony size early in the cycle is a predictor of eventual colony size (worker number) at reproduction, which correlates closely with reproductive success (Muller & Schmid-Hempel 1992). ‘Fast-paced’ colonies with an increased death rate of workers, for this reason, should produce more workers over the colony cycle to achieve similar sizes when compared to colonies with a low turnover rate. For given resources or equal per capita workload hence, these more numerous workers reared in ‘fast-
paced’ colonies might be of lower quality (e.g. reduced immunity see Chapter 3) compared to workers with a lower turnover rate. No evidence to this effect is as yet available in our system. However, a comparable pattern of immune investment can be observed within species and among castes of social insect. Short-lived male ants, for instance, have a lower immune response than the longer-lived workers and queens (Baer et al. 2005).

While shifting the timing of reproduction requires collective decision-making in infected colonies (Moret & Schmid-Hempel 2004), the worker turnover rate most likely depends on the queen. The role of the queen as the pacemaker of the colony, as an aside, is an old idea going back to Schneirla’s studies on army ants (Schneirla 1944) and was taken up repeatedly over the last decades mainly focusing on the queen as the central force that initiates activity, coordinates work or determines the timing of sexual reproduction (Reeve & Gamboa 1983; Premnath et al. 1995; Jha et al. 2006; Souza & Prezoto 2011; Holland et al. 2013). Whether the observed increase in worker turnover rate is the result of more efficient brood raising by workers, of how the queen allocates her resources and hence might be a response to parasitism, or simply reflects the queen’s high or low quality remains to be seen. For example, we would expect that for given resources a queen produces either more short-lived workers at the expense of a reduced immune system, or few long-lived workers that may have a good immune system to counter their prolonged period of transmission.

Here we conclude that a high worker turnover rate could be a life-history strategy that protects a bumblebee colony against the spread and transmission of an infectious parasite. This increase in the turnover rate may be a direct response - plastic or selected - to parasitic infections, or may indirectly be caused by factors that decrease worker longevity such as food shortage or queen quality. Parasite-imposed selection for short-lived workers should in any case contribute towards worker lifespan variation in social insects.
References


Biological sciences, 76, 121.


3. WORKER LONGEVITY AND ITS RELATION TO COLONY RESISTANCE AGAINST INFECTIOUS PARASITES IN A SOCIAL INSECT

Abstract
Social insects should face a classical trade-off when investing in their workforce, just as solitary animals do when investing in their offspring. To sustain colony growth and reproduction, resources may be invested in either many ‘low quality’ workers or few ‘high quality’ workers. Worker quality moreover may manifest in anything that improves survival. Here we use the annual bumblebee *Bombus terrestris* and its gut parasite *Crithidia bombi* to study the proposed quality/quantity trade-off. In particular we measure quality as a worker's capacity to respond to immune challenge, which seems plausible as immunity plays an integral part in self-maintenance and is inherently costly. We first measured variation in intrinsic worker lifespan (quality) and the number of workers produced over the colony cycle (quantity). In a second experiment, we infected workers with *C. bombi* and measured the standing expression of bumblebee immune genes, to test for differential immune investment among colonies with different worker longevity. We found that across bumblebee colonies, immunity indeed is linked to worker longevity, such that individuals from colonies that produced longer-lived workers had lower *C. bombi* infection intensity Colonies with short-lived workers tended to be small, which is usually associated with low reproductive success. The standing variation in colony size and worker longevity may thus be maintained by the relation of longevity to colony size and their linked consequences on individual resistance and epidemic dynamics within the colony.

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*A version of this Chapter is in preparation as S. D Buechel, S.M. Barribeau & P. Schmid-Hempel.*
Introduction
There is striking variation in individual longevity, within and between species, among most multicellular organisms (Carey & Judge 2000; De Magalhaes & Costa 2009) including the social insects (Calabi & Porter 1989; Tsuji et al. 1996; Chapuisat & Keller 2002; Omholt & Amdam 2004; Rueppell et al. 2009). Longevity is a life history trait that is under selection and evolutionary and mechanistic theories of ageing (Medawar 1952; Williams 1966; Hamilton 1966; Kirkwood 1977; Stearns 1992) can help to explain the observed variation. Resource allocation to fecundity, growth, and maintenance (Stearns 1992; Roff 1993) against the background of extrinsic mortality is thereby considered to be a key element of longevity. Increased investment into growth and maintenance, for example, can be adaptive if the likelihood to survive to old age is high. The organism then lives longer, grows larger and therefore produces more, or better quality offspring as compared to short-lived, smaller parents. The reverse were true if the chances to survive to old age is small (Stearns 1992). This principle of optimal resource investment not only explains the range of life histories observed in solitary species but is also applicable to lifespan variation among the non-reproductive worker caste of insect societies (Kramer & Schaible 2013).

Analogous to the predicted strategies for solitary organisms, colonies subject to low extrinsic mortality should invest into colony growth and maintenance and thus become long-lived, whereas colonies that are subject to high mortality should invest in early reproduction. This is because, as with body size for solitary species, colony size in social insects is positively associated with reproductive output and competitiveness (Hölldobler & Wilson 1990; Adams 1990; Muller & Schmid-Hempel 1992; Schmid-Hempel et al. 1993; Palmer 2004; Lopez-Vaamonde et al. 2009). Additionally, and essential to our understanding of worker longevity, investment in growth and maintenance of the colony may be seen as investment in the physiological maintenance and thus a prolonged lifespan of workers. As workers provide most functions needed for colony maintenance and colonies with long-lived workers are usually large colonies (Carey 2001), such an investment could be selected for (Kramer & Schaible 2013). Moreover, it is likely that in most species the
queen is in control of important steps in the life cycle of the colony (Holland et al. 2013), and she might therefore also control investment. Empirical data supports these predictions for the reproductive caste (Keller & Genoud 1997; Keller 1998; Carey 2001) and suggests that in ants increased worker lifespan tends to be associated with low levels of extrinsic mortality, and vice versa (Tsuji et al. 1996; Chapuisat & Keller 2002; Hartmann & Heinze 2003; Rueppell et al. 2007). No such correlation, however, is as yet studied between worker longevity and another key trait, colony size - defined as the number of workers present at any one time. In wasps, however, there is a positive association between colony size and worker lifespan (O'Donnell & Jeanne 1992). Evidence in honeybees is equivocal as large colonies were reported to have short-lived workers (see Rueppell et al. 2009).

During colony development, social insect colonies may face a classical trade-off for worker quality versus worker quantity with repercussions on colony size and colony life span. The choice may be to produce either few ‘high-quality’ workers (which then are long-lived) or many ‘low-quality’ workers (which are short-lived). In this context we define worker quality as relating to anything that improves survival; in particular, we here measure "quality" as the worker's capacity to resist infection by a common parasite and the ability to respond to immune challenges. Although this covers just one aspect of "quality", it is a plausible and practical choice as immunity is an integral part of self-maintenance and since an immune response is inherently costly (Moret 2000; Sadd & Schmid-Hempel 2008). On the other hand, and from the perspective of the epidemiology of an infectious disease spreading through the colony, short-lived workers reduce the infectious period in any one host and thus the chances to encounter and transmit a parasite suggesting that a short worker lifespan per se may be a colony defence strategy against a spreading parasite (Chapter 2).

The bumblebee, *Bombus terrestris*, and its trypanosomatid gut parasite, *Crithidia bombi*, are a model for host-parasite studies in evolutionary and ecological immunology. The extensive toolbox available to study this host-parasite system and the presence of annual colonies that vary in worker lifespan makes this system a good candidate to study the expected quality/quantity
trade-off with respect to investment into immune functions. *C. bombi* is a directly transmitted trypanosomatid, most closely related to the *Leishmania* group (Schmid-Hempel & Tognazzo 2010), that induces severe fitness losses in young queens by reducing their likelihood of successfully founding colonies (Brown et al. 2003). *C. bombi* infects *per os*, can be picked up via contaminated flowers (Durrer & Schmid-Hempel 1994) and spread through the colony by infectious cells shed in the feces of infected bees. Hence, cell count (infection intensity) in the gut is a good proxy for the transmission success of the parasite as it correlates with cell numbers in faeces (Sadd 2011).

Here we report the variation in intrinsic worker longevity (quality) and the number (quantity) of workers produced over the colony cycle and assess how this colony level investment into worker quality determines *C. bombi* infection susceptibility and the expression of 27 immunologically important genes.

**Materials and Methods**

*Experimental procedure*

We did this experiment in two consecutive years using laboratory colonies started from spring queens caught in 2011/2012, originating from two populations in Switzerland (near Aesch BL; and Neunforn TG). Colonies were reared in circular perlite nests (Pomeroy & Plowright 1980) and kept under standard laboratory conditions (28 ± 2 °C, 60% RH ) with pollen and sugar water provided *ad libitum*. We carefully checked the queens for natural *Crithidia bombi* infections and only used uninfected colonies. We started the experiment by collecting callow workers (young, not yet sclerotized adults) on a daily basis when colonies reached a size of 12-15 workers; we stopped collecting when the first males and gynes (daughter queens) were produced. Callows were housed in boxes, in groups of two to six per colony and collection day, until the age of eight days when we randomly assigned them to one of three treatments: (1) lifespan monitoring, (2) *C. bombi* infection, and (3) immune gene expression. Bees that were used for the assessment of variation in worker lifespan between colonies (treatment no. 1) were kept singly in boxes and
checked for their survival twice a day. For the screening of differential immune gene expression (treatment no. 3), bees were snap frozen in liquid nitrogen at noon and stored at -80 °C for RNA extraction. Note that we only extracted RNA of workers from the five colonies with the longest and shortest-lived workers (N = 5 colonies each, N = 46 workers). With treatment no. 2, we examined the correlation between resistance to *C. bombi* infection (measured as infection intensity in the gut, 7 days post-infection) and mean worker lifespan in *B. terrestris* colonies. We infected workers with a cocktail of six genetically distinct *C. bombi* strains. To infect, we starved workers for two hours before presenting them with 10 µl of inoculum containing 18'000 cells in medium and sugar water (1:1 ratio; 3'000 cells per strain). Bees that did not consume the inoculum within 1 h were excluded from the study to ensure primary infections and viability of the parasite cells. We individually kept the remaining (infected) bees for 7 days before we assessed faeces cell counts (infection intensity reflecting parasite transmission success) and sampled them for genetic analysis. Parasite strains used for infections were sourced from queens caught in 2010 from the same two populations we used for this experiment. In addition, single cells were isolated from the queens' faeces, cloned and maintained as cultures as described in (Salathé et al. 2012). The same experimental procedure was re-done in 2012 with no gene expression data collected, but callows were marked to monitor the total number of workers produced in these colonies.

**Quantification of infections**

We dissected out the entire guts of workers to extract the DNA with a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and amplified a partial sequence of the *C. bombi* Cyt b gene (primers CB-Cytb2-F, CB-Cytb 2-B; (Schmid-Hempel & Tognazzo 2010) so as to visualize the infection status of workers (1.5 % agarose gel). For infection quantification we run quantitative real-time PCRs (qPCR) using Fast EvaGreen Master Mix (Biotium, Hayward, CA, USA) with primers designed to amplify a fragment conserved in the *C. bombi* 18sRNA (Ulrich et al. 2011). The reaction was set up in 10 µL reaction volume and included 2 µL eluted DNA 0.2 µM of each primer and 2 µL HOT FIREPol EvaGreen qPCR Mix. We used the thermal profile
described in (Ulrich et al. 2011). Each biological sample was run in technical duplicates with negative controls (distilled H$_2$O) and positive controls (bumblebee DNA). A dilution series of DNA extracted from a known number of *C. bombi* cells was included as a standard to calculate the relative infection intensities in workers, see Supplementary information figure S1. We included the same dilution series for each qPCR run. This allowed us to standardize our quantifications across plates.

**RNA preparation and quantification of candidate genes**

We used the protocol in Brunner *et al.* (2013) to measure the expression of candidate genes. In brief, we extracted RNA (RNeasy Plus Mini kit, Qiagen) from 92 homogenized worker abdomen (N= 46 workers from n=5 short-lived and long-lived colonies) using an Omni Bead Ruptor 24 Homogenizer at -4°C (OMNI International), confirmed RNA integrity for a subsample of n=12, on a 2100 Bioanalyzer (Agilent Technologies) and evaluated quantity and purity of extracted RNA with the Nanodrop 8000 (ThermoScientific). For six contaminated samples, with a low 260/280 nm or 260/230 nm ratios, we re-extracted RNA from the homogenized abdomen preparation kept for this purpose. We reverse transcribed 0.4 µg RNA from each sample (Quantitect reverse transcription kits, Qiagen) and amplified the cDNA product with intron specific primers to confirm the absence of genomic DNA. We measured the expression of 27 genes (*c.f.* Table 1) on a Fluidigm 96.96 Dynamic Array (with EvaGreen DNA Binding Dye, Biotium) with each biological sample measured over three technical replicates and the average of each triplicate was used as the observed expression value (Ct).

We selected a set of candidate genes to cover known aspects of insect immunity, representing the classical immune response cascades (recognition, signaling, effectors) of three main insect immune pathways (Toll, Imd, JAK/STAT). Because the workers were not immune-challenged, the expression profiles represent the non-induced, constitutive levels of gene activities. We included genes relevant for viral infection, bacterial infection, metabolic activities, reactive oxygen species, general stress response, melanisation and antimicrobial peptides; the included
genes were PGRP-S3, PGRP-LC, BGRP1, BGRP2, PGRP-LB, abaecin, apidaecin, defensin, hymenoptaecin, argonaute, aubergine, relish, hopscotch, TEPA, catsup, yellow, punch, prophenoloxidase, vitellogenin, apolipophorin III, cytochrome P450, serpin 27a, peroxiredoxin5, jafrac, lysozyme3, transferrin, ferritin - as well as five reference genes (AK, PLA2, RPL13, Perit, ef.1f.a2). We preferentially included genes that were published in previous studies and are considered relevant for parasite defense (Riddell et al. 2009; Vermeulen et al. 2009; Li et al. 2010; Schlüns et al. 2010; Radyuk et al. 2010; Vogel et al. 2011; Erler et al. 2011; Riddell et al. 2011). We used primers published in (Brunner et al. 2013), except for vitellogenin (Li et al. 2010) b and relish (Schlüs et al. 2010). Note that primers for, yellow, and PGRB-LB, apolipophorin III, cytochrome P450 and prophenoloxidase were designed following the same protocol. NCBI accession numbers and primer sequences for all genes can be found in the Supplementary information Table S1.

Analysis and statistics

For each colony we first calculated the mean worker lifespan and infection intensity from the experiment, as measured with qPCR, and tested the relation of resistance to C. bombi and longevity of workers (adult life span after emergence, in days) using a linear regression model with "infection intensity" (measured as total number of parasite cells per bee) included as response variable. In a similar approach we regressed the total number of workers that each colony produced against the mean worker lifespan to test whether colonies with short-lived workers tend to produce more workers compared to colonies with long-lived workers. Linear regressions were performed in IBM SPSS Statistics version 19.0, and data were log-transformed to meet the normality criterion (Shapiro-Wilk-test). To analyze the expression of our candidate genes in colonies that produced long-lived or short-lived workers, we used the Ct values (see above) of candidate genes normalized against the geometric mean of the four most stable reference genes (PLA2, RPL13, Perit, ef.1f.a2; determined with GeNorm, Biogazelle). We Yeo-Johnson-transformed the data to improve normality and performed a classical multivariate analysis of variance (MANOVA) on all candidate genes including longevity groups (long-lived
workers and short-lived workers, n=5 colonies each) and colony identity as a fixed factor. Different levels of expression in individual genes were explored with univariate variance analysis (ANOVA). We additionally performed a linear discriminant analysis on all candidate genes to determine different gene expressed among workers from colonies with long-lived and short-lived workers. We analyzed these data with R (2.15.2, (R Core Team 2012).

Results
We found that adult worker lifespan varied greatly among B. terrestris colonies (N = 35 colonies, N = 409 workers). Worker life span was, on average, 34.5 ± 5.44 days for the colony with shortest-lived workers, and 82.0 ± 9.11 days for the colony with the longest-lived workers (Figure 1).

Figure 1. The average worker lifespan (mean ± S.E. in days) in B. terrestris colonies (colony identity indicated by letters) collected in 2010 and 2012 and reared under standard laboratory conditions. Colonies marked with solid circles were subsequently used for the analysis of immune gene expression. Small numbers below lines indicate sample sizes (number of workers monitored for their entire life span).
As hypothesized, we found that across colonies worker longevity correlates with resistance to experimental infections by *C. bombi* with infection intensity inversely correlated to worker longevity (Figure 2; linear regression: $F_{1,16} = 5.29, r^2 = 0.218, P = 0.03$), indicating that colonies with long-lived workers also showed higher resistance. Colonies with long-lived workers also generally differed in their constitutive immune gene expression from colonies with short-lived workers (MANOVA for all genes combined: $F_{27,87} = 4.099, P < 0.001$). More specifically, genes involved in parasite recognition (PGRB-LB), melanisation (yellow, prophenoloxidase), and stress response (cytochrome P450) were expressed at a higher level in colonies with long-lived workers. The pattern was reversed - with higher expression in colonies with short-lived workers - for the antimicrobial peptide abaecin, the antiviral gene aubergine, and the key protein vitellogenin (Figure 3 & Table 1). A total 72% of workers moreover were correctly classified to the respective longevity scheme in their colony based on scores of the linear discriminant analysis for the expression levels of candidate genes. Contrary to our expectations from a hypothetical quality-
quantity trade-off, short-lived workers were produced at lower numbers compared to long-lived workers as indicated by a positive relationship between the number of workers produced in a colony and average worker longevity (linear regression: F$_{1,11}$ = 8.11, r$^2$ = 0.425, P= 0.016, Figure 4).

\[\Delta T_{Ct} \]

Figure 3. Relative expression levels (see Methods) of 7 bumblebee immune genes that were differentially expressed between colonies that produced short-lived workers (shaded boxes) and colonies that produced long-lived workers (open boxes). Box plot showing median (line) 25-75% quartiles (boxes) ranges (whiskers) and extreme values (circles). Sample sizes are added in small numbers (workers from which expression level of the respective gene was assessed).
Table 1. ANOVA results for differential gene expression between colonies with short-lived workers versus long-lived workers. Tested were 27 genes with known functions in insect immunity, metabolism and stress response. These genes were involved in three main immune pathways of insects (Toll, Imd, Jak/stat) and normalized against 4 common housekeeping genes of *B. terrestris*.

<table>
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<th>Class</th>
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<th>P</th>
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* indicates significantly or marginally increased level of expression in the respective colonies, long-lived = colonies that produced long-lived workers, short-lived = colonies that produced short-lived workers.

b vitellogenin has pleiotropic effects (see text)
Discussion
We tested a hypothesized quality vs. quantity trade-off, where "quality" was here measured as the ability of workers to resist a common parasite and reduce infection intensity; this would also limit the transmission success of the parasite. These measures were additionally highlighted by a gene expression study. We found that across bumblebee colonies, resistance is indeed linked to worker longevity, such that individuals from colonies that produced short-lived workers showed lower resistance to *C. bombi* infection. But they also expressed higher levels of antimicrobial (abaecin) and antiviral defence (aubergine) genes compared to individuals from colonies with long-lived workers. The latter being phenotypically better protected against *C. bombi*, (showing lower infection intensities) also expressed genes involved in parasite recognition (PGRB), stress response (Cyt P450), and the general ‘non-specific’ melanisation response (PPO) at higher levels. Although the detailed conclusions must remain preliminary for the time being, the differences in gene expression patterns may indicate differences in how parasites are controlled - either early in
the defence cascade (e.g. by strongly expression recognition, in colonies with long-lived workers) or later in the cascade (e.g. by anti-microbial effectors such as abaecin, in colonies with short-lived workers). Importantly and in contrast to predictions made based on the quality quantity trade-off; short-lived workers generally were from colonies having lower workers numbers and, hence, small colony size.

Colony size is a key life-history parameter in social insects, affecting a wide range of characteristic, such as division of labour and social structure (e.g. in ants; (Holbrook et al. 2013), and a correlate of parasite load, infection rate and transmission dynamics (Strassmann 1981; Muller & Schmid-Hempel 1992; Schmid-Hempel 1995; 1998; Naug & Camazine 2002; Pie et al. 2004). Interestingly, body size of nurses is also positively correlated with the number of eggs laid by the queen and the number of new workers emerging (Cnaani & Hefetz 1994), indicating that large-sized workers (having received more investment as larvae) are also more productive for the colony.

The positive relationship between worker longevity and colony size might be important to understand differential investment in worker quality, e.g. in the context of parasite resistance. For example, long-lived workers are more likely to live in large colonies and, therefore, may encounter more parasites brought in by the many others, and have more opportunities to transmit an infection to nest mates. On the other hand, we find them better defended individually. Individual worker life span, individual resistance to infection, colony size and epidemic potential could thus act together to control infectious diseases via demography and appropriate immune responses. In fact, immune defences and social organisation may not always be independent (Castella et al. 2010). Our previous work on worker turnover rate found that artificially increased birth and death rate reduced parasite spread in groups of bumblebee workers Chapter 2. Investment in worker defences - as it associates with worker longevity (Figure 2) - for this reason should thus influence the respective epidemiological potential of a parasite (high with long lived workers) in relation to worker longevity and colony size (Figure 5). With these considerations,
low quality colonies (queens) that produce only few poorly protected workers could be maintained by their reduced epidemic potential.

Figure 5. A sketch showing how colonies may vary in overall susceptibility to an infectious parasite spreading within the colony as a function of worker life span (longevity). The sketch is based on the current findings and those reported in Chapter 2. The solid lines illustrate the assumption that "epidemiological resistance" decreases with worker longevity, i.e. an infectious parasite can spread more easily within the colony. This is because long-lived workers allow longer infection times and more transmission events; in addition, long-lived workers are found in larger colonies. These factors make it more likely that a parasite can maintain itself and spread in the colony. The solid curves illustrate the assumption that long-lived workers have higher individual resistance to infection, as found in this study. This would increase the overall resistance of the colony as a whole - based on host resistance per se. For illustrative purposes only, we assume that the resulting overall colony resistance is a combination of individual resistance and colony-level "epidemiological resistance". To keep it simple, and for the sake of argument, the two components will combine to yield maximum overall resistance where the two processes (line and curve) intersect (point X). The two panels (a, b) demonstrate how variation in worker life span affects this overall resistance In situation (a), individual worker resistance may be the parameter most sensitive to changes in life spans (e.g. the solid curve changes to the dotted curve) whereas the "epidemiological resistance" is not affected (line remains the same). If so, the new point of maximum overall resistance will have moved to X1, predicting longer life span. In situation (b), epidemiological resistance (via larger colony size, longer transmission windows) may be most sensitive to changes in life spans (solid line changes to dotted line) whereas individual worker resistance remains the same (solid curve). If so, worker lifespan should get shorter to yield maximum colony resistance (point X1). Variation in worker life span can thus be driven by various processes that link to overall resistance of a colony.

Based on the differences in immune gene expression, colonies with short-lived workers may perhaps be prepared to defend themselves against different groups of parasites than colonies with long-lived workers. For example, colonies with short-lived workers could defend themselves primarily against rapidly spreading pathogens with immediate fitness detriments to colony growth and reproduction such as viruses and bacteria. For example, honeybees viruses such as DWV (Deformed Wing Virus) and ABPV (Acute Bee Paralysis Virus) account for the most prevalent...
but mainly non-apparent infections, yet causing fatal effects for colonies under stressful conditions (e.g. when co-infected with mites or under reduced food supply (reviewed in (Chen & Siede 2007)). These viruses may also play a role in bumblebee disease (Genersch et al. 2006; Singh et al. 2009). Colonies with short-lived workers indeed show higher expression for genes involved in RNA-silencing (aubergine, argonaut) - a key antiviral defence - a pattern that perhaps may reflect this threat. Additionally, these colonies had higher expression levels of vitellogenin, a general yolk precursor, up-regulated together with transferritin (P = 0.07; in our study), mostly needed when bees develop their ovaries (Koywiwattrakul & Sittipranceed 2008). In social insects such as the honeybee, vitellogenin is a central pace maker and also acts as antioxidant to prolong queen and worker longevity (Corona et al. 2007) and strongly affects social organization (Nelson et al. 2007). Colonies with long-lived workers, by contrast, may be more generally protected. Parasites might be detected and controlled at an early step of infection. The high expression of PPO and yellow suggests effective melanisation response to foreign particles including wound healing upon injury. Activation of the prophenoloxidase however can cause self-harm. Considering that long-lived workers should have an increased risk of parasites and predators, investing in general and costly defence might be relevant.

In conclusion we found that colonies with different worker longevity differed in immune investment and colony characteristics. Overall, colonies with short-lived workers are small colonies, which, at least in the absence of parasites, have low reproductive success (Hölldobler & Wilson 1990; Adams 1990; Muller & Schmid-Hempel 1992; Schmid-Hempel et al. 1993; Palmer 2004). Variation in colony size and worker longevity, such as those observed in Figure 1, therefore may be maintained by parasite diversity and its effect on colony size and success and the drawbacks in terms of facilitating an epidemic within the nest.
References


Supplementary Information

Figure S1. The Ct values for a single logarithmic dilution series of a known concentration of C. bombi cells from 4 qPCR runs performed on a ABI 7500 Real-Time PCR System (Applied Biosystems, Rotkreuz, Switzerland)
Table S1. Details for primers of candidate genes. Primers with no reference were developed for this study according to the protocol in Brunner et al. 2013.

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Table S1. (Continued) Details for primers of candidate genes. Primers with no reference were developed for this study according to the protocol in Brunner et al. 2013.

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4. WORKER LONGEVITY IN A PRIMITIVELY EUSOCIAL INSECT: IMMUNE SENESCENCE DOES NOT AFFECT WORKER RESISTANCE TO AN ECOLOGICALLY RELEVANT PARASITE.§

Abstract
In insect societies, worker lifespan strongly affects colony success. Long-lived workers may be favoured as they allow for large colony size, which typically correlates with higher reproductive success. At the same time, long-lived and therefore, on average, older workers should pose an increased risk of parasite transmission within the colony. This is because older workers are more likely to have encountered a parasite, and defence (immune) functions commonly decline with age. To counter this epidemic risk, social insects may have evolved adaptations related to worker age. Old workers, for example, tend to be foragers and often have limited contact with younger workers engaged in in-hive tasks. Our study organisms, the bumblebees, show little such age-related changes in behaviour and might be especially vulnerable to infection through old workers in the colony. Using the bumblebee, Bombus terrestris, and its trypanosomatid gut parasite, Crithidia bombi, we experimentally tested the role of worker age for the resistance to this ecologically important parasite. Contrary to expectations, we found that older workers were less heavily infected, with fewer infective cells in the gut but nonetheless were also more likely to transmit the parasite. Whereas this increased transmission could not be explained by different strains infecting worker of different ages, it may be due behavioural changes. We conclude that immune senescence may not necessarily affect resistance to this important parasite. Age effects, however, may be relevant for the spread of the infection through the colony and may be mediated by behaviour rather than by infection intensity. Adaptive demography in social insects for this reason should be analysed, too, in the light of preventing or controlling disease epidemics.

§ A version of this Chapter is in preparation as S. D. Buechel, M. Arnoldini & P. Schmid-Hempel.
**Introduction**

Lifespan is a highly variable trait that differs among and within species (Keller & Genoud 1997; Carey & Judge 2000; De Magalhaes & Costa 2009). Life history theory explains variation in longevity by selection acting to shape individual life history such as to maximise Darwinian fitness. A long life, for example, implies investment into body maintenance and should be favoured only if the likelihood to later reproduce and thus to survive to an older age is high. If instead adult survival were low, organisms should sacrifice prolonged lifespan for early reproduction (Williams 1966; Stearns 1992).

Eusocial species such as the ants, bees, wasps and termites live in highly integrated societies that often consist of one reproductive queen (and king in termites) and many sterile workers that perform all non-reproductive tasks needed for the colony to survive and grow; this includes nest construction, foraging, maintenance and caring for the queen and her brood (Hölldobler & Wilson 1990). Colony fitness, hence, depends crucially on worker performance (Bourke 1995). Among other worker characteristics, variation in worker lifespan should therefore be selected so as to contribute to the colony performance (Schmid-Hempel 1992; Moret & Schmid-Hempel 2009; Armitage & Boomsma 2010; Helft et al. 2012). For example, long-lived workers might be beneficial as they allow the colony to grow large and thus be better defended, be ergonomically more efficient, and ultimately rear more males and daughter queens that contribute to the next generation (Hölldobler & Wilson 1990; Adams 1990; Muller & Schmid-Hempel 1992; Carey 2001; Palmer 2004; Lopez-Vaamonde et al. 2009). But long-lived workers may also be costly - in terms of their "production cost" (i.e. producing higher "quality" workers) as well as through secondary effects. For example, we expect a secondary cost, as colonies with long-lived workers might be more vulnerable to parasites. This is because colonies with long-lived workers will, on one hand, have relatively more old workers than colonies with short-lived workers. Old workers may be more likely to have encountered parasites and thus be infected, but may also be less protected as the immune system undergoes senescence over an organisms life (Franceschi et al. 2000; Haussmann et al. 2005; Stanley 2012). Furthermore, increased foraging in bumblebees - as
is typical for older workers in general - correlates with a decreased immune response to an implanted object (Konig & Schmid-Hempel 1995; Doums & Schmid-Hempel 2000); this age effect may be mediated by the reduced number of haemocytes (immune cells involved in encapsulation response) in old workers (Moret & Schmid-Hempel 2009). Similar patterns of a decline in important immune function with age have been found in honeybees (Amdam et al. 2005; Schmid et al. 2008) and ants (Helft et al. 2012) - although as of yet there is no direct evidence for diminishing resistance to real pathogenic infections with older age structure of the colony. Nevertheless, it is conceivable that worker longevity should associate with higher vulnerability of the colony to infection. On the other hand, long-lived workers would pose an increased epidemic threat to the colony, both, by being more vulnerable individually and by allowing for longer infection periods (e.g. leading to higher parasite loads and longer transmission windows). Additionally, the resulting larger colony sizes also generate more opportunities for transmission within the colony, perhaps assisted by higher within-colony densities and more frequent behavioural interactions (Schmid-Hempel 1998; Naug & Camazine 2002; Pie et al. 2004; Otterstatter & Thomson 2007).

Importantly, social insects have additionally evolved efficient behavioural defences based on collective decision-making and altruistic behaviour (e.g. hygienic behaviour and mutual grooming of diseased workers (Cremer et al. 2007; Parker et al. 2011). These social defences complement the individual immune system, can counter the defence cost to large colonies, and may be another key to the spectacular ecological dominance of social insects. Taken longevity and social defences together, we might, for example, imagine that advanced insect societies mitigate the threat posed by senescing workers through age-based polytheism (Schmid-Hempel & Schmid-Hempel 1993; Calderone 1995; Calderone & Page 1996; Seid & Traniello 2006; Camargo et al. 2007; Johnson 2008; Mersch et al. 2013). In such a system, young workers would perform tasks in disease-privileged areas of the colony, such as assisting the queen or brood. Older, presumably less protected, workers are more likely to be foragers or seek other tasks outside the colony,
where they would be more likely to encounter parasites but are less likely to directly transmit them to important colony members.

In this paper we investigate the role of age-related resistance of workers to an ecologically important infectious parasite. We use the primitively eusocial bumblebee *Bombus terrestris* and its trypanosomatid gut parasite *Crithidia bombi*, a well-studied model for host–parasite studies in evolutionary and ecological immunology. Their life cycles are simple: after hibernation mated bumblebee queens found colonies in spring that grow in numbers of workers until sexuals (males and gynes) are produced and the colony declines. *C. bombi*, in turn, is picked up *per os* via contaminated flowers and spreads through the colony by infectious cells shed in the faeces of workers (Durrer & Schmid-Hempel 1994). It has only one host in its life cycle, is not vectored, but can infect virtually all species of *Bombus*.

Bumblebees are primitively eusocial insects that did not, or - as far as known - only partly evolved social defences against parasites (Müller & Schmid-Hempel 1993; Korner-Nievergelt 2003). Workers also show no clear-cut division of labour according to age (Cameron 1989; Cameron & Robinson 1990; O'Donnell et al. 2000; Jandt & Dornhaus 2009; Jandt et al. 2009) but task affinity based on body size is common. In particular, small workers are more likely to perform hive tasks whereas bigger workers forage or guard the nest (Goulson et al. 2002; Goulson 2003; Yerushalmi et al. 2006). With respect to infectious parasites, bumblebees should thus be susceptible to a demographic shift towards old workers and we could expect that individual, physiological immunity should be maintained at high levels throughout worker life (see (Schmid-Hempel 1992; Moret & Schmid-Hempel 2009; Armitage & Boomsma 2010; Helft et al. 2012).

We were thus interested in testing whether in this system worker age has an effect on elements that affect the risk of an infection and spread of a parasite. In a first experiment we infected old and young workers with *C. bombi* to test for age-related differences in resistance, infection load, and parasite diversity and identity. We then grouped old and young infected workers with naïve, uninfected nest mates to test the age specific potential to transmit the parasite. In a second experiment we aimed to investigate differences in the behavioural repertoire of old and young
workers likely to influence their transmission potential. We video tracked worker groups that included an infected young or old focal bee and analysed their respective activity and the distance kept between workers.

**Methods**

*Collection and culturing*

We collected wild queens of *B. terrestris* in spring 2012 from two distinct populations in Switzerland (near Aesch BL; and Neunforn TG) and allowed them to establish colonies in the lab. As soon as the first workers emerged, we transferred the colonies to a circular perlite nests (Pomeroy & Plowright 1980) and kept them under standardized laboratory conditions (28 ± 2 °C, 60% RH) with constant red light illumination and pollen and sugar water provided *ad libitum*. Colonies were carefully checked for the presence of parasites in their faeces and only uninfected colonies were used in these two experiments.

The parasite, *C. bombi* originated from naturally infected queens, sampled from the same two populations in 2010 as from where the queens originated. Single infective cells were isolated and maintained clonally in liquid medium (Salathé et al. 2012). The five strains used in this experiment were shown to be equally infective in an earlier experiment (Chapter 2) and had distinct multi-locus genotypes at three polymorphic microsatellite loci that allowed distinguishing them by genetic markers.

*Experiment 1: Age related resistance and the potential to transmit C. bombi to naïve workers*

We marked young callow workers (not yet sclerotized adults that had emerged within the last 24 hours) daily in 21 colonies and randomly assigned them to two treatment groups - infected when they were 4 days (young workers) or when 18 days old (old workers). We chose these particular days to ensure that the infection did not interfere with worker development, that it approached a natural setting (with adults living for 3-4 weeks and becoming infected at various times) as well as for practical reasons (e.g. to ensure enough bees surviving to the end of the experiment at age
of 31 days (Chapter 3). At their respective age of infection workers were isolated and starved for 2 hrs and subsequently fed with a cocktail of 40’000 infectious *C. bombi* cells containing five distinct parasite strains. Workers that did not ingest the cocktail after 1 h were excluded from the experiment so as to ensure primary infections and viability of the cells. Workers were then kept individually to prevent cross infection; 8 d later their faeces were visually checked for the presence of *C. bombi* cells, confirming their infection status. For later use, "resistant" was defined by the absence of infective cells in the faeces. To test the transmission potential of young and old workers we grouped workers that were successfully infected at either young or old age with six naïve (uninfected) workers from the respective colony. The artificial group thus consisted of 1 infected and 6 naïve workers and was housed in a plastic box (15 x 20 cm) for a period of 6 days, which allowed transmission to naïve workers (as tested in a preliminary study; data not shown) but prevented cross infection. Bees were then isolated for another 8 days, for the parasite to establish and be readily detected with molecular tools using *C. bombi* specific primers (see below). We also measured the infection intensity of young and old workers as the total number of infective *C. bombi* cells in the gut (correction for body size did not change the results, as size was anyway randomized across treatments), and we also genotyped the *C. bombi* strains that successfully infected young and old workers.

**Molecular analysis**

We dissected out the whole gut of all workers and extracted genomic DNA following the protocol for Blood & Tissue samples from Qiagen (Hilden, Germany). To visualise the infection status of workers we then amplified a partial sequence of the *C. bombi* Cyt b gene (primers CB-Cytb2-F, CB-Cytb2-F; (Schmid-Hempel & Tognazzo 2010) and run the amplification product on an 1.5 % agarose gel. To quantify the infection intensity, we measured the number of *C. bombi* cells in the guts of young and old workers using quantitative real time PCRs (qPCR) based on EvaGreen (Biotium, Hayward, CA, USA) and primers that were designed to amplify a fragment conserved in the *C. bombi* 18s RNA (Primers CriRTF2, CriRTR2 (Ulrich et al. 2011). The qPCRs were performed on an ABI 7200 Fast Real-time PCR System (Applied Biosystems Rotkreuz
In 10 µl reaction volumes containing 2µL eluted DNA, 0.2 µL of each primer (10 µM) and 2µL HOT FIREPol EvaGreen qPCR Mix. We used the thermal profile described in Ulrich et al. (2011). A dilution series of DNA from a known number of *C. bombi* cells was included and served as a standard to calculate the relative infection intensities in the guts of young and old workers. Including the same dilution series over all plates also allowed standardizing our quantification across plates. Finally, we genotyped the *C. bombi* strains that successfully infected young and old workers at the *C. bombi* microsatellite markers Cri4, Cri4G9 and Cri1B6 (Schmid-Hempel & Reber Funk 2004). For this, two multiplex reaction were performed on a ABI 3730xl DNA sequencer (Applied Biosystems, Rotkreuz, Switzerland) in 10 µL reaction volumes including 2 µL eluted DNA in 2 µL 5 x reaction buffer, 0.5 µL dNTPS (2.5 mM), 0.05 µL GoTaq polymerase (5U/µL) and (1) 0.1 µL of the primers Cri4 and Cri4G9, (2) 0.06 µL of the primer Cri1B6. PCR conditions were the same as described in (Koch & Schmid-Hempel 2011). The microsatellites were scored with Peakscanner software V1.0 (Applied Biosystems, Rotkreuz, Switzerland) and only samples where strains could be clearly identified at all three markers were included in the analysis.

**Experiment 2: Behavioural assay and transmission potential**

We used the same colonies and an identical infection protocol to compare the transmission potential of young and old workers (tested as described above) with their behavioural activities and the physical distance they kept from naïve nest mates. These measures were found to be relevant (Pie et al. 2004; Otterstatter & Thomson 2007) and seem plausible considering the transmission mode of the parasite (infective cell shed in faeces and taken up *per os*). For the experiment, we marked callows daily over 5 consecutive days and infected them at the age of 4, respectively 18 days (as above). Workers where then housed with 3 naïve workers each in a box (as above), as a group of 4 workers could technically be readily tracked (see below). After 6 days - a time chosen to allow the parasitic infection to develop and hosts becoming highly infectious for others - the group was recorded twice for 15 min under red light illumination. For the analysis we chose recordings taken at similar daytimes. We did not analyse the first and last 2.5 min of
each recoding to eliminate the effects of possible differences in handling and timing for each box. Custom software was developed by one of us (AM) using MATLAB (The Mathworks Inc.) to analyze movies. The analysis proceeded in several steps. First, automated individual recognition of bumblebees based on their grey values was performed. Second, recognition errors were corrected manually. Third, the movement of individual bumblebees was tracked by connecting bumblebees in the single images over time based on proximity. In a fourth step, this tracking was checked and errors again corrected manually. These procedures resulted in data on activity and distance maintained between the workers in the analysed movies.

Analyses and statistics

We tested the effect of worker age on resistance to *C. bombi* infection, defined as the absence of parasite cells in faeces, using a linear mixed model with binomial error distribution. The model used worker age (4 days, and 18 days at infection) as independent fixed variable and the identity of the colony that workers originated from as random variable. The infection intensity of young and old workers was measured as the number of parasite cells in the gut and was analysed with a linear mixed model using worker age and colony identity as fixed and random variables, respectively. The number of parasite cells in workers as determined with quantitative PCR was log-transformed so that the residuals met the normality criterion. We tested for parasite diversity, defined as the number of strains that persisted in young and old workers at time of checking, with a nonparametric Mann-Whitney U-test, and for the identity of the strains that successfully infected workers of different age (i.e. strain number) by Fisher's exact test. Finally, we tested the age effect on the potential to transmit *C. bombi* to naïve nest mates as follows: the number of infected workers that had been housed with young or old workers was the dependent variable in a binomial logit link model with worker age as fixed and colony identity as random variable. To correct for over-dispersed data we included an additional random variable at the level of the individual observation (the number of data points), which allows to model extra variance in the data. Behavioural data included the activity (total distance covered during observation) of the focal bee (young or old infected worker), the "cohesiveness" of the group (average distance
between individual workers), and the average distance of the focal bee from naïve workers. These measures were averaged over time intervals of 30 s (corresponding to 225 frames) and analysed with linear models that used the worker age as fixed variable and time interval (20 intervals of 30 s), and the identity of the tracked group as random factor. Statistical analyses were carried out in IBM SPSS Statistics version 19.0, or R statistical software version 2.15.2 (R Core Team 2012). Note that all interaction terms were not significant and therefore removed from the models presented here.

Results
To begin with, both, old (proportion infected = 0.7) and young workers (proportion infected = 0.6) were equally likely to contract the experimental infection (GLMMs: \( F_{1,184} = 1.862, p > 0.1 \), Figure 1). Upon primary infection though, old workers were more efficient in clearing the infection as indicated by a reduced number of parasite cells measured in their gut at 8 d post-infection than younger workers (number of cells old workers: \( 6.35 \times 10^3 \pm 1.26 \times 10^3 \) versus young workers \( 3.21 \times 10^3 \pm 0.55 \times 10^3 \); LMM: \( F_{1,20} = 7.189, p = 0.01 \), Figure 2). Yet, low infection intensity in old workers did not directly result in a low transmission potential to the surrounding naïve nest mates. To the contrary, more workers were infected in groups with old focal workers compared to groups with young focal workers (proportion of naïve workers infected = 0.21 ± 0.05 S.E. for old workers, and 0.12 ± 0.035 for young workers; GLMMs: \( z = 2.022, p = 0.04 \), Figure 3). This increased risk to transmit the parasite was not explained by differences in parasite diversity, as the number of strains did not differ according to age (old workers: 1.92 ± 0.199 S.E. versus young 1.96 ± 0.204; Mann-Whitney U test: \( p = 0.9 \)), nor could it be assigned to different parasites strains having infected workers of young or old age (Fishers exact test: \( p = 0.9 \)). Rather, behavioural differences may have accounted for the increased transmission.
Figure 1. The proportion of young (open bar) and old workers (filled bar) with established infections upon experimental exposure, defined as the presence or absence of *C. bombi* cells in their faeces 8 days post-infection (GLMMs; $F_{1,184} = 1.862, p > 0.1$). Lines represent 95% C.I., small numbers indicate sample sizes (number of workers).

Figure 2. The number of parasite cells in the gut of young (open bars) and old workers (filled bar), as measured with qPCR, 13 days post-infection (LMM; $F_{1,20} = 7.189, p = 0.01$). Bar plot showing mean number of cells according to worker age, S.E. (lines) and sample sizes (workers, small numbers).
Figure 3. The proportion of naïve workers that were infected when grouped 6 days with young infected worker (open bar) and old infected workers (filled bar), (GLMMs; \( z = 2.022, p = 0.04 \)). Groups contained a total of 7 workers and the infection status was determined using *C. bombi* specific primers. Bars represent mean infections of groups, and lines show S.E. Sample sizes (workers) are given in small numbers.

Figure 4. Percentage of different *C. bombi* strains (shades of grey, letters A-F) present in young workers (open bar) or old workers (filled bars), (Fisher's exact test; \( p = 0.9 \)). Strains were typed 13 days post-infection.
We found that old workers showed higher levels of activity (distance in cm old workers: 24.68 ± 1.02 S.E. and young workers: 16.82 ± 0.67; LMM: F_{1,22} = 6.109, p = 0.02, Figure 5a) which may have led to a less cohesive group structure (group with old focal worker 5.70 ± 0.09 S.E. versus young focal worker: 4.7 ± 0.09; LMM: F_{1,22} = 4.871, p = 0.03, Figure 5b) and a tendency to keep more distance to naïve workers (old workers: 16.75 ± 0.29 S.E. and young workers: 13.98 ± 0.33; LMM: F_{1,22} = 3.499, p = 0.07, Figure 5c).
Figure 5. Measures of activity and proximity of young and old infected focal workers, grouped together with 6 naïve workers from their respective colony (n= 12 colonies for both groups). Data from half-automated video tracking was obtained for: (a) the total distance (in cm) the focal workers walked during the 10 min of tracking ("activity measure", old workers: LMM; $F_{1,22} = 6.109$, $p = 0.02$, Figure 5a), (b) the cohesiveness of the group measured as the mean pairwise distances (in cm) of all workers in groups (LMM; $F_{1,22} = 4.871$, $p = 0.03$, Figure 5b), and (c) the distance focal workers kept from the surrounding naïve workers (LMM; $F_{1,22} = 3.499$, $p = 0.07$, Figure 5c). The analysis is based on averages from of 30 s intervals (see text). Bars represent the estimated marginal means ($\pm$ S.E.) derived from a model using worker age as fixed factor and the time intervals as well as the video identity as random factor (see text).
Discussion
Contrary to expectation, we found that older bumblebee workers seemed to have cleared the infection better as compared to young workers. Despite a lower number of infectious cells in the gut and thus in their faeces, old workers nevertheless infected more naïve workers than did their younger sisters. This high potential to transmit the parasite could not be explained by parasite diversity nor could it be assigned to different parasites strains infecting young or old workers. Rather, increased transmission may have resulted from age-related differences in the behavioural repertoire of bumblebees, for example, increased levels of activity in old workers infected with \textit{C. bombi}.

At first sight, bumblebees therefore maintain functional immunity against this parasite as they age. Low infection intensity in old workers however, does not primarily contradict the observation that the immune system of bumblebees declines with age, but rather suggests that at least against \textit{C. bombi} infections, immune senescence may be less relevant. One possible, although not yet tested and therefore hypothetical scenario may be that old workers are protected against \textit{C. bombi} by means of the microbial gut community (Koch & Schmid-Hempel 2011), whose composition may differ from that of young workers. Also not all immune functions necessarily always decline with worker age. In the case of prophenoloxidase, an enzyme relevant for melanisation response, higher levels were measured in older ants and honey bees (Schmid et al. 2008; Armitage & Boomsma 2010).

We may hypothesize that resistance to \textit{C. bombi} infection is also explained by selection on worker longevity acting at the colony level. Long-lived workers in the colony would allow achieving big colony sizes (Carey 2001), which ultimately results in increased colony fitness (Muller & Schmid-Hempel 1992; Schmid-Hempel et al. 1993; Lopez-Vaamonde et al. 2009). That old bumblebees nevertheless infected more naïve workers may at first be an intriguing disadvantage, as it would allow a parasite spreading through the colony more easily. In the present case, and considering the virulence pattern of \textit{C. bombi}, however, the virulence costs of an infection are mainly paid by the young, daughter queens that if infected, are less likely to found
their own colony next year (Brown et al. 2003). Interestingly, young queens seem also more resistant to *C. bombi* infection than workers are and can only be infected upon ingestion of a high number of cells (S. Barribeau, pers. comm.). Low infection intensity in older workers may thus in the first place be relevant to reduce the risk of infecting daughter queens, which emerge only at the end of colony cycle when many older workers are present. On one hand, therefore, and besides other constraints, the appearance of sexual brood late in the colony cycle (as also determined by the development time for the sexual brood) could also function as temporal buffer against the presence of younger workers and thus a high number of infectious cells that circulate in the colony. We could not test here whether the above-mentioned changed behaviour of older workers would also mean increased contacts with daughter queens of the colony and, on balance, would thus still lead to more transmission. As to the males, these are produced along with last workers before young queens emerge (Goulson 2003). Males may be especially prone to infection (Vainio et al. 2003; Gerloff et al. 2003; Baer et al. 2005) but leave the colony only few days after emergence before shedding infective cells in the colony. It thus remains to further investigate whether colony demography in *B. terrestris* - with many older and less infected workers towards the end - may also be understood as adaptation to the threat posed by infectious parasites for an annual life cycle of the colony. Alternatively, and in contrast to our finding, old bumblebee workers were often reported to be inactive (Jandt & Dornhaus 2009) which is also true, for example, for ants (Cole 1992; Pinter-Wollman et al. 2012). At least in some bumblebee colonies, workers tend to shift their spatial zone of activity further away from the colony centre as they age (Jandt & Dornhaus 2009). In our experimental setup, increased activity in old workers may be a result of them seeking distance from their nest mates. Groups with old infected workers indeed showed lower cohesiveness and old infected workers tended to keep more distance to all other workers in the groups compared to younger workers (*P* = 0.07).

In this paper we aimed to test the effect of worker age resistance to an ecologically relevant parasite in the light of worker longevity in primitively eusocial insects. We conclude that immune senescence may not be relevant against *C. bombi* infection in bumblebees. Age effects, however,
can become relevant for the spread of a disease in the colony, but this may be mediated by behavioural changes rather than infection loads *per se*. Adaptive demography in social insects should thus be analysed, too, in the light of preventing or controlling disease epidemics.
References


Mersch, D. P., Crespi, A. & Keller, L. 2013. Tracking Individuals Shows Spatial Fidelity Is a Key Regulator of Ant Social Organization.


5. GENERAL DISCUSSION

In this thesis I made use of a primitively eusocial bumblebee and its infectious gut parasite *C. bombi* to investigate the importance of worker longevity on the infection and transmission of this ecologically relevant parasite. In particular I tested how life span variation at the colony level and the underlying colony demography relates to the within colony epidemics and the potential threat to the colony's survival and reproduction. I thereby considered the epidemic effect of long versus short worker lifespans and tested how lifespan may be associated with physiological immunity. The role of senescence was thereafter assessed with respect to immunity and for the potential to transmit a disease. A ‘healthy’, reproductive colony in any case should maximize colony size, while keeping a within-colony epidemic low.

As a starting point, long-lived, and thus old workers, may be a risk for the colony exposed to parasites. This is because older workers have an increased probability to have already encountered a parasite brought into the colony by nest mates. Furthermore, longer lifespan means long transmission periods that may additionally render the colony vulnerable to the spread of diseases (Chapter 2). Individual resistance as a possible response may mitigate this risk if positively related to worker longevity (Chapter 3, 4); it should also be important to sustain colony growth in bumblebees (Chapter 2). (Table 1 summarizes these findings). In more detail, and in an epidemiological context, lifespan (here the time an adult worker spends working for the colony) determines the time of residence of a given parasite in a host individual (e.g. leading to higher infection intensities) and the length of time the host can transmit the disease. In Chapter 2, I investigated these patterns.

A long lifespan of workers resulted in more other infected workers in the group. By keeping group size constant and experimentally altering worker lifespan and birth rate, I controlled for density effects and the immunological background of workers. This high epidemic potential of long-lived workers would become even more relevant at high densities, yet another characteristic that facilitates parasite spread (Naug & Camazine 2002; Pie et al. 2004). Such a positive
relationship between colony size and worker longevity was proposed before (O'Donnell & Jeanne 1992; Carey 2001) and may suggest selection for increased individual immunity in long-lived workers. This was shown by workers from colonies with long-lived workers being more resistant to experimental infections and their colonies also having larger size (Chapter 3). But how about variation in individual resistance? Resistance as a plastic, density dependent response was shown in this system before (Moret & Schmid-Hempel 2009; Ruiz-Gonzalez et al. 2009). Moreover, immune responses were often shown to change over an individual’s life and especially showing senescence in later life (Doums et al. 2002; Moret & Schmid-Hempel 2009) The increased immune investment associated with long-lived workers therefore may not be relevant to decrease parasite transmission in colonies with long-lived workers. In Chapter 4, I found the opposite. Immune senescence did not affect functional immunity at old age but resistance to *C. bombi* was upheld in older workers. This finding highlights the importance for old workers to be well protected. Nevertheless, in Chapter 4, I also found that older workers were more likely to transmit the parasite. This overall effect must therefore be due to changes beyond the individual immune defences. A likely candidate is changed behaviours.

Table 1. Summary of epidemic characteristics of colonies with long-lived workers and colonies with short-lived workers.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Long-lived workers</th>
<th>Short-lived workers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 2 Transmission potential</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Chapter 3 Resistance</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Chapter 3 Colony size (Density)</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Chapter 4 Effect of Immune senescence</td>
<td>Low</td>
<td>Low</td>
</tr>
</tbody>
</table>

**A hypothetical scenario: How parasite pressure may act as driving force for colony social organization**

Social insects evolved from having simple, small societies to having large colonies with complex colony structure and an efficient system for division of labour. These transitions were associated with changes in the mating system, the evolution of caste/colony organisation, and are typically
considered to have evolved for ergonomic benefits (Oster & Wilson 1978), that is, increasing the economic benefits for the colony. Evolution along this trajectory was favoured by kinship effects e.g. (Hughes et al. 2008). However, the role of parasites for the evolution of social insect colony organisation is probably often underestimated. Based on the results and insights presented in this thesis, I here suggest a hypothetical scenario of how parasite pressure can act as a selective force for the evolution of colony organization.

To start with, a few preconditions are necessary for this scenario to be plausible. These can be summarized as follows:

(1) It is assumed that social evolution went from small to large colonies, from singly mated to multiply mated queens, from monogyny to polygyny, and from simple colony structure to complex structure, eventually with the presence of defined castes (Bourke 1999; Hughes et al. 2008; Boomsma 2009). (2) Social evolution offered conditions for increased longevity as a longer working life is the main contribution of individuals towards their own (inclusive) success (Keller & Genoud 1997; Keller 1998; Carey 2001). (3) Long-lived workers may also add to larger colony size as discussed in (Carey 2001). Empirical evidence is given in Chapter 2 and by (O'Donnell & Jeanne 1992). (4) Parasites are ubiquitous and have important selective effects on social insects. This argument was first summarized by P. (Schmid-Hempel 1998) and is supported by an ever increasing literature e.g. (Boomsma et al. 2005). (5) The risk of contracting an infection from the outside increases with colony size. This is shown, for example, for bumblebees (Muller & Schmid-Hempel 1992), social spiders (Hieber & Uetz 1990) and colonial birds (Brown & Brown 1986). (6) Parasite transmission is more frequent in large colonies (Naug & Camazine 2002; Pie et al. 2004; Otterstatter & Thomson 2007) and among long-lived workers (7) (discussed in Chapter 2).
The scenario

Given the above-mentioned preconditions, the evolution towards more complex social systems may have been driven by parasite pressure according to the following steps: (visualized in Figure 1).

**Figure 1.** A sketch showing how colony size, worker longevity, and parasite pressure may have influenced the evolutionary path towards more complex social structure. Firstly, several steps (i-iii, trajectory A) mark the origin of sociality to the evolution of complex colonies and may have led to an increase in worker longevity and colony size. At a certain point (B) the colony reaches the critical epidemic potential in terms of size and worker longevity. The benefits of colony size at this point may no longer outweigh the cost of increased parasite transmission and the higher chances of sustaining an epidemic (shaded grey areas). From there, social insect evolution may have followed different paths as dictated, among other things by the critical density of workers within a colony. Above this density, parasite transmission is too high and the colony risks serious systemic effects of an infection; hence, regions C, D, E are not available for evolution. Critical density is affected by colony size and is inversely correlated with worker lifespan, as more workers are present in the colony at any one time. From point B, evolution could maintain colonies at critical colony size and worker lifespan (option B). Alternatively, selection for larger colony size could persist, which increases efficiency and thus reproductive output. But selection for large colony size may have decreased worker longevity (C&C) resulting in a high worker turnover rate, or it may have favoured additional, behavioural adaptations (D) to counter the additive epidemic potential imposed by colony size and worker longevity, leading to large complex colonies. Evolution towards small colony size (E’) seems unlikely, as this would entail a loss of reproductive capacity. In all, parasite pressure could generate a range of worker longevities and colony sizes, depending in the nature of the selective forces and the relationship of colony size with worker longevity and epidemic risks.
Along the evolutionary pathway from the origin of sociality to advanced systems, the reproductive division of labour, may first have put selection on worker longevity towards longer-lived worker and, consequently, larger colony size (A in Figure 1: trajectory from the origin towards point B).

The steps that perhaps prolonged worker longevity have to some extent been envisioned in (Carey 2001) and, in brief, include:

(i) Nest as locus: The nest originated as a protected microclimate for a singly mated female, her brood and food provisions. As this female spent longer time in the protected nest, mortality rates due to predation were reduced, and there was selection for decreased rate of senescence. The female thus may have become longer lived (Williams 1966; Stearns 1992) and more likely to be alive when the brood emerged to form a social group. Along with selection on the female (queen), protection, shelter and food provisioning, may also have improved larvae and adult survival and resulted in offspring staying longer in the nest. This in turn allowed for further selection to decrease senescence rate in adults; thus, as workers evolved, they became longer-lived, too.

(ii) Overlapping generations: Females may have reared their own brood in the sheltered nest, occasionally providing their sister’s brood. This adaptation likely allowed females to spend on the average more time in the nest. Extrinsic mortality once more may have been decreased.

(iii) Reproductive division of labour: The transition to sterile worker castes according to (Bourke 1999) and (Rodriguez-Serrano et al. 2012) may be a consequence of increased colony size and competition over reproduction. As the number of individuals increases chances to be part of the reproductive caste decrease. Loss of reproduction may have saved resources for body maintenance and repair, prolonging individual life span at the proximate level (Stearns 1992). Colony size increases as sisters rear brood more efficiently.
(b) At a certain point, evolution may reach a critical point (B in Figure 1). This is because the "epidemic potential", i.e. the probability of contracting an infectious parasite and to sustain an epidemic within the colony increases with colony size and worker longevity. This is augmented, for instance, when the individual immune defence capacity reaches its physiological maximum. A colony reaching this point may have several evolutionary options. It could, for example, maintain a critical colony size and worker lifespan (point B, Figure 1) Alternatively, selection for larger colony size could continue, which increases efficiency and thus reproductive output. If so, selection for large colony size could have decreased worker longevity (C'&C in Figure 1), or may have favoured additional adaptations (e.g. changed behaviours) to compensate for the increased risk of an epidemic (D in Figure 1). Selection for long-lived workers at the expense of colony size is unlikely, as reproductive output in social insect colonies should largely depend on the workforce present at time of reproduction (e.g. workers that rear brood) (Muller & Schmid-Hempel 1992). Scenario E and E' (Figure 1) are thus not very realistic (c.f. Figure 1).

(c) A plausible way to sustain colony growth while not having too long-lived workers may be to increase birth rate and thus worker turnover rate (C'& C in Figure 1). In Chapter 2, I demonstrated that many short-lived workers might effectively protect against a spreading disease. Colonies with many short-lived workers may even reach a higher critical colony size; this, however, depends on the relative epidemic potential of factors colony size and worker longevity (Chapter 3). Above the critical density however (C’, Figure 1), further anti-parasite behaviour may have evolved due to a possible cost associated with increased worker production (low individual resistance, Chapter 3).

(d) Strong selection for increased colony size and thus long-lived workers (O'Donnell & Jeanne 1992; Carey 2001) may have favoured additional anti-parasite adaptations to overcome the cumulative effects epidemic effects of density and worker lifespan (D, Fig 1.) These behavioural adaptations may include:
(i) Changes in the mating system (polygyny, polyandry) that increase genetic diversity and thus decrease the potential for parasite spread (Shykoff & Schmid-Hempel 1991; Liersch & Schmid-Hempel 1998; Baer & Schmid-Hempel 1999; Tarpy 2003; Hughes & Boomsma 2004; Tarpy & Seeley 2006). Genetic heterogeneity may also have allowed for more elaborate caste structure (colony organization) in these colonies. There is indeed evidence that task affiliation can have a genetic component (see (Page et al. 1995) and references therein).

(ii) Behavioural adaptations that reduce the uptake, establishment and spread of the parasite in the colony (e.g. avoidance behaviour, removal and inactivation of infectious spores, nest hygiene and immunity transfer by social interactions (Cremer et al. 2007; Walker & Hughes 2009; Bos et al. 2011; Konrad et al. 2012; Tragust et al. 2013). Age related behaviour moreover may have introduced heterogeneity in an otherwise homogenous structure (e.g. as in the conveyer belt model (Schmid-Hempel 1998). Overall, the transmission of a parasite may become more difficult as colony structure gains in complexity (Naug & Camazine 2002).

Together this scenario suggests that parasite pressure against colony size and worker longevity could act as a selective force for the evolution towards complex social structure, eventually leading to caste formation, with ergonomic benefits following suite rather than be the prime or only source of selection. A comparative study on colony size worker longevity and the degree of complexity among social insect species would allow further assessing the applicability of this idea. However, while this is an idealized sketch and not all lineages may have followed this path, this scenario integrates the research I did in this thesis and recalls the importance of parasitism on colony structure, patterns and processes that are worthwhile for further investigation.
References


