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Pheromonal dominance and the selection of a socially parasitic honeybee worker lineage (*Apis mellifera capensis* Esch.)

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Abstract

The recent invasion by self-replicating socially parasitic Cape honeybee workers, *Apis mellifera capensis*, of colonies of the neighbouring African subspecies *Apis mellifera scutellata* represents an opportunity to study evolution of intraspecific parasitism in real time. As honeybee workers compete pheromonally for reproductive dominance, and as *A. m. capensis* workers readily produce queen-like pheromones, we hypothesized that these semiochemicals promoted the evolution of intraspecific social parasitism. Remarkably, the offspring of a single worker became established as a parasite in *A. m. scutellata*'s range. This could have resulted from extreme selection among different clonal parasitic worker lineages. Using pheromonal contest experiments, we show that the selected parasitic lineage dominates in the production of mandibular gland pheromones over all other competitors to which it is exposed. Our results suggest that mandibular gland pheromones played a key role in the evolution of intraspecific social parasitism in the honeybee and in the selection of a single genotype of parasitic workers.

Introduction

Parasitism constitutes a major evolutionary force driving phenomena like speciation, adaptation, biodiversity, competition among species and the evolution of sociality (Keymer & Read, 1990; Bermudes & Joiner, 1993; Renaud *et al.*, 1996; O'Donnell, 1997; Hudson & Greenman, 1998; Schmid-Hempel, 1998, 2001; Summers *et al.*, 2003). In the social insects, parasitism is regarded as selecting for increased genetic diversity within a society, influences colonial organization as well as the sex ratio of the alates produced (Bourke & Franks, 1995; O'Donnell, 1997; Schmid-Hempel, 1998, 2001; Aaron *et al.*, 1999; Foitzik & Heinze, 2000; Hughes & Boomsma, 2006; Tarpy & Seeley, 2006). Social parasitism is a form of parasitism in which individuals take advantage of the interactions with their social host and exploit the well-organized division of labour within the host colony to increase their own fitness (Wilson, 1971; Hölldobler & Wilson, 1990; Schmid-Hempel, 1998). Social parasitism can occur between two species (= interspecific) or within a single species (= intraspecific). Interspecific social parasitism by mated females is widespread in the social Hymenoptera and several species have been studied in detail (reviewed in Wilson, 1971; Buschinger, 1986; Roubik, 1989; Hölldobler & Wilson, 1990; Bourke & Franks, 1995; Schmid-Hempel, 1998). By contrast, intraspecific social parasitism is difficult to recognize and is consequently less well documented and understood. For instance, foreign reproductive workers have been shown to function as intraspecific social parasites only in a few species of social bees (Onions, 1912; Neumann & Hepburn, 2002; Birmingham *et al.*, 2004; Lopez-Vaamonde *et al.*, 2004; Nanork *et al.*, 2005) and probably also occur in ants (Tsuji, 1995; Sasaki & Tsuji, 2003). Reproduction by parasitic workers might have been overlooked as the tools that make their detection possible have only

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recently become available (Lopez-Vaamonde *et al.*, 2004; Nanork *et al.*, 2005; Härtel *et al.*, 2006a). This suggests that intraspecific social parasitism by laying workers is more widespread and significant in the social insects than previously thought.

Emery's rule states that parasites and hosts are closely related (Emery, 1909; LeMasne, 1956; Ward, 1996; Lowe *et al.*, 2002) and several evolutionary scenarios have been proposed to explain speciation of the parasites and the phylogenetic relationships between the interacting species (Bourke & Franks, 1991; Lowe *et al.*, 2002). Where the parasite is the host's closest phylogenetic relative (Emery's rule in the strict sense), it is likely to have originated through sympatric speciation. However, the reproductive isolation mechanisms required for sympatric speciation are complex (Bourke & Franks, 1991) and this type of speciation is rarely supported by phylogenetic studies (for exceptions, see Goff *et al.*, 1997; Sumner *et al.*, 2004). In contrast, when parasites and hosts are close relatives, but not sibling species (loose version of Emery's rule), they are likely to originate from allopatric speciation, following straightforward evolutionary scenarios. Evidence for such speciation events are numerous (Carpenter *et al.*, 1993; Ward, 1996; Goff *et al.*, 1997; Lowe & Crozier, 1997; Sanetra & Buschinger, 2000; Lowe *et al.*, 2002; Parker & Rissing, 2002; Janda *et al.*, 2004; Sumner *et al.*, 2004; Pitts *et al.*, 2005). However, to determine the actual evolutionary scenario that produced extant host–parasite systems can only be speculative because of the lack of historical information on the origins of and interactions between the species (Via, 2001). Therefore, situations in which two protagonists have recently developed a host–parasite relationship are of interest if one wants to understand the evolution of social parasitism. Such a rare system can be found in the honeybee, *Apis mellifera* (Neumann & Moritz, 2002). In the Cape honeybee, *A. m. capensis*, thelytokous parthenogenesis is the dominant mode of worker reproduction (Onions, 1912; Hepburn & Crewe, 1991; Neumann *et al.*, 2000), resulting in female clonal worker lineages (Moritz & Haberl, 1994; Baudry *et al.*, 2004). Workers of *A. m. capensis* function as facultative intraspecific social parasites when entering colonies of their own (Härtel *et al.*, 2006a) and other *A. mellifera* subspecies where they replicate themselves (Lundie, 1954; Neumann & Hepburn, 2002). Parasitism in honeybees has evolved both in species in which the mode of worker reproduction is arrhenotoky or thelytoky (Nanork *et al.*, 2005; Härtel *et al.*, 2006a). Here, we use a thelytokous subspecies in which intense reproductive conflicts are expected (Greeff, 1996) to lead to high virulence, making it possible to identify the mechanisms underlying intraspecific social parasitism more easily. The natural invasion of *A. m. scutellata* by Cape honeybee workers is probably prevented by the introgression zone separating the two neighbouring subspecies (Neumann *et al.*, 2001, Fig. 1). In 1990, migratory beekeepers transported approximately

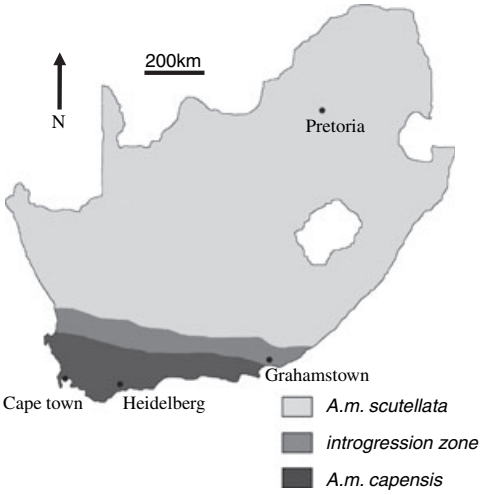

Fig. 1 Map of South Africa showing the natural distribution of *Apis mellifera scutellata* and *Apis mellifera capensis* with the introgression zone. This map is based on the morphological characters of both subspecies (after Hepburn *et al.*, 1998).

400 *A. m. capensis* colonies for pollination purposes across this natural barrier (Hepburn & Crewe, 1991; Allsopp, 1993, 1995). Since then, socially parasitic *A. m. capensis* workers have spread widely in the range of *A. m. scutellata*, causing significant losses of host colonies (tens of thousands of colonies per year, Allsopp & Crewe, 1993). Ten years after the initial introduction, only a single clonal lineage of socially parasitic *A. m. capensis* workers (hereafter designated as the invasive lineage) was found in infested *A. m. scutellata* colonies (Kryger, 2001a,b; Baudry *et al.*, 2004; Härtel *et al.*, 2006b), despite the initial introduction of approximately 12 000 000 potentially parasitic worker genotypes (~400 colonies translocated, each with ~30 000 workers). If humans provided the opportunity for *A. m. capensis* to parasitize *A. m. scutellata*, the extreme selection process that established a single lineage in the parasite population was a natural process. There is hitherto no explanation for the monopoly of a single invasive lineage in the parasitic population.

In honeybee workers, reproduction is associated with the secretion of queen-like pheromones (Velthuis & van der Kerk, 1988; Hepburn, 1992; Simon *et al.*, 2005). The production of these semiochemicals is involved in the acquisition of reproductive status and allows individuals to inhibit the reproductive development of other workers (Velthuis *et al.*, 1965; Crewe &Velthuis, 1980; Moritz *et al.*, 2000, 2004; Simon *et al.*, 2005). *A. m. capensis* workers can develop a queen-like mandibular gland pheromonal bouquet much more readily than workers of other subspecies (Hemmling *et al.*, 1979; Crewe & Velthuis, 1980; Velthuis *et al.*, 1990; Hepburn & Allsopp, 1994; Wossler, 2002). This ability could represent a feature of standard social life that became the starting point for parasitic evolution (Buschinger, 1986).

We hypothesize that the monopoly of the invasive lineage in the parasitic population is due to the ability of this lineage to out-compete other *A. m. capensis* lineages through a superior or more rapid ability to produce mandibular gland pheromones. We suggest that the competition for reproductive dominance occurred at two levels during the selection process of this lineage: (a) *intracolonial level*: there is strong competition between individual workers for reproductive dominance within queenless colonies of Cape honeybees and of other subspecies (Page & Robinson, 1994; Moritz *et al.*, 1996; Martin *et al.*, 2004; Härtel *et al.*, 2006a). This competition results in the reproductive domination by one or a few patrilines in a given colony (Moritz *et al.*, 1996). This probably also occurs in multiply infested *A. m. scutellata* colonies, where a few thelytokous *A. m. capensis* lineages can dominate reproduction (as they pheromonally out compete *A. m. scutellata*, see above). (b) *population level*: winners of intracolonial competition are likely to compete directly with each other for limited host resources, because the colonies they infest eventually die and they have to find new host colonies (Martin *et al.*, 2002a; Neumann & Hepburn, 2002). As thelytoky promotes more intense conflicts over reproduction between workers than arrhenotoky (Greeff, 1996), and because we assume workers from the introgression zone (thereafter designated as hybrids for brevity; Fig. 1) to be intermediate in this respect, we expect *A. m. scutellata* workers to be reproductively subordinate to hybrid workers and the latter to be subordinate to *A. m. capensis* workers. As there is high genetic variance for traits related to worker reproduction in *A. m. capensis* (up to $h^2 = 0.89$, Moritz & Hillesheim, 1985; see also Simon *et al.*, 2005), the ability of queen offspring to acquire reproductive dominance is variable. On the other hand, clonal worker offspring are expected to be reproductively dominant because they are likely to have inherited the trait from their parents, as the latter were selected at the intracolonial level (Moritz *et al.*, 1996). *A. m. capensis* worker offspring should therefore, on average, out-compete *A. m. capensis* queen offspring. Finally, as it is likely that workers from the invasive lineage have out competed all the other *A. m. capensis* parasitic lineages at the population level, we expect them to out compete all the other groups. In summary, we expect the following increasing order of reproductive dominance among honeybee workers: (1) *A. m. scutellata*, (2) hybrids, (3) native *A. m. capensis* queen offspring, (4) native *A. m. capensis* worker offspring and (5) invasive *A. m. capensis* lineage found in infested *A. m. scutellata* colonies.

If this hierarchy of reproductive dominance can be demonstrated, it would clarify the role of the mandibular gland pheromones in the evolutionary processes that resulted in social parasitism and in the selection of a single worker lineage. In order to do this, we measured mandibular gland pheromone production by pairs of workers during pheromonal contests (Moritz *et al.*, 2000, 2004). This bioassay was designed to determine the outcome of pheromonal competition between colony members. We now use it to investigate competition between honeybee workers of different subspecies or lineages. This reflects a situation, where individuals from different origins are placed in close proximity in apiaries and contributes to our understanding of the evolution of social parasitism by workers. Our results showed that the invasive lineage pheromonally dominates all other groups after only four days and that the observed order of pheromonal dominance corresponds to that predicted on theoretical grounds. This supports the idea that a superior ability to produce queen-like mandibular gland secretions played a key role in the selection of a single parasitic lineage. Pheromonal dominance (through selection for high reproductive dominance) can therefore constitute a proximate mechanism for the allopatric evolution of social parasites with secondary sympatry.

Material and methods

Study populations, sampling and experimental set up

Three colonies of native *A. m. capensis* from Heidelberg (Western Cape province, South Africa) were used for this experiment. The colonies were split into a queenright and a queenless part of equal size. Requeening was prevented in the queenless splits by removing all queen cells so that laying worker offspring could be obtained.

Three *A. m. scutellata* colonies from Pretoria (Gauteng province, South Africa) were used. Prior to sampling, these colonies were screened for signs of infestation by socially parasitic *A. m. capensis* workers (Neumann & Hepburn, 2002; Neumann & Moritz, 2002; Härtel *et al.*, 2006b). All three colonies used were free of parasites.

Additionally, two queenright colonies of naturally occurring hybrids between *A. m. scutellata* and *A. m. capensis* from the introgression zone were used. The colonies sampled were located in Grahamstown (Eastern Cape province, South Africa). This location is close to the *A. m. capensis* distribution area and the characteristics of the hybrids are thus predominantly influenced by *A. m. capensis* (Hepburn & Radloff, 1998, 2002; Neumann *et al.*, 2000). It must be noted that there is no bee breeding practised in these regions. The colonies thus represent unrelated and authentic samples of the natural populations (Hepburn *et al.*, 2004). All colonies sampled had been caught in trap boxes (which is standard beekeeping practice in South Africa, Tribe & Allsopp, 2001) and were queenright.

Finally, three queenless *A. m. scutellata* colonies, heavily infested by the socially parasitic *A. m. capensis* lineage, were obtained from Pretoria. Infested *A. m. scutellata* colonies can be easily distinguished by the occurrence of multiple eggs in worker brood cells, a scattered brood

pattern and raised brood cell cappings (Neumann & Hepburn, 2002; Härtel *et al.*, 2006b).

In order to evaluate the pheromonal dominance between individual workers, contests were performed by using a standard protocol (Moritz *et al.*, 2000, 2004). Frames with sealed worker brood were taken from each colony or colony split and individually stored in an incubator (34 °C, 60% humidity) until adult emergence. Pairs of workers were introduced into Petri dishes. They were provided with food (honey/icing sugar/pollen mix) *ad libitum* and placed in a dark storeroom at room temperature for four or seven days. To evaluate individuals with potentially different levels of pheromonal dominance, workers of the following origins were tested against each other:

1. *A. m. capensis* workers from the Western Cape, queen offspring;
2. *A. m. capensis* workers, from the Western Cape, laying worker offspring;
3. *Apis mellifera capensis* workers of the invasive lineage, laying worker offspring;
4. Hybrid workers from the introgression zone, queen offspring;
5. *Apis mellifera scutellata* host workers, queen offspring.

A total of 198 pairs were kept for four days, with 8–24 replicates per combination between workers of different origins. Only those pairs for which the pheromonal dominance was unresolved after four days [i.e. that did not yield significant differences in quantity of 9-ODA or 9-ODA/(9-ODA + 10-HDA) ratio produced, see below] were tested for seven days ($n = 80$ pairs, with 11–14 replicates per combination between workers of different origins).

GC analyses

After four or seven days, the workers were frozen and decapitated. Their heads were placed in 200 μL dichloromethane for at least 24 h to extract compounds of the mandibular gland. The extracts were then evaporated to dryness under a stream of nitrogen. The residues were redissolved in 20 μL of an internal standard (octanoic acid and tetradecane in dichloromethane) and 20 μL of bis-trimethylsilyltrifluoroacetamide. One microlitre of this solution was injected into an HP 5890 gas chromatograph in the split-less mode. The capillary column used was a methyl silicone coated fused silica column (HP-1, 25 m × 0.32 mm). Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The temperature of the oven was maintained at 60 °C for 1 min, and then increased to 100 °C at 50 °C min^{-1} and to 220 °C at a rate of 3 °C min^{-1}. The final temperature was maintained for 10 min. Chromatograms were recorded and peak areas determined by using HP CHEMSTATION software. The mandibular gland compounds were identified based on the retention times of synthetic compounds and on their retention times compared with the internal standards (Simon *et al.*, 2001). The 'queen substance' 9-keto-(E)-2-decenoic acid (9-ODA) and the 'worker substance' 10-hydroxy-(E)-2-decenoic acid (10-HDA) (Callow *et al.*, 1959; Barbier & Lederer, 1960; Slessor *et al.*, 1988; Pankiw *et al.*, 1996) were quantified by using peak areas and the relative mass ratios calculated relative to tetradecane (Simon *et al.*, 2001). A standard solution containing the 9-ODA and 10-HDA were run daily to ensure that relative mass ratios were within the limit of the variability found in the series of standard runs (Crewe, 1988; Moritz & Crewe, 1988). We also calculated the quantitative ratios of 9-ODA/(9-ODA + 10-HDA) to assess how 'queen-like' a mandibular gland pheromonal bouquet was (Moritz *et al.*, 2000, 2004; Schäfer *et al.*, 2006). A ratio close to one indicates a queen-like blend, whereas a ratio close to zero indicates a worker-like blend.

Data analysis

The selection process studied here resulted in a single lineage of parasitic workers; we thus considered lineages as units of replication. As the tested workers were not genotyped, we estimated the probability of re-sampling, a given pair of patrilines $P(p_{ab})$ by using the average frequency of the most abundant patriline. Only a few patrilines dominate reproduction in queenless colonies of *A. m. capensis* (Moritz *et al.*, 1996) and the probability of sampling the most abundant patriline corresponds to its frequency in the colony (mean of 70%, Moritz *et al.*, 1996).

The probability of drawing a particular patriline a (p_a) out of a particular colony (i) is:

$$P(C_i p_a) = P(C_i) \cdot P(p_a) \tag{1}$$

Where $P(C_i)$ is the probability of sampling one colony out of N colonies, with $P(C_i) = \frac{1}{N}$ and $P(p_a)$ the probability of sampling a particular patriline a, which is equivalent to the frequency of patriline a in the colony.

The probability of drawing patriline a $P(p_a)$ from colony i (C_i) and patriline b (p_b) from colony j (C_j) is:

$$P(p_{ab}) = P(p_a \cap p_b) = P(C_i p_a) \cdot P(C_j p_a) \tag{2}$$

The probability of drawing twice or more times the same pair of patrilines is:

$$P(2 \cdot p_{ab}) = \sum_{i=2}^{m} P(p_{ab})^i \cdot (1 - P(p_{ab}))^{m-i} \tag{3}$$

With m being the number of replicates of this pheromonal contest. In this estimate, the variability originating in the matriline is not accounted for in the uniqueness of the lineage. We will therefore obtain a conservative estimate of the probability of pseudo replication.

Our worst case scenario (where probability of re-sampling workers from similar lineages is higher)

corresponds to the pheromonal competition between parasitic workers from the invasive lineage with *A. m. capensis* worker offspring. The parasitic workers of this lineage are clones (Kryger, 2001a,b; Baudry *et al.*, 2004; Härtel *et al.*, 2006b) and all individuals within a colony are identical which results in a frequency of 1 for this patriline [$P(p_{\text{parasite}}) = 1$]. This also means that there are no genetic differences among individuals collected in different infested colonies (the three colonies sampled here only introduced variability for developmental differences). For this calculation, we consider that $N_{\text{parasite}} = 1$. The *A. m. capensis* worker offspring was sampled from three different colonies ($N_{\text{capensis worker offspring}} = 3$) and the frequency of the most abundant patriline is 0.7 [$P(p_{\text{capensis worker offspring}}) = 0.7$; Moritz *et al.*, 1996].

Using equation (2) gives the following probability of drawing a particular patriline from the parasitic colonies and a particular patriline from the *A. m. capensis* worker offspring colonies.

$$\begin{aligned} &P(p_{\text{parasitic/capenisis worker}}) \\ &\quad = P(C_{\text{parasitic}} p_{\text{parasitic}})^{i} \cdot P(C_{\text{capensis worker}} C_{\text{capensis worker}}) \\ &\quad = (1 \cdot 1) \cdot (0.7 \cdot \frac{1}{3}) = 0.23. \end{aligned}$$

This pheromonal contest was replicated 16 times ($m = 16$). Therefore, the probability of sampling the most abundant patriline 2 or more times is, according to equation (4):

$$\begin{aligned} &P(2 \cdot p_{\text{parasitic/capensis worker}}) \\ &\quad = \sum_{i=2}^{16} P(p_{\text{parasitic/capensis worker}})^{i} \cdot \\ &\qquad (1 - P(p_{\text{parasitic/capensis worker}}))^{16-i} < 0.002. \end{aligned}$$

In our worst case scenario, the probability of sampling the same combination of worker lineages (pseudo replication) is therefore negligible, even though one of the groups tested in a pair has no genetic variability (parasitic lineage) and the other one weak variability (*A. m. capensis* worker offspring). Therefore, it is safe to consider each pair tested as an independent replicate. Wilcoxon matched pair tests were performed to evaluate differences in 9-ODA/(9-ODA + 10-HDA) ratio produced by each individual of a pair. All statistical tests were performed by using the program STATISTICA© (Statsoft, Tulsa, OK, USA).

Results

A total of 396 individual workers were tested. We found significant differences in the ratios of the mandibular gland compounds produced by different groups (Table 1). In particular, workers of the invasive lineage had a significantly more queen-like blend than all other groups after only four days (Table 1). The *A. m. capensis* worker offspring from the Western Cape dominated *A. m. scutellata* workers within four days (Table 1), but neither dominated hybrid workers nor *A. m. capensis* queen offspring, even after seven days. The *A. m. capensis* queen offspring from the Western Cape dominated the hybrids within four days (Table 1) and *A. m. scutellata* within seven days (Table 1). The hybrids dominated *A. m. scutellata* within four days (Table 1). Finally, *A. m. scutellata* workers were always pheromonally subordinate.

Although *A. m. capensis* worker offspring did not dominate *A. m. capensis* queen offspring even after seven days, we can rank them relatively to their ability to dominate the *A. m. scutellata* workers. Indeed, *A. m. capensis* worker offspring dominated *A. m. scutellata* workers within four days, whereas it took between four and seven days for *A. m. capensis* queen offspring to do so.

Table 1 Results of pheromonal contests between five groups of workers after four days. If a tie was obtained after this four-day period, the experiment was repeated and extended to seven days.

	Dominant				
Subordinate	*Apis mellifera scutellata*	Hybrids	*Apis mellifera capensis* queen offspring	*A. m. capensis* worker offspring	Invasive lineage
A. m. scutellata	–	0.19 ± 0.27* 4 days $n = 10$	0.04 ± 0.14* 7 days $n = 14$	0.15 ± 0.25** 4 days $n = 13$	0.82 ± 0.25** 4 days $n = 13$
Hybrids	+	–	0.36 ± 0.30** 4 days $n = 11$	0.11 ± 0.33 7 days $n = 13$	0.68 ± 0.29** 4 days $n = 16$
A. m. capensis queen offspring	+	+	–	0.31 ± 0.54 7 days $n = 14$	0.44 ± 0.41** 4 days $n = 14$
A. m. capensis worker offspring	+	0	0	–	0.62 ± 0.28** 4 days $n = 16$
Invasive lineage	+	+	+	+	–

The mean ± SD of the difference in quantitative ratio 9-ODA/(9-ODA + 10-HDA) between the individuals in each pair are given. Sample size is given in each cell. Significant differences between 9-ODA/(9-ODA + 10-HDA) ratios of the individuals in a pair at the $P < 0.05$ and $P < 0.01$ levels are indicated by * and ** respectively, using Wilcoxon paired tests. In the lower part of the matrix, '+' and '0' indicate whether the hypothetical order of pheromonal dominance for each contest was supported by the data or not respectively.

Thus, the increasing pheromonal dominance order obtained is the following: (1) *Apis mellifera scutellata* workers, (2) hybrid workers, (3) Western Cape *A. m. capensis* queen offspring, (4) Western Cape *A. m. capensis* worker offspring and (5) invasive *A. m. capensis* lineage.

Discussion

Our data confirmed that *A. m. capensis* is reproductively dominant over *A. m. scutellata* (see Introduction). Moreover, we showed that the invasive lineage of *A. m. capensis* workers established pheromonal dominance over all tested groups within four days. This is in line with studies suggesting that rapid development of pheromones is important in establishing reproductive dominance among honeybee workers (Hepburn, 1992; Moritz *et al.*, 2000, 2004; Simon *et al.*, 2005). In addition, our proposed pheromonal dominance order based on the levels of competition was supported in broad terms by the results: *A. m. capensis* workers from the Western Cape were dominated by the selected parasitic lineage, but dominated *A. m. scutellata*.

Evolution of social parasitism by honeybee workers

Natural invasions of *A. m. scutellata* populations by *A. m. capensis* parasitic workers are probably prevented by an introgression zone between the two subspecies (Hepburn & Radloff, 2002). The stability of this zone could be explained if native *A. m. capensis* workers are unable to infest colonies of the hybrid population. However, our results showed that the hybrids can be dominated pheromonally by *A. m. capensis* workers from the native range and by the invasive lineage. This suggests that natural infestations by socially parasitic workers are possible in the introgression zone just as they are in the endemic range of the Cape honeybee (Härtel *et al.*, 2006a). Thus, the stability of the introgression zone is more likely to be due to sparse population density, rendering host finding more difficult (Neumann *et al.*, 2001).

The naturally occurring hybrids were pheromonally subordinate to *A. m. capensis* queen offspring, as predicted, and they seemed to be subordinate to worker offspring as well, although the difference in pheromonal secretion was not significant. However, contrary to our hypothesis, there were no differences in mandibular gland compound ratios between *A. m. capensis* worker and queen offspring after seven days. These results reflect the relative reproductive dominance that occurs at the colony level, and results from competition for access to reproduction after queenloss (Moritz *et al.*, 1996). Indeed, a worker lineage that gains reproductive dominance in one colony may not necessarily gain it in another, when it is exposed to other, putatively more dominant lineages. Moreover, the hybrid colonies originated from an area where traits of *A. m. capensis* dominate over those of *A. m. scutellata* (Hepburn & Radloff, 1998). This contributes to a reduced probability of finding significant differences in pheromonal dominance between these hybrids and *A. m. capensis*. Nevertheless, our hypothesis is supported because *A. m. capensis* worker offspring dominated *A. m. scutellata* within four days, whereas *A. m. capensis* queen offspring established dominance only between four and seven days. This underlines the importance of fast pheromonal development for reproductive dominance in honeybee workers (Moritz *et al.*, 2004; Simon *et al.*, 2005).

After queen loss, which is an integral part of infestations by socially parasitic workers (Martin *et al.*, 2002a; Neumann & Hepburn, 2002), pheromonal dominance amongst workers governs access to reproduction (Simon *et al.*, 2005). The data show that *A. m. scutellata* workers were always pheromonally subordinate as predicted by our hypothesis. This confirms earlier studies that workers of *A. m. scutellata* develop queen-like mandibular gland pheromonal bouquets less readily than *A. m. capensis* (see Introduction).

Secretion of queen-like pheromonal bouquets represents an advantage for several reasons. In recently queenless colonies of *A. m. capensis*, substantial aggression may occur among workers, causing the death of many individuals (Anderson, 1968; Tribe, 1981, 1983). Such aggression between *A. m. capensis* workers is also likely to occur in infested host colonies and clearly only surviving individuals can dominate reproductive competition. It has been observed that attacking workers back away from pheromonally developed individuals, as they do when approaching a queen (Tribe, 1981; van der Blom, 1991; = protection function). In contrast, workers eliminate *A. m. capensis* workers that are not fully pheromonally developed (Velthuis, 1976). Production of queen-like mandibular secretions also allows workers to obtain food by trophallaxis with increased probability (Hillesheim *et al.*, 1989) and this sustains the protein need for their oogenesis (Schäfer *et al.*, 2006; = nutritional function). In addition, workers producing these secretions can prevent pheromonal and ovarian development in other workers (Velthuis *et al.*, 1965, 1990; Hepburn & Radloff, 1998; Neumann & Moritz, 2002; Wossler, 2002; = regulatory function). Together with other factors linked to reproduction (e.g. reduced worker policing, Neumann *et al.*, 2001, 2003; Martin *et al.*, 2002b), and given its significant advantage for the acquisition of reproductive dominance, the fast production of queen mandibular pheromones could represent the main factor that promoted a parasitic association between these two subspecies.

Thelytoky by honeybee workers other than *A. m. capensis* is rare but has been described repeatedly (Mackensen, 1943; Tucker, 1958; DeGrandi-Hoffman *et al.*, 1991). However, none of these occurrences evolved into stable thelytokous or parasitic populations. Although thelytoky promotes the evolution of reproductive

dominance and social parasitism (Greeff, 1996; this study), these systems must be lacking other traits necessary to allow for the fixation of the parasitic behaviour. In the case studied here, the invasive lineage probably lacks an efficient host finding mechanism because the phenomenon is sustained by beekeepers' activities (Moritz, 2002; Dietemann *et al.*, 2006a). In the other cases where thelytoky appeared, the local potential host was certainly not susceptible enough to allow parasitism to evolve or the thelytokous strains lacked other necessary traits associated with successful reproduction (e.g. laying acceptable eggs, Martin *et al.*, 2002b). Alternatively, parasitic lineages may occur at a low frequency and could have remained undetected (cf. Introduction; Härtel *et al.*, 2006a).

In sharp contrast to other social parasites (Buschinger, 1986; Hölldobler & Wilson, 1990; Schmid-Hempel, 1998), the invasive *A. m. capensis* worker lineage is not rare and its occurrence results in dramatic losses of host colonies (Allsopp & Crewe, 1993). This is likely to be due to the recent establishment of the parasite and the lack of a co-evolutionary history between host and social parasite. Introduction of the parasite by beekeepers in its new range occurred in 1990 (Dietemann *et al.*, 2006a) and this has not been long enough for resistant *A. m. scutellata* colonies to be selected and spread in the population. Thus, we are only witnessing the very first steps in the evolution of a social parasite and this represents a good model to study evolutionary processes as they occur (Neumann & Moritz, 2002). This invasive lineage can be compared to a queen-intolerant inquiline microgyne (Wilson, 1971; Hölldobler & Wilson, 1990). Indeed, it is an obligate parasite because it cannot establish new colonies and does not forage efficiently (Martin *et al.*, 2002a; Neumann & Hepburn, 2002). The parasites belong to the worker caste and only reproduce by thelytokous parthenogenesis without mating. There is therefore no need to evade the queen determination mechanism of the host to obtain reproductive parasites (Bourke & Franks, 1991; Aaron *et al.*, 1999). Although the host queen dies a few weeks after infestation (Martin *et al.*, 2002a), attacks by the worker do not seem to be the usual cause of her death. In contrast, it is the host queen that attacks the parasites (Moritz *et al.*, 2003; V. Dietemann unpubl.). In fact, the queen's death is likely to be a consequence of the exploitation of the reproductive regulation mechanism by the social parasite (Neumann & Moritz, 2002), as host workers attempt to regulate the number of reproductive individuals in their colonies. Host workers might favour the survival of social parasitic workers who secrete queen-like pheromones over that of a queen whose health is degrading following a succession of fights (Moritz *et al.*, 2003).

Selection of a single parasitic lineage

Socially parasitic lineages which reach reproductive status rapidly should have a reproductive head start and could therefore spread faster in the host population. Our data give strong support to a selection scenario that favored such fast pheromonal development: invasive parasitic *A. m. capensis* workers pheromonally dominating *A. m. capensis* worker offspring in each pair and queen offspring in most of the pairs after only four days. This indicates that the invasive lineage has a superior ability to produce the pheromones associated with reproduction and can produce them faster than other strains. In addition, the early production of these semiochemicals confers a head start on them to inhibit the onset of reproductive activity in other individuals (Velthuis *et al.*, 1965, 1990; Moritz *et al.*, 2000, 2004; Simon *et al.*, 2005). Thus, fast pheromone production could have given this particular lineage a fitness advantage that allowed it to outcompete other parasitic *A. m. capensis* lineages in the range of *A. m. scutellata*. Moreover, both automictic parthenogenesis through central fusion (Verma & Ruttner, 1983) and the reduced crossing over rate in thelytokous worker reproduction (Baudry *et al.*, 2004) favours the selection of beneficial traits and therefore the fixation of a lineage with high fitness. Indeed, in this situation co-adapted gene complexes are not disrupted, as can occur with sexual recombination (Dobzhansky, 1970).

Different selection scenarios for *A. m. capensis* laying workers in its native range and for the invasive lineage in *A. m. scutellata* range may have promoted the differences in pheromonal competitiveness observed within *A. m. capensis*: whereas *A. m. scutellata* colonies appear to be susceptible hosts (Martin *et al.*, 2002a; Neumann & Hepburn, 2002; Wossler, 2002; Neumann *et al.*, 2003), native *A. m. capensis* colonies are naturally less often infested by socially parasitic workers of their own subspecies (Härtel *et al.*, 2006a). Thus, considerably more parasitic offspring are produced in the new range compared with the native distribution area of the Cape honeybee. In the *A. m. capensis* range, massive colony losses because of parasitic workers have not been reported and therefore virulence seems to be low. A large number of parasites in the *A. m. scutellata* population should lead to higher competition for host resources during multiple infestations of colonies or apiaries with different parasitic worker lineages. Moreover, infested *A. m. scutellata* colonies eventually die or abscond (= nonreproductive swarming; Neumann & Hepburn, 2002), resulting in a more limited time window for successful parasite reproduction. This probably further enhanced selection for fast pheromone production and high reproductive competitiveness in the social parasite population in its new range. We therefore suggest that selection between lineages at the population level is much stronger within the *A. m. scutellata* host populations compared with native *A. m. capensis* populations and favoured the emergence of a highly virulent social parasite.

Conclusion

Our experiments investigated pheromonal contests among workers to determine how parasitism evolved once sympatry was (artificially) restored between *A. m. capensis* and *A. m. scutellata* and to determine how a single parasitic worker lineage was selected. However, this study does not clarify the proximate mechanisms of the parasitic interaction. Individuals from the invasive lineage are able to take over *A. m. scutellata* host colonies despite the presence of a reproducing queen and brood (Neumann & Hepburn, 2002; Neumann & Moritz, 2002; Wossler, 2002). Moritz *et al.* (2003) contributed to the understanding of the interactions between parasitic workers and queens by demonstrating the occurrence of physical fights, but parasites and host queens can also compete pheromonally (Dietemann *et al.*, 2006b). Investigating both the behavioural and pheromonal interactions in the host–parasite relationship will help determine how reproductive take over of host colonies occurs. Knowledge of interactions with parasitic competitors, host queens and host workers are necessary to understand the evolution of this host–parasite relationship fully.

Our data suggest that the parasite has the ability to exploit the hosts' communication system that mediates regulation of reproduction and that a unique lineage that possesses a genotype yielding particularly rapid pheromonal dominance has been selected. We believe that our bioassay accurately reflected the different levels of competition the invasive lineage was exposed to in the early years after introduction into the host's range and that the pheromonal dominance order revealed in our experiments corresponds with the outcome of the natural selection of the invasive lineage. Mandibular gland pheromones are likely to have played a central role in the evolution of social parasitism in honeybees. The importance of these pheromones is based on their multiple functions in determining reproductive status and allowing individuals to prevent reproduction by their nestmates (Velthuis *et al.*, 1990; Simon *et al.*, 2005). Despite the uniqueness of the honeybee system, our findings contribute to the understanding of the evolution of intraspecific social parasitism in the social insects.

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