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Female lines in social insects – a homage to the Croziers' mitochondria

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Abstract

Mitochondrial genes are passed on in the female line only. Furthermore, in social hymenoptera, such as bees, wasps, and ants, daughters have (genetically) twice the reproductive value of males, because of the complementary ("haplo-diploid") sex determination system. Mitochondrial genes are also typically involved in coding for the pathways of the energy metabolism; this takes on special significance as defence against parasitic infections, for example, has been shown to be energetically costly. Despite these significant elements, the functional study of mitochondrial variation is almost absent in social insects. As a case study, we therefore investigated the distribution and dynamics of mitochondrial haplotypes (mitotypes) in natural populations of *Bombus terrestris* in a field population in Switzerland. Our data show a diversity of extant mitotypes with two types being dominant. A field experiment demonstrated that contrary to simple expectations of parasite-driven negative frequency-dependent selection, colony fitness surrogates were correlated with mitotype frequency. These findings are valued by reference to the literature, which shows that this subject matter is virtually unexplored as yet; especially, there is very little knowledge on the functional significance of different mitochondrial lines in one of the best studied groups of social insects – the ants, but see (HASEGAWA & al. 2011) in this volume.

Key words: Mitochondrion, mitotype, diversity, fitness, colony size, *Bombus terrestris*.

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Introduction

In eukaryotic cells, mitochondria contain additional extranuclear genetic information that is transmitted solely via the female line. In the case of hymenopteran social insects such as the ants, bees, and wasps, the mother queen(s) pass on their mitochondrial type as haplotypes (the mitotypes) to their worker and sexual offspring. Much of this mitochondrial genetic information is coding for enzymes that are affecting the metabolic networks, and, by implication, the energetic performance of its carriers. Natural populations contain a certain range of mitotypes and this has been used to differentiate populations and to reconstruct their phylogenetic history (AVISE 1994). In social insects, mitochondrial markers to study population structure and history have been used quite extensively.

The genome of a hymenopteran mitochondrion was first sequenced by the group of Ross Crozier in the honeybee in the early 1990's (CROZIER & CROZIER 1993); a look into GenBank shows that since then several others have been added (e.g., CHA & al. 2007). Surprisingly little use seems to have been made of this information beyond the question of population structure, however. This is remarkable as, by way of example, the relevance of metabolic enzyme activity has been demonstrated for foraging strategies (PRYS-JONES 1986), for the ability to carry the costs of immune defences (MORET & SCHMID-HEMPEL 2000, MORET 2003), or for keeping sperm alive during storage over many years as is common in ant queens (BAER & al. 2006). A closer inspection of the variability and functional significance of mitotype diversity in natural populations of social insects therefore seems to be appropriate.

In this contribution, we report a pilot study that seeks to link mitochondrial haplotypes to phenotypic traits of interest. In particular, we studied the diversity of mitotypes in a natural population of the European bumblebee, *Bombus terrestris*, and carried out a field experiment testing for the fitness effects that are associated with different mitotypes. The study was initially informed by considerations on antagonistically coevolving host-parasite systems. In such systems, parasites reduce the fitness of their hosts (and, vice versa, by way of host defence), but parasites are thought to adapt more rapidly to their hosts. Such an asymmetric scenario generates time-lagged negative frequency-dependent selection (the Red Queen scenario, HAMILTON 1980, PETERS & LIVELY 1999) and could, at least under some conditions (GANDON & MICHALAKIS 2002, MORGAN & al. 2005), lead to the parasite population being adapted to locally common host genotypes (DYBDAHL & LIVELY 1996, OSNAS & LIVELY 2005, GREISCHAR & KOSKELLA 2007). Common host genotypes should therefore have a lower fitness than rare ones, at least during certain episodes of the co-evolutionary cycle. However, a number of obvious difficulties arise with this scenario. Among those, it is not clear what time points or states of the ongoing co-evolutionary process can and should be investigated, what parasites might be the most relevant ones, and – last but not least – what the "genotype" under scrutiny means.

In obligately out-crossing organisms, such as in the social insects, any nuclear genotype will be destroyed sooner or later by segregation and meiotic recombination (i.e., by cross-overs). Hence, unless a complex of tightly linked genes

relevant for the outcome of host-parasite interactions can be defined, the method of choice is to trace genetic markers such as microsatellites or SNPs that are hopefully closely associated with the genes and the phenotype of interest. The situation is different for the mitotypes. Mitochondrial DNA (mtDNA) is maternally inherited, haploid, typically is not subject to recombination, but has a rate of nucleotide substitution 5 - 10 times higher than found in nuclear DNA (BROWN & al. 1979). Therefore it lends itself well to being used as a marker (BEHURA 2006). The drawback is that there is no proven link as yet to how resistance against parasitic infections is affected by different mitotypes, or how many other traits of interest are connected to mtDNA variation. However, with strong selection from parasites, mitotypes might be selected and could become common, for example, due to temporary linkage with "good" genes coding for parasite resistance elsewhere in the genome. Mitotypes coupled with less effective parasite resistance genes will in turn become, or remain, rare. This loose form of linkage with a phenotype is expected to hold for at least a short time, for instance one season, until it is broken up by reproduction and recombination. Furthermore, in bumblebees, and social insects more generally, the importance of the matriline (represented by the mitotypes) is especially pronounced because only queens overwinter and found new colonies in the spring (or bud off to found a new colony as happens in other social insects). But very few colonies are successful enough to produce daughter queens (DONOVAN & WIER 1978, MÜLLER & SCHMID-HEMPEL 1993, IMHOOF & SCHMID-HEMPEL 1998), and so the populations will be dominated by a few matrilines. Moreover, the main fitness effect of a major parasite in the current study system, *Crithidia bombi*, is to reduce queen colony founding success (BROWN & al. 2003). Those mitotypes that are common in a population in spring queens may thus not remain common over the summer season, as infected queens are unsuccessful at starting their colony and if this is varying with mtDNA variation. Many more colonies produce males, which do not transmit the mitotype – although in other species occasional leakage of paternal mitochondrial DNA has been recorded (KONDO & al. 1990). Together, mitotypes might therefore be a valuable source of information to trace the fate of different lines in a natural situation.

Here, we adopt the background working hypothesis that mitotype, perhaps through variation in metabolic performance, is relevant for the outcome of host-parasite interactions, and for variation in fitness effects more generally. This is made likely by the relevance of energy metabolism for defence against parasites (MORET & SCHMID-HEMPEL 2000, SCHMID-HEMPEL 2003) as well as for many other traits of interest (e.g., VOGT 1986). In this study, we explicitly looked at common and rare mitochondrial haplotypes within a population of bumblebees, *Bombus terrestris*, in Northeastern Switzerland. As we will show, mitotype frequency is predictive for surrogates of colony fitness and seems to be associated with parasite defence, too.

Material and methods

(a) Experimental procedures. Spring queens were collected from an area of Northeastern Switzerland (location near Neunforn, Thurgau) in March 2002. The queens were brought into the laboratory, placed in breeding boxes under red light and kept under standard conditions (24˚C, 70 - 80% r. H.). Queens were fed ApiInvert® sugar solution diluted 1:1 with water, and offered pollen pellets. Queens that did not start colonies were removed from the experiment; the others were allowed to develop their incipient colony in the laboratory. Afterwards, the colonies were placed into field nests (supplied by Schwegler company, Schorndorf, Germany). We placed the colonies into the same field population from where they were sampled. In particular, 27 colonies raised from spring queens containing one of the two common haplotypes, and 4 colonies from a mixture of rare haplotypes (see below) were placed in the field in the grounds of Kartause Ittingen in Thurgau, NE Switzerland.

Colonies were placed in the field when they had produced at least 10 workers (setting the time of field placement). The first colonies were placed in the field on 8 May 2002, and the last colonies were placed in the field on 26 June 2002. Colonies with common and rare haplotypes were matched for date of field placement. The mean Julian day of colony placement was 144.47 days (± 2.77 standard error, S.E.), which corresponds to 24 May 2002. The colonies were monitored once a week, the number of workers counted and 10% per week removed for dissection. Colonies and samples were given "blind numbers", i.e., identities were unknown to the observer during the later dissections and analyses. When sexuals were produced, the colonies were checked twice weekly, and the newly produced sexuals counted and removed.

We dissected all removed bees for parasites, checking for external mites, internal macroparasites such as thoracic mites (*Bombacarus buchneri*; Acarina, Podapolipodidae), conopid fly eggs and larvae (Diptera, Conopidae) (SCHMID-HEMPEL & SCHMID-HEMPEL 1996), and the protozoan parasites *Nosema bombi* (Microsporidia, Nosematidae) and *Crithidia bombi* (Trypanosomatidae, Zoomastigophorea). We here report on two measures of parasite load: prevalence of *C. bombi* and *N. bombi* (the proportion of parasitized workers per colony). Other measures were used, too (e.g., infection intensity, number of parasite species per bee or colony, etc.), but they did not reveal any pattern and are not reported here. To determine fitness, daughter queens and males were counted and weighed to the nearest mg (as well as queen size measured, i.e., the length of the radial cell of the right wing in mm). Average radial cell size of workers was 2.82 ± 0.022 mm, S.E., $n = 288$, but it had no effects or relationships with any other measure, and so will not be reported further here.

(b) Defining the mitotypes. We extracted DNA from legs of a sample of unsuccessful queens and from workers of successfully founded colonies using 10% Chelex (500 µl for a worker leg and 700 µl for a queen leg) (WALSH & al. 1991, SCHMID-HEMPEL & SCHMID-HEMPEL 2000). The mitochondrial types (the mitotypes) were defined by an area of coding (5'-end of COI) plus non-coding DNA (intergenic space) between the COI and COII genes (which code for parts of the cytochrome oxidase enzyme) that is unique to bees (CROZIER & al. 1989, CROZIER & CROZIER 1993). This intergenic sequence shows size and sequence variation in both honeybees (CORNUET & GARNERY 1991, CORNUET & al. 1991) and in bumblebees. This area of the genome is strongly linked to the rest of the mitochondrial genome by way of its clonal inheritance. A 500 bp fragment was amplified using the primer pair BB_IGSF1 (forward: 5'-GGA GCA ATA ATT TCA ATA AAT AG-3') and BB_

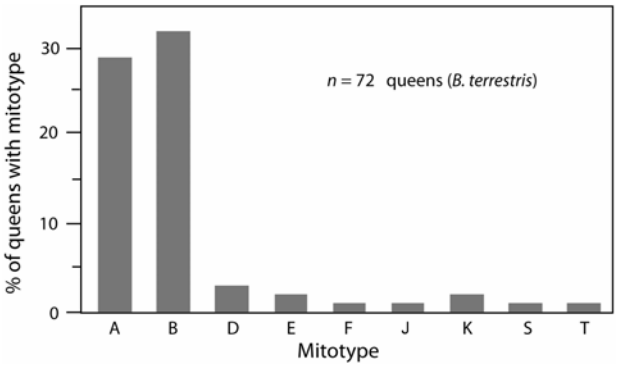

Fig. 1: Frequency distribution of mitotypes (A to T) observed among n = 72 typed bumblebee queens collected in spring 2002 in Northeastern Switzerland. For molecular sequence of haplotypes, see Appendix as digital supplementary material to this article; available at the journal's web pages.

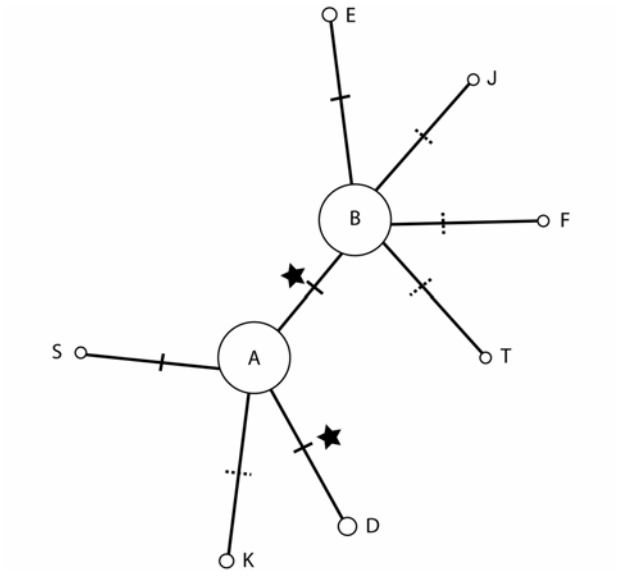

Fig. 2: The haplotype network of this study. The graph was generated using distances calculated with dnadist from Phylip and the tree generated by neighbour-joining. The sizes of the circles indicate the frequencies of the mitotypes. Bars indicate one base pair substitution each. Solid bars are substitutions observed in the 5'-end of the coding region for COI; broken lines are substitutions in the intergenic region, and asterisks denote a change in the amino acid (in both cases, the change affects a conversion between Ile and Val).

COIIB3 (reverse: 5'-TTA TGA AAT GAA ATT AAA TTA TCA G-3'). PCR reactions were carried out with a final reaction volume of 100 µl, containing 10 µl DNA, 1 × reaction buffer including MgCl2 (Promega), 5 µl of dNTPs of 2.5 mM each, 2.0 µl of each primer of 10 µM, and 2.5 U Taq polymerase. The thermocycling profile consisted of a 3-step PCR with 37 cycles, an annealing temperature of 48°C, and an elongation time of 1 min. The PCR yielded a fragment of the 5'-end of COI plus the intergenic sequence (CROZIER & CROZIER 1993). The relationships among haplotypes were analysed with Phylip 3.6 (FELSENSTEIN 2005) generating pair-wise distances; distances calculated with dnadist using the default maximum likelihood, DNAML with F84 model, and the tree generated by neighbour-joining. GenBank numbers for haplotypes are given in Appendix, as digital supplementary material to this article, at the journal's web pages.

(c) Fitness and immunological measures. The production of sexuals (males and daughter queens) was used as a fitness measure and weighted as described below. In addition, we took three measures that reflect immunological responsiveness. (1) Antibacterial activity of the haemolymph after a standard challenge with LPS (lipopolysaccharides) and according to standard protocols (KORNER & SCHMID-HEMPEL 2004). The measure is given as the diameter of the zone of inhibition caused by a drop of haemolymph on an agar plate coated with the bacterium *Arthrobacter globiformis*. (2) Activity of the key enzyme phenoloxidase that is involved in the melanization and encapsulation cascade. The procedure followed standard protocols (KORNER & SCHMID-HEMPEL 2004) and is given as the slope, v_{max}, of the line that characterizes the enzyme's capacity to transform substrate into product over time (in arbitrary units). (3) The concentration of haemocytes from a blood sample (KORNER & SCHMID-HEMPEL 2004), given as arbitrary units of cells per counting square. All statistical measures are given as average ± S.E. if not specified otherwise. For the tests, we used Welch's t for unequal variances where the data allowed and if not specified otherwise; statistics were done with Jump 8.0.2 for Macintosh.

Results

(a) Frequency of mitotypes. We identified a total of nine different mitotypes sampled from n = 72 spring queens in 2002. Among those, two mitotypes (A, B) made up approximately two thirds (84.7%, 61 individuals) of all identified types. The residual one third was distributed among seven different, rare mitotypes represented in only one or at most two spring queens (Fig. 1). The mitotypes formed a small network where the mitotypes differed only slightly by one base change each among neighbours. Furthermore, it suggested that from a core group of (abundant) A- and B-types, further mitotypes descended by single base pair changes with some of them in the coding region of the 5'-end of COI (Fig. 2).

(b) Mitotypes and colony success. We compared measures of colony success with the kind of mitotype (rare / common) that the colony queen was carrying. Firstly, we observed that colony founding success of queens in the laboratory varied with mitotype. Of a total of 19 mitotype-A queens, 15 founded a colony (53.6%), of 18 mitotype-B queens 13 were successful (46.4%), and of the 11 remaining rare-mitotype queens four managed to start a colony (12.5%). Overall, queens with common (28 of 37 successful) mitotypes were thus more successful than those with rare mitotypes ($\chi^2 = 5.630$, $df = 1$, $p = 0.018$).

The eventual reproductive success of a colony (its fitness) 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 body mass), as they are much larger and cost more energy to produce (DUCHATEAU & VELTHUIS 1988, BAER & SCHMID-HEMPEL 1999). Most importantly, the (diploid) queens have twice the genetic value as compared to (haploid) males. From our data, we found

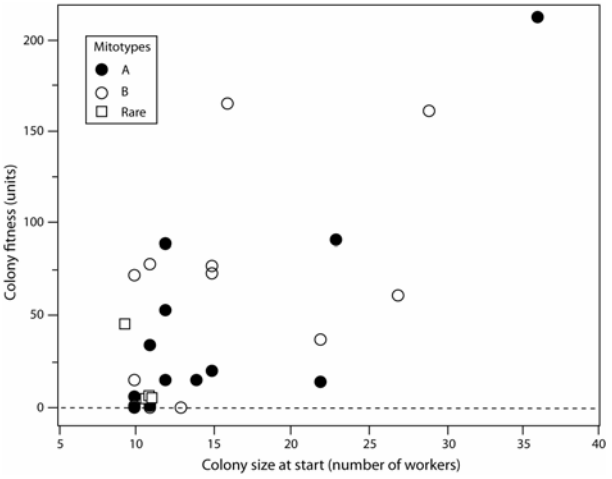

Fig. 3: Fitness is correlated with colony size at field placement (r^2 = 0.490, $F_{1,29}$ = 27.938, p < 0.001, n = 31 colonies).

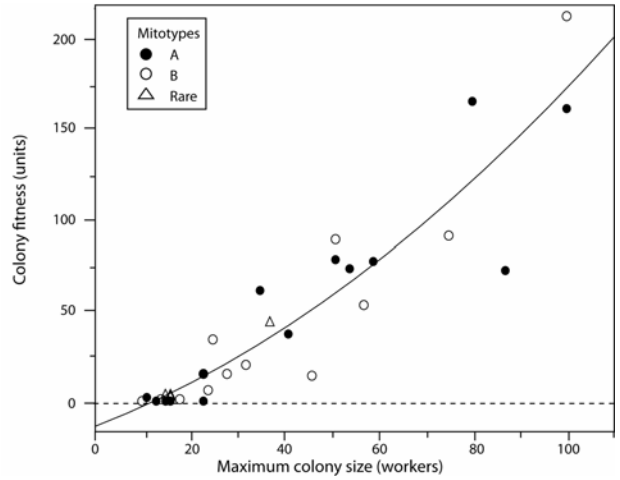

Fig. 4: Fitness is highly correlated with largest colony size recorded. The line is a quadratic fit with r^2 = 0.856, $F_{2,28}$ = 83.587, p < 0.0001, n = 31 colonies. Note that all mitotypes follow the same basic relationship, which is: fitness (measured in units, see text) = -26.714 (± 7.09) + 1.666 (± 0.21) W + 0.0085 (± 0.001) W^2, where W is the number of workers; values in parentheses indicate S.E. of the estimate for the respective coefficients.

that queens had a fresh mean body mass of 0.734 ± 0.007 g, and males weighed a mean of 0.273 ± 0.003 g, which gives a relation of closer to queens being 2.5 × heavier than males. However, the analyses lead to the same overall conclusions if queen number was weighted by a factor 2.5 rather than a factor of 2.0. Colony nr. 13 (with mitotype A) was taken over by a cuckoo bumblebee (*Psithyrus* sp.) by the 27 June, and at a stage when it had 24 workers. Colony nr. 13 therefore was removed from the analyses past the initial stages.

The number of workers in the colony (colony size) when it was placed in the field (size at field placement) had a significant effect on the colonies' eventual fitness and was therefore taken into account as a covariate in further analyses (Fig. 3). The range of colony sizes at field placement of all of the colonies was 10 to 36 workers, with a mean of 14.53 ± 1.41 workers, n = 32 colonies. The size at field placement was highly correlated with the largest recorded size of the colony, i.e., maximum size (Spearman's r = 0.574, p < 0.001, n = 31), which in turn is correlated highly with fitness (Fig. 4). Colony size has been previously shown to be extremely important in the eventual reproduction of bumblebee colonies (POMEROY & PLOWRIGHT 1982, MÜLLER & SCHMID-HEMPEL 1992, 1993). Out of a total of 31 colonies that successfully completed this experiment, 10 produced queens (32.3% of colonies) and 24 produced males (77.4% of colonies). These proportions are higher than those found by SHYKOFF & MÜLLER (1995) and IMHOOF & SCHMID-HEMPEL (1998). Only one colony produced one queen but not males.

Colonies of rare mitotypes did not show the full range of sizes at field placement (see Fig. 3). Common-mitotype colonies had a larger mean size at field placement of 15.15 ± 1.24 workers (n = 27) compared to rare mitotypes (10.75 ± 3.25 workers, n = 4) (t = 3.276, df = 27.59, p = 0.003). But there was no significant difference in the Julian day of field placement (mean Julian day of field placement for common-mitotype colonies: 144.82 ± 3.02 d; for rare-mitotype colonies: 144.50 ±7.846 d; t = 0.047, df = 4.86, p = 0.96). Hence, common mitotypes appeared to enjoy an advantage during the early colony stage and while still in the laboratory. This early stage has been shown to be crucial for bumblebee colony success (MÜLLER & SCHMID-HEMPEL 1993). After being placed in the field, common mitotypes also reached a larger maximum colony size (common: 41.15 ± 5.30 workers, n = 27; rare: 21.0 ± 5.34, n = 4; t = 2.678, df = 10.64, p = 0.022). Overall, colonies of common mitotypes had higher fitness than colonies from rare mitotypes (Fig. 5). In line with the colony size differences, this effect depends on taking into account size at field placement as a covariate (ANCOVA for fitness, mitotype class t = 0.39, p = 0.69, size at field placement: t = 4.98, p < 0.0001, n = 31 colonies). Hence, again, the main effect of mitotype seems to be very early acting, such that the rare types grow more slowly and do not reach such a large col-

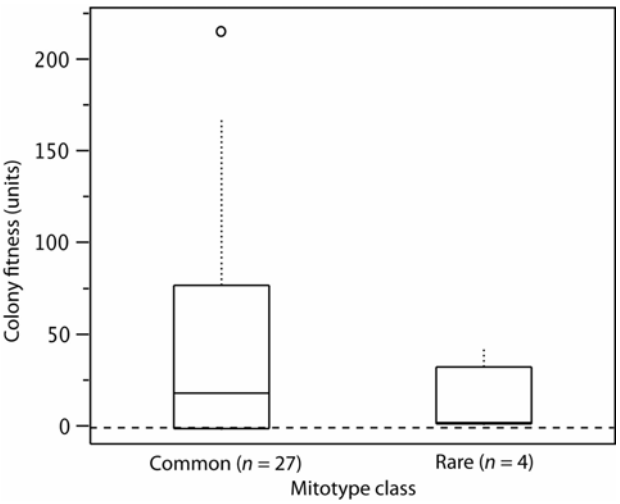

Fig. 5: Boxplot for colony fitness (in units, see text). Colonies having common mitotypes (mean fitness: 47.85 ± 11.03 units, n = 27 colonies) were somewhat fitter than colonies harbouring rare types (13.75 ± 10.09 units, n = 4) (t = 2.282, df = 12.411, p = 0.041). The rare mitotypes in this experiment were K (2 colonies), S, and T.

ony size (Figs. 3, 4); subsequently, they do not produce as many sexuals. It is however important to note from Figure 4 that all colonies follow the same pattern, i.e., they fall along the same curved relationship between maximum colony size and fitness. Both of the common mitotypes (A and B) show the full range of size and fitness, whereas rare types are constrained at approximately 40 workers. Considering the number of queens produced per colony, common-mitotype colonies produced a mean of 6.55 ± 3.11 queens (n = 27), which was not quite significantly more than those with a rare mitotype (0.25 ± 0.25 queens, n = 4) (t = 2.02, df = 26.32, p = 0.054). However, a clear difference existed when considering the total biomass of sexuals (males and queens) that were produced by common-mitotype colonies (6.703 ± 2.26 g, n = 27) as compared to rare-mitotype colonies (0.298 ± 5.56 g, n = 4) (t = 2.819, df = 26.46, p = 0.009).

(c) Mitotypes and parasites. In the year of study, we found an unusually low prevalence of parasites in the dissected workers. Overall, 3.12% of workers were infected with *Crithidia bombi* (10 workers from a total of 321 investigated), divided between six colonies from a total of 32 that went into the experiment. Furthermore, 3.43% of workers were infected with the microsporidian, *Nosema bombi* (in 11 bees from a total of 312), divided between five colonies from the 32 in the experiment. No thoracic mites were found, and three bees each (1.96% of workers) contained eggs or larvae of conopid flies (SCHMID-HEMPEL & SCHMID-HEMPEL 1996). Colony nr. 13 (with mitotype A) was taken over by a cuckoo bumblebee (*Psithyrus* sp.) by 27 June and removed from the analyses as mentioned above.

For colonies, no difference in the prevalence of *Crithidia bombi* according to mitotype (common: 4 out of 27 = 12.9 % of colonies infected; rare: none of 4 infected) (χ^2 = 1.190, p = 0.28) was found. Also, for *Nosema bombi*, common-mitotypes colonies (5 out of 27 infected, 16.1%) were not different from rare ones (none infected, n = 11) (χ^2 = 1.517, p = 0.21). Hence, mitotype had no strong effects on the presence of the parasites. Similarly, none of the immunological parameters differed between colony workers of rare and common mitotypes. In particular, there was no difference in the colony-average strength of the antibacterial activity of the haemolymph (common-mitotype colonies: 5.06 ± 0.41 mm, n = 27; rare: 4.01 ± 1.07 mm, n = 4) (t = 0.716, df = 3.474, p = 0.52), in the enzymatic activity of phenoloxidase (common: slope = 2.709 ± 0.10 units, n = 27; rare: 2.675 ± 0.25 units, n = 4) (t = 0.077, df = 3.231, p = 0.94), or in the concentration of haemocytes from blood samples (common: 116.63 ± 7.16 cells, n = 27; rare: 108.06 ± 18.61 cells, n = 4) (t = 0.543, df = 4.840, p = 0.61).

Among all colonies, in general no relationships were found between the strength of the immune responses and parasite prevalence, or between strength and colony-fitness measures. However, colonies infected by *Nosema bombi* seemed to deviate in this respect. For example, they had larger maximum colony sizes (infected: 81.60 ± 8.48 workers, n = 5; non-infected: 30.27 ± 3.72, n = 26) (t = 4.96, df = 4.88, p = 0.007), higher fitness (infected: 129.40 ± 17.93 units, n = 5; non-infected: 26.92 ± 7.86, n = 26) (t = 4.96, df = 4.88, p = 0.007), but were not larger at field placement. No such differences were observed for colonies infected or not infected by *Crithidia bombi*, but there was a trend that infected colonies had lower fitness values.

(d) Differences between the two common mitotypes (A vs. B). Looking at the two common mitotypes, there were not many differences. For example, these haplotypes were similar for colony size at placement in field (mitotype-A colonies had a mean of 15.07 ± 1.71 workers at field placement, n = 14; mitotype-B colonies had 15.23 ± 2.10 workers, n = 13). We also found no statistically established differences between A- and B-types for various other measures of interest, for example: mitotype-A colonies produced 35.93 ± 12.86 males, n = 14 colonies, whereas mitotype-B produced 33.46 ± 13.34 males, n = 13. For queen production, the numbers were: type A produced 8.50 ± 4.36 queens, n = 14, and type B produced 4.46 ± 4.53 queens, n = 13. Similarly, fitness was the same for mitotype A (52.93 ± 15.15 units, n = 14) and mitotype B (42.38 ± 16.57, n = 11); and also the same total biomass of sexuals that was produced (type A: 8.475 ± 3.16 g, n = 14; type B: 4.795 ± 3.28 g, n = 13). In all of these cases, a lack of statistical significance for the difference was certainly due to considerable variation among the colonies.

Interestingly, two of the three immunological measures taken from the workers while still in the laboratory suggested that mitotype A might be better defended than B in our tests because antibacterial activity (average for type A colonies: 5.87 ± 0.50 mm, n = 14; type B: 4.19 ± 0.52 mm, n = 13; t = 2.315, df = 24.872, p = 0.029) and the activity of the key enzyme, phenoloxidase (type A, slope 2.92 ± 0.11 n = 14; type B: 2.48 ± 0.09 mm, n = 13; t = 2.851, df = 24.269, p = 0.009) were both stronger in A (no difference in haemocyte count was found for type A with 115.75 ± 8.63 cells, n = 14, as compared to type B: 117.59 ± 12.47 cells, n = 13). Also no difference was found for the prevalence of *Nosema bombi* (type A: 4 of 14 colonies infected; type B: 1 of 13 infected) or *Crithidia bombi* infections (type A: 2 of 14 colonies infected; type B: 2 of 13 infected).

Discussion

The experiment reported here refers to a possible relationship of different mitochondrial haploytpes, the mitotypes, with colony success, parasitic infections and immunological parameters in the European bumblebee, *Bombus terrestris*. It emerged that mitotype diversity is considerable (with a total of 10 types among 73 individuals, Fig. 1) but that only two mitotypes were common (types A and B). At the DNA sequence level, A and B differ by one amino acid. Functionally, these common mitotypes differed from the rarer ones primarily by showing a faster colony development during the early colony stage. In our experiment, this stage unfolded under favourable laboratory conditions, so it is not yet clear whether this would also be the case under field conditions. However, laboratory differences normally become inflated under the harsher field conditions. This is shown in the somewhat higher fitness of common-type colonies as compared to rare-type colonies (Fig. 5). Furthermore, colony size at the date when the colony was transferred to field conditions was essential for eventual fitness, with larger colonies being more successful. A quadratic relationship between maximum colony size and fitness supports the relevance of colony size for fitness more generally (Fig. 4). Looking at measures of parasite presence and immunological parameters, no remarkable differences among mitotypes were observed. But, in all, the mitotype appears to be a useful candidate as a surrogate for

colony fitness. If future studies support these findings, a queen's mitotype could be an informative predictor for her colony's success and so provide a hitherto overlooked marker for fitness. Remarkably, we found that mitotype colonies infected by *Nosema bombi* where larger in size and had higher fitness, regardless of mitotype. But without experimental infections it is not possible to distinguish between cause and effect. Yet, interestingly, this finding matches earlier observations in the same system (IMHOOF & SCHMID-HEMPEL 1998).

Mitochondrial genetic markers have been used extensively for studies of population structure and population history, not only in bees (e.g., PIROUNAKIS 1995, ARIAS & SHEPPARD 1996) but also including the ants (SHOEMAKER & al. 1996, CAHAN & al. 2006, PUSCH & al. 2006, ABBOTT & al. 2007, SUNI & al. 2007). However, these markers have usually been treated as neutral with respect to the question under scrutiny. Here, we take another approach by asking whether different mitotypes are associated with different fitness of colonies. This subject seems understudied. On the other hand, the activity of metabolic enzymes, well represented by the mitochondrial genome, has been connected to eventual performance and fitness measures (PRYS-JONES 1986, ASKEW & al. 2010). Several observations additionally suggest a potentially important role for mitochondrial genes and their haploytpes. For example, mitochondrial gene expression seems associated with queen ageing (CORONA & al. 2005), which is a relevant process that determines colony longevity and, implicitly, success. Quite analogous findings have been made in *Drosophila* where mitochondrial variants lead to differential effects when situated into different nuclear-genetic backgrounds (RAND & al. 2006). Female-line transmitted parasites such as *Wolbachia* might also, in principle, affect the success of different mitotypes (SHOEMAKER & al. 2003, VILJAKAINEN & al. 2008) and thus population structure (ENGELSTÄDTER & TELSCHOW 2009); to date, no evidence for *Wolbachia* being present in *Bombus terrestris* exists (C. Reber Funk, R. Schmid-Hempel, unpubl.). In the closest, comparable study, two mitotypes of *Drosophila subobscura* differed in fitness components, such as egg-to-larva and larva-to-adult survivorship, longevity, or desiccation resistance, with one haplotype performing 10 - 20% better than the alternative one under laboratory conditions (CHRISTIE & al. 2004). Furthermore, mitochondrial genes show signs of selection when compared among different species of the wasp *Nasonia* (see DEODORO & al. 2008). It is also remarkable that, at least in the honeybee, many elements of the mitochondrial genome have – over evolutionary times – become transferred to the nuclear genome (PAMILO & al. 2007). The functional significance of these processes is unclear but it would lessen the signal of selection and the effect of different mitotype variants on the fitness of its carriers. Furthermore, in a recent study of the parthenogenetic ant *Pristomyrmex punctatus* reported in this volume (HASEGAWA & al. 2011), normal and cheater morphs (that are larger and have high fecundity) were shown to differ in their mitochondrial sequence. All of these findings suggest that mtDNA variation has interesting functional consequences that are worthy of studying.

A trivial message from our findings is that fitter mitotypes (A, B) are more common. However, this static picture does not cover the question of why there is such a diversity of rare mitotypes, and why rare types have not been eliminated by selection from the population in the first place. It remains possible that rare mitotypes emerge by mutation events from the common ones. But perhaps the lower parasite load observed for the rarer mitotypes could constitute a balancing benefit that is needed to maintain the mitochondrial polymorphism in the population. Whatever the story, there is a need for a more thorough analysis of mitotype variation with respect to fitness of its carriers. This seems especially relevant for social insects such as bees or ants, where the type of the mother queen determines the type of the workers that form the bulk of all offspring ever produced and that, by their own activities, determine the success of the founding female or females. Clearly, the pioneering molecular genetic work of Ross and Ching Crozier has opened new doors to as yet unknown worlds.

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