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Host variability and parasitism in *Bombus terrestris*

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For Mike Johnson
1937-2006

"I beg a million pardons. Abuse me to any degree, but forgive me: it is all an illusion (but almost excusable) about the bees. I do so hope that you have not wasted any time from my stupid blunder. I hate myself, I hate clover, and I hate bees."

Charles Darwin

Letter to John Lubbock (Lord Avebury).
Cliff Cottage, Bournemouth,
Wednesday, September 3rd 1862.

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Zusammenfassung

Es wird angenommen, dass Parasiten und die genetische Variation in ihren Wirten sich gegenseitig beeinflussen. Parasiten müssen sich in einem Wirt fortpflanzen und auf neue Wirte überspringen, wenn sie überleben wollen. Parasiten können theoretisch leichter zwischen genetisch ähnlichen (homogenen) Wirten übertragen werden, und können sich auch leichter an die entsprechenden Genotypen anpassen und sie effizienter ausbeuten. Auf diese Weise werden sie in einem bestimmten Genotyp virulenter, auf Kosten der Fähigkeit, auch andere Genotypen effizient ausbeuten zu können. Eine heterogene Wirts-Umgebung limitiert theoretisch diese Entwicklung hin zu grösserer Virulenz, indem sie den Parasiten zwingt, eine generalistische anstatt einer spezialisierten Strategie auszubilden.

Parasitismus könnte ebenso zur Erhaltung einer genetischen Diversität in Wirts-Populationen führen. Es wird erwartet, dass die Anpassung der Parasiten an genetische Variationen in Wirts-Populationen zu negativer frequenzabhängiger Selekion und damit zu Zyklen in den Wirts-Genotypen führt (die “Red Queen”-Hypothese). Häufige Wirts-Genotypen leiden an übermässiger Infektion durch gut adaptierte Parasiten und erleiden dadurch einen Verlust an Fitness, was wiederum zur Ausdünnung des Genotyps führt. Seltene Genotypen leiden weniger an den Beeinträchtigungen durch Parasiten, da weniger Parasiten an sie angepasst bleiben, und kommen dadurch zunehmend häufiger vor. Diese Dynamik wurde von der Theorie vorhergesagt, und könnte eine wichtige Rolle in der Wahrung sexueller Fortpflanzung und Rekombination spielen, sie wurde jedoch kaum tatsächlich in der Natur nachgewiesen.

In dieser Arbeit stelle ich Untersuchungen zu diesen zwei Aspekten der wirtsgenetischen Heterogenität und des Parasitismus anhand der Erdhummel *Bombus terrestris* vor. Ich habe anhand des Darmparasiten *Crithidia bombi*, der in Population von entweder heterogenen oder homogenen Wirten weitergegeben wurde, nicht feststellen können, dass wirtsgenetische Heterogenität eine Einschränkung für die Entwicklung von Virulenz darstellt. Entgegen meinen Erwartungen zeigt *Crithidia bombi* nach mehreren Durchgängen durch entweder heterogene (Hummeln aus zwei Kolonien) oder homogene (Hummeln aus einer Kolonie) Umgebungen keine Unterschiede in der Virulenz, wie anhand der Grösse der Eierstöcke der Hummeln, ihrem Körperfettanteil und ihrer Überlebensdauer bei Nahrungsentzug festgestellt wurde. Da die Hummeln mit einem Cocktail von verschiedenen Stämmen infiziert wurden, könnte die Übertragung der Parasiten in einer Bevorzugung einzelner Stämme aufgrund starker genetischer Interaktion zwischen Wirt und Stamm resultieren, anstatt in einer direkten Konkurrenz zwischen Stämmen oder einer Anpassung an den Wirts-Hintergrund und der Entwicklung höherer Virulenz.

Ich stelle ausserdem die Resultate zweier Untersuchungen vor, die sich mit einigen der Vorhersagen der Red Queen-Hypothese befassen. Im Red Queen-Szenario wären mehrere Genotypen von Hummeln in einer Population zu erwarten, einige davon häufig und andere selten. Die häufigen Genotypen sollten mit Parasiten überinfiziert sein, und alle Genotypen sollten aufgrund des parasitären Einflusses unter negativer frequenzabhängiger Selektion stehen. Ich habe eine Sektion mitochondrialer DNS (die nicht-kodierende intergenetische Sequenz zwischen den Cytochrom-Oxidase-Genen I und II) benutzt, um Hummeln jeweils einem Haplotypen zuzuordnen. Ich habe in beiden untersuchten Population – Zürich, Schweiz und Gotland, Schweden – mehrere verschiedene Haplotypen unterschieden, wobei zwei Haplotypen in beiden Populationen häufig auftraten. Ein Experiment zur Feststellung der Infektionsraten und der Fitness dieser verschiedenen Haplotypen hat gezeigt, dass häufige Haplotypen in der Tat einen Trend zu mehr Parasiten aufweisen, dass jedoch die seltenen Haplotypen in Bezug auf Grösse der Kolonien und sexueller Reproduktion schlechter gestellt sind. Die Daten zeigen weiterhin einen Trend in einem der häufigen Haplotypen – Haplotyp A – zu grösserer Fitness und höherer Parasiten-Belastung als im anderen häufigen Haplotyp, Haplotyp B.

In Zürich variiert die Häufigkeit der Haplotypen von einem Jahr zum nächsten, obschon das in keinem offensichtlichen Zusammenhang zum Parasitenbefall zu stehen scheint. In den meisten Jahren ist Haplotyp A der häufigere. In Gotland ist in den meisten untersuchten Jahren und Jahreszeiten B der häufigste Haplotyp; im Sommer 2002 ändert sich dies jedoch dramatisch, und Haplotyp A ist der häufigste. Wiederum scheint dieser Wechsel nicht vom Einfluss der Parasiten abzuhängen. Diese Resultate werden in Anbetracht möglicher Unterschiede in der mitochondrialen Enzymaktivität diskutiert, sowie der vielversprechenden Möglichkeit, neu entwickelte “Quantitative Trait Loci”-Marker zu benutzen, um Hummeln in Kategorien einzuteilen, die in direkterem Zusammenhang zu in Wirts-Parasiten-Interaktionen involvierten Genen stehen.

Summary

Host genetic variation and parasites are thought to interact in a number of ways. Parasites must reproduce inside the host and transmit to new hosts if they are to survive. When hosts are genetically similar (homogeneous), parasites can in theory transmit more easily, and can also adapt to better exploit that specific genotype. They thus become more virulent in this one genotype at the expense of being able to exploit other host genotypes. A heterogeneous host environment theoretically constrains this evolution of virulence by forcing the parasites to adopt a generalist rather than specialist strategy.

Parasitism could also lead to the maintenance of host genetic diversity within populations. Parasite adaptation to genetic variation within host populations is expected to lead to negative frequency-dependent selection and therefore cycles of host genotypes (the Red Queen hypothesis). Common host genotypes will suffer from overinfection by adapted parasites and suffer a concomitant loss of fitness, driving them to become rare. Rare genotypes escape the fitness consequences of parasitism as fewer parasites remain adapted to them, and will become more common. These dynamics have been predicted in theory, and could have an importance in the maintenance of sexual reproduction and recombination, but have rarely been shown to be occuring in nature.

In this thesis I present investigations into these two aspects of host genetic heterogeneity and parasitism in the bumblebee, *Bombus terrestris*. I found that host genetic heterogeneity does not constrain the evolution of virulence when the trypanosome *Crithidia bombi*, a gut parasite of bumblebees, is serially passaged through a population of heterogeneous versus a population of homogeneous hosts. Contrary to my expectations, *Crithidia bombi* shows no difference in virulence after several passages in either heterogeneous (bumblebees from two colonies) or homogeneous (bumblebees from one colony) environments as shown by host ovariole size, fat body weight and survival under starvation conditions. As the bumblebees were infected with a cocktail of several strains, the passage of parasites may result in strain sorting caused by strong genetic interactions between host and strain, rather than direct strain competition or adaptation to the host background and the evolution of higher virulence.

I also present results of two investigations into some of the predictions made by the Red Queen hypothesis. Under a Red Queen scenario, one would expect to see multiple genotypes of bumblebees within a population, some common and some rare. The common genotypes should be overinfected by parasites, and genotypes should be under negative frequency-dependent selection due to this parasite pressure. I used a section of mitochondrial DNA (the

non-coding intergenic sequence between cytochrome oxidase I and II genes) to assign bumblebees to haplotype. I found multiple haplotypes within both the populations studied – Zürich, Switzerland and Gotland, Sweden – with two haplotypes being common in both populations. An experiment to ascertain the infection rates and fitness of these different haplotypes showed that common haplotypes do indeed show a trend to having more parasites, but that rare haplotypes fare much worse in terms of colony size and sexual production. The data shows a trend that one of the common haplotypes – haplotype A – has higher fitness and higher parasite loads than the other common haplotype, haplotype B.

Across years in Zürich, the frequency of haplotypes A and B varies, although this does not seem to be obviously related to parasites. In most years haplotype A is the most frequent. In Gotland, haplotype B is is the most common in most years and seasons sampled, although in summer 2002 this changes dramatically, and haplotype A is the most common. Again, this change does not appear to be due to parasite pressure. These results are discussed with reference to possible differences in mitochondrial enzyme activity, and the exciting possibility of using newly developed Quantitative Trait Loci markers to assign bumblebees into types which are more directly linked to genes involved with host-parasite interactions.

Chapter 1

Introduction

Almost every known species suffers from at least one parasite or pathogen, ranging from large ectoparasitic mites to parasitic fragments of DNA or protein. This means that the vast majority of extant species are parasitic (Price 1980) – with important consequences for evolution. Traditionally, change through natural selection was seen in terms of adaptation to the physical environment and to food availability; now antagonistic coevolution with other organisms is recognised as a major driving selection pressure. Examples include conflicts between males and females, and interactions of parasites and hosts or predators and prey, and vice versa.

This thesis examines the effect host genetic diversity has on parasite virulence, the effect of parasites on host genetic diversity, and how host genetics change over time. The specific aim has been to test the Red Queen hypothesis, which states that parasites cause dynamic cycling of host genotypes, as parasites adapt to the common types, causing a reduction in fitness (Howard & Lively 1998). The study system I used is the bumblebee species *Bombus terrestris*, and its parasites — mainly *Crithidia bombi*, but also examining *Nosema bombi* and various mites, nematodes and parasitoids.

The introduction is divided into three main sections — the first focusses on host-parasite interactions: explaining the nature of virulence and describing the Red Queen model of host-parasite coevolution. The second main section details the system of *Bombus* and its parasites. I then give brief chapter outlines and a summary of my research questions and aims.

Host–parasite interactions

Virulence

Virulence is a common term that has a rather general meaning — in the Oxford Dictionary it is defined as "*adj* **1** [esp attrib] (of a disease or poison) extremely harmful or deadly: *a virulent strain of flu.* **2** (*fml*) strongly and bitterly hostile: *virulent abuse* ○ *make a virulent attack on the press* ○ *a particularly virulent form of racism.*". In addition, it is colloquially used to denote not

only a debilitating disease, but one that spreads quickly and easily (ie. has a high transmission rate). Different areas of science also define it in different ways. In the field of plant pathology, for example, virulence is simply the ability of a pathogen strain to infect the host and reproduce, in other words, to defeat the resistance genes of a particular host (Schaner et al. 1992). An avirulent strain does not overcome this resistance, and cannot infect the host at all.

Here I will use the standard evolutionary definition of virulence, as a parasite mediated reduction in host fitness (Bull 1994). This refines the concept of harm — somatic damage is immaterial in evolutionary terms if it does not affect the ability of an individual to pass on its' genes to future generations. An avirulent strain under this definition could still infect a host and reproduce, but it would cause no reduction in host fitness. The cause of this fitness reduction varies with the life histories of host and parasite. Parasites can cause a reduction in host fitness in a variety of ways, for example, they can cause the host to mount a costly immune response (Moret & Schmid-Hempel 2000); they may reduce host fitness by changing host behaviour (e.g. Müller 1994).

It was previously believed that a pathogen would always become less virulent as it adapts to its host, and that host–parasite relationships would thus become mutualistic or symbiotic in time. The underlying assumption was that reducing host fitness reduces the parasite's own fitness. However, both theoretical and empirical studies have shown that if the parasite can be transmitted on shorter time–scales than the host's lifespan, then it can pay the parasite to increase it's virulence and even seriously harm the host (Frank 1996). Intra-host competition of parasite strains also typically leads to an increase in virulence in models, irrespective of whether this increases transmission rate (Levin & Bull 1994, Nowak & May 1994).

Now it is understood that there is usually a relationship between transmission and virulence (Anderson & May 1982, Ganusov & Antia 2003, Galvani 2003). Virulence generally confers a transmission benefit as well as a cost and this prevents the evolution of zero virulence in most systems. The cost of virulence is less important to the parasite if it can transmit quickly. Transmission can be facilitated by high population density, high frequency of susceptible hosts within the population and certain transmission pathways. In general, parasites that can live outside the host, or become dormant (for example as spores) for long periods can also be very virulent because they do not need to keep the host alive in order to be transmitted. If a host population is decimated, they can simply remain dormant until another suitable host appears (Bonhoeffer et al. 1996). Under such circumstances, much more virulent parasites can evolve. This can be seen in human populations, where in historical times the growth of cities has allowed the development of increasingly virulent diseases than a hunter-gatherer or subsistence farming population at much lower population densities allows. Examples of this include cholera outbreaks and the plague in medieval europe (Ewald 1994).

The relationship between virulence and transmission, and how this influences virulence evolution has been formalised by R_0, the basic reproductive rate of a parasite or pathogen (Anderson & May 1979a, Anderson & May 1979b, Anderson & May 1982, May & Anderson 1983). This is the number of secondary infected hosts when one infected individual is placed into an entirely susceptible host population. R_0 must be greater than one in order for a parasite to establish an infection within a new population, as the parasite produces more than one infected host. As sexual organisms must produce at least 2 offspring to maintain population size, so a parasite needs an R_0 greater than one to establish itself within a host population.

$$R_0 = \frac{\lambda(\alpha,N)}{\alpha + b + v(\alpha)}$$

λ is the transmission rate, N the host population size, α the virulence (here the increase in the death rate caused by the parasite), b the *per capita* death rate in the absence of infection and v the recovery rate of infected hosts. From this equation it can be seen that R_0 increases with increased transmission rate, and with increased population size (see p. 84, May & Anderson 1983), as well as with increased virulence. It also shows that transmission rate and recovery rate are also dependent upon the virulence — transmission rate increasing with increased virulence and recovery rate decreasing with increased virulence. If this were not the case, then virulence would indeed always evolve to zero. The level of virulence that is expected to evolve is that which maximises R_0 (the optimal level of virulence: see Figure 1.1, page 3). Evidence for the evolution to optimal virulence is scant (Bull 1994), but has been recently found in a castrating parasite (Jensen et al. 2006).

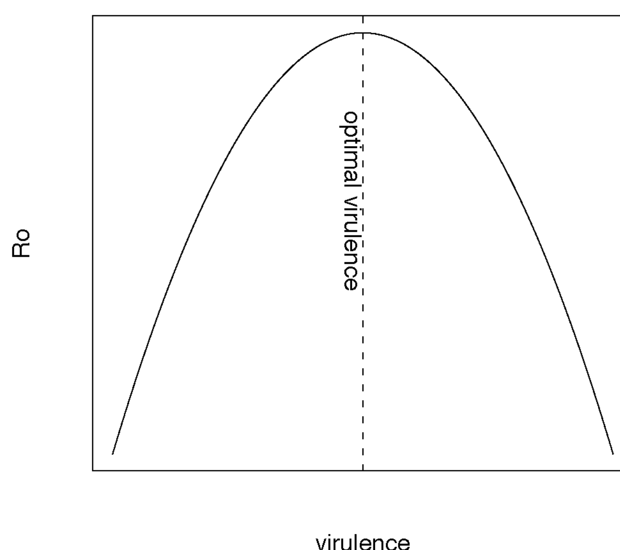

Figure 1.1: *Optimal virulence and R_0 — before optimal virulence is reached, an increase in virulence affects transmission more than host mortality. R_0 then decreases after optimal virulence is reached, as infected individuals die before they have a chance to transmit the parasite to new hosts.*

Within-host competition between parasite strains can increase virulence to “maladaptive” (with respect to R_0) levels, because a faster reproducing genotype gets a larger share of host resources, and will outcompete less aggressive strains, even if this reduces its hosts lifespan to such a degree that it is not transmitted to any new hosts. Hence, if multiple infections are common, then virulence may be higher than expected to optimise transmission (Nowak & May 1994). Host heterogeneity is expected to limit the evolution of virulence, as either the parasite will adapt to one type and be concomitantly less effective in others (Ebert & Hamilton 1996, Ebert 1998, Yourth & Schmid-Hempel 2006) or will adopt a generalist strategy to be able to infect both or all types equally (Regoes et al. 2000). However, at least one set

of models predicts that virulence in terms of host mortality may be increased by host heterogeneity (Ganusov et al. 2002).

The assumptions of the classical R_0 models is that hosts live in a freely-mixing population. However, host population structure can play an important role in the evolution of virulence, because it determines how a parasite adapts to the population (Boots et al. 2004). Spatial structure can make it more difficult for a parasite to be transmitted (for example, see Herre 1993).

The Red Queen

Coevolution occurs when one species imposes a selection pressure on another species, leading to evolutionary change and a reciprocal selection pressure on the first species (Janzen 1980, Thompson 1989). A typical example of this is between host and parasite. For example, Hudson et al. (1998) showed that fluctuations in grouse populations were stopped by treatment against a parasitic nematode.

When parasites cause a a reduction in host fitness (are virulent), hosts are under selection to overcome this fitness cost. When the hosts defences cause a reduction in fitness in the parasite, this leads to selection on parasites (see Webster et al. 2004).

Host responses involve the immune system (especially if one considers skin or exoskeleton as part of that system). The host mounts either a generic response or develops a specific response to that parasite strain, and the parasite must try to escape this.

There are clasically two main models of how the underlying genetics determine the direct interactions of parasite and host. Gene-for-gene interactons, which is generally assumed in the plant literature (Flor 1956, Thompson & Burdon 1992, Frank 1992), involves parasites having virulence alleles which allow the parasite to infect more host types, and hosts having resistance alleles, allowing them to escape from being infected. Matching alleles (Frank 1993), which is commonly assumed when studying invertebrates, is based on the self-nonself recognition system (Grosberg & Hart 2000) and considered to reflect the biochemistry of parasite recognition by a host (Frank 1994). This involves a range of host resistance alleles, which each confer resistance to one of a range of parasite alleles. There is limited knowledge of what these relationships actually look like in natural populations (Sorci et al. 1997, Kover & Caicedo 2001). These two models can be seen as a continuum, with pure gene-for-gene interactions at one extreme, and pure matching-alleles at the other, but with all other shades in between (Agrawal & Lively 2002). It is also possible that both are appropriate, in different contexts, for example gene-for-gene could govern infection and matching-alleles determine whether the parasite reproduces or not (Agrawal & Lively 2003).

The Red Queen Hypothesis was formulated originally by Leigh Van Valen to explain his "law of constant extinction" — based on patterns of species extinction in the geological record (Van Valen 1973) — which suggests that species go extinct mainly because of competition with other species (Anon 1973). He suggested that species competing within a particular environment are engaged in a zero-sum game where an improvement in fitness of one species is met with a concomitant reduction in fitness spread over all of the other species in that environment. The "law" that he put forward, as well as whether the Red

Queen hypothesis explained the findings, was the subject of much debate at the time (Foin et al. 1975, Van Valen 1975, Hallam 1976, Van Valen 1976, Maynard Smith 1976)

The Red Queen is a character from Alice Through the Looking Glass by Lewis Carroll:

> "Alice looked round her in great surprise. 'Why, I do believe we've been under this tree the whole time! Everything's just as it was!'
>
> 'Of course it is,' said the Queen, 'what would you have it?'
>
> 'Well, in OUR country,' said Alice, still panting a little, 'you'd generally get to somewhere else – if you ran very fast for a long time, as we've been doing.'
>
> 'A slow sort of country!' said the Queen. 'Now, HERE, you see, it takes all the running YOU can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!'
>
> 'I'd rather not try, please!' said Alice. 'I'm quite content to stay here...' "

The Red Queen metaphor was later taken up by Bell (1982). It describes a situation where outwardly parasite virulence and population size do not seem to change, but the parties involved (host and parasite) are both "running" as fast as they can in evolutionary terms, in order that one does not gain the advantage. He also points out that under this scenario the purpose of sex and recombination is the production of rare genotypes. This idea was previously pointed out by Jaenike (1978), although he did not explicity link this idea to the Red Queen hypothesis. The idea of the Red Queen was extended by Hamilton to explain the effects of parasites on a host species, especially with regards to recombination and sexual reproduction (Hamilton 1980, Hamilton et al. 1990, Ebert & Hamilton 1996).

The Red Queen Hypothesis is one of the leading theories to explain the evolution and maintenance of sexual reproduction and recombination. Theoretical models show that increased recombination can be selected for under changing environments such as parasitism (Hamilton et al. 1981, Barton 1995, Otto & Michalakis 1998, Peters & Lively 1999) and some field and experimental studies show that increased recombination is selected for under parasitism (Kovalchuk et al. 2003, Fischer & Schmid-Hempel 2005), and that genetic diversity, at least in immune alleles such as MHC in vertebrates, is advantageous under parasite pressure (e.g. Wegner et al. 2003). For example, studies of New Zealand freshwater snails, which can self-fertilise or mate with another individual, have found that the most common clonal types within a lake are the most parasitised, and that those areas under highest parasite pressure show the highest rates of sexual reproduction (Dybdahl & Lively 1998, Lively & Dybdahl 2000). It is likely that the Red Queen is a useful explanatory model for the maintenance of sex, especially when combined with other models (see West et al. 1999, Milinski 2006).

Although the Red Queen paradigm is mainly used in the study of the evolution and maintenance of sex, Hamilton also proposed that the coevolution of species could maintain genetic diversity within populations (Hamilton 1982, Judson 1995). This is irrespective of sexual reproduction, as just as much diversity could be maintained in clonal populations through this mechanism (Haag & Ebert 2004, Sasaki et al. 2002).

Mathematical models of host-parasite coevolution predict dynamic cycling of host genotypes within a population (Seger 1988, Seger & Hamilton 1988). This is due to time lagged

negative frequency-dependent selection. Rare host genotypes escape parasitism, and related costs, and so increase in frequency in the population. The common genotypes suffer a concomitant increased cost of parasitism because of parasite adaption, and become rare. There is then a time-lagged adaptation of parasites to the currently common host genotype. This seems to be held up in a few studies (for example Dybdahl & Lively 1998). Rare types could be created faster with sexual reproduction, but clonal types within a population would also experience such dynamic frequency dependent selection pressure. This sequence is illustrated simply in Figures 1.2 and 1.3.

The Red Queen hypothesis has mostly been studied in facultatively sexual species, to evaluate its relevence to the evolution and maintenance of sex. In my thesis I examine Red Queen dynamics in a natural population of the sexual bumblebee.

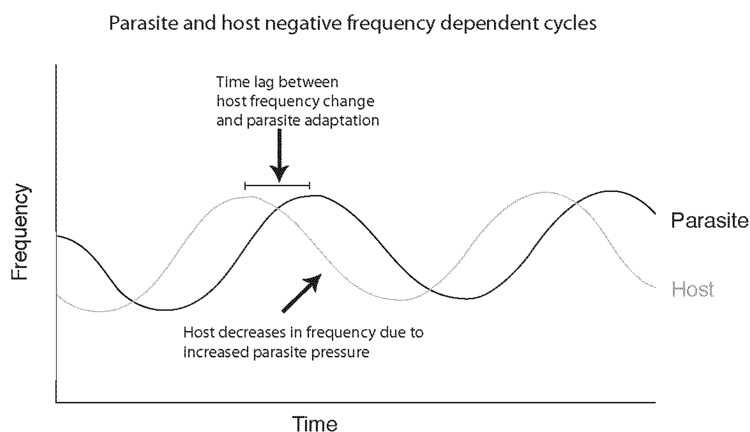

Figure 1.2: *Negative frequency dependent cycles of host and parasite*

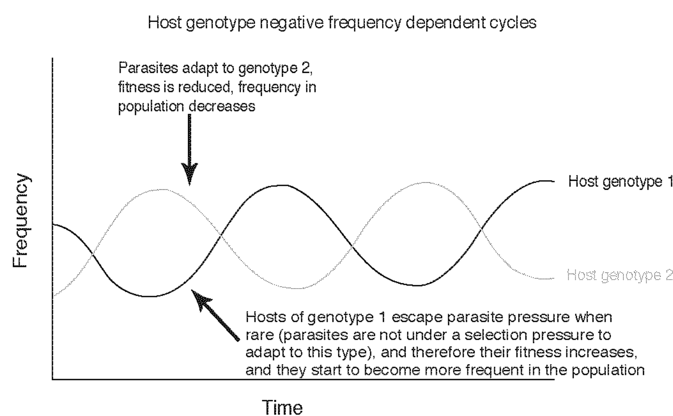

Figure 1.3: *Negative frequency dependent cycles of host genotypes. Over time a high diversity of host genotypes is maintained; loss is rare.*

The System: *Bombus*

Bumblebees occur naturally on all continents, apart from sub-Saharan Africa and Australasia — although European bumblebees have been introduced to New Zealand and Tasmania. There are probably around 250 separate species worldwide (Williams 1998) with vastly differing ecologies, ranging from those found in the Himalaya region to those in the Amazon rainforest. I have worked on European (Palearctic region) bumblebees, and almost exclusively on the buff-tailed bumblebee (German: Dunkle Erdhummel), *Bombus terrestris* (Linnaeus). This section will therefore cover the annual life cycle and parasites of this particular species.

Why bumblebees?

Bumblebees are a well studied system (Alford 1975, Goulson 2003, Heinrich 2004) which are amenable both to being reared in laboratory conditions, and to field studies. The bumblebee is an economically important pollinator of fruit and tomato plants (Velthuis & van Doorn 2006). A greater understanding of how genetic diversity in hosts interacts with parasites will hopefully allow better breeding and rearing methods to be developed. Bumblebees are also genetically close to honey bees (they both belong to the Family Apidae - long-tongued bees), and any insights from the bumblebee system may be generalisable to honeybees as well.

Life cycle

The annual life cycle of the bumblebee and of its microparasites is shown in Figure 1.4, page 9.

Queens emerge from hibernation in spring. They search for a site to found a colony and start to forage. The queen lays a first clutch of eggs and rears the initial brood of workers. Then the queen remains in the nest as the workers forage to raise more workers, and eventually sexuals. A colony can grow to between 100 and 600 individuals in *Bombus terrestris*, and in rare cases reach 800 to 1000 workers (von Hagen & Aichhorn 2003). In mid- to late summer, the colony — if successful — will start production of sexuals. Many, but not all, colonies will produce males, but queens are very costly, and many colonies will not produce any. Males set up patrolling areas and wait for queens to visit and mate. Most central European bumblebees are singly mated, with *Bombus hypnorum* as a notable exception (Schmid-Hempel & Schmid-Hempel 2000). After mating, queens search for a hibernacle, and are dormant through the winter. All workers and males die at the end of the summer (Prŷs-Jones & Corbet 1991).

Parasites

The bumblebee suffers from a range of parasites (MacFarlane et al. 1995, Schmid-Hempel 2001), which is encouraged by the nest environment. Once in a nest, a parasite can easily spread from one individual to another, as they are genetically similar. The main parasites studied are protozoan gut parasites, parasitoid flies and ectoparasites (Schmid-Hempel 1998). Unfortunately very little is known about the bacteria or viruses present in bumblebees, although

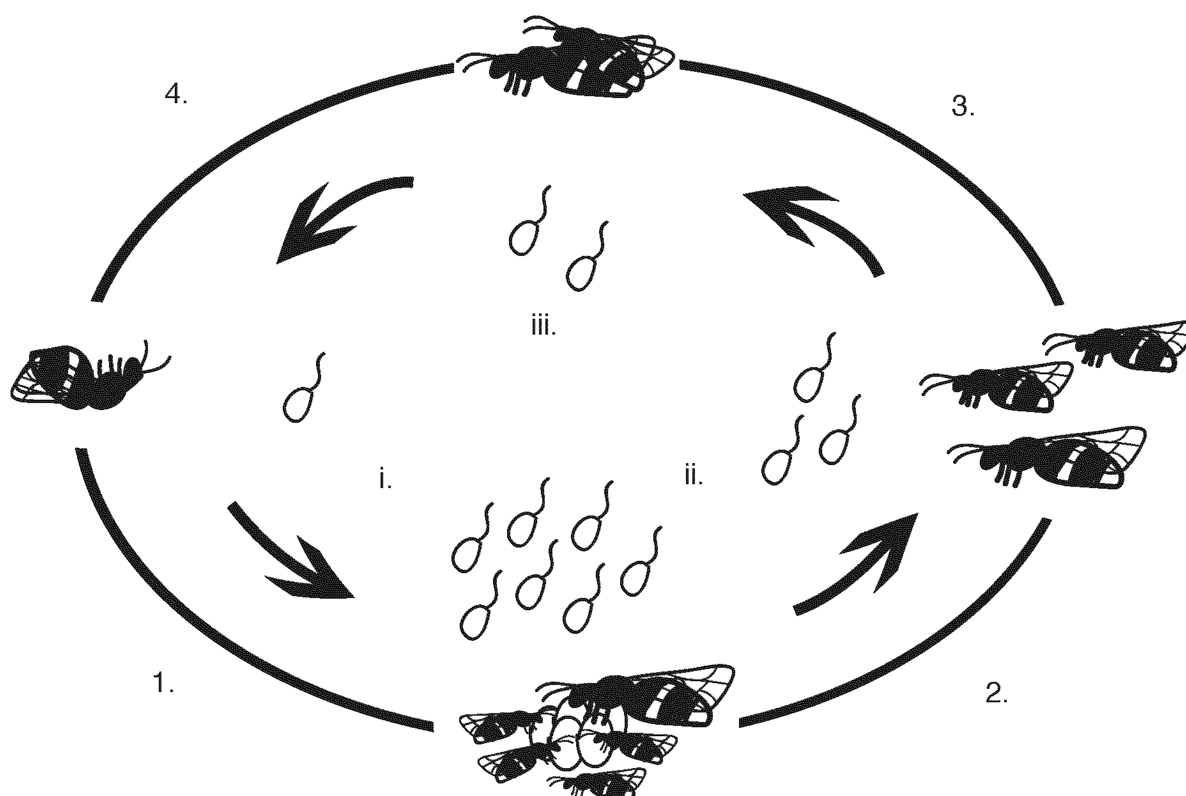

Figure 1.4: *The life cycle of the bumblebee and of its microparasites. 1. Emergence of queens, colony founding; 2. Colony growth; 3. Production of sexuals; 4. Hibernation; i.-iii. show microparasites which increase in numbers throughout the colony cycle, but can only pass through queens from one generation to the next*

it is known that the haemolymph contains a potent antimicrobial agent (Schmid-Hempel 2005). Social insects are interesting from the point of view of host-parasite evolution because they have high population densities within colonies of genetically similar individuals, allowing, presumably, easy transmission between hosts for a parasite (Schmid-Hempel 1998). *Bombus terrestris* is a primitively eusocial insect. A colony is founded by a single queen who has mated once, which means that genetic variability within *Bombus terrestris* colonies is not very high. It has been shown that artificially creating polyandrous colonies reduces parasite loads within colonies (Baer & Schmid-Hempel 1999). In honeybees, which are arguably under greater threat from parasites due to the size and longevity of their colonies, a queen can mate naturally with up to 15 – 20 males (Fuchs & Moritz 1998), which has been shown to decrease parasite load in the colony (Tarpy & Seeley 2006, Seeley & Tarpy 2007).

Infectious microparasites which don't form spores can only survive the winter in a host (ie a hibernating queen), and are therefore most likely subjected to genetic bottle necks (see Figure 1.4, page 9). Parasites that are fortunate enough to make it through the winter in a queen cannot guarantee that this queen will produce a colony, and certainly not sexuals, at the end of a summer season.

When infected queens start feeding the parasites may be passed on to uninfected individu-

als through flowers. The microparasite *Crithidia bombi* has been shown to be passed between individuals when foraging (Durrer & Schmid-Hempel 1994). *Nosema* is less well understood, but it is assumed that they are passed between individuals in the same way, although it appears to be more difficult. Microparasites benefit from the summer increase in host numbers within the colony in which individuals are genetically similar and therefore transmission presumably easy, and in the general population (Imhoof & Schmid-Hempel 1999).

My main study parasite, *Crithidia bombi* (Lipa & Triggiani 1980), exhibits conditional virulence. When an infected bee is kept in the lab, and fed *ad libitum*, we do not see a negative effect of infection. However, during the colony founding stage of the host *Bombus terrestris*, as queens emerge from hibernation (Brown, Schmid-Hempel & Schmid-Hempel 2003), *Crithidia* infection reduces the queen's ability to start a colony. Under difficult conditions, such as lack of food (Brown et al. 2000), *Crithidia* increases mortality rate by 50%. *Nosema bombi*, another important parasite of bumblebees, has a complex virulence, sometimes seeming to increase the number of queens produced by a colony (Imhoof & Schmid-Hempel 1998b) and other times decimating a colony entirely (Otti, pers. comm.).

Thesis outline

My work concentrated on the effect of host genotype and variability in host genotype on parasite characteristics such as virulence and prevalence, as well as the reverse questions of the effects of parasites on the fitness and variability of host genotypes. Chapter 2 covers an experiment on host genetic variability and its effect on parasite virulence measured as host mortality under starvation conditions. We found no difference in virulence between parasites serially passaged through host bees from the same colony – genetically homogeneous – and those passaged through bees taken from two colonies.

The rest of my work (Chapters 3 and 4) used a haplotype marker (a section of mitochondrial DNA) to assess bumblebee "types" within populations. Chapter 3 details a field experiment carried out to test fitness differences and parasite rates of common and rare *Bombus terrestris* haplotypes. The main parasites I investigated were *Crithidia bombi* and *Nosema bombi*. Chapter 4 looks at changes in host haplotype frequency and parasites over time in an island population (Gotland, Sweden) and a continental population (Zürich, Switzerland).

Chapter 2

Virulence evolution in homogenous and heterogenous host environments

Introduction

Virulence is usually defined in evolutionary biology as parasite-mediated host morbidity or mortality (Poulin & Combes 1999, Galvani 2003), that is, the negative effects of a parasite on host fitness. Recent theoretical and empirical studies suggest that virulence can be considered an evolved trait that maximizes parasite fitness in a given host population (Ebert 1998), the exact level being dependent on a multitude of factors. Amongst those, heterogeneity, and especially genotypic heterogeneity, is one of the most obvious factors that characterise host populations. However, few theoretical and empirical studies address the effect of host heterogeneity on the evolution of virulence (Pfennig 2001). It has been predicted that when in a homogeneous host population, virulence should increase, but that when a parasite evolves in a heterogeneous host environment virulence increase is constrained (Regoes et al. 2000). This effect is due to a trade-off between the exploitation of different host types. If a parasite can specialise on one host line, then it can adapt to that line and increase its virulence. Host heterogeneity constrains this by forcing the parasite to adopt a more generalist strategy. Here, we experimentally tested whether the virulence of a parasite changes in relation to host genetic heterogeneity.

In our study organism – the bumblebee, *Bombus terrestris* – previous studies have shown that an increase in genetic variation within a colony leads to lower parasite loads in the field (Liersch & Schmid-Hempel 1998, Baer & Schmid-Hempel 1999, Baer & Schmid-Hempel 2001). Bumblebees are annual social insects. The queen emerges from hibernation in the spring and forages before looking for a site to found her colony. When she has enough resources and has found a suitable site (an old mouse nest, hollow tree etc.) she will start to build her nest and lay eggs. She provisions the larvae with pollen and nectar, and roughly a month later the first generation of workers will eclose and take over foraging duties. Towards the middle to end of the summer, if the colony has grown large enough, sexuals may be produced. These sexuals will stay in the nest for a short while before embarking on a search for a mate (*B. terrestris* queens mate with only one male). Queens will then search for a hibernation site and bed down for the winter, whilst the old queens, workers and males will die. The cycle begins anew

in spring.

In the field, bumblebees are host to a range of parasites (MacFarlane et al. 1995). In particular, the intestinal trypanosome, *Crithidia bombi* (Lipa & Triggiani 1980), is very common (Shykoff & Schmid-Hempel 1991b). Workers pick up novel infections during flower visits (Durrer & Schmid-Hempel 1994), such that most colonies in natural populations will eventually become infected during the seasonal cycle (Imhoof & Schmid-Hempel 1998b). *C.bombi* affects the fat body and ovary development in workers (Shykoff & Schmid-Hempel 1991a) and has been shown to exhibit condition-dependent virulence depending on the state of the bee (Brown et al. 2000). In addition, the prevalence and intensity of infections by this intestinal trypanosome is significantly affected by the genotypic variance within a colony (Baer & Schmid-Hempel 1999) and by the identity of particular genetic lines (Baer and Schmid-Hempel, in prep.). Several studies have directly demonstrated the underlying genotype - genotype interactions with its host, *B. terrestris* (Schmid-Hempel & Schmid-Hempel 1993, Schmid-Hempel et al. 1999, Schmid-Hempel 2000).

Here, we report the results of two independent experiments carried out using a serial passage set up, to test the prediction that virulence evolves to lower values in genetically heterogeneous populations as compared to virulence in homogeneous populations. This prediction matches empirical evidence that serially passaging parasites through a single host type can increase virulence towards that particular host type while it lowers virulence in the former host (Ebert 1998).

Materials and Methods

Two independent experiments were carried out, the first one of which in the winter of 1997. Twelve laboratory colonies of *B. terrestris* were used as sources for uninfected animals. For this purpose, queens were reared in the lab (using 2nd generation queens derived from field-caught mother colonies) in October/November 1997 under standard conditions (20°C, 60% humidity, food ad libitum). Workers were removed from their colony, starved for two hours and then fed with 15 μl (5 μl medium – plus a standard inoculum of around 300,000 *C. bombi* cells for infected treatments – and 10 μl sugarwater) according to the treatment. Experimental boxes were set up which contained either four workers from one uninfected colony (homogeneous treatment), or two workers each from two uninfected colonies (heterogeneous treatment) (see 2.1). These were set up in a pairwise fashion, so each uninfected colony contributed to one homogeneous and one heterogeneous treatment. The cocktail of *Crithidia*, which contained equal numbers of cells collected from each of six infected colonies, was fed to each of the four starting bees in sugar water. Two bees were removed at random from the boxes after five days, and replaced with new bees. This then proceeded at five day intervals, removing the oldest two bees, five times.

At the end of the period of selection, faeces were taken from the remaining bees, and the *Crithidia* was used to infect eight test bees from each colony, of a standard age (four days). The test bees were infected either with homogenous treatment *Crithidia*, heterogeneous treatment *Crithidia* or were left uninfected. They were then placed singly in plastic boxes (12 $\times$ 10 $\times$ 7 cm) and freeze-killed after 8 days. Before the experiment began a test was also carried out on the pre-selection *Crithidia* cocktail. The bees were kept in a freezer (-20°C) and dissected

later. Wings were embedded in Eukitt® and the length of the radial cell of the right wing was measured as a size control (Owen 1988). As tokens for individual host condition, the three largest oocytes were measured during dissection, and then the fat content was measured by drying the abdomen for three days at 70 °C, weighing for pre-fat-removal weight, then placing in 2 ml of ether for 24 hours. The abdomens were then washed with fresh ether and dried in the oven once more for three days at 70 °C. The weight after this gives the post-fat-digestion weight, and the difference is the amount of fat (Ellers 1996).

The second experiment, using different measures of host condition, was carried out using spring queens of *B. terrestris*, collected from around Zürich and Basel, Switzerland in March 2000. Colonies were raised as above. Twelve uninfected colonies and six infected colonies were then chosen at random for this experiment. Selection proceeded as explained above.

At the end of the period of selection, faeces were taken from the remaining bees, and the *Crithidia* numbers were standardised to around 10,000 cells per bee, and used to infect eight test bees from each colony (which were not age controlled as above). These were kept for 5 days and then placed in boxes without food, and time until death from starvation was measured. They were checked every half an hour until all bees had died. Before the selection procedure, four bees from each colony were infected with the pre-selected parasite cocktail, and starved as above.

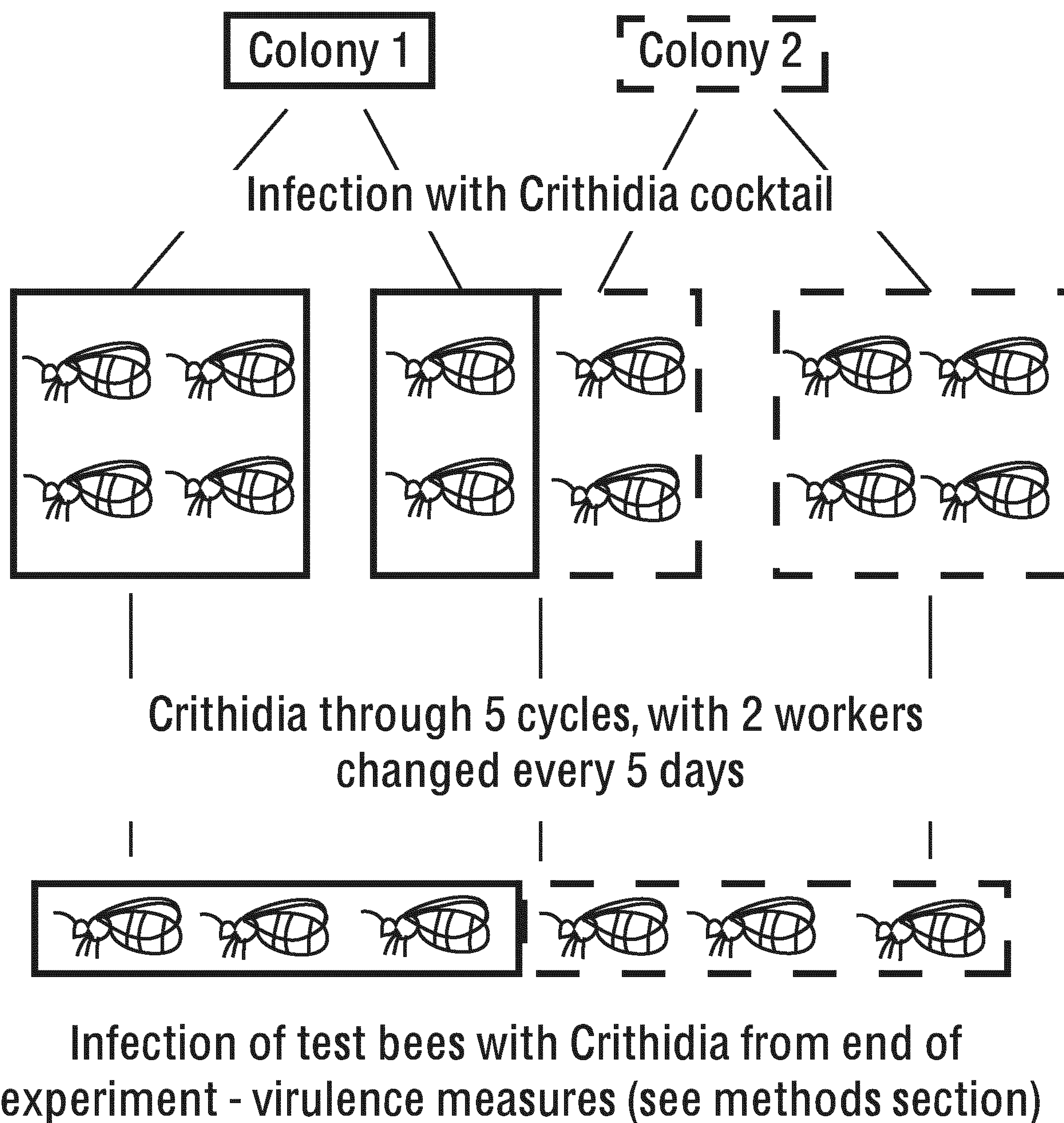

Figure 2.1: *A generalised scheme of the serial passage design for both experiments.*

Results

A MANOVA on the results from the first experiment showed no significant difference between treatments in the measures of host condition – ovariole size or fat body size ($Wilks\lambda = 0.993, df = 2, p < 0.689$; see also Figure 2.2 and 2.1), although a significant effect of colony is detected ($Wilks\lambda = 0.594, df = 8, p < 0.001$).

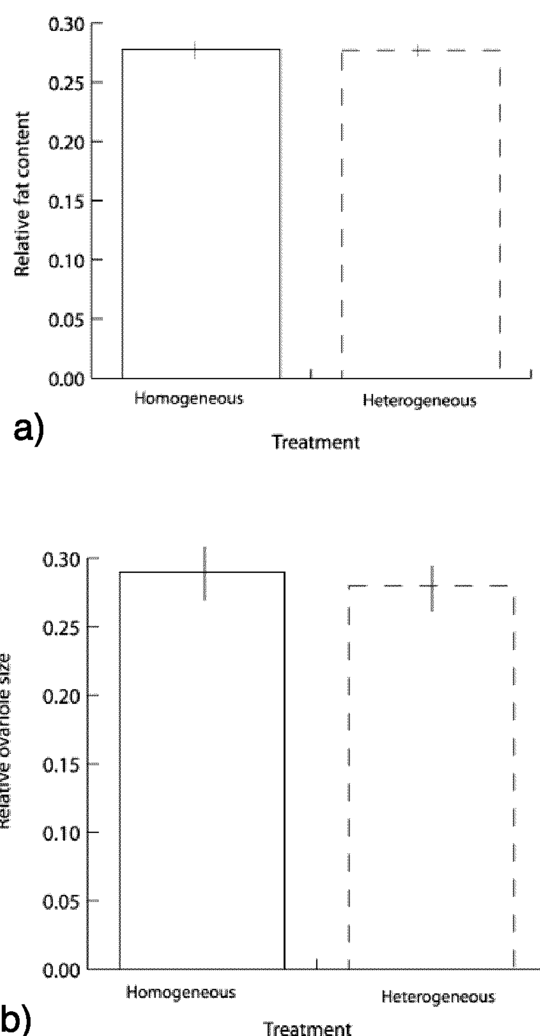

Figure 2.2: *Relative fat content and relative ovary size of hetero- and homogeneous treatment groups in the first experiment (see Table 2.1)*

The survival data from the second experiment were analysed with a Cox regression. It included the factors "treatment", "colony of worker origin" and "colony by treatment" interaction. Colony and colony by treatment interaction are included in the model ($\chi^2 = 122.436, df = 22, p < 0.001$; forward stepwise likelihood ratio regression). There was no significant effect of treatment on survival ($p = 0.845$; see 2.3 and 2.2). The significance of colony effects was mainly due to colonies 1, 2, 11 and 12, with colony 8 showing borderline significance. The colony by treatment interaction effect was mainly due to colony 1, 4 and 5 (1 and 4 are significant in the opposite direction than predicted, i.e. that the bees from these colonies show increased

Table 2.1: *MANOVA for dependent variables relative ovariole size and relative fat content with fixed factor treatment and random factor colony.*

Effect	Wilks λ	df (effect, error)	F	P	Univariate F (P) ovariole	Univariate F (P) fat
Treatment	0.993	2, 100	0.378	0.689	0.331 (0.566)	0.439 (0.509)
Colony	0.594	8, 200	7.447	< 0.001	13.375 (< 0.001)	3.477 (0.011)
Treatment*colony	0.968	8, 200	0.406	0.917	0.138 (0.968)	0.681 (0.607)

mortality in the heterogeneous treatment).

Table 2.2: *Results of the Cox regression model for survival, showing only those results which are statistically significant or borderline significant*

Factors	B	S.E.	Wald	df	p	Odds
Colony			31.753	11	<0.001	
1	0.738	0.345	4.574	1	0.032	2.091
2	1.255	0.351	12.788	1	<0.001	3.508
8	-0.668	0.345	3.754	1	0.053	0.513
11	-0.813	0.346	5.510	1	0.019	0.444
12	-0.938	0.345	7.390	1	0.007	0.392
Colony by treatment (t)			25.639	11	0.007	
1*t	-0.750	0.426	3.100	1	0.078	0.472
4*t	-1.303	0.439	8.809	1	0.003	0.272
5*t	1.490	0.446	11.156	1	0.001	4.439

Further analysis was carried out to test for the power of the experiment. Survival data was normalized ($ln(1+x)$), and then residuals from an ANOVA with colony as a factor were used in a two-tailed independent samples t-test to test the differences between the mean of the heterogeneous and homogeneous treatments. There was no significant difference between them ($t = 1.861, df = 157.842, p = 0.069$) and the slight significance in one tail is in the opposite direction than expected (i.e. towards decreased survival in the heterogeneous as opposed to the homogeneous treatment). In addition, a power analysis was carried out using G*Power software. The analysis suggested a modest level of power for the experiments ($1 - beta = 0.4237$). Hence, we cannot exclude a difference between treatments with a high level of confidence. On the other hand, the inference of no difference gains substantial support from the fact that the two independent experiments produced the same result.

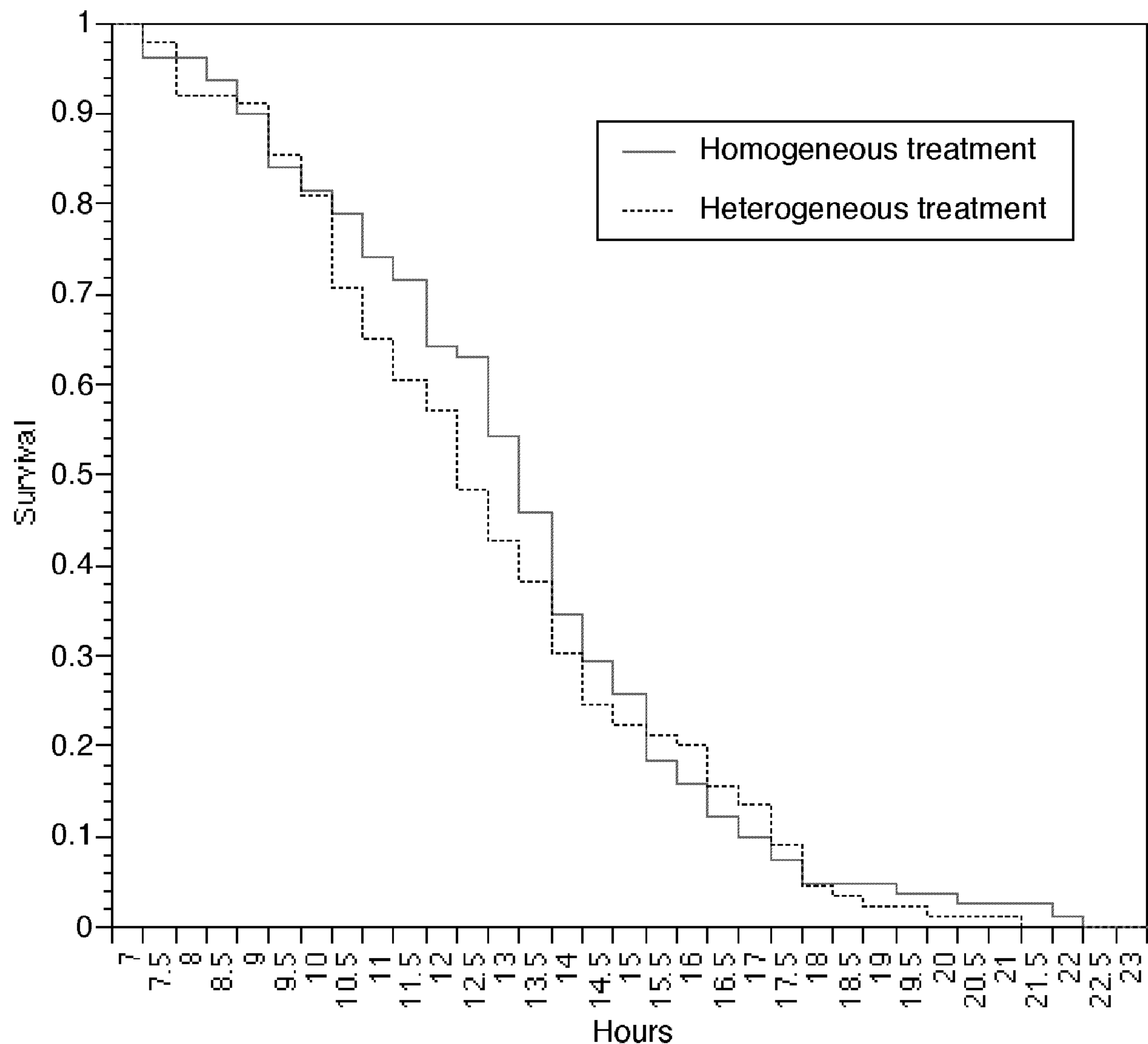

Figure 2.3: *Survival curve from experiment 2 showing proportion of bees left alive at each time point, with separate curves for the hetero- and homogeneous treatments (Cox regression with factors "treatment", "colony of worker origin" and "colony by treatment" interaction: colony and colony by treatment interaction are included in the model:*$\chi^2 = 122.436, df = 22, p < 0.001$*; forward stepwise likelihood ratio regression.*

Discussion

The expectation that virulence would increase more after serial passage through a homogeneous host population than through a heterogeneous one has not held up in this study. This could be due to experimental conditions, for example, we did not use age controlled bees for the survival experiment (although bees were assigned at random). This was in order to increase the sample size, but it may be that the age differences of the bees surpassed and masked any differences between the evolved parasites. As a bumblebees ability to mount an immune response decreases with age (Doums et al. 2002), we would perhaps see older bees dying sooner regardless of parasite strain. Although as the average age of the bees will increase with colony age, we should see higher mortality in both the heterogeneous and homogeneous treatments compared to the pre-selection treatment, which we did not. A repetition with age-controlled bees may nevertheless be worthwhile to exclude this possibility.

Theory suggests that a mixed strain infection should select for an increase in average virulence (for example Frank 1992, Nowak & May 1994, Frank 1996, Ebert 1999, Read et al. 2002). In our experiment, this could come about by elimination of less virulence strains that attain lower parasite densities within the host. However, it may not be true that more virulent strains have a within-host competitive advantage (Read & Taylor 2001). Ganusov et al. (2002) found that, depending on the within-host interactions of the host and parasite, virulence could evolve to higher levels in heterogeneous host backgrounds. Studies such as this are critically important to ascertain the effect of host genetic heterogeneity *in vivo*.

Where there are specific responses to parasites, a more virulent parasite is more likely to be the subject of an immune response, which would then benefit less competitive strains (see de Roode et al. 2004). Also, if there are strong genotypic interactions between host types and parasite strains, competition within host (and increase in virulence) may play a minimal role in mixed-strain infection dynamics (Wille et al. 2002, de Roode et al. 2004). In our system, we know that there is a strong colony by strain effect in virulence (Imhoof & Schmid-Hempel 1998b). The mixture of strains that we initially infected with may or may not contain strains able to compete and increase in virulence in the specific colonies used in the experiment.

It is also possible that there is indeed strain sorting of the parasite as suggested by the results of Yourth & Schmid-Hempel (2006). If strains from the initial cocktail are being filtered, there hasn't been an increase in virulence due to an intitial loss of those strains not suited to that particular colony in the homogeneous environments or those particular colonies in the heterogeneous treatment, and no actual adaptation of individual strains. Yourth & Schmid-Hempel (2006) found that passaging *Crithidia bombi* through workers of the same colony did not increase the parasites success in that colony (as evidenced by infection intensity), but did reduce their success in an unrelated colony. By having a heterogeneous treatment, we may simply retain more of the strains from the original cocktail. It is interesting to note that the only experimental treatment which lost the infection was a homogeneous treatment. This raises the possibility that there may have been no strains in the original cocktail that could persist in this host background.

As colony by strain genetic effects between *B. terrestris* and *C. bombi* have previously been shown to be very pronounced (Schmid-Hempel et al. 1999, Schmid-Hempel & Funk 2004) it is not surprising to find significant differences between colonies with respect to fitness parameters. There is also great variability between colonies and matrilines in fitness parameters in

the absence of parasites (Gerloff & Schmid-Hempel 2005).

In this study we could not find a difference in virulence between parasites passaged through heterogeneous or homogeneous host backgrounds. Such a failure was also the case in Imhoof & Schmid-Hempel (1998b). Hence, it is possible that in this system *C. bombi* does not react to host population structure. As the main virulence effect is on spring queens (Brown, Moret & Schmid-Hempel 2003), which have a different physiology, we may only see the effect of homogeneous versus heterogeneous treatments on colony founding. However, Yourth (2004) found no effect of previous host background in cross-infections of queens with *Crithidia bombi*.

Chapter 3

Fitness and parasitism in common and rare haplotypes of *Bombus terrestris*

Introduction

In a coevolving host parasite system, parasites which are detrimental to host fitness will adapt to locally common host genotypes (Hamilton 1980, Dybdahl & Lively 1995), giving rare host genotypes a selective advantage. We would therefore expect common host genotypes to be over-infected with parasites and rare host genotypes to be under-infected (Dybdahl & Lively 1995, Siemens & Roy 2005). The Red Queen hypothesis (Bell 1982, Hamilton 1982) predicts time–lagged frequency–dependent selection (for example Seger 1988, Dybdahl & Lively 1998) between host genotype and parasites. This complicates our expectations, as in a phase where a previously common host is now rare, the parasites may not yet be adapted to the newly common host genotype (see Dybdahl & Lively 1998, Woolhouse & Webster 2000, Little 2002). However we expect that, on average or at an arbitrary time point, host fitness will generally be inversely related to genotype frequency, with those common types having reduced fitness due to parasite pressure (Siemens & Roy 2005).

In order to study the Red Queen hypothesis, we must be able to quantify common and rare genotypes within host populations and measure their respective frequencies, parasite loads and fitness (Lively & Dybdahl 2000, Siemens & Roy 2005). In this experiment, we looked at common and rare mitochondrial haplotypes within a population of bumblebees, *Bombus terrestris*, around Zürich, Switzerland. The area of mitochondrial DNA we used to classify bumblebee haplotypes is an area of non-coding DNA between the COI and COII genes (which code for parts of the cytochrome oxidase enzyme) that is unique to bees (Crozier et al. 1989). This intergenic sequence shows size and sequence variation in both honeybees (Cornuet et al. 1991, Cornuet & Garnery 1991) and in bumblebees (R. Schmid-Hempel, pers. comm., unpublished data). Mitochondrial DNA (mtDNA) is maternally inherited, haploid, is not subject to recombination and has a rate of nucleotide substitution 5-10 times higher than that of nuclear DNA (Brown et al. 1979). Therefore it lends itself well to being used as a marker (Behura 2006). This area of the genome is both non-coding and mtDNA is presumably not directly related to parasite resistance (Crozier & Crozier 1993). However, we assume that given strong selection

from parasites, haplotypes become common because of temporary linkage with "good" genes coding for parasite resistance. Haplotypes which are coupled with less effective parasite resistance genes will become, or remain, rare. This loose form of linkage is expected to hold for at least a short time, for instance one season, until it is broken up by reproduction and recombination. For example, Oliver et al. (2002) found that in natural populations of *Drosophila subobscura* there is linkage disequilibrium between mitochondrial haplotypes and chromosomal arrangements. In bumblebees the importance of matriline (represented by the haplotypes) is especially pronounced because only queens overwinter, and must found new colonies in the spring (Alford 1975). Very few colonies are successful enough to produce daughter queens (Donovan & Wier 1978, Müller & Schmid-Hempel 1993, Imhoof & Schmid-Hempel 1999), and so populations will be dominated by a few matrilines.

In this experiment, we hoped to test the predictions that colonies from rare haplotypes show lower infection rates with parasites in the field, and have concomitantly higher fitness than colonies from common haplotypes.

Materials and Methods — Mitochondrial marker

Spring queens were collected from an area of northeastern Switzerland in March 2002. They were brought into the lab, placed in breeding boxes and kept in the dark or under red light at a constant temperature of 24 °C with high relative humidity. Queens were fed ApiInvert® sugar solution diluted 1:1 with water and pollen pellets made from blended pollen and ApiInvert®. Those which did not start colonies were frozen. We extracted DNA from legs of a sample of unsuccessful queens and workers from successful colonies using 10 % Chelex (500 μl for a worker leg and 700 μl for a queen leg; N = 73, (Walsh et al. 1991, Schmid-Hempel & Schmid-Hempel 2000)).

A 500 base pair fragment was amplified using the forward primer IGS F1 (5' – GGA GCA ATA ATT TCA ATA AAT AG – 3', A. Widmer, pers. comm.) and reverse primer COII B3 (5' – TTA TGA AAT GAA ATA AGA TTA TCA G – 3', R. Schmid-Hempel, pers. comm., see Appendix A). PCR reactions were carried out with a final reaction volume of 100 μl, containing 1 x reaction buffer including $MgCl_2$ (Promega), 0.3 μM each of forward and reverse primers, 100 μM of each of dNTP, and 5 U of Taq polymerase (Promega). The thermocycling profile consisted of a 3-step PCR with 37 cycles, an annealing temperature of 52 °C, and an elongation time of 1 minute.

The PCR yielded a fragment of the intergenic sequence (Crozier & Crozier 1993). Samples were cleaned with Qiagen DNeasy™kits and then sent to Microsynth GmbH for sequencing. Haplotypes and their frequencies within the sample were analysed by aligning the sequences with MacVector 7.0 software (Accelrys) using a ClustalW alignment (Thompson et al. 1994) with standard parameters.

Results — Mitochondrial marker

We found polymorphism in mitochondrial haplotype. In accordance with the predictions from the Red Queen hypothesis, we found both common and rare haplotypes. There are two common haplotypes (haplotype A = 31.5% of individuals sequenced, N = 23, haplotype B = 37.0% individuals, N = 27) and 20 different rare haplotypes (haplotypes C to Z; 31.5%, N = 23; see Table 3.1). All differences recorded were point mutations: haplotypes A and B varied only at one position and rare haplotypes showed differences at a variety of positions (see Table 3.2 for the sequences of the haplotypes used in this experiment, Appendix B, Table B.1 for all the aligned haplotype sequences, and Appendix B, Table B.2 for all the aligned sequences of the individual bees).

Table 3.1: *Haplotypes found in northeastern Switzerland, Spring 2002.*

Type	No. of queens	Frequency (%)	Queen No.	Field colony no. (queen no.)
A	23	31.5	27, 42, 75, 93, 99, 101, 109, 131, 135, 139, 145, 172, 175, 176, 177, 180, 181, 184, 186, 191, 192, 196, 273	1 (93), 4 (196), 9 (109), 12 (131), 13 (27), 18 (184), 19 (135), 21 (192), 23 (139), 29 (101), 31 (181), 32 (75)
B	27	37.0	8, 22, 29, 31, 46, 60, 71, 82, 87, 91, 114, 116, 119, 130, 148, 149, 152, 159, 162, 164, 165, 170, 171, 195, 200, 208, 264	2 (171), 3 (8), 7 (87), 8 (159), 10 (164), 14 (29), 16 (200), 22 (165), 24 (130), 25 (82), 26 (71), 30 (162)
C	1	1.37	111	—
D	2	2.74	106, 125	—
E	1	1.37	194	—
F	1	1.37	51	—
G	1	1.37	10	5 (10)
H	1	1.37	160	—
I	1	1.37	55	—
J	1	1.37	270	15 (270)
K	1	1.37	52	11 (52)
L	2	2.74	96, 136	—
M	1	1.37	120	17 (120)
N	1	1.37	190	27 (190)
O	1	1.37	67	6 (67)
S	1	1.37	50	28 (50)
T	1	1.37	215	20 (215)
U	1	1.37	147	—
V	1	1.37	167	—
W	1	1.37	100	—
X	1	1.37	37	—
Y	1	1.37	138	—
Z	1	1.37	94	—

Table 3.2: *Haplotype sequences found in NE Switzerland, Spring 2002 and used in the experiment.*

Haplotype A:	TTTCAATAAA	TAGAATATTA	TTTTTAATTT	TCATTATTTT	TGAAAGATTA	ATTTCTAAAC	GATTAATTTT	ATTTAAATTC	CATCAATCAT	CACTTGAATG	ATTAAATAAT
Haplotype B:	....	....	....	....	....	....	....	....	....	....	....
Haplotype G:	....	....	....	....	....	....	....	....	....	....	....
Haplotype J:	....	....	....	....	....	....	....	....	....	....	....
Haplotype K:	....	....	....	....	....	....	....	....	....	....	....
Haplotype M:	....	....	....	....	....	....	....	....	....	....	....
Haplotype N:	....	....	....	....	....	....	....	....	....	....	....
Haplotype O:	....	....	....	....	....	....	....	....	....	....	....
Haplotype S:	....	....	....	....	....	....	....	....	....	....	....
Haplotype T:	....	....	....	....	....	....	....	....	....	....	..G.......

Haplotype A:	TATCCTCCTT	ATGATCACTC	ATTAATTGAA	ATTCCATTAA	-TTTCAAAAA	ATAAAATTAA	AAATATTTTC	AATAAATAAA	TTACCCTTTT	AATATAAATT	TAACATTTAA
Haplotype B:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype G:	....	....	....	....	G.........	.....G....	....	....	....	....	....
Haplotype J:	....	....	....	....	....	....	....	....	....	....	....
Haplotype K:	....	....	....	....	....	....	....	....	....	....	....
Haplotype M:	....	....	....	....	....	....	....	....	....	....	....
Haplotype N:	....	....	....	....	....	....	....	....	....	....	....
Haplotype O:	....	....	....	....	....	....	....	....	....	....	....
Haplotype S:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype T:	....	....	....	....	....	.....G....	....	....	....	....	....

Haplotype A:	TATAATATTA	ATATTGCAAA	TTAATGCCTT	GAACTTAAAA	TTCAACTATA	AAGATTATTT	TT-CTTTTAT	TAATATAAAT	ATTTAATATA	ATATTAATAT	GGC-AGATTA
Haplotype B:	....	....	....	....	....	....	....	....	....	....	....
Haplotype G:	....	....	....	....	....	....	....	....	....	....	....
Haplotype J:	....	....	.....A....	....	....	....	....	....	....	....	....
Haplotype K:	....	....	.....A....	....	....	....	..T.......	....	....	....	....
Haplotype M:	....	....	....	....	....	....	..T.......	....	....	....	....
Haplotype N:	....	....	....	....	....	....	....	....	....	....	...C......
Haplotype O:	....	....	....	....	....	....	....	....	....	....	....
Haplotype S:	....	....	....	....	....	....	....	....	....	....	....
Haplotype T:	....	....	....	....	....	....	....	....	....	....	....

Haplotype A:	GTGC-TTTGA	ATTTAAAATT	CAACTATAAA	GATTATTTTT	C-TTTTATTA	ATATAAATAT	TTAATAAATC	AATATTTTGA	ATTGAATT
Haplotype B:	....	....	....	....	....	....	....	....	....
Haplotype G:	....	....	....	....	....	....	....	....	....
Haplotype J:	....	....	....	....	....	....	....	....	....
Haplotype K:	....	....	....	....	....	....	....	....	....
Haplotype M:	....	....	....	....	....	....	....	....	...
Haplotype N:	....C.....	....	....	....	....	....	....	....	....
Haplotype O:	....	....	....	....	.C........	....	....	....	....
Haplotype S:	....	....	....	A.........	....	....	....	....	....
Haplotype T:	....	....	....	....	....	....	....	....	....

Materials and Methods — Field experiment

To test whether fitness varies between common and rare haplotypes, we placed a sample of colonies from both groups into the population from where they came. Twelve colonies raised from spring queens of each of the two common haplotypes and eight colonies from a mixture of rare haplotypes were placed in the field at Kartause Ittigen in Thurgau, NE Switzerland (a site in the same area where the spring queens were collected). Colonies were placed in the field when they had produced at least 10 workers. The first colonies were placed in the field on the 8th May 2002, and the last colonies were placed in the field the 26th June 2002. The mean Julian day of colony placement was 144.47 days ($\pm$ 2.70, S.E.) which corresponds to the 24th May 2002. The colonies were monitored once a week, the number of workers counted and 10% per week removed for dissection (these were given "blind numbers", i.e. identities were unknown to the observer during the later dissections). When sexuals were produced, the colonies were checked twice weekly, and the new sexuals counted and removed (see Figure 3.1).

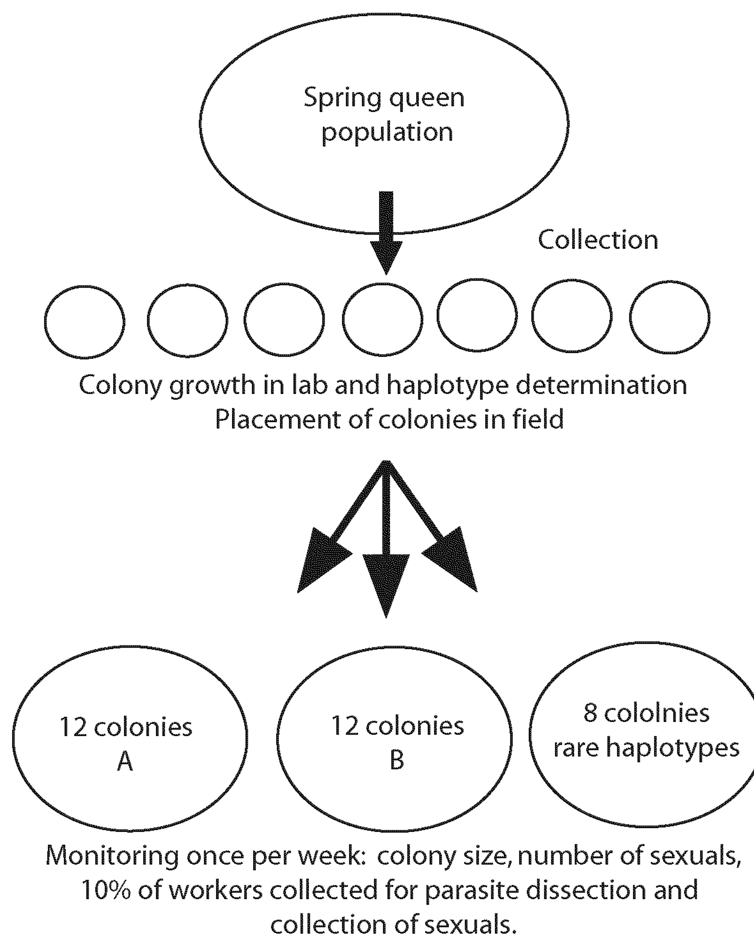

Figure 3.1: *Design of the field experiment*

We dissected the removed bees for parasites, checking for external mites, internal macroparasites such as thoracic mites (*Bombacarus buchneri*; Acarina, Podapolipodidae)

(Stammer 1951) and conopid fly eggs and larvae (Diptera, Conipidae) (Schmid-Hempel & Schmid-Hempel 1988, Schmid-Hempel & Schmid-Hempel 1989, Schmid-Hempel et al. 1990), and the protozoan gut parasites *Nosema bombi* (Microsporidia, Nosematidae) (de Jonghe 1986, Fisher & Pomeroy 1989) and *Crithidia bombi* (Trypanosomatidae, Zoomastigophorea) (Lipa & Triggiani 1980, Gorbunov 1987). We examined four measures of parasitism: parasite richness (the number of parasite species per colony), parasite load (the average number of parasite species per worker per colony), prevalence of *Crithidia bombi* and *Nosema bombi* (the proportion of parasitised workers per colony) and the intensity of infection of *C. bombi* and *N. bombi* (the average number of parasites within the workers of a colony).

Young queens and males were weighed to the nearest mg and queen size measured (the length of the radial cell of the right wing in mm). Analyses were carried out using SPSS 11 for Macintosh. Figures are means $\pm$ S. E. when not otherwise specified.

Results — Field experiment

The fitness of a colony was defined as the number of males produced by that colony plus twice the number of queens produced. This formula takes into account how much more "expensive" queens are (from dry weight), as they are much larger and cost more energy to produce (Duchateau & Velthuis 1988, Baer & Schmid-Hempel 1999). In addition, the (diploid) queens have twice the genetic value as compared to (haploid) males. From our data, we found that queens had a fresh weight of mean of 0.734 g $\pm$ 0.007g, and males weighed a mean of 0.273 g $\pm$ 0.003 g, which gives a relation of closer to queens being 2.5 x heavier than males. However, the analyses lead to the same conclusions if male and queen number are weighted equally, or if queen number was weighted by a factor 2.5 rather than a factor of 2.

We found a low incidence of parasites in general in the dissected workers. 3.12% of workers were infected with *C. bombi* (10 workers from a total of 321 investigated), divided between 6 colonies from the 32 in the experiment. 3.43% of workers were infected with *N. bombi* (11 bees from a total of 312), divided between 5 colonies from the 32 in the experiment. No thoracic mites were found, and three bees each contained conopid eggs or larvae (1.96% of workers). Colony number 13 (haplotype A) was taken over by a cuckoo bumblebee (*Psithyrus* spp. Lepeletier) by the 27th June, when it had 24 workers, and has been removed from the analysis.

The number of workers in the colony when it was placed in the field (size at field placement) has a significant effect on the colonies eventual fitness and was therefore taken into account as a covariate in further analyses (see Figure 3.2; R square = 0.489, df = 1, F = 28.656, $p < 0.001$). The range of size at field placement of all of the colonies is 10 to 36 workers, with a mean of 14.53 $\pm$ 1.14 workers. The size at field placement was also highly correlated with the largest recorded size of the colony (Pearsons correlation coefficient = 0.654, $p < 0.001$, N = 31), which in turn is correlated highly with fitness (Pearsons correlation coefficient = 0.920, $p < 0.001$, N = 31; see Figure 3.3). Colony size has been previously shown to be extremely important in the eventual reproduction of bumblebee colonies (Pomeroy & Plowright 1982, Müller & Schmid-Hempel 1993).

Out of 32 colonies in this experiment, 11 produced queens (34.38% of colonies) and 25

produced males (78.13% of colonies). These numbers are higher than found by Shykoff & Müller (1995) and Imhoof & Schmid-Hempel (1999).

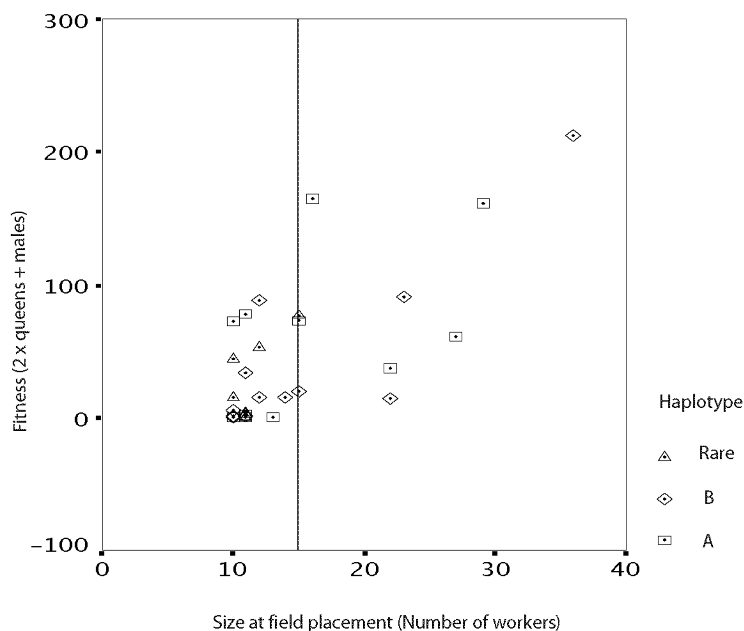

Figure 3.2: *Fitness is highly correlated to size at field placement (Pearsons correlation coefficient=0.700, p<0.001, N=31). The vertical line shows the maximum size out of any rare haplotype colony.*

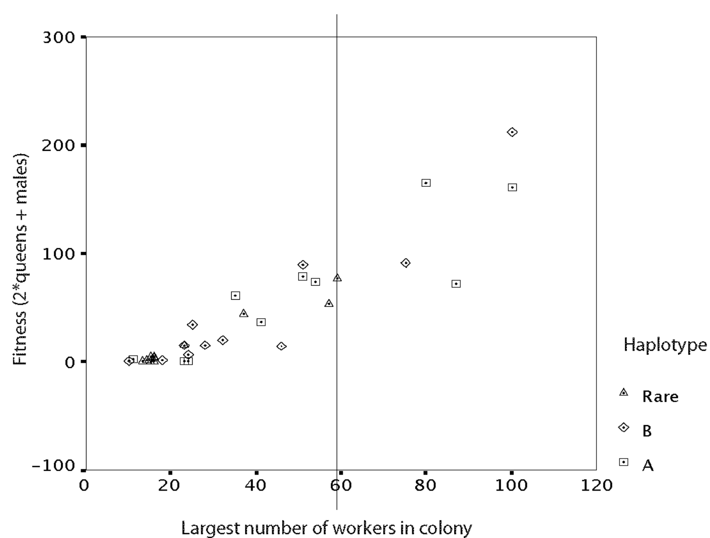

Figure 3.3: *Fitness is highly correlated with largest size recorded (Pearsons correlation coefficient=0.920, p<0.001, N=31). The vertical line shows the maximum size any rare haplotype colony reached.*

Results between common and rare haplotypes

Colonies of rare haplotypes do not show the full range of size at field placement (see Figure 3.2). Common haplotype colonies have a mean size at field placement of 15.58 workers (± 1.45), and a range of 10 to 36. Rare haplotype colonies have a mean size out of 11.38 workers (± 0.56) and a range of size at field placement 10 to 15. The difference between common and rare haplotype colonies in size at field placement is statistically significant (T-test: t = 2.871, df = 22.968, p = 0.009, Levene's test for equality of variances indicated that variances are not equal: F = 13.144, p < 0.001; see Figure 3.4). As there are no significant differences in the Julian day of field placement (mean Julian day of field placement for common haplotype colonies = 144 ± 3; mean Julian day of field placement for rare haplotype colonies = 146 ± 6; T-test: t = -0.325, df = 30, p = 0.748), this suggests that rare haplotype colonies grow more slowly at the early stage of colony growth than common haplotype colonies. This early stage is crucial for bumblebee colony success (Müller & Schmid-Hempel 1992). This suggests that the difference between the common and rare haplotypes shows itself very early on in the colony cycle. It can also be seen that the rare haplotype colonies do not grow as large as the common haplotype colonies (see Figure 3.3). The maximum recorded size of a rare haplotype colony is 59 workers (the mean is 29.88 ± 6.66), and the maximum size for a common haplotype colony is 100 workers (the mean is 40.83 ± 5.79; T-test between means: t=1.015, df=30, p=0.318).

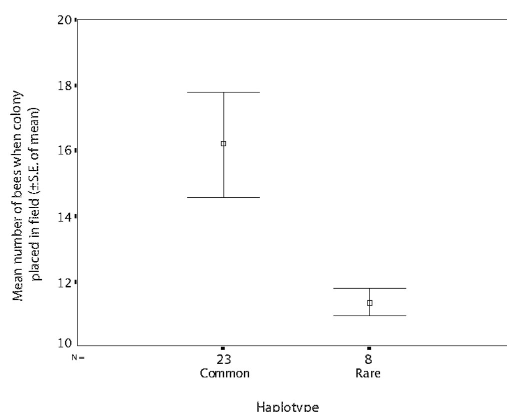

Figure 3.4: *Common haplotype colonies were significantly larger (16 workers ± 2) at the time of field placement than rare haplotype colonies (11 workers ± 1; T-test: t = 2.871, df = 22.968, p = 0.009, equal variances not assumed — Levene's test for equality of variances: F = 13.144, p < 0.001)*

Colonies of rare haplotypes show lower fitness than colonies from common haplotypes (T-test: t=2.313, df=29.4, p=0.028, see Figure 3.5). This effect disappears when size at field placement is put into the analyses as a covariate (see Figures 3.2 and 3.4 and Table 3.3, ANOVA: Size at field placement: F=23.582, df=1, p<0.001; Common or rare haplotype: F=0.040, df=1, p=0.843).

The only parasite measures that show borderline statistical significance between common haplotype colonies and rare haplotypte colonies are *Crithidia bombi* prevalence (Mann Whitney U-test: z = -1.704, p = 0.085) and infection intensity (U-test: z = -1.703, p = 0.088; see Table 3.4). Only one rare haplotype colony showed infection with *Crithidia bombi* compared to three common haplotype colonies.

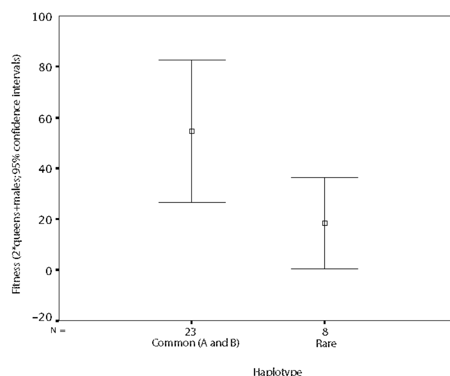

Figure 3.5: *Common types (pooled data, with a mean fitness of 49.87 ± 12.65) are marginally significantly fitter than rare types (with a mean fitness of 22.22 ± 9.50; T-test equal variances assumed — Levene's test for equality of variances: F=3.911, p=0.057: t=1.302, df=30, p=0.203)*

Table 3.3: *ANOVA examining the effect on fitness (2 x queens + males) of common and rare haplotypes as a fixed factor, and size at placement in field as a covariate.*

Source	SS	df	Mean square	F	p
Corrected model	44537.660	2	22268.830	13.527	< 0.001
Intercept	8735.393	1	8735.393	5.306	0.029
Size out	38822.130	1	38822.130	23.582	< 0.001
Common/rare	65.987	1	65.987	0.040	0.843
Error	46096.017	28	1646.286		
Total	149163.000	31			
Corrected total	90633.677	30			

The main effect of haplotype seems to be very early acting — that they grow more slowly, do not reach such a large size (see Figure 3.3), and do not produce as many sexuals. It is important to note in Figure 3.3 that all colonies follow the same pattern with regards to the relationship between largest number of workers in a colony and fitness, and both of the common haplotypes (A and B) show the full range of size and fitness, rare types are constrained at approximately 60 workers.

Table 3.4: *Summarised differences between common and rare genotype colonies with respect to parasite parameters with Mann Whitney U-test results.*

Haplotype		Common (N = 24) (mean ± S.E.)	Rare (N = 8) (mean ± S.E.)	Mann Whitney U-test and significance
Parasite richness		0.75 ± 0.17	0.25 ± 0.16	z = -1.548 p = 0.174
Parasite load		0.13 ± 0.05	0.03 ± 0.02	z = -1.628 p = 0.143
Prevalence:	*Crithidia*	0.03 ± 0.01	0.00 ± 0.00	z = -1.704 p = 0.085
	Nosema	0.06 ± 0.05	0.07 ± 0.07	z = -0.408 p = 0.684
Infection intensity:	*Crithidia*	9.86 ± 5.27	0.00 ± 0.00	z = -1.703 p = 0.088
	Nosema	210.99 ± 209.56	652.71 ± 652.71	z = -0.377 p = 0.734

Results between two common haplotypes

Colonies from haplotype A and haplotype B do not show any difference in size at placement in field (see Table 3.5).

Not all colonies produce sexuals. More colonies of haplotype B produced males (11 out of 12 colonies) than haplotype A colonies (7 out of 12 colonies; borderline significant: χ^2 = 3.556, df = 1, p = 0.059). More colonies of haplotype A produced queens (7 out of a total of 12 colonies) than of haplotype B (3 out of a total of 12 colonies; although this is not statistically significant: χ^2 = 2.743, df = 1, p = 0.098). Haplotype A colonies also tended towards higher and heavier queen and male production, although this is not statistically significant (see Table 3.5).

More colonies of type A are infected with *Nosema bombi* (4 out of a total of 12 colonies) than of type B (1 out of 12, although this is not significant; χ^2 = 2.274, df = 1, p = 0.132). Colonies from haplotype A and colonies from haplotype B have no difference in size at field placement (haplotype A colonies have a mean of 15.91 ± 2.10 workers at field placement; haplotype B colonies have a mean of 15.15 ± 2.11 workers at field placement).

As can be seen in Table 3.5, colonies from haplotype A and B do not differ significantly in any of the fitness or parasite parameters. However, there is borderline significance of number of queens produced per colony, with haplotype A colonies producing a mean 10 queens (± 6) and haplotype B colonies producing a mean of 5 queens (± 3; Mann Whitney U-test: z = -1.812, p = 0.070), as well as parasite load, with haplotype A colony workers having a higher parasite load than colony B workers (mean for A = 0.22 ± 0.10; mean for B = 0.05 ± 0.02; Mann Whitney U-test: z = -1.827, p = 0.068). Although non-significant, all results tend in the same direction – to haplotype A colonies having higher fitness, more parasites and a greater diversity of parasites than haplotype B colonies.

Table 3.5: *Differences between haplotypes A and B. Results are summarised means ± S.E. for the colonies within each haplotype. None of these differences are statistically significant - tests shown are either from an ANOVA (F-statistic) for normally distributed data or a Mann Whitney U-test (z statistic) for non-normally distributed data.*

		Haplotype A (N=11)	Haplotype B (N=12)	Statistic (F or z)	p
Number of males		34 ± 14	32 ± 15	z=-1.105	0.269
Number of queens		10 ± 6	5 ± 3	z=-1.812	0.070
Fitness (2*queens + males)		54.08 ± 17.35	41.50 ± 17.99	F=1.210	0.333
Total sexual weight (g)		8.98 ± 4.25	5.15 ± 2.79	F=0.243	0.795
Mean queen weight (g)		0.704 ± 0.021	0.663 ± 0.086	F=0.211	0.818
Mean male weight (g)		0.291 ± 0.016	0.269 ± 0.012	F=1.282	0.371
Mean radial cell size (mm)		3.11 ± 0.16	2.94 ± 0.12	F=0.288	0.764
Infection intensity:	*Crithidia*	9.78 ± 5.51	8.31 ± 8.26	z=-1.066	0.286
	Nosema	873.88 ± 594.24	2.47 ± 2.47	z=-1.672	0.094
Prevalence:	*Crithida*	0.03 ± 0.01	0.02 ± 0.01	z=-1.105	0.269
	Nosema	0.15 ± 0.09	0.01 ± 0.01	z=-1.672	0.094
Parasite load		0.22 ± 0.10	0.05 ± 0.02	z=-1.827	0.068
Parasite richness		1 ± 0.25	0.50 ± 0.23	z=-1.709	0.119

Discussion

We carried out this experiment to examine the difference in fitness between genotypes of different frequencies within a natural population of *Bombus terrestris*. An important prediction of the Red Queen hypothesis is that common genotypes should have higher parasite loads, and concomitantly lowered fitness. We tested this in a snapshot of a natural population of *Bombus terrestris*. In order to study the negative frequency dependence that the Red Queen hypothesis predicts, there must be differences in frequencies of genotypes. We did find differences in frequencies of genotypes. We found two common haplotypes and many rare ones within the population when it was sampled in spring 2002. We also confirmed that these results are shown to be stable over years in a long-term study of two different populations (see Chapter 4).

Common host haplotypes do have an increased parasite load and higher infection intensities. There is an overall trend in the data towards common haplotype colonies being under higher parasite pressure. This pattern is also found in the two common haplotypes, with A having more parasites than B. However, the overall levels of infection were lower than previ-

ously recorded in field studies in this system (Imhoof & Schmid-Hempel 1999, Baer & Schmid-Hempel 1999), and may have affected the results. Previous studies have shown that as many as 80 % of *Bombus terrestris* workers caught in the field may be infected by *Crithidia bombi* (Shykoff & Schmid-Hempel 1991b), and from field experiments that almost all colonies were soon infected (Imhoof & Schmid-Hempel 1999). In this experiment we found only 3.12 % of workers were infected with *C. bombi*, divided between 6 colonies from the 32 in the experiment. Imhoof & Schmid-Hempel (1999) found that all but one colony became infected with *C. bombi*. Shykoff & Schmid-Hempel (1991b) also found that 14.8% of *B. terrestris* individuals were infected with *Nosema bombi*. From our colonies, 3.43 % of workers were infected with *N. bombi*, divided between 5 colonies from the 32 in the experiment (compared to 17 out of 32 colonies showing *N. bombi* infection from Imhoof & Schmid-Hempel (1999)).

The difference in infection rates between the two common haplotypes A and B may be due to intrinsic differences between the haplotypes, or to stochasticity. In this case, an experiment in another year may yield different results. However, as all of the results show a tendency in the same direction, it is likely that there is an actual difference between the two haplotypes. In Spring 2002, both of these haplotypes were approximtely equally common (see Chapter 4). In the previous two years, haplotype A was more common (see Figure 4.3), and in the following year, the frequency of haplotype B was severely reduced. This fits with our observation of lower fitness, and especially lower queen production, in haplotype B colonies. If fitness and parasite load are related to the haplotype frequency in the previous year, then we might expect the haplotype which had been more common the previous year to be more at risk from parasites. As A was more common in the previous year, it is expected that colonies of haplotype A will suffer more from parasites, which we see. However, the effect is confounded by larger colony size, as larger colonies pick up more parasites when workers forage, but have the capability to overcome the parasite pressure and still produce sexuals.

Common host haplotypes have a higher fitness than rare ones, which is unexpected. This is not necessarily in conflict with the Red Queen hypothesis, as at different times in the cycle different patterns will be observed (Dybdahl & Lively 1998). However, that the rare haplotype colonies did so much worse than the common haplotype colonies seems to suggest that some other selection is at work here. More colonies in our experiment produced sexuals than found by Imhoof & Schmid-Hempel (1999), perhaps related to size at field placement (the colonies from Imhoof & Schmid-Hempel (1999) had an average of 12.9 workers at field placement, and a minimum of 7; our colonies had an average of 14.53 workers at field placement and a minimum of 10).

The large fitness differences found between haplotypes are mainly due to size at field placement. The common haplotypes grew more quickly in the lab. We did not remove workers to equalise the numbers, as we did not want to interrupt early stage fitness, given its importance in colony life-history (Müller & Schmid-Hempel 1993). The effect of colony size at field placement on fitness agrees with the findings of previous studies of bumblebees (Pomeroy & Plowright 1982, Fisher & Pomeroy 1989, Müller & Schmid-Hempel 1993, Imhoof & Schmid-Hempel 1999) — that early colony growth is an important factor in colony success. We had expected rare types to do well by escaping parasitism, but it seems that there is selection against rare haplotypes, and that this happens early on in the colony cycle, even when conditions are generally favourable, such as in lab conditions.

The differences in fitness between haplotypes may also be caused by mutations in functional mitochondrial genes causing lowered or increased metabolic rates. This could be tested

by investigating the mitochondrial enzyme amounts and activities in bees from different haplotypes (such as is done with honeybees by Suarez et al. 2000). However, this is the first time that a genetic quantity (haplotype frequency) has become a correlate to understand colony size, which is one of the most important factors determining the ecology and life history of social insects (e.g. Schmid-Hempel et al. 1993). Mitochondrial enzymes are particularly important for honeybee queens (Corona et al. 1999), and presumably bumblebee queens as well.

These results cannot show conclusively that a Red Queen scenario is not occurring. Haplotypes are possibly not the best markers to use as a proxy for underlying interactions with parasites, as they may be linked to genes under directional selection (Bazin et al. 2006). Using a marker linked closely with genes involved in parasite resistance or immune response may give clearer results (Little 2002). Wilfert et al. (2007) have developed Quantative Trait Loci (QTLs) in the bumblebee that are involved with *Crithidia* resistance and encapsulation response. By using markers which are linked directly to the genes in question, we may be able to categorise bees into more "types" which are more directly involved with parasite interactions, and see if the outcome fits the predictions of the Red Queen hypothesis more closely.

Chapter 4

Haplotype dynamics of European *Bombus terrestris*

Introduction

The Red Queen hypothesis predicts that there is dynamic cycling of host genotypes within a population with time-lagged adaptation of parasites to the most common host genotypes (Hamilton 1982, Seger 1988, Dybdahl & Krist 2004). We would expect that those host genotypes which are common in a population will have more parasites than those which are rare (Dybdahl & Lively 1995, Lively & Dybdahl 2000, Siemens & Roy 2005). We tested the predictions that host genotype frequency would show cycles where different haplotypes are common and rare at different times, and that these are related to parasite pressure. We followed two populations of *Bombus terrestris* for three and four years, looking at haplotype frequency and parasite infection.

We used the intergenic sequence in the mitochondrial genome between cytochrome oxidase I and II genes (Crozier et al. 1989, Crozier & Crozier 1993, Cornuet et al. 1991) to categorise *Bombus terrestris* bees by haplotype in Gotland, Sweden (an island population on the edge of *B. terrestris*'s range (Løken 1973, Pekkarinen & Kaarnama 1994)), and near Zürich, Switzerland (a mainland population in the main area of *B. terrestris*' range, see Figure 4.1). Although the mitochondrial haplotype is not likely to be directly linked with resistence genes, we nevertheless considered that it would be a good marker, due to the maternal inheritance of mitochondria and their increased mutation rate compared to the nuclear genome. In the *Bombus* system, queens, which transmit the haplotype, are only produced by a relatively small percentage of colonies (Müller & Schmid-Hempel 1993) which have been successful in growth and fighting off parasites during the summer season. Many more colonies produce males, which do not transmit the haplotype – although in other species occasional leakage of paternal mitochondrial DNA has been recorded (e.g. Kondo et al. 1990). Therefore, only those genotypes which have been successful at producing queens will appear in the haplotype frequencies of the next year. If parasites exert a significant selection pressure on hosts, we would expect that over one or two seasons, mitochondrial haplotypes will form a loose linkage with resistance genes. As the main fitness effect of our study parasite *Crithidia bombi* occurs in

queens (Brown, Schmid-Hempel & Schmid-Hempel 2003), those haplotypes which are common in a population in spring queens may not remain common over the summer season, due to infected queens being unsuccessful at colony foundation.

We expected to find multiple matrilines within populations of *Bombus terrestris*, and that these matrilines would change frequency over the course of a season and/or across years. This would then allow us to examine if this change is related to parasite pressure and whether there are time lags between parasite adaptation and host genotype frequency change. We would also expect populations with a higher diversity of matrilines to have low parasite loads. These predictions from the Red Queen hypothesis have rarely been tested explicitly in the field (Little 2002), exceptions being a partially sexual aquatic snail system studied by Lively and Dybdahl (Dybdahl & Lively 1995, Dybdahl & Lively 1998, Lively & Dybdahl 2000, Lively et al. 2004, Dybdahl & Krist 2004) and by Siemens & Roy (2005), who studied these questions in an asexual plant species. Lively et al. have found that common snail clones are overparasitised by trematode parasites, that rare clones have a fitness advantage and that populations under greater pressure from parasites show higher rates of sex. Siemens & Roy (2005) found that rust fungus showed local adaptation and tracked common host genotypes. To our knowledge, this is the first study conducted on these questions on a fully sexual species.

Materials and Methods

We collected samples from Gotland, Sweden over a period of 3 years, and from the region around Zürich, Switzerland over a period of 4 years. The Swiss population was sampled once per year, in spring, over the years 2000 to 2003. The spring queens from Zürich were used to form laboratory colonies. The individuals used for the year 2002 were not the same individuals used for the experiment detailed in Chapter 3, but show the same frequency results. The Gotland population was sampled either once per year in spring (year 2001) , or twice — once in spring and once towards the end of the summer (years 2002 and 2003). The bees were frozen straight from the field using liquid nitrogen and stored at -80 $^{\circ}$C and later dissected. DNA was extracted either from the queen herself or from one of her offspring and used to categorise the bumblebees into haplotypes. Two methods of DNA extraction were used: 10% Chelex (Bio-Rad) was used for extraction from legs (see Chapter 3; Walsh et al. 1991, Schmid-Hempel & Schmid-Hempel 2000); or DNA was extracted with Invisorb DNA Tissue HTS 96 Kits/C (24) (Invitek) from both legs and from gut samples.

For ease of visualisation of haplotypes, we used the single-strand conformation polymorphism (SSCP) method (see Sunnucks et al. 2000). The single-strand conformation polymorphism method was developed for detection of point mutations and DNA polymorphisms (Orita et al. 1989). It uses the folding properties of denatured DNA — and their differing electrophoretic mobility. Different sequences will fold in different ways, and therefore run through a gel at different rates, showing different banding patterns. The DNA must be run slowly through a gel to allow the differences to become apparent. The SSCP method is both specific and sensitive to point mutations (Hayashi & Yandell 1993).

For economic reasons we used only a part of the sequence characterised in Chapter 3 (see Appendix B). We developed primers with MacVector 7 (Accelrys) software (forward primer IGS2 F6: 5'- AAT TTT CAT TAT TTT TGA AAG -3'; reverse primer IGS2 B1: 5'- ATA GTT GAA

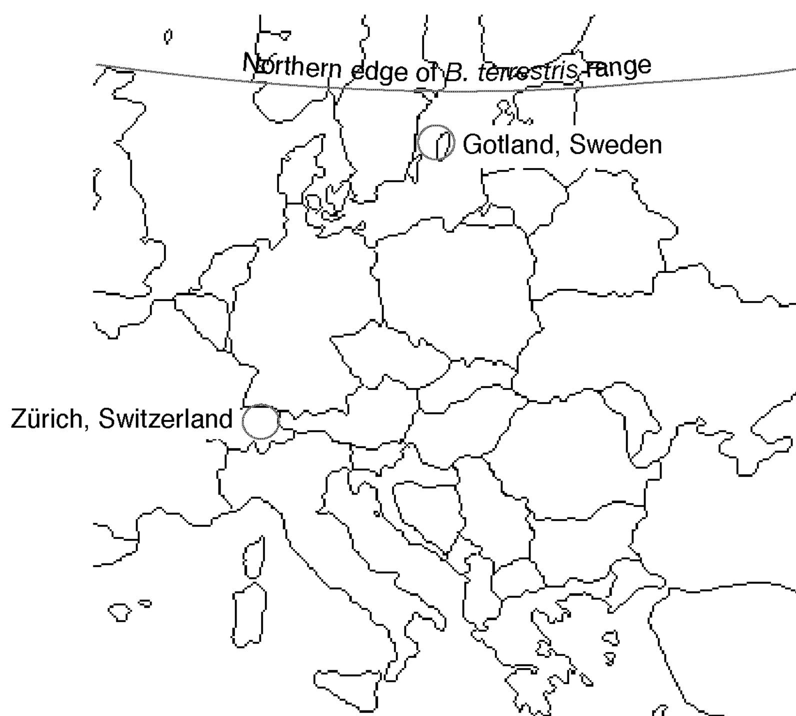

Figure 4.1: *Collection areas*

TTT TAA GTT CAA -3') within this region to create a section the correct size for the SSCP gels. The ideal size is between 150–300 base pairs — our section is around 300 base pairs long). This shorter region still includes the main areas of variation.

For SSCPs, PCR reactions were carried out using a final reaction volume of 10 μl PCR containing 1 x reaction buffer (containing $MgCl_2$) (Promega), 0.3 μM each of forward and reverse primers, 100 μM of each of dNTP, and 0.5 U of Taq polymerase (Promega). The thermocycling profile consisted of a 3-stage PCR with 35 cycles, an annealing temperature of 52 °C for 30 seconds, and an elongation time of 30 seconds.

Amplifications were checked on 1.5% agarose gel. 5 μl of the PCR product was mixed with 7 μl of loading buffer, and 10 μl was loaded. The gel was run for 40 minutes with a standard voltage, and then the bands visualised with either ethidium bromide or GelRed™(Biotium). Those samples which amplified were then run on SSCP gels (GMA™Gels, Elchrom Scientific, Switzerland).

We used SEA 2000 apparatus (Elchrom Scientific, Switzerland), with precast GMA™(Gene Mutation Analysis) gels from Elchrom Scientific (Switzerland). 3 μls of the DNA fragment is first denatured with 7 μls of formamide containing 10mM sodium hydroxide at 95 °C for 4 – 5 minutes, then cooled on ice and loaded into the gel. The chamber was run at 5 – 6 V/cm at approximately 5 °C for 15 hours. The gels were then stained with SYBR Gold and photographed (see Figure 4.2). A sample of the individuals was sent to Microsynth GmbH to be sequenced, to check the validity of the SSCP banding. In this case, the PCR reaction volume used was 100

μl, and after checking with an agarose gel, the remaining product was cleaned with Qiagen DNeasy™kits.

Those queens which were bred for lab colonies were regularly checked for *Crithidia* and *Nosema* by looking at faeces samples. Field caught bees which were frozen were dissected and examined for parasites, both by checking guts under the microscope, and by molecular detection with *Crithidia* microsatellite primers (Schmid-Hempel & Funk 2004) or *Crithidia* 18S ribosomal subunit primers (Tognazzo 2006).

Results

For economic reasons, we used an SSCP protocol for haplotype identification as an alternative to sequencing all samples. The shorter section of intergenic DNA used for this study contains most of the areas found to be polymorphic in the longer sequence used in Chapter 3. However, there has been some loss of variation, and some of the rare haplotypes now resolve into one of the two common types (see Table 4.1 and aligned sequences and primers in Appendix B, Table B.3). We found a total of 4 genotypes (2 common and 2 rare) in Gotland, Sweden, and 6 haplotypes (2 common and 4 rare) in Zürich, Switzerland across the years. The two common haplotypes in both populations are the haplotypes A and B, and only two of the rare genotypes are the same as those found in Chapter 3. Through sequencing of individuals which appeared unique on the gels, we found four new haplotypes (labelled IGS2I to IGS2V) not seen in the bees sampled in Chapter 3.

There are two main haplotypes, and in both the Swiss and the Swedish populations the two main haplotypes are the same ones (haplotype A and haplotype B). Throughout my study period, haplotype A was usually more common in the Swiss population (between 57 and 83%; apart from in the year 2002 where A stands at 42% of the bees sampled, and B at 49%; see Table 4.2 and Figure 4.3). Haplotype B is between 6 and 37% in the years sampled apart from 2002.

In Gotland across years in spring, haplotype B is generally more common (at a frequency of between 83 and 94% of the sampled population). The frequency of haplotype A in spring in Gotland is low – between 6 and 12%. The haplotype frequencies change dramatically between season in Gotland in 2002, however, when the frequency of haplotype B was reduced to 33% and the frequency of haplotype A increased to 67% (see Table 4.2 and Figure 4.7).

The exceptions in both our populations occur in the year 2002. This raises the interesting question of what difference the year 2002 presented to both populations. Perhaps climatic factors played into the differences in haplotype frequencies we saw in that year, although this is outside the scope of our data, and we cannot answer these questions. This highlights the importance of long term studies on ecological data. These populations are still being followed and data from further years could clarify the picture.

As we would expect from the Red Queen hypothesis, the common types have a higher parasite prevalence in the Swiss population. There is a significant correlation between haplotype frequency and frequency of *Crithidia bombi* within haplotype (Spearmans r = 0.701, p = 0.02, N = 17). However, there is no correlation in the Gotland population between haplotype

Table 4.1: *Haplotype as found from IGS2 F6 and IGS2 B1 primers, and corresponding haplotypes from IGS F1 and COII B3 primers*

Haplotype IGS2 F6 and IGS2 B1	Haplotype IGS F1 and COII B3
A	A, O, L, M, H
B	B, S, W, Y, U
C	C
D	D
E	E
F	F
G	G
I	I
J	J, K
T	T
V	V
X	X

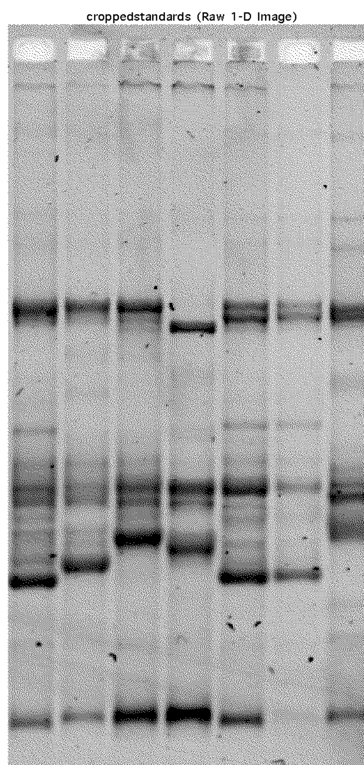

Figure 4.2: *Variability of banding patterns on an SSCP gel. Example shows seven of the haplotypes identified in the previous chapter: from left to right A, B, E, I, J, K, X.*

Table 4.2: *Gotland and Swiss haplotypes, haplotype frequencies and frequencies of Crithidia bombi and Nosema bombi within each haplotype.*

Location	Year	Season	Haplotype	Frequency (of *Crithidia*/ of *Nosema*)		N (with *Crithidia*/ with *Nosema*)	
Gotland	2001	Spring	A	0.12	(0.00 / 0.00)	7	(0 / 0)
			B	0.88	(0.17 / 0.02)	52	(9 / 1)
	2002	Spring	A	0.06	(0.00 / 0.00)	2	(0 / 0)
			B	0.83	(0.10 / 0.00)	29	(3 / 0)
			IGS2I	0.11	(0.00 / 0.00)	4	(0 / 0)
		Summer	A	0.67	(0.27 / 0.00)	41	(11 / 0)
			B	0.33	(0.40 / 0.00)	20	(8 / 0)
	2003	Spring	A	0.06	(0.00 / 0.00)	2	(0 / 0)
			B	0.94	(0.00 / 0.00)	30	(0 / 0)
		Summer	A	0.07	(0.88 / 0.00)	8	(7 / 0)
			B	0.87	(0.46 / 0.03)	90	(41 / 3)
			E	0.02	(0.00 / 0.50)	2	(0 / 1)
			IGS2I	0.04	(1.00 / 0.00)	4	(4 / 0)
NE Switzerland	2000	Spring	A	0.57	(0.00 / 0.00)	26	(0 / 0)
			B	0.37	(0.00 / 0.00)	17	(0 / 0)
			IGS2III	0.02	(0.00 / 0.00)	1	(0 / 0)
			IGS2IV	0.02	(0.00 / 0.00)	1	(0 / 0)
			IGS2V	0.02	(0.00 / 0.00)	1	(0 / 0)
	2001	Spring	A	0.60	(0.06 / 0.06)	31	(2 / 2)
			B	0.37	(0.11 / 0.05)	19	(2 / 1)
			IGS2I	0.02	(0.00 / 0.00)	1	(0 / 0)
			IGS2III	0.04	(0.00 / 0.00)	2	(0 / 0)
	2002	Spring	A	0.42	(0.05 / 0.00)	22	(1 / 0)
			B	0.49	(0.08 / 0.00)	26	(2 / 0)
			IGS2III	0.02	(0.00 / 0.00)	1	(0 / 0)
			IGS2V	0.02	(0.00 / 0.00)	1	(0 / 0)
			V	0.06	(0.00 / 0.00)	3	(0 / 0)
	2003	Spring	A	0.83	(0.07 / 0.00)	15	(2 / 0)
			B	0.06	(0.00 / 0.00)	1	(0 / 0)
			IGS2III	0.11	(0.00 / 0.00)	2	(1 / 0)

frequency and frequency of *Crithidia bombi* within haplotype (Spearmans r = 0.093, p = 0.764, N = 13). We could find no correlations between *Nosema bombi* frequency in haplotype and haplotype frequency.

There are also no statistically significant correlations between the change in haplotype frequency and *Crithidia bombi* frequency within haplotype (see Tables 4.4 and 4.3). Haplotype frequency change was considered between years in the Swiss population and between seasons in the Gotland population. There is no difference when one considers *Crithidia bombi* from the same season or from the previous season, and no differences are found when using absolute change or relative change in haplotype frequencies. There were not enough haplotypes found infected with *Nosema bombi* to repeat the analysis using this parasite.

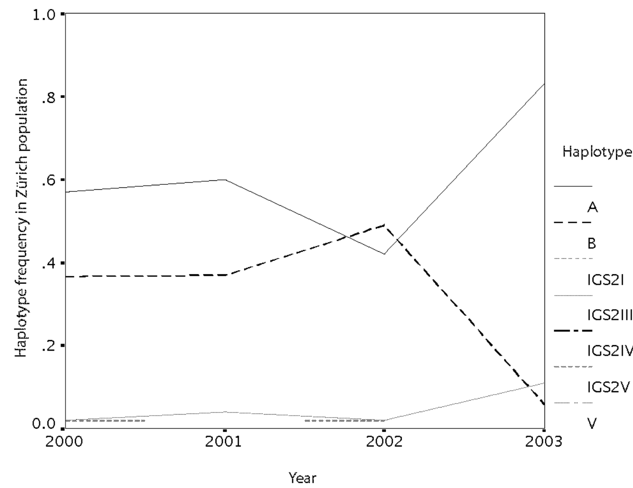

Figure 4.3: *Frequencies of haplotypes in the Swiss population over time. Most of the rare haplotypes are too infrequent to show in the graph. The two common ones are haplotype A and haplotype B.*

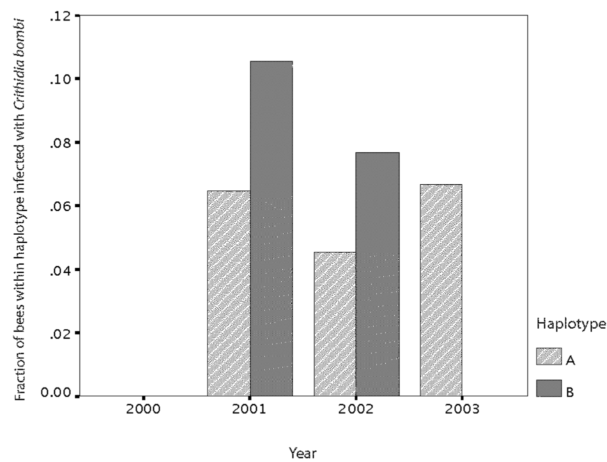

Figure 4.4: *Frequencies of Crithidia bombi in the two common haplotypes in the Swiss population.*

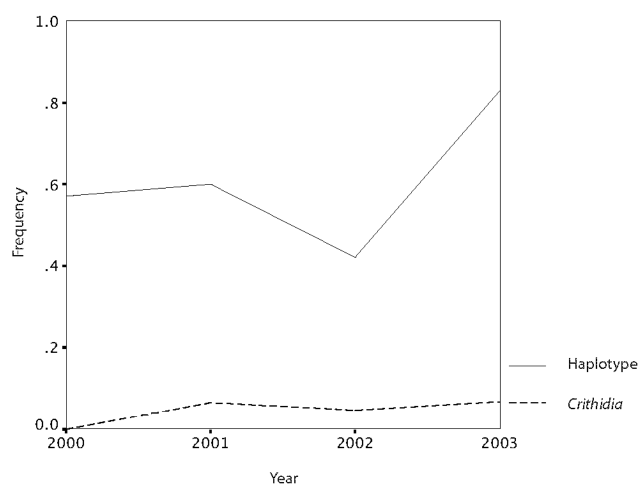

Figure 4.5: *Frequency change of haplotype A and Crithidia bombi presence in haplotype A over time in the Swiss population.*

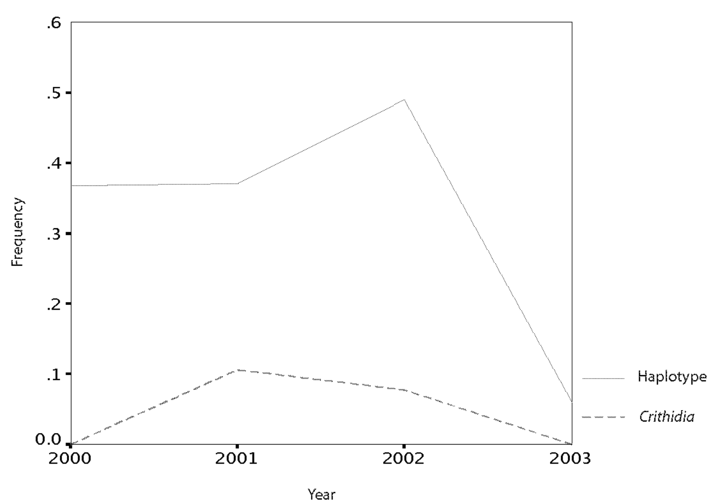

Figure 4.6: *Frequency change of haplotype B and Crithidia bombi presence in haplotype B over time in the Swiss population.*

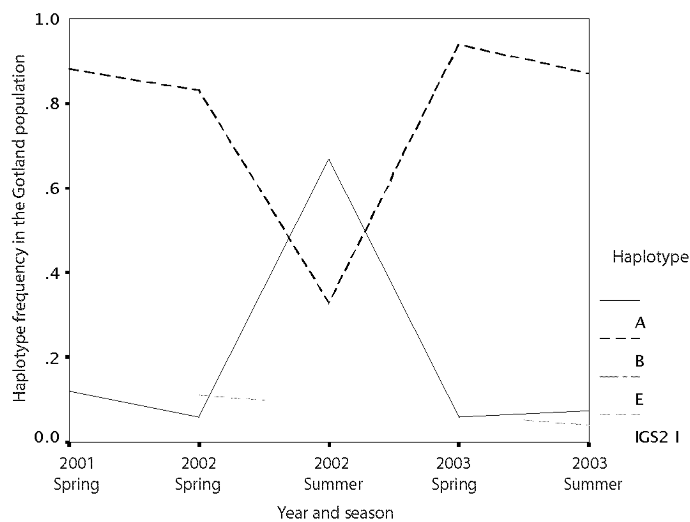

Figure 4.7: *Frequencies of haplotypes Gotland over time.*

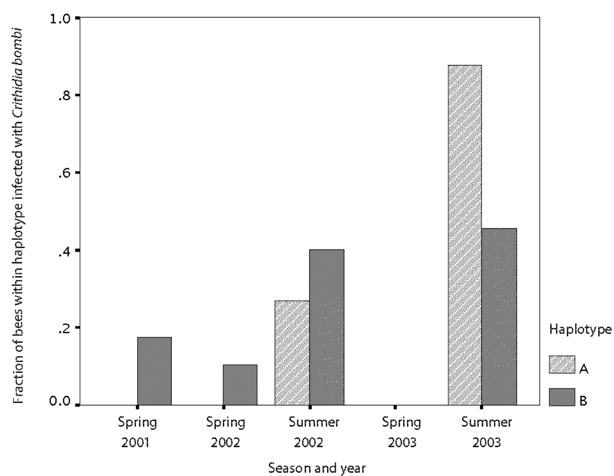

Figure 4.8: *Frequencies of Crithidia bombi in the two common haplotypes in Gotland.*

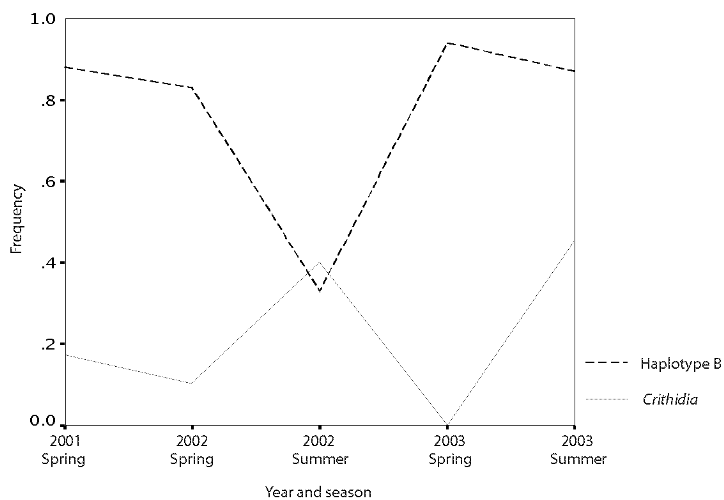

Figure 4.9: *Frequency change of haplotype B and Crithidia presence in haplotype B over time in Gotland.*

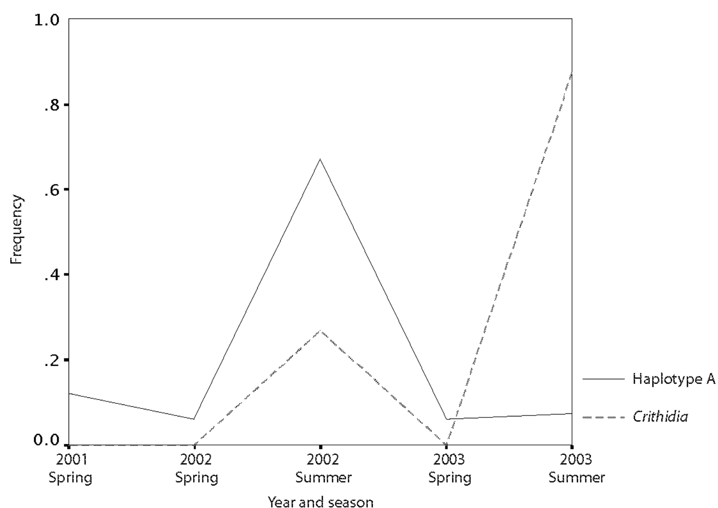

Figure 4.10: *Frequency change of haplotype A and Crithidia presence in haplotype A over time in Gotland*

Table 4.3: *Correlation coefficients (Spearmans r), Significance (2-tailed) and N for Zürich haplotype frequencies and Crithidia frequencies*

Haplotype frequency *Crithida* frequency	Change [a] (current-previous)	Relative change [a] (current - previous)/previous
Same year	0.125, 0.644, 16	0.226, 0.530, 10
Previous year	-0.123, 0.735, 10	-0.266, 0.457, 10

[a] for the years 2000, 2001, 2002 and 2003

Table 4.4: *Correlation coefficients (Spearmans r), Significance (2-tailed) and N for Gotland haplotype frequencies and Crithidia frequencies*

Haplotype frequency *Crithida* frequency	Change [a] (summer-spring)	Relative change [a] (summer-spring)/spring
Summer	-0.071, 0.856, 9	-0.258, 0.742, 4
Spring	-0.429, 0.249, 9	-0.4, 0.6, 4

[a] for the years 2001, 2002 and 2003

Discussion

These are preliminary, exploratory data to look for the Red Queen in the field. Unfortunately, the data do not generate statistically significant patterns. However, they give interesting hints as to what may be occurring in populations of *Bombus terrestris*.

As these are two geographically separate populations, and given the non-coding nature of the intergenic sequence and the high rate of mtDNA mutation, it is surprising that we find the same common haplotypes in both populations. Zürich is in the centre of *Bombus terrestris*'s range, and is in the middle of continental Europe, facilitating movement and gene transfer throughout the population (Estoup et al. 1996, Widmer et al. 1998). On the other hand, Gotland is not only an island population, which is presumably somewhat isolated from the mainland Europe populations, but is also at the northern edge of the species distribution. These ecological factors may play a role in the dynamic observed in the data, and that a different haplotype is common in each population. However, Estoup et al. (1996) and Widmer et al. (1998) both found that there is little nuclear genetic variation in microsatellites, or in a section of mitochondrial COII gene, within Europe. Island populations differ very slightly, but given the strong flying ability of bumblebee queens, and the observation of long distance migrations (Mikkola 1978, Mikkola 1984), we cannot treat the Gotland population as totally isolated. Interestingly, the most common haplotype differs between the populations – in the Swiss population in most years it is haplotype A and in Gotland in all samples except that of summer 2002 it is haplotype B.

Haplotype frequencies vary little between years in both populations between springs, although in the Swiss population in spring 2003 we see a previously rare haplotype (IGS2III) overtake haplotype B in frequency (although sample size here is rather low; see Figure 4.3). This fits well with the results from the field experiment detailed in Chapter 3 carried out in the year 2002. We found that colonies from haplotype B had a lower fitness, particularly producing fewer and lighter queens than colonies from haplotype A. These differences do not seem to be caused by parasites, either from the observed frequencies here or the infection rates recorded

in the field experiment in Chapter 3.

The parasite frequencies within each haplotype also vary and may play a role in the change of haplotype frequencies, although this cannot be shown conclusively in this data set. In accordance with our expectations, we find that individuals from the common haplotypes are more infected with *Crithidia bombi*, although we cannot show the expected overinfection of common haplotypes.

The drastic changes of haplotype frequency between spring and summer 2002 in Gotland indicates that there is some selection pressure at work, as does the change in the frequencies of common haplotypes in the Swiss population. The year 2002 is an exception in both populations, where the normally less common haplotype becomes common. This may have been due to weather conditions in Europe in this year. The haplotype which is normally common in the Swiss population does well in summer 2002 in Gotland, however it does not persist in being so common to the next spring.

The prediction that rare types are at a selective advantage is not held up in this study. Rare haplotypes appear to stay rare. This may be due to mutation within functional mitochondrial genes (see Chapter 3). There does seem to be some cycling between the two common genotypes in both places, which may be indicative of a Red Queen process occurring, even though the role of parasites must remain conjectural at the moment. These results, and those of Chapter 3, are indicative of some kind of directional selection on mitochondrial haplotype. Indeed, there is evidence that mitochondrial markers are under greater directional selection than previously assumed (Bazin et al. 2006).

Another opportunity for categorising bumblebees to types to study the Red Queen hypothesis has become avaiable in the meantime. Quantitative Trait Loci developed for *Crithidia resistance* and encapsulation response (Wilfert et al. 2007) could be used on the same samples presented here to compare what is occurring near to loci involved with *Crithidia* resistance. Hopefully, this will give a more definitive set of results. It would also be possible to examine the *Crithidia bombi* genetic variation using microsatellites to look at the strains in both populations and in the host "types" (Schmid-Hempel & Funk 2004). This would be extremely interesting, as *Crithidia bombi* is known to have strong genotypic interactions with its *Bombus terrestris* hosts (Schmid-Hempel & Schmid-Hempel 1993, Schmid-Hempel & Loosli 1998, Schmid-Hempel et al. 1999, Schmid-Hempel 2001, Mallon et al. 2003).

Chapter 5

General Discussion

Host genetic heterogeneity is predicted to interact with parasites in a variety of ways — both holding back the evolution of virulence (Regoes et al. 2000), and creating cycles of host genotypes and parasite adaptation (Bell 1982, Hamilton 1982, Seger 1988). Here I examine both in the model system of *Bombus* and its parasites, both experimentally and in the field.

I found that in our study system, host heterogeneity did not appear to affect the evolution of virulence. This could be due to a variety of experimental factors. For example, starting with a mixture of parasite strains might mean that strains are sorted before any change in virulence is witnessed (Yourth & Schmid-Hempel 2006). It is possible that not age-controlling the test bees in the survival experiment masked the effect of parasites, and we primarily see the effect of age of the bee when the survival experiment was carried out. However, the two separate experiments carried out in this thesis both lead to the conclusion that host heterogeneity does not reduce virulence in *Crithidia bombi*. This agrees with other experimental findings that the host background that *Crithidia bombi* comes from does not affect various virulence measures (Yourth 2004, Yourth & Schmid-Hempel 2006). However, in natural populations of *Bombus terrestris* and *Crithidia bombi* local adaptation has been found to leads to lower level of virulence when bees are infected with sympatric strains as opposed to allopatric strains (Imhoof & Schmid-Hempel 1998a), which may suggest that adaptation to a particular host environment leads to reduced virulence of *C. bombi*. This also suggests that host and parasite do show adaptation to each other in this system, but perhaps on longer timescales than those of a laboratory experiment.

Perhaps looking at a more directly virulent pathogen would yield clearer answers on virulence evolution. Trypanosomatids such as *Crithidia bombi* often colonise the gut and show low levels of pathogenicity (Schaub 1994). It may be that viral or bacterial pathogens which disrupt biochemical pathways in the host would be more amenable to such studies. It is also possible that host heterogeneity does not constrain the evolution of virulence. This may seem intuitively correct and is supported by theory (Regoes et al. 2000), but other theory points to a more complicated relationship between host heterogeneity and virulence evolution, in some cases leading to higher virulence than in homogeneous host backgrounds (Ganusov et al. 2002).

In Chapters 3 and 4, I investigated some of the predictions of the Red Queen hypothesis

(Bell 1982, Hamilton 1982), which states that host genotypes will be under parasite-mediated frequency dependent selection. Social insects such as bumblebees may be particularly interesting for the study of the Red Queen hypothesis as they live in colonies of closely related individuals with overlapping generations and brood care. Bumblebees, in particular, are subject to a seasonal genetic bottleneck with only young queens overwintering. This means that even relatively benign parasites such as *Crithidia bombi* can exert a large selection pressure on host genotypes. Agrawal (2006) suggests that parasites of low pathogenicity in host populations where hosts live in genetically similar groups and are even slightly more likely to become infected by their mother (a description which well fits the *Bombus-Crithidia* study system), select for the maintenance of sex in the hosts (part of the Red Queen hypothesis). This may be a factor in maintaining Red Queen negative frequency dependent cycles of host genotypes in natural populations.

To study the dynamics of host genotypes, I used mitochondrial DNA to assign bumblebees to haplotypes. Using mitochondrial DNA as a marker has advantages and disadvantages in this system. It is maternally inherited, although paternal mtDNA leakage has been observed (Kondo et al. 1990), which enabled me to follow matrilines within two populations of *Bombus terrestris*. It is known that there are strong genotypic interactions between colony and parasites in this system (Schmid-Hempel & Schmid-Hempel 1993, Schmid-Hempel & Loosli 1998, Schmid-Hempel et al. 1999, Schmid-Hempel 2001, Mallon et al. 2003). It has also been shown that colony founding and early growth is both one of the most important predictors of colony succes (Müller & Schmid-Hempel 1993). Colony founding is also the time that *Crithidia bombi* exerts its main selection pressure (Brown, Schmid-Hempel & Schmid-Hempel 2003). Most colonies will never produce queens (Müller & Schmid-Hempel 1993), and so we used haplotypes as a proxy for the success of nuclear genes in the colony. I found both common and rare haplotypes within the populations in north-eastern Switzerland and Gotland, Sweden. The same two haplotypes (with the same sequence and banding patterns on SSCP gels) were present as the most common genotypes in both populations, despite the geographical separation of the populations. It is still unclear how far bumblebee queens can migrate, although they have been observed to travel great distances in spring in northern Europe (Mikkola 1978, Mikkola 1984), and genetic studies suggest that the continental European populations of *Bombus terrestris* and *Bombus pascuorum* are fairly homogeneous (Estoup et al. 1996, Widmer et al. 1998, Widmer & Schmid-Hempel 1999). This would explain why the same haplotypes are found in both populations, but not why different ones are common and rare in each population.

In Chapters 3 and 4, I show that there are multiple matrilines within two populations of *Bombus terrestris*, and that there are some frequency changes within populations of these haplotypes, in Zürich across years and in Gotland within years. These particular assumptions of the Red Queen hypothesis are, therefore, met. The predictions of the Red Queen hypothesis we investigated in relation to haplotype frequency were fitness and parasite infestation. We expected that parasites would overinfect common haplotypes, and that there would be an inverse correlation between haplotype frequency and fitness. Common haplotypes do show higher infection rates than rare haplotypes, but also have higher fitness. It seems that rare haplotypes tend to do badly, both in terms of fitness (as shown in the field experiment in chapter 3) and in terms of frequency within populations (Chapter 4). It is possible that these rare types also contain mutations in functional mitochondrial genes, which means that they are unable to found colonies, or if they do found colonies, do extremely poorly and are unable to produce queens. This could be tested by looking at the amount or activity of the cytochrome oxidase enzyme, or other mitochondrial enzymes (Hess & Pope 1953, Suarez et al. 2000). If

mutations in functional genes are present, the ability of rare colonies to produce workers and males but not queens could reflect the increased importance of mitochondrial enzymes for queens in bees (Corona et al. 1999).

I found no evidence to link changes of haplotype frequency with parasitism. The rare genotypes do badly and stay rare within populations. Of the two common haplotypes, we do not see overinfection of the more common haplotype (Chapter 4), or a loss of fitness due to higher levels of parasitism (Chapter 3). As I found the same two common haplotypes within both populations, I was unable to test the prediction that populations with a higher diversity of matrilines would be less affected by parasites (Siemens & Roy 2005). I was also unable to see time-lagged frequency dependent selection. It could be that we are looking at the wrong level of host diversity, or diversity in the wrong area of the genome. The mitochondrial marker, although a non-coding area, may be too closely linked to genes under strong selection to show any more subtle effects (Gemmell & Allendorf 2001, Bazin et al. 2006). Although both Zürich and Gotland populations contain the same two common haplotypes, the frequencies are reversed, with haplotype A being more common than haplotype B in Zürich (from spring collections in most years in my study – with 2002 being an exception), and haplotype B being more common in Spring in Gotland. Haplotype A then becomes more common in Gotland over the course of the summer. It is possible that these two haplotypes are associated with bumblebees with different strategies, with B being able to withstand cooler temperatures, for example. We may see functional differences between these two haplotypes in terms of mitochondrial enzyme activity.

The relationship of host genetic heterogeneity to parasitism is complex. Taking together all the results in my thesis, I showed that host heterogeneity does not necessarily constrain the evolution of parasite virulence as one might expect. As we do see local adaptation by *Crithidia bombi* to hosts (Imhoof & Schmid-Hempel 1998a), it may be that we are looking at a system where virulence evolves only slowly. This could affect the development of host genotype dynamics in this system. It is assumed that in a Red Queen scenario, the parasites can adapt to the hosts quickly. If *Crithidia bombi* is a parasite that evolves increased virulence only slowly, then to see host genotype cycles we may have to observe populations for longer than the 5 to 7 host generations estimated for a single cycle (Barton 1995).

Appendix A

Primers and sequences

Primers IGSF1 and IGSR1 were originally designed by Alex Widmer from *Apis mellifera*. To design new primers that also included the whole IGS region we joined the sequence of fragment IGSF1/IGSR1 and sequence (Accession) from GenBank. Primer COIIB3 was designed from this “artificial” sequence. Primer pair IGSF1 x COIIB3 then amplified a fragment including 3’end of COI, the complete IGS-region and the 5’end of COII. Primer pair IGSF2.6 x IGS2.B1 was designed for SSCP analysis (haplotype study).

The aligned primers are shown below on sequences from *Bombus terrestris* and *Bombus moderatus*. Primer IGSR1 is not found on the sequence of B. moderatus

***Bombus terrestris*: IGSF1 - COIIB3**

```
Primer IGSF1                           Primer IGS2.F6
GAGGCAATAATTTCAATAAATAGAATATTATTTTTAATTTTCATTATTTTTGAAAGATTAATTTCTAAACGATTAATTTT

ATTTAAATTCCATCAATCATCACTTGAATGATTAAATAATTATCCTCCTTATGATCACTCATTAATTGAAATTCCATTAA

TTTCAAAAAATAAAGTTAAAAATATTTTCAATAAATAAATTACCCTTTTAATATAAATTTAACATTTAATATAATATTAA
                  Primer IGS2.B1
TATTGCAAATTAATGCCTTGAACTTAAAATTCAACTATAAAGATTATTTTTCTTTTATTAATATAAATATTTAATATAAT
          Primer IGSR1
ATTAATATGGCAGATTAGTGCTTTGAATTTAAAATTCAACTATAAAGATTATTTTTCTTTTATTAATATAAATATTTAAT

AAATCAATATTTTGAATTGAATTGAAAATTCAAATAAATTTTATTAATTATTAAAAATTTCTACATGAAACATATTTTTA
                           Primer COIIB3
TTTCAAGATTCAAATTCTTTTTACTCTGATAATCTTATTTCATTTCATAA
```

Bombus moderatus: IGSF1 - COIIB3

```
Primer IGSF1                       Primer IGS2.F6
GGAGCAATAATTTCAATAAATAGAATATTACTTTTAATTTTCATTATTTTTGAAAGATTAATTTCTAAACGATTAATTTT

ATTTAAGTTCCATCAATCATCTCTTGAATGATTAAATAATTATCCACCTTTAGATCACTCACTAGTAGAAATTCCGTTAA

TTTCAAAAAATAAAATTAAAAATATTTTCAATAAATAATTATATATATATATATATAATTAATATGGCAGAATTAGTGCC
Primer IGS2.B1
TTGAACTTAAAATTCAACAATAAAGATATATTTTCTTTTATTAATATTAATATTTTAATAAATCAATATATATATATATA

TATATATATTATTTTGAATTGAATTTTAAATTCAAATAAATTTTTCTAATTATTAAAAATTTCTACATGAAATATATTTT
                          Primer COIIB3
TATTTCAAGATACAAATTCTTTTTACTCTGATAATCTTATTTCATTTCATAA
```

Apis mellifera ligustica

Complete sequence COI to COII (Crozier & Crozier 1993) showing the placement of the *Bombus* primers IGSF1 (A. Widmer, pers. comm.), COIIB3 (R. Schmid-Hempel, pers. comm.) and E2 and H2 (Estoup et al. 1996).

```
        10        20        30        40        50        60
CTTAATAATAAAGTGATTCATATCAACCAATCATAAAAATATTGGGATCTTGTATATTAT
GAATTATTATTTCACTAAGTATAGTTGGTTAGTATTTTTATAACCCTAGAACATATAATA
     I  I  K  *  F  I  S  T  N  H  K  N  I  G  I  L  Y  I  I>
    ___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME____>
____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

        70        80        90       100       110       120
TCTAGCTTTATGATCTGGAATACTAGGATCATCAATGAGACTTATTATTCGAATAGAATT
AGATCGAAATACTAGACCTTATGATCCTAGTAGTTACTCTGAATAATAAGCTTATCTTAA
  L  A  L  *  S  G  I  L  G  S  S  M  R  L  I  I  R  I  E  L>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       130       140       150       160       170       180
AAGATCCCCAGGATCATGAATTAGCAATGATCAAATTTATAATACAATTGTTACTAGTCA
TTCTAGGGGTCCTAGTACTTAATCGTTACTAGTTTAAATATTATGTTAACAATGATCAGT
  R  S  P  G  S  *  I  S  N  D  Q  I  Y  N  T  I  V  T  S  H>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       190       200       210       220       230       240
TGCATTCCTAATAATTTTTTTTATAGTTATACCATTTTTAATTGGAGGATTTGGAAATTG
ACGTAAGGATTATTAAAAAAAATATCAATATGGTAAAAATTAACCTCCTAAACCTTTAAC
  A  F  L  I  I  F  F  I  V  I  P  F  L  I  G  G  F  G  N  W>
```

```
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       250       260       270       280       290       300
GCTTATTCCTTTAATACTAGGATCACCTGATATAGCATTCCCCCGAATAAATAATATTAG
CGAATAAGGAAATTATGATCCTAGTGGACTATATCGTAAGGGGGCTTATTTATTATAATC
  L  I  P  L  I  L  G  S  P  D  I  A  F  P  R  I  N  N  I  R>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       310       320       330       340       350       360
ATTTTGATTACTTCCTCCCTCATTATTTATACTTTTATTAAGAAATTTATTTTATCCAAG
TAAAACTAATGAAGGAGGGAGTAATAAATATGAAAATAATTCTTTAAATAAAATAGGTTC
  F  *  L  L  P  P  S  L  F  I  L  L  L  R  N  L  F  Y  P  R>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       370       380       390       400       410       420
ACCAGGAACTGGATGAACAGTATATCCACCATTATCAGCATATTTATATCATTCTTCACC
TGGTCCTTGACCTACTTGTCATATAGGTGGTAATAGTCGTATAAATATAGTAAGAAGTGG
  P  G  T  G  *  T  V  Y  P  P  L  S  A  Y  L  Y  H  S  S  P>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       430       440       450       460       470       480
TTCAGTAGATTTTGCAATTTTTTCTCTTCATATATCAGGAATTTCCTCAATTATAGGATC
AAGTCATCTAAAACGTTAAAAAAGAGAAGTATATAGTCCTTAAAGGAGTTAATATCCTAG
  S  V  D  F  A  I  F  S  L  H  I  S  G  I  S  S  I  I  G  S>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       490       500       510       520       530       540
ATTAAACTTAATAGTTACAATTATAATAATAAAAAATTTTTCTATAAATTATGACCAAAT
TAATTTGAATTATCAATGTTAATATTATTATTTTTTAAAAAGATATTTAATACTGGTTTA
  L  N  L  I  V  T  I  I  I  I  K  N  F  S  I  N  Y  D  Q  I>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       550       560       570       580       590       600
TTCATTATTTCCATGATCAGTTTTTATTACAGCAATTTTATTAATTATATCATTACCTGT
AAGTAATAAAGGTACTAGTCAAAAATAATGTCGTTAAAATAATTAATATAGTAATGGACA
  S  L  F  P  *  S  V  F  I  T  A  I  L  L  I  I  S  L  P  V>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       610       620       630       640       650       660
ATTAGCTGGAGCAATTACTATACTATTATTTGATCGAAATTTTAATACATCATTTTTCGA
TAATCGACCTCGTTAATGATATGATAATAAACTAGCTTTAAAATTATGTAGTAAAAAGCT
  L  A  G  A  I  T  I  L  L  F  D  R  N  F  N  T  S  F  F  D>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       670       680       690       700       710       720
TCCTATAGGAGGTGGAGATCCAATTCTTTATCAACATTTATTTTGATTTTTTGGTCATCC
AGGATATCCTCCACCTCTAGGTTAAGAAATAGTTGTAAATAAAACTAAAAAACCAGTAGG
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  P  I  G  G  G  D  P  I  L  Y  Q  H  L  F  *  F  F  G  H  P>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       730       740       750       760       770       780
AGAAGTTTATATTTTAATTTTACCTGGATTTGGATTAATCTCTCATATTGTAATAAATGA
TCTTCAAATATAAAATTAAAATGGACCTAAACCTAATTAGAGAGTATAACATTATTTACT
  E  V  Y  I  L  I  L  P  G  F  G  L  I  S  H  I  V  I  N  E>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       790       800       810       820       830       840
AAGAGGAAAAAAAGAAATTTTTGGTAATTTAAGAATAATTTATGCAATATTAGGAATTGG
TTCTCCTTTTTTTCTTTAAAAACCATTAAATTCTTATTAAATACGTTATAATCCTTAACC
  R  G  K  K  E  I  F  G  N  L  R  I  I  Y  A  I  L  G  I  G>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       850       860       870       880       890       900
ATTTCTAGGTTTTATTGTTTGAGCACATCACATATTTACAGTCGGATTAGATGTTGATAC
TAAAGATCCAAAATAACAAACTCGTGTAGTGTATAAATGTCAGCCTAATCTACAACTATG
  F  L  G  F  I  V  *  A  H  H  I  F  T  V  G  L  D  V  D  T>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       910       920       930       940       950       960
TCGAGCATATTTTACTTCAGCAACAATAATCATTGCTGTACCAACAGGAATTAAAGTTTT
AGCTCGTATAAAATGAAGTCGTTGTTATTAGTAACGACATGGTTGTCCTTAATTTCAAAA
  R  A  Y  F  T  S  A  T  I  I  I  A  V  P  T  G  I  K  V  F>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

       970       980       990      1000      1010      1020
TAGATGATTAGCAACTTATCATGGTTCAAAATTAAAATTAAATATTTCAATTTTATGATC
ATCTACTAATCGTTGAATAGTACCAAGTTTTAATTTTAATTTATAAAGTTAAAATACTAG
  R  *  L  A  T  Y  H  G  S  K  L  K  L  N  I  S  I  L  *  S>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1030      1040      1050      1060      1070      1080
ACTAGGTTTTATTATACTATTTACTATTGGTGGATTAACAGGAATTATATTATCAAATTC
TGATCCAAAATAATATGATAAATGATAACCACCTAATTGTCCTTAATATAATAGTTTAAG
  L  G  F  I  I  L  F  T  I  G  G  L  T  G  I  I  L  S  N  S>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1090      1100      1110      1120      1130      1140
TTCTATTGATATTATTCTTCATGATACATATTACGTTGTTGGACATTTTCATTATGTTCT
AAGATAACTATAATAAGAAGTACTATGTATAATGCAACAACCTGTAAAAGTAATACAAGA
  S  I  D  I  I  L  H  D  T  Y  Y  V  V  G  H  F  H  Y  V  L>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1150      1160      1170      1180      1190      1200
TTCAATAGGTGCAGTATTTGCAATTATTTCAAGATTTATTCATTGATATCCATTAATTAC
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AAGTTATCCACGTCATAAACGTTAATAAAGTTCTAAATAAGTAACTATAGGTAATTAATG
  S  I  G  A  V  F  A  I  I  S  R  F  I  H  *  Y  P  L  I  T>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1210      1220      1230      1240      1250      1260
TGGATTATTATTAAATATTAAATGATTAAAAATTCAATTTATTATAATATTTATTGGAGT
ACCTAATAATAATTTATAATTTACTAATTTTTAAGTTAAATAATATTATAAATAACCTCA
  G  L  L  L  N  I  K  *  L  K  I  Q  F  I  I  I  F  I  G  V>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1270      1280      1290      1300      1310      1320
AAATCTAACTTTCTTTCCTCAACATTTTTTAGGACTAATATCTATACCACGACGTTATTC
TTTAGATTGAAAGAAAGGAGTTGTAAAAAATCCTGATTATAGATATGGTGCTGCAATAAG
  N  L  T  F  F  P  Q  H  F  L  G  L  I  S  I  P  R  R  Y  S>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>
                                                Primer IGSF1
      1330      1340      1350      1360      1370      1380
AGACTATCCAGATTCTTATTACTGTTGAAATTCAATTTCATCTATAGGATCAATAATTTC
TCTGATAGGTCTAAGAATAATGACAACTTTAAGTTAAAGTAGATATCCTAGTTATTAAAG
  D  Y  P  D  S  Y  Y  C  *  N  S  I  S  S  I  G  S  I  I  S>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1390      1400      1410      1420      1430      1440
ATTAAATAGAATAATTTTTTTAATTTTTATTATTTTAGAAAGATTAATTTCTAAACGAAT
TAATTTATCTTATTAAAAAAATTAAAAATAATAAAATCTTTCTAATTAAAGATTTGCTTA
  L  N  R  I  I  F  L  I  F  I  I  L  E  R  L  I  S  K  R  I>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1450      1460      1470      1480      1490      1500
ATTATTATTTAAATTCAACCAATCATCACTTGAATGATTAAATTTTTTACCACCTCTAGA
TAATAATAAATTTAAGTTGGTTAGTAGTGAACTTACTAATTTAAAAAATGGTGGAGATCT
  L  L  F  K  F  N  Q  S  S  L  E  *  L  N  F  L  P  P  L  D>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1510      1520      1530      1540      1550      1560
TCATTCACATTTAGAAATTCCATTATTAATTAAAAATTTAAATTTAAAATCAATTTTAAT
AGTAAGTGTAAATCTTTAAGGTAATAATTAATTTTTAAATTTAAATTTTAGTTAAAATTA
  H  S  H  L  E  I  P  L  L  I  K  N  L  N  L  K  S  I  L  I>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>
            Primer E2
      1570      1580      1590      1600      1610      1620
TAAATTTTAATATGGCAGAATAAGTGCATTGAACTTAAGATTCAAATATAAAGTATTTTT
ATTTAAAATTATACCGTCTTATTCACGTAACTTGAATTCTAAGTTTATATTTCATAAAAA
  K  F  *>
__________>
     ___PRODUCT=TRNA-OTHER; CODON RECOGNIZED: AAU; ANTIC____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>
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      1630      1640      1650      1660      1670      1680
AAACTTTTATTAAAATTTCCCCACTTAATTCATATTAATTTAAAAATAAATTAATAACAA
TTTGAAAATAATTTTAAAGGGGTGAATTAAGTATAATTAAATTTTTATTTAATTATTGTT
___PRODUCT=____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1690      1700      1710      1720      1730      1740
TTTTTAATAAAATAAATAATTAATTTTATTTTTATATTGAATTTTAAATTCAATCTTAAA
AAAAATTATTTTATTTATTAATTAAAATAAAAATATAACTTAAAATTTAAGTTAGAATTT
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1750      1760      1770      1780      1790      1800
GATTTAATCTTTTTATTAAAATTAATAAATTAATATAAAATAAAACAAAATATAACAGAA
CTAAATTAGAAAAATAATTTTAATTATTTAATTATATTTTATTTTGTTTTATATTGTCTT
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1810      1820      1830      1840      1850      1860
TATATTTATTAAAATTTAATTTATTAAAATTTCCACATGATTTATATTTATATTTCAAGA
ATATAAATAATTTTAAATTAAATAATTTTAAAGGTGTACTAAATATAAATATAAAGTTCT
                             I  S  T  *  F  I  F  I  F  Q  E>
                            ___CODON_START=1; TRANSL_TAB____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>
                Primer COIIB3
      1870      1880      1890      1900      1910      1920
ATCAAATTCATATTATGCTGATAATTTAATTTCATTTCATAATATAGTTATAATAATTAT
TAGTTTAAGTATAATACGACTATTAAATTAAAGTAAAGTATTATATCAATATTATTAATA
  S  N  S  Y  Y  A  D  N  L  I  S  F  H  N  I  V  I  I  I  I>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1930      1940      1950      1960      1970      1980
TATTATAATTTCAACATTAACTGTATATATTATTTTAGATTTATTTATAAACAAATTCTC
ATAATATTAAAGTTGTAATTGACATATATAATAAAATCTAAATAAATATTTGTTTAAGAG
  I  I  I  S  T  L  T  V  Y  I  I  L  D  L  F  I  N  K  F  S>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      1990      2000      2010      2020      2030      2040
AAATTTATTTTTATTAAAAAATCATAATATTGAAATTATTTGAACAATTATTCCAATTAT
TTTAAATAAAAATAATTTTTTAGTATTATAACTTTAATAAACTTGTTAATAAGGTTAATA
  N  L  F  L  L  K  N  H  N  I  E  I  I  *  T  I  I  P  I  I>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      2050      2060      2070      2080      2090      2100
TATTCTATTAATTATTTGTTTTCCATCATTAAAAATTTTATATTTAATTGATGAAATTGT
ATAAGATAATTAATAAACAAAAGGTAGTAATTTTTAAAATATAAATTAACTACTTTAACA
  I  L  L  I  I  C  F  P  S  L  K  I  L  Y  L  I  D  E  I  V>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      2110      2120      2130      2140      2150      2160
AAATCCTTTTTTTTCAATTAAATCAATTGGTCATCAATGATATTGATCATATGAATATCC
TTTAGGAAAAAAAAGTTAATTTAGTTAACCAGTAGTTACTATAACTAGTATACTTATAGG
  N  P  F  F  S  I  K  S  I  G  H  Q  *  Y  *  S  Y  E  Y  P>
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___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      2170      2180      2190      2200      2210      2220
AGAATTTAATAATATTGAATTTGATTCATATATACTAAATTATAATAATTTAAACCAATT
TCTTAAATTATTATAACTTAAACTAAGTATATATGATTTAATATTATTAAATTTGGTTAA
  E  F  N  N  I  E  F  D  S  Y  I  L  N  Y  N  N  L  N  Q  F>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      2230      2240      2250      2260      2270      2280
TCGTTTACTAGAAACTGATAATCGAATAGTAATTCCAATAAAAATCCCACTACGTTTAAT
AGCAAATGATCTTTGACTATTAGCTTATCATTAAGGTTATTTTTAGGGTGATGCAAATTA
  R  L  L  E  T  D  N  R  I  V  I  P  I  K  I  P  L  R  L  I>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>
           Primer H2
      2290      2300      2310      2320      2330      2340
TACAACATCAACAGATGTAATTCATTCATGAACAGTTCCATCCTTAGGTATTAAAGTTGA
ATGTTGTAGTTGTCTACATTAAGTAAGTACTTGTCAAGGTAGGAATCCATAATTTCAACT
  T  T  S  T  D  V  I  H  S  *  T  V  P  S  L  G  I  K  V  D>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      2350      2360      2370      2380      2390      2400
TGCAGTTCCAGGACGAATTAATCAATTAAATTTAATTAGAAAACGTCCAGGAATTTTTTT
ACGTCAAGGTCCTGCTTAATTAGTTAATTTAAATTAATCTTTTGCAGGTCCTTAAAAAAA
  A  V  P  G  R  I  N  Q  L  N  L  I  R  K  R  P  G  I  F  F>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      2410      2420      2430      2440      2450      2460
TGGTCAATGTTCAGAAATTTGTGGTATAAATCATAGATTTATACCAATTATAATTGAATC
ACCAGTTACAAGTCTTTAAACACCATATTTAGTATCTAAATATGGTTAATATTAACTTAG
  G  Q  C  S  E  I  C  G  I  N  H  R  F  I  P  I  I  I  E  S>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=CYTOCHROME C O____>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE=MITOCHON____>

      2470      2480      2490      2500      2510
AACTTCATTTCAATATTTTTTAAATTGAGTAAATAAACAAATCTAAAAAAT
TTGAAGTAAAGTTATAAAAAATTTAACTCATTTATTTGTTTAGATTTTTTA
  T  S  F  Q  Y  F  L  N  *  V  N  K  Q  I  *>
___CODON_START=1; TRANSL_TABLE=5; PRODUCT=____>
                                            _______>
___ORGANISM=APIS MELLIFERA LIGUSTICA; ORGANELLE____>
```

Appendix B

Aligned sequences

Table B.1: *Aligned sequences of all haplotypes found in northeast Switzerland 2002 (IGSF1 and COIIB3 primers; Chapter 3).*

Haplotype A:	TTTCAATAAA	TAGAATATTA	TTTTTAATTT	TCATTATTTT	TGAAAGATTA	ATTTCTAAAC	GATTAATTTT	ATTTAAATTC	CATCAATCAT	CACTTGAATG	ATTAAATAAT
Haplotype B:	....	....	....	....	....	....	....	....	....	....	....
Haplotype C:	....	....	....	....	....	....T.....	....	....	....	....	....
Haplotype D:	....	....	....	....	....	....	.....G....	....	....	....	....
Haplotype E:	....	....	....	....	....	....	....	....	....	.T........	....
Haplotype F:	....	....	....	....	....	....	....	....	....	....	....
Haplotype G:	....	....	....	....	....	....	....	....	....	....	....
Haplotype H:	....	....	....	....	....	....	....	....	....	....	....
Haplotype I:	....	....	....	....	....	....	....	....	....	....	....
Haplotype J:	....	....	....	....	....	....	....	....	....	....	....
Haplotype K:	....	....	....	....	....	....	....	....	....	....	....
Haplotype L:	....	....	....	....	....	....	....	....	....	....	....
Haplotype M:	....	....	....	....	....	....	....	....	....	....	....
Haplotype N:	....	....	....	....	....	....	....	....	....	....	....
Haplotype O:	....	....	....	....	....	....	....	....	....	....	....
Haplotype S:	....	....	....	....	....	....	....	....	....	....	....
Haplotype T:	....	....	....	....	....	....	....	....	....	....	..G.......
Haplotype U:	....	....	....	....	....	....	....	....	....	....	....
Haplotype V:	....	....	....	....	....	....	.....G....	....	....	....	....
Haplotype W:	....	....	....	....	....	....	....	....	....	....	....
Haplotype X:	....	....	....	....	....	....	....	....	....	....	....
Haplotype Y:	....	....	....	....	....	....	....	....	....	....	....
Haplotype Z:	....	....	....	....	....	....	....	....	....	.T........	....

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Table B.1: *Aligned sequences of all haplotypes found in northeast Switzerland 2002 (IGSF1 and COIIB3 primers; Chapter 3). – continued*

Haplotype A:	TATCCTCCTT	ATGATCACTC	ATTAATTGAA	ATTCCATTAA	TTTCAA-AAA	ATAAAATTAA	AAATATTTTC	A-ATA-AATA	AATTACCCTT	TTAATATAAA	TTTAACATTT
Haplotype B:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype C:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype D:	....	....	....	....	....	....	....	....	....	....	....
Haplotype E:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype F:	........C.	....	....	......NN..	......C...	.....G..NN	....	.C...T...T	........C.	....	....
Haplotype G:	....	....	....	....	G..TC.A...	.....G....	....	....	....	....	....
Haplotype H:	....	....	....	....	....	....	....	....	....	....	....
Haplotype I:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype J:	....	....	....	....	....	....	....	....	....	....	....
Haplotype K:	....	....	....	....	....	....	....	....	....	....	....
Haplotype L:	....	....	....	....	....	....	....	....	....	....	....
Haplotype M:	....	....	....	....	....	....	....	....	....	....	....
Haplotype N:	....	....	....	....	....	....	....	....	....	....	....
Haplotype O:	....	....	....	....	....	....	....	....	....	....	....
Haplotype S:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype T:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype U:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype V:	....	....	....	....	....	....	....	....	....	....	....
Haplotype W:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype X:	....	....	....	....	....	.....G....	....	.G........	....	....	....
Haplotype Y:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype Z:	....	....	....	....	....	.....G....	....	....	....	....	....

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Table B.1: *Aligned sequences of all haplotypes found in northeast Switzerland 2002 (IGSF1 and COIIB3 primers; Chapter 3). – continued*

Haplotype A:	AATATAATAT	TAATATTGCA	AATTAATGCC	-TTGAAC-TT	AAAATTCAAC	TATAAAG-AT	TATTTTT-CT	TTTATTAATA	TAAATATTTA	ATATAATATT	AATATGGC-A
Haplotype B:	....	....	....	....	....	....	....	....	....	....	....
Haplotype C:	....	....	....	....	....	....	....	....	....	....	....
Haplotype D:	....	....	....	....	....	....	....	....	....	....	....
Haplotype E:	....	....	....	....	....	....	....	....	....	....	....
Haplotype F:	....	....	....	....	....	....	....	....	....	....	....
Haplotype G:	....	....	....	....	....	....	....	....	....	....	....
Haplotype H:	....	....	....	....	....	....	....	....	....	....	....
Haplotype I:	....	....	...C......	....	....	....	....	....	....	....	....
Haplotype J:	....	....	.......A..	....	....	....	....	....	....	....	....
Haplotype K:	....	....	.......A..	....	....	....	.......T..	....	....	....	....
Haplotype L:	....	....	....	C.........	....	....	.......T..	....	....	....	....
Haplotype M:	....	....	....	....	....	....	.......T..	....	....	....	....
Haplotype N:	....	....	....	....	....	....	....	....	....	....	........C.
Haplotype O:	....	....	....	....	....	....	....	....	....	....	....
Haplotype S:	....	....	....	....	....	....	....	....	....	....	....
Haplotype T:	....	....	....	....	....	....	....	....	....	....	....
Haplotype U:	....	....	....	....	....	....	....	....	....	....	....
Haplotype V:	....	....	....	C.........	....	....	....	....	....	....	....
Haplotype W:	....	....	....	.......C..	....	.......G..	.......T..	....	....	....	....
Haplotype X:	....	....	....	....	....	....	....	....	....	....	....
Haplotype Y:	....	....	....	....	....	....	....	....	....	....	....
Haplotype Z:	....	....	....	....	....	....	.......T..	....	....	....	....

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Table B.1: *Aligned sequences of all haplotypes found in northeast Switzerland 2002 (IGSF1 and COIIB3 primers; Chapter 3). – continued*

Haplotype A:	GATTAGTGC-	TTTGAATTTA	AAATTCAACT	ATAAAGATTA	TTTTTC-TTT	TATTAATATA	AATATTTAAT	AAATCAATAT	TTTGAATTGA	ATT
Haplotype B:	....	....	....	....	....	....	....	....	....	...
Haplotype C:	....	....	....	....	....	....	....	....	....	...
Haplotype D:	....	....	....	....	....	....	....	....	....	...
Haplotype E:	....	....	....	....	....	....	....	....	....	...
Haplotype F:	....	....	....	....	....	....	....	....	....	...
Haplotype G:	....	....	....	....	....	....	....	....	....	...
Haplotype H:	....	....	....	....	....	....	....	....	....	...
Haplotype I:	....	....	....	....	....	....	....	....	....	...
Haplotype J:	....	....	....	....	....	....	....	....	....	...
Haplotype K:	....	....	....	....	....	....	....	....	....	...
Haplotype L:	....	....	....	....	....	....	....	....	....	...
Haplotype M:	....	....	....	....	....	....	....	....	....	
Haplotype N:	C	....	....	....	....	....	....	....	....	...
Haplotype O:	....	....	....	....	......C...	....	....	....	....	...
Haplotype S:	....	....	....	.....A....	....	....	....	....	....	...
Haplotype T:	....	....	....	....	....	....	....	....	....	...
Haplotype U:	....	....	....	....	....	....	....	....	....	...
Haplotype V:	....	....	....	....	....	....	....	....	....	...
Haplotype W:	....	....	....	....	......C...	....	....	....	....	...
Haplotype X:	....	....	....	....	....	....	....	....	.....G....	...
Haplotype Y:	....	....	....	....	....	....GGAGC.	.T.....C.-	-..A....G	AAAA..G..	
Haplotype Z:	....	....	....	....	....	....	....	....	....	...

Table B.2: *Aligned sequences of all individuals used to determine haplotype frequencies (Chapter 3).*

02.008:	TTTCAATAAA	TAGAATATTA	TTTTTAATTT	TCATTATTTT	TGAAAGATTA	ATTTCTAAAC	GATTAATTTT	ATTTAAATTC	CATCAATCAT	CACTTGAATG	ATTAAATAAT
02.010:	....	....	....	....	....	....	....	....	....	....	....
02.022:	....	....	....	....	....	....	....	....	....	....	....
02.027:	....	....	....	....	....	....	....	....	....	....	....
02.029:	....	....	....	....	....	....	....	....	....	....	....
02.037:	....	....	....	....	....	....	....	....	....	....	....
02.042:	....	....	....	....	....	....	....	....	....	....	....
02.046:	....	....	....	....	....	....	....	....	....	....	....
02.050:	....	....	....	....	....	....	....	....	....	....	....
02.051:	....	....	....	....	....	....	....	....	....	....	....
02.052:	....	....	....	....	....	....	....	....	....	....	....
02.055:	....	....	....	....	....	....	....	....	....	....	....
02.060:	....	....	....	....	....	....	....	....	....	....	....
02.067:	....	....	....	....	....	....	....	....	....	....	....
02.071:	....	....	....	....	....	....	....	....	....	....	....
02.087:	....	....	....	....	....	....	....	....	....	....	....
02.091:	....	....	....	....	....	....	....	....	....	....	....
02.093:	....	....	....	....	....	....	....	....	....	....	....
02.094:	....	....	....	....	....	....	....	....	....	.T........	....
02.096:	....	....	....	....	....	....	....	....	....	....	....
02.099:	....	....	....	....	....	....	....	....	....	....	....
02.100:	....	....	....	....	....	....	....	....	....	....	....
02.101:	....	....	....	....	....	....	....	....	....	....	....
02.106:	....	....	....	....	....	....	.....G....	....	....	....	....
02.109:	....	....	....	....	....	....	....	....	....	....	....
02.111:	....	....	....	....	....	....T.....	....	....	....	....	....
02.114:	....	....	....	....	....	....	....	....	....	....	....
02.116:	....	....	....	....	....	....	....	....	....	....	....
02.119:	....	....	....	....	....	....	....	....	....	....	....
02.120:	....	....	....	....	....	....	....	....	....	....	....
02.125:	....	....	....	....	....	....	.....G....	....	....	....	....
02.130:	....	....	....	....	....	....	....	....	....	....	....
02.131:	....	....	....	....	....	....	....	....	....	....	....
02.136:	....	....	....	....	....	....	....	....	....	....	....
02.138:	....	....	....	....	....	....	....	....	....	....	....
02.139:	....	....	....	....	....	....	....	....	....	....	....

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02.008: TTTCAATAAA TAGAATATTA TTTTTAATTT TCATTATTTT TGAAAGATTA ATTTCTAAAC GATTAATTTT ATTTAAATTC CATCAATCAT CACTTGAATG ATTAAATAAT
02.145: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.147: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.148: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.149: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.152: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.155: .......... .......... .......... .......... .......... .......... .....G.... .......... .......... .......... ..........
02.159: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.160: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.162: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.163: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.164: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.165: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.167: .......... .......... .......... .......... .......... .......... .....G.... .......... .......... .......... ..........
02.170: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.171: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.172: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.175: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.176: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.177: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.180: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.181: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.184: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.186: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.190: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.191: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.192: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.194: .......... .......... .......... .......... .......... .......... .......... .......... .......... .T........ ..........
02.195: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.196: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.200: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.208: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.215: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..G.......
02.264: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.270: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.273: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
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Table B.2: *Aligned sequences of all individuals used to determine haplotype frequencies (Chapter 3). – continued*

02.008:	TATCCTCCTT	ATGATCACTC	ATTAATTGAA	ATTCCATTAA	TTTCAA-AAA	ATAAAGTTAA	AAATATTTTC	A-ATA-AATA	AATTACCCTT	TTAATATAAA	TTTAACATTT
02.010:	....	....	....	....	G..TC.A...	....	....	....	....	....	....
02.022:	....	....	....	....	....	....	....	....	....	....	....
02.027:	....	....	....	....	....	.....A....	....	....	....	....	....
02.029:	....	....	....	....	....	....	....	....	....	....	....
02.037:	....	....	....	....	....	....	....	.G........	....	....	....
02.042:	....	....	....	....	....	.....A....	....	....	....	....	....
02.046:	....	....	....	....	....	....	....	....	....	....	....
02.050:	....	....	....	....	....	....	....	....	....	....	....
02.051:	........C.	....	....	......NN..	......C...	........NN	....	.C...T...T	........C.	....	....
02.052:	....	....	....	....	....	.....A....	....	....	....	....	....
02.055:	....	....	....	....	....	....	....	....	....	....	....
02.060:	....	....	....	....	....	....	....	....	....	....	....
02.067:	....	....	....	....	....	.....A....	....	....	....	....	....
02.071:	....	....	....	....	....	....	....	....	....	....	....
02.087:	....	....	....	....	....	....	....	....	....	....	....
02.091:	....	....	....	....	....	....	....	....	....	....	....
02.093:	....	....	....	....	....	.....A....	....	....	....	....	....
02.094:	....	....	....	....	....	....	....	....	....	....	....
02.096:	....	....	....	....	....	.....A....	....	....	....	....	....
02.099:	....	....	....	....	....	.....A....	....	....	....	....	....
02.100:	....	....	....	....	....	....	....	....	....	....	....
02.101:	....	....	....	....	....	.....A....	....	....	....	....	....
02.106:	....	....	....	....	....	.....A....	....	....	....	....	....
02.109:	....	....	....	....	....	.....A....	....	....	....	....	....
02.111:	....	....	....	....	....	....	....	....	....	....	....
02.114:	....	....	....	....	....	....	....	....	....	....	....
02.116:	....	....	....	....	....	....	....	....	....	....	....
02.119:	....	....	....	....	....	....	....	....	....	....	....
02.120:	....	....	....	....	....	.....A....	....	....	....	....	....
02.125:	....	....	....	....	....	.....A....	....	....	....	....	....
02.130:	....	....	....	....	....	....	....	....	....	....	....
02.131:	....	....	....	....	....	.....A....	....	....	....	....	....
02.136:	....	....	....	....	....	.....A....	....	....	....	....	....
02.138:	....	....	....	....	....	....	....	....	....	....	....
02.139:	....	....	....	....	....	.....A....	....	....	....	....	....

02.008:	TATCCTCCTT	ATGATCACTC	ATTAATTGAA	ATTCCATTAA	TTTCAA-AAA	ATAAAGTTAA	AAATATTTTC	A-ATA-AATA	AATTACCCTT	TTAATATAAA	TTTAACATTT
02.145:	....	....	....	....	....	.....A....	....	....	....	....	....
02.147:	....	....	....	....	....	....	....	....	....	....	....
02.148:	....	....	....	....	....	....	....	....	....	....	....
02.149:	....	....	....	....	....	....	....	....	....	....	....
02.152:	....	....	....	....	....	....	....	....	....	....	....
02.155:	....	....	....	....	....	.....A....	....	....	....	....	....
02.159:	....	....	....	....	....	....	....	....	....	....	....
02.160:	....	....	....	....	....	.....A....	....	....	....	....	....
02.162:	....	....	....	....	....	....	....	....	....	....	....
02.163:	....	....	....	....	....	....	....	....	....	....	....
02.164:	....	....	....	....	....	....	....	....	....	....	....
02.165:	....	....	....	....	....	....	....	....	....	....	....
02.167:	....	....	....	....	....	.....A....	....	....	....	....	....
02.170:	....	....	....	....	....	....	....	....	....	....	....
02.171:	....	....	....	....	....	....	....	....	....	....	....
02.172:	....	....	....	....	....	.....A....	....	....	....	....	....
02.175:	....	....	....	....	....	.....A....	....	....	....	....	....
02.176:	....	....	....	....	....	.....A....	....	....	....	....	....
02.177:	....	....	....	....	....	.....A....	....	....	....	....	....
02.180:	....	....	....	....	....	.....A....	....	....	....	....	....
02.181:	....	....	....	....	....	.....A....	....	....	....	....	....
02.184:	....	....	....	....	....	.....A....	....	....	....	....	....
02.186:	....	....	....	....	....	.....A....	....	....	....	....	....
02.190:	....	....	....	....	....	.....A....	....	....	....	....	....
02.191:	....	....	....	....	....	.....A....	....	....	....	....	....
02.192:	....	....	....	....	....	.....A....	....	....	....	....	....
02.194:	....	....	....	....	....	....	....	....	....	....	....
02.195:	....	....	....	....	....	....	....	....	....	....	....
02.196:	....	....	....	....	....	.....A....	....	....	....	....	....
02.200:	....	....	....	....	....	....	....	....	....	....	....
02.208:	....	....	....	....	....	....	....	....	....	....	....
02.215:	....	....	....	....	....	....	....	....	....	....	....
02.264:	....	....	....	....	....	....	....	....	....	....	....
02.270:	....	....	....	....	....	.....A....	....	....	....	....	....
02.273:	....	....	....	....	....	.....A....	....	....	....	....	....

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Table B.2: *Aligned sequences of all individuals used to determine haplotype frequencies (Chapter 3). – continued*

02.008:	AATATAATAT	TAATATTGCA	AATTAATGCC	-TTGAAC-TT	AAAATTCAAC	TATAAAG-AT	TATTTTT-CT	TTTATTAATA	TAAATATTTA	ATATAATATT	AATATGGC-A
02.010:	....	....	....	....	....	....	....	....	....	....	....
02.022:	....	....	....	....	....	....	....	....	....	....	....
02.027:	....	....	....	....	....	....	....	....	....	....	....
02.029:	....	....	....	....	....	....	....	....	....	....	....
02.037:	....	....	....	....	....	....	....	....	....	....	....
02.042:	....	....	....	....	....	....	....	....	....	....	....
02.046:	....	....	....	....	....	....	....	....	....	....	....
02.050:	....	....	....	....	....	....	....	....	....	....	....
02.051:	....	....	....	....	....	....	....	....	....	....	....
02.052:	....	....	.......A..	....	....	....	.......T..	....	....	....	....
02.055:	....	....	...C......	....	....	....	....	....	....	....	....
02.060:	....	....	....	....	....	....	....	....	....	....	....
02.067:	....	....	....	....	....	....	....	....	....	....	....
02.071:	....	....	....	....	....	....	....	....	....	....	....
02.087:	....	....	....	....	....	....	....	....	....	....	....
02.091:	....	....	....	....	....	....	....	....	....	....	....
02.093:	....	....	....	....	....	....	....	....	....	....	....
02.094:	....	....	....	....	....	....	.......T..	....	....	....	....
02.096:	....	....	....	C.........	....	....	.......T..	....	....	....	....
02.099:	....	....	....	....	....	....	....	....	....	....	....
02.100:	....	....	....	.......C..	....	.......G..	.......T..	....	....	....	....
02.101:	....	....	....	....	....	....	....	....	....	....	....
02.106:	....	....	....	....	....	....	....	....	....	....	....
02.109:	....	....	....	....	....	....	....	....	....	....	....
02.111:	....	....	....	....	....	....	....	....	....	....	....
02.114:	....	....	....	....	....	....	....	....	....	....	....
02.116:	....	....	....	....	....	....	....	....	....	....	....
02.119:	....	....	....	....	....	....	....	....	....	....	....
02.120:	....	....	....	....	....	....	.......T..	....	....	....	....
02.125:	....	....	....	....	....	....	....	....	....	....	....
02.130:	....	....	....	....	....	....	....	....	....	....	....
02.131:	....	....	....	....	....	....	....	....	....	....	....
02.136:	....	....	....	C.........	....	....	.......T..	....	....	....	....
02.138:	....	....	....	....	....	....	....	....	....	....	....
02.139:	....	....	....	....	....	....	....	....	....	....	....

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02.008: AATATAATAT TAATATTGCA AATTAATGCC -TTGAAC-TT AAAATTCAAC TATAAAG-AT TATTTTT-CT TTTATTAATA TAAATATTTA ATATAATATT AATATGGC-A
02.145: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.147: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.148: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.149: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.152: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.155: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.159: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.160: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.162: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.163: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.164: .......... .......... .......... C......... .......... .......... .......... .......... .......... .......... ..........
02.165: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.167: .......... .......... .......... C......... .......... .......... .......... .......... .......... .......... ..........
02.170: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.171: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.172: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.175: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.176: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.177: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.180: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.181: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.184: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.186: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.190: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ........C.
02.191: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.192: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.194: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.195: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.196: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.200: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.208: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.215: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.264: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
02.270: .......... .......... .......A.. .......... .......... .......... .......... .......... .......... .......... ..........
02.273: .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
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Table B.2: *Aligned sequences of all individuals used to determine haplotype frequencies (Chapter 3). – continued*

02.008:	GATTAGTGC-	TTTGAATTTA	AAATTCAACT	ATAAAGATTA	TTTTTC-TTT	TATTAATATA	AATATTTAAT	AAATCAATAT	TTTGAATTGA	ATT
02.010:	....	....	....	....	....	....	....	....	....	...
02.022:	....	....	....	....	....	....	....	....	....	...
02.027:	....	....	....	....	....	....	....	....	....	...
02.029:	....	....	....	....	....	....	....	....	....	...
02.037:	....	....	....	....	....	....	....	....	....G.....	...
02.042:	....	....	....	....	....	....	....	....	....	...
02.046:	....	....	....	....	....	....	....	....	....	...
02.050:	....	....	....	....A.....	....	....	....	....	....	...
02.051:	....	....	....	....	....	....	....	....	....	...
02.052:	....	....	....	....	....	....	....	....	....	...
02.055:	....	....	....	....	....	....	....	....	....	...
02.060:	....	....	....	....	....	....	....	....	....	...
02.067:	....	....	....	....	......C...	....	....	....	....	...
02.071:	....	....	....	....	....	....	....	....	....	...
02.087:	....	....	....	....	....	....	....	....	....	...
02.091:	....	....	....	....	....	....	....	....	....	...
02.093:	....	....	....	....	....	....	....	....	....	...
02.094:	....	....	....	....	....	....	....	....	....	...
02.096:	....	....	....	....	....	....	....	....	....	...
02.099:	....	....	....	....	....	....	....	....	....	...
02.100:	....	....	....	....	......C...	....	....	....	....	...
02.101:	....	....	....	....	....	....	....	....	....	...
02.106:	....	....	....	....	....	....	....	....	....	...
02.109:	....	....	....	....	....	....	....	....	....	...
02.111:	....	....	....	....	....	....	....	....	....	...
02.114:	....	....	....	....	....	....	....	....	....	...
02.116:	....	....	....	....	....	....	....	....	....	...
02.119:	....	....	....	....	....	....	....	....	....	...
02.120:	....	....	....	....	....	....	....	....	....	
02.125:	....	....	....	....	....	....	....	....	....	...
02.130:	....	....	....	....	....	....	....	....	....	...
02.131:	....	....	....	....	....	....	....	....	....	...
02.136:	....	....	....	....	....	....	....	....	....	...
02.138:	....	....	....	....	....	....GGAGC.	.T.....C.-	-..A....G	AAAA..G..	
02.139:	....	....	....	....	....	....	....	....	....	...

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02.008:  GATTAGTGC-  TTTGAATTTA  AAATTCAACT  ATAAAGATTA  TTTTTC-TTT  TATTAATATA  AATATTTAAT  AAATCAATAT  TTTGAATTGA  ATT
02.145:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.147:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.148:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.149:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.152:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.155:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.159:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.160:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.162:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.163:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.164:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.165:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.167:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.170:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.171:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.172:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.175:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.176:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.177:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.180:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.181:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.184:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.186:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.190:  .........C  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.191:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.192:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.194:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.195:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.196:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.200:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.208:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.215:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.264:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.270:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
02.273:  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...
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Table B.3: *IGS2 primers (forward = IGS2F6, reverse = IGS2B1) aligned with IGSF1 x COIIB3 haplotype sequences from Zürich 2002, showing which variablility is retained in the shorter sequence.*

IGS2.B1:											
IGS2.F6:			AATTT	TCATTATTTT	TGAAAG						
Haplotype A:	TTTCAATAAA	TAGAATATTA	TTTTTAATTT	TCATTATTTT	TGAAAGATTA	ATTTCTAAAC	GATTAATTTT	ATTTAAATTC	CATCAATCAT	CACTTGAATG	ATTAAATAAT
Haplotype B:	....	....	....	....	....	....	....	....	....	....	....
Haplotype C:	....	....	....	....	....	....T.....	....	....	....	....	....
Haplotype D:	....	....	....	....	....	....	.....G....	....	....	....	....
Haplotype E:	....	....	....	....	....	....	....	....	....	.T........	....
Haplotype F:	....	....	....	....	....	....	....	....	....	....	....
Haplotype G:	....	....	....	....	....	....	....	....	....	....	....
Haplotype H:	....	....	....	....	....	....	....	....	....	....	....
Haplotype I:	....	....	....	....	....	....	....	....	....	....	....
Haplotype J:	....	....	....	....	....	....	....	....	....	....	....
Haplotype K:	....	....	....	....	....	....	....	....	....	....	....
Haplotype L:	....	....	....	....	....	....	....	....	....	....	....
Haplotype M:	....	....	....	....	....	....	....	....	....	....	....
Haplotype N:	....	....	....	....	....	....	....	....	....	....	....
Haplotype O:	....	....	....	....	....	....	....	....	....	....	....
Haplotype S:	....	....	....	....	....	....	....	....	....	....	....
Haplotype T:	....	....	....	....	....	....	....	....	....	....	..G.......
Haplotype U:	....	....	....	....	....	....	....	....	....	....	....
Haplotype V:	....	....	....	....	....	....	.....G....	....	....	....	....
Haplotype W:	....	....	....	....	....	....	....	....	....	....	....
Haplotype X:	....	....	....	....	....	....	....	....	....	....	....
Haplotype Y:	....	....	....	....	....	....	....	....	....	....	....
Haplotype Z:	....	....	....	....	....	....	....	....	....	.T........	....

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Table B.3: *IGS2 primers (forward = IGS2F6, reverse = IGS2B1) aligned with IGSF1 x COIIB3 haplotype sequences from Zürich 2002, showing which variablility is retained in the shorter sequence. – continued*

IGS2.B1:											
IGS2.F6:											
Haplotype A:	TATCCTCCTT	ATGATCACTC	ATTAATTGAA	ATTCCATTAA	TTTCAA-AAA	ATAAAATTAA	AAATATTTTC	A-ATA-AATA	AATTACCCTT	TTAATATAAA	TTTAACATTT
Haplotype B:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype C:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype D:	....	....	....	....	....	....	....	....	....	....	....
Haplotype E:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype F:	........C.	....	....	......NN..	......C...	.....G..NN	....	.C...T...T	........C.	....	....
Haplotype G:	....	....	....	....	G..TC.A...	.....G....	....	....	....	....	....
Haplotype H:	....	....	....	....	....	....	....	....	....	....	....
Haplotype I:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype J:	....	....	....	....	....	....	....	....	....	....	....
Haplotype K:	....	....	....	....	....	....	....	....	....	....	....
Haplotype L:	....	....	....	....	....	....	....	....	....	....	....
Haplotype M:	....	....	....	....	....	....	....	....	....	....	....
Haplotype N:	....	....	....	....	....	....	....	....	....	....	....
Haplotype O:	....	....	....	....	....	....	....	....	....	....	....
Haplotype S:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype T:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype U:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype V:	....	....	....	....	....	....	....	....	....	....	....
Haplotype W:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype X:	....	....	....	....	....	.....G....	....	.G........	....	....	....
Haplotype Y:	....	....	....	....	....	.....G....	....	....	....	....	....
Haplotype Z:	....	....	....	....	....	.....G....	....	....	....	....	....

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Table B.3: *IGS2 primers (forward = IGS2F6, reverse = IGS2B1) aligned with IGSF1 x COIIB3 haplotype sequences from Zürich 2002, showing which variablility is retained in the shorter sequence. – continued*

IGS2.B1:				TTGAAC-TT	AAAATTCAAC	TAT				
IGS2.F6:										
Haplotype A:	AATATAATAT	TAATATTGCA	AATTAATGCC	-TTGAAC-TT	AAAATTCAAC	TATAAAG-AT	TATTTTT-CT	TTTATTAATA	TAAATATTTA	ATATAATATT	AATATGGC-A
Haplotype B:	....	....	....	....	....	....	....	....	....	....	....
Haplotype C:	....	....	....	....	....	....	....	....	....	....	....
Haplotype D:	....	....	....	....	....	....	....	....	....	....	....
Haplotype E:	....	....	....	....	....	....	....	....	....	....	....
Haplotype F:	....	....	....	....	....	....	....	....	....	....	....
Haplotype G:	....	....	....	....	....	....	....	....	....	....	....
Haplotype H:	....	....	....	....	....	....	....	....	....	....	....
Haplotype I:	....	....	...C......	....	....	....	....	....	....	....	....
Haplotype J:	....	....	.......A..	....	....	....	....	....	....	....	....
Haplotype K:	....	....	.......A..	....	....	....	.......T..	....	....	....	....
Haplotype L:	....	....	....	C.........	....	....	.......T..	....	....	....	....
Haplotype M:	....	....	....	....	....	....	.......T..	....	....	....	....
Haplotype N:	....	....	....	....	....	....	....	....	....	....	........C.
Haplotype O:	....	....	....	....	....	....	....	....	....	....	....
Haplotype S:	....	....	....	....	....	....	....	....	....	....	....
Haplotype T:	....	....	....	....	....	....	....	....	....	....	....
Haplotype U:	....	....	....	....	....	....	....	....	....	....	....
Haplotype V:	....	....	....	C.........	....	....	....	....	....	....	....
Haplotype W:	....	....	....	.......C..	....	.......G..	.......T..	....	....	....	....
Haplotype X:	....	....	....	....	....	....	....	....	....	....	....
Haplotype Y:	....	....	....	....	....	....	....	....	....	....	....
Haplotype Z:	....	....	....	....	....	....	.......T..	....	....	....	....

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Table B.3: *IGS2 primers (forward = IGS2F6, reverse = IGS2B1) aligned with IGSF1 x COIIB3 haplotype sequences from Zürich 2002, showing which variablility is retained in the shorter sequence. – continued*

IGS2.B1:										
IGS2.F6:										
Haplotype A:	GATTAGTGC-	TTTGAATTTA	AAATTCAACT	ATAAAGATTA	TTTTTC-TTT	TATTAATATA	AATATTTAAT	AAATCAATAT	TTTGAATTGA	ATT
Haplotype B:	....	....	....	....	....	....	....	....	....	...
Haplotype C:	....	....	....	....	....	....	....	....	....	...
Haplotype D:	....	....	....	....	....	....	....	....	....	...
Haplotype E:	....	....	....	....	....	....	....	....	....	...
Haplotype F:	....	....	....	....	....	....	....	....	....	...
Haplotype G:	....	....	....	....	....	....	....	....	....	...
Haplotype H:	....	....	....	....	....	....	....	....	....	...
Haplotype I:	....	....	....	....	....	....	....	....	....	...
Haplotype J:	....	....	....	....	....	....	....	....	....	...
Haplotype K:	....	....	....	....	....	....	....	....	....	...
Haplotype L:	....	....	....	....	....	....	....	....	....	...
Haplotype M:	....	....	....	....	....	....	....	....	....	
Haplotype N:	C	....	....	....	....	....	....	....	....	...
Haplotype O:	....	....	....	....	......C...	....	....	....	....	...
Haplotype S:	....	....	....	.....A....	....	....	....	....	....	...
Haplotype T:	....	....	....	....	....	....	....	....	....	...
Haplotype U:	....	....	....	....	....	....	....	....	....	...
Haplotype V:	....	....	....	....	....	....	....	....	....	...
Haplotype W:	....	....	....	....	......C...	....	....	....	....	...
Haplotype X:	....	....	....	....	....	....	....	....	.....G....	...
Haplotype Y:	....	....	....	....	....	....GGAGC.	.T.....C.-	-..A....G	AAAA..G..	
Haplotype Z:	....	....	....	....	....	....	....	....	....	...

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August 2003	ASAB Travel Bursary to attend the ESEB conference in Leeds, UK
August 2001	ETH Travel Bursary to attend the ESEB conference in Århus, Denmark
June 2001	Royal Swedish Academy of Sciences scholarship to undertake fieldwork at the Abisko Scientific Research Station, Sweden
September 1999	British Ecological Society Attendance Grant for Tropical Biology Association course in Tanzania
July 1999	The Percy Sladen Memorial Fund award for a project investigating the effect of a sexually transmitted mite on male two-spot ladybirds
June to August 1998	UCL Summer Bursary to investigate the relationship between lifespan, nutrition and reproduction in female fruitflies
September 1997	UCL Travel and Subsistence Bursary to attend the British Association Festival of Science

Courses and Workshops Attended

February 2002	Molecular techniques used for studying social insects, Oulu University, Finland
October 2000	Model Selection in Ecological Statistics Course, University of Zürich, Switzerland
August 2000	Evolutionary Ecology of Sex Summer School and Conference, Groningen University, The Netherlands
September 1999	Tropical Biology Association Darwin Course, Amani, Tanzania

Talks and poster presentations

August 2003	ESEB International meeting, Leeds, UK (poster) "Different fitness of common and rare bumblebee haplotypes"

February 2002 INSECTS EU Network workshop on Molecular Techniques (talk)
"Molecular techniques used in the study of bumblebees and parasites"

August 2001 ESEB International meeting, Århus, Denmark (poster)
"Evolution of virulence in a heterogeneous host environment"

August 2000 Evolutionary Ecology of Sex conference, Groningen, Netherlands (poster)
"The effect of antibiotics on the sex ratio trait of *Drosophila bifasciata*"

Positions of responsibility

October 2001 – February 2003 Co-founder and chair of the Zürich Interaction Seminar

March 1998 – January 1999 Biology Representative on the Student Union Council at University College, London

February 1998 – May 1999 Student Co-Chairman on the Staff-Student Consultative Committee for the Biology Department at University College, London

November 1997 – November 1998 President of the Conservation Society at UCL and London Contact/Co-ordinator for the Green Student Network

Professional Memberships

International Union for the Study of Social Insects

British Ecological Society

European Society for Evolutionary Biology

Association for the Study of Animal Behaviour

Institute of Biology

Other

Computer skills Operating systems: MacOSX (including Darwin UNIX core), Windows, Linux (Debian)
Basic programmes: MS Office, OpenOffice, TeX, EndNote, Adobe Illustrator and Photoshop, various internet browsers and email applications
Analysis: SPSS, R, Quantity One, MacVector
Programming: Basic HTML

Languages fluent German, good Thai, basic French

Outside interests singing, playing guitar, sailing, snowboarding, cooking, gardening