Insect. Soc. 53 (2006) 80–85
0020-1812/06/010080-6
DOI 10.1007/s00040-005-0838-5

Insectes Sociaux

Research article

Intra-colonial variation of the sting extension response in the honey bee *Apis mellifera*

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Received 29 April 2005; revised 8 July 2005; accepted 25 July 2005.

Abstract. The workers' sting extension in response to noxious stimulations is a common test used to study physiological modulations of behaviour in the honey bee. In this study, we investigated the variation of the sting extension response between honey bee workers from different patrilines in a colony with a naturally mated queen. We found that the sting extension response varied according to patrilines, indicating a genetic contribution to the intra-colonial variation of this behaviour. Patrilines differed in their responses during successive stimulations applied at a constant level: bees belonging to some patrilines exhibited a constant level of response during repeated stimulations, while others showed a decreasing response under the same conditions. These results fit well with the models of division of labour based on differences in response thresholds among workers of different subfamilies.

Keywords: Sting extension test, patrilines, defensive behaviour, response threshold, honey bees.

Introduction

Disentangling the complex determinism of behavioural specialisation is one of the most challenging questions concerning division of labour in honey bee colonies. The division of labour among workers in a colony is explained not only by the general age-related pattern of the polyethism, but also by physiological factors and by genetic predispositions (Fahrbach and Robinson, 1996; Robinson and Page, 1989). Many studies on the genetic basis of the division of labour have involved intracolonial comparisons of workers' behaviour. As a result of polyandry, a honey bee colony consists of different patrilines which can exhibit different genetically-based behaviour. Paternal origin of workers within a colony has been found to influence brood rearing and grooming behaviour, corpse removal, comb shaping, guarding of the nest entrance, and foraging for pollen or for nectar (Calderone and Page, 1988; Calderone et al., 1989; Frumhoff and Baker, 1988; Robinson and Page, 1988). More recent studies have provided evidence of a genetic determinism on neurological and physiological aspects of the division of labour such as learning performance related to foraging (Scheiner et al., 2001) and flight metabolism (Harrison and Fewell, 2002).

Defence of the hive should not be seen as a single behaviour; as with major functions such as brood rearing or foraging, it is divided into different tasks (Breed et al., 1990). At least two groups of workers are involved in the defence of the hive. A first group of workers performs the guarding task by patrolling at the nest entrance and preventing alien bees from entering the hive. These guards, generally less than one hundred per colony (Moore et al., 1987), detect a potential

aggressor and alert other bees by visual and pheromonal stimuli. The second group of workers consists of thousands of bees. These defenders play a defensive role in the event of a major disturbance, trying to deter the aggressor by stinging.

Most studies of honey bee defensive behaviour were based on a field test which presented a moving black leather target in front of the hive. This procedure makes it possible to quantify the level of bee attack by counting the stings remaining on the target (Guzmán-Novoa and Page, 1993; Millor et al., 1999). Laboratory observations of caged bees also were used to quantify the defensive behaviour (Collins, 1980; Collins and Rothenbuhler, 1978). Response intensity was estimated by the proportion of bees that reacted to alarm chemicals, a measure which was particularly applied to study the influence of age (Collins, 1980) and geographical race (Collins et al., 1982; 1987) on defensive behaviour. Other procedures are based on individual level experiments in the laboratory. These procedures allow one to control for environmental conditions and for the possibility of group effects, as well as to measure response of individuals. The first type of individual assay was developed by Kolmes and Fergusson-Kolmes (1989), who used electrical stimulation to elicit stinging behaviour. Using this procedure, Paxton et al. (1994) showed that both within-colony environment and age highly influenced the workers' stinging behaviour: the response threshold notably reached a minimum around 20 days of age. A slightly different procedure allows one to measure the extent to which the sting is extruded on restrained bees stimulated with a mild electric shock (Balderrama et al., 2002; Nuñez et al., 1983, 1998). This sting extension response, which can also be obtained on isolated abdominal preparations (Burrell and Smith, 1994; 1995), reproduces under highly controlled conditions one component of the natural alarm display, i.e., the opening of the sting chamber and the protraction of the sting (Balderrama et al., 2002; Nuñez et al., 1983, 1998). The extent to which the sting was extruded was proved powerful to differentiate between the individual response thresholds to various noxious stimuli (Balderrama et al., 2002; Nuñez et al., 1983, 1998).

The genetic determinant of defensive behaviour has been studied mainly at the colony level. Heritability estimates first revealed that the defensive behaviour has a genetic component (Collins, 1979; Collins et al., 1984). In colonies established from queens artificially inseminated with semen from three unrelated males, the different patrilines were shown to contribute unequally to guards and defenders (Breed et al., 1990; Robinson and Page, 1988). In order to select and compare some defensive behavioural traits, Guzmán-Nova et al. (2002) and Arechavaleta-Velasco et al. (2003) produced colonies with singly-mated queens by controlled artificial insemination. They showed that several quantitative trait loci influenced the expression of guarding and stinging behaviour of the bees. In the present study, we address the question of the patriline variation in the sting extension response among workers from a colony with a queen naturally mated with unselected drones in number or quality.

Methods

Honey bees

Experiments were conducted on bees reared in control conditions in order to discriminate between patriline effect and other confounding effects such as age effect (Collins, 1980; Paxton et al., 1994) or effect of previous exposure to alarm pheromone which could affect the sting response. A total of 660 workers was obtained from a single colony reared under natural conditions. Newly emerged bees were collected from combs of capped brood and were caged in groups of 70 individuals of the same age (Pain, 1966). Caged bees were maintained in an incubator (temperature 33 °C, relative humidity 55 %, darkness). They were fed with a sugar solution and water *ad libitum*, and with pollen during the first eight days. Experimental tests were conducted on all the 12-day-old caged worker bees (*Apis mellifera* L.). As this is the median age at which workers become guards (Sakagami, 1953; Winston, 1987), bees can be considered capable of stinging.

Behavioural study

The procedure and apparatus for the study of the sting extension response were adapted from Nuñez et al. (1983; 1998). Each worker was placed on a holder, without prior anaesthesia, with its back on a plastic plate (figures of the holder can be found in Nuñez et al. (1983; 1998) and Balderrama et al. (2002)). A metallic plate with a notch constituted the first electrode. This was placed between the head and the thorax. A second metallic electrode was located at the bee peduncle. Conductive gel (Spectra electrode gel, contents 8.5 OZ, 250 GMS, salt free, Parker Laboratories Inc.) was applied to each electrode to allow an optimal contact between the electrodes and the cuticle of the bee. The bee was maintained in this position with a metal strip exerting mild pressure on the mesosternum. The tip of the abdomen was immobilized by a small plate with a hole of 2-mm diameter, through which the sting could be extruded. Bees were restrained in the device for 1 min before stimulation. Each bee was then subjected to four 2-s electric stimulations (4 volts) with a 1-min inter-stimulation interval. Responses to each stimulation were recorded as the extent to which the sting was extruded. In previous works in which the same experimental design was used (Balderrama et al., 2002; Nuñez et al., 1983, 1998), the response was binary scored, i.e., it was scored 1 when the sting was fully exposed and the sting chamber was open during the entire stimulation or it was scored 0. Burrell and Smith (1994; 1995) presented a more detailed method of scoring using 3 levels of response detection: "no response", "partial" and "full" response. In order to account for the variability of responses which were below the maximal one, we added intermediate levels of response, expanding the previous scoring systems. The absence of any response was scored 0, the response was scored 1/3 when the sting extended less than the half of its length, it was scored 2/3 when it extended between half and all of its length without opening of the sting chamber, and finally, the response was scored 1 when the sting was fully extended and the sting chamber was completely open (Fig. 1).

The determination of patrilines

Patrilines were determined by analysis of nuclear DNA microsatellite markers. Among the numerous microsatellites available in the honey bee (Solignac et al., 2003), three loci (Table 1) provided sufficient genetic variability to classify the workers into distinct patrilines within the studied colony. DNA extractions were performed from a fragmented hind leg by a rapid Chelex method (Franck et al., 1999). Extracted DNA was then amplified by radioactive PCR processed in 10 µl containing 5–10 ng of isolated DNA, 400 nM of each primer, 25 µM of each dGTP, dCTP and dTTP, 6 µM of dATP, 0.15 µCi of [^{33}P]dATP, 20 mg.ml^{-1} of bovine serum albumine, 1.2–1.5 mM $MgCl_2$, 1 µl of 10X Mg-free reaction buffer (Promega) and 0.4 unit of Taq DNA polymerase (Promega). Amplifica-

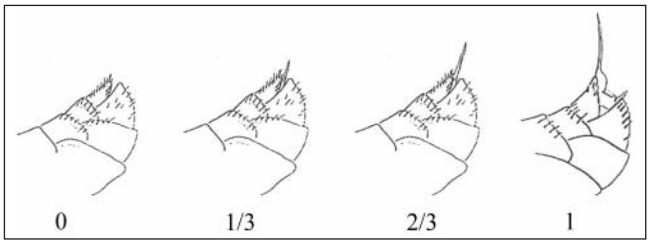

Figure 1. Diagram of the tip of the abdomen exhibiting different degrees of response to 4-volt electrical stimulation (modified from Nuñez et al. (1983; 1998) and corresponding scoring system.

tions were processed through 35 cycles consisting of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. PCR products were run on 6 % polyacrylamide gels and the alleles were visualised by autoradiography. Patrilines are easily identified due to haplodiploid determination of sex (Estoup et al., 1994; Moritz et al., 1995). Using the genotypes of all the workers, it is possible to infer the maternal alleles (either two alleles in equal proportions among workers when the queen is heterozygous at the considered locus, or the same allele in all the workers when the queen is homozygous at the considered locus). Then paternal haplotypes are deduced by subtraction. We used MatSoft Software (Moilanen et al., 2004) (http://www.zi.ku.dk/personal/jspedersen/matesoft.htm) to determine patrilines. We also calculated the effective paternity (m_e) (Starr, 1984) to confirm the representativeness of our colony.

Statistical analysis

The effect of the patrilines on the sum of the individual levels of responses was analysed using non-parametric tests. The Kruskal-Wallis test indicated whether the responses of the bees differed significantly between groups. Non-parametric Wilcoxon pairwise comparisons were then applied in order to identify the groups that differed. Pairwise comparisons were conducted according to the method proposed by Conover (1980), ensuring that the experiment-wise alpha level was 0.05. To analyse the evolution of the sting extension response over the successive simulations, Friedman tests were applied. Statistical analyses were made using the S-plus software (Venables and Ripley, 1999).

Results

Variability of the sting extension response

Total numbers of each response score are presented in Table 2. Between 70 % and 80 % of workers did not completely extend their sting. The most frequently occurring response for each stimulation was that scored as 2/3 (at least 46 % of the responses whatever the stimulation). A zero response was quite rare with a maximum occurrence of 4.24 % during the fourth stimulation.

Sting extension responses by patrilines

Of the 660 tested bees, 632 were genotyped for the three microsatellites loci. The 28 missing bees could not be genotyped due to technical problems. We detected 16 different patrilines within the hive and the effective paternity (m_e) is 9.67 (numbers of bees belonging to each patriline are reported on Fig. 2). The number of patrilines as the effective paternity are in the range of usual polyandry estimates in the honey bee (Tarpy and Nielsen, 2002).

Some patrilines (for instance, patrilines 8, 12, 4, 6 and 13) showed a low level of response (mean of the response over the four stimulations <0.65) whereas some others (especially patrilines 7, 1, 9, 5, 10 and 15) showed a high level of response (Fig. 2). This mean individual response to the four stimulations differed significantly according to the different patrilines (Kruskal-Wallis test, $N = 632$, $\chi^2 = 25.85$, $df = 15$, $P = 0.0397$). The responses of workers belonging to patriline 8 differed significantly from those of patrilines 14 (Wilcoxon pairwise comparisons, $P = 0.0306$), 2 ($P = 0.0226$), 7 ($P = 0.0074$), 1 ($P = 0.0162$), 9 ($P = 0.0112$), 5 ($P = 0.0163$), 10 ($P = 0.0047$) and 15 ($P = 0.0016$). Responses of workers belonging to patriline 12 differed from those of patrilines 2 ($P = 0.0413$), 7 ($P = 0.0136$), 1 ($P = 0.0280$), 9 ($P = 0.0201$), 5 ($P = 0.0282$), 10 ($P = 0.0082$) and 15 ($P = 0.0027$). Finally, the responses of workers belonging to patriline 15 differed not only from those of patrilines 8 and 12 but also from patrilines 4 ($P = 0.0358$), 6 ($P = 0.0162$) and 13 ($P = 0.0283$). Other comparisons between patrilines led to non-significant differences.

In addition, patrilines can be distinguished by the degree to which their sting extension responses changed over the four stimulations. Six patrilines (1, 6, 7, 8, 11 and 12) (Friedman test, patriline 1: $\chi^2 = 9.35$, $df = 3$, $P = 0.0249$; patriline 6: $\chi^2 = 15.44$, $df = 3$, $P = 0.0015$; patriline 7: $\chi^2 = 25.34$, $df = 3$, $P = 0.0001$; patriline 8: $\chi^2 = 13.92$, $df = 3$, $P = 0.0030$; patriline 11: $\chi^2 = 16.73$, $df = 3$, $P = 0.0008$; patriline 12: $\chi^2 = 20.51$, $df = 3$, $P = 0.0001$) exhibited responses that decreased

Table 1. Core sequence and PCR conditions for the three microsatellites used.

Locus	core sequence	sequence of primers	annealing Temperature	[$MgCl_2$] (mM)
B124	$(CT)_8$ … $(CT)_{14}$ CCTC$(GC)_3$ … $(GGCT)_8$	5'-GCAACAGGTCGGGTTAGAG-3' 5'-CAGGATAGGGTAGGTAAGCAG-3'	55 °C	1.5
Ap33	$(CT)_{15}$	5'-TTTCTTTTTGTGGACAGCG-3' 5'-AAATATGGCGAAACGTGTG-3'	54 °C	1.2
Ap19	$(TC)_{11}$	5'-CTCGTTTCTTCCATTGCG-3' 5'-CGGTACGCGGTAGAAAGA-3'	56 °C	1.2

Table 2. Total number of each level of sting extension responses at each of the four successive 4-volt stimulations. The absence of any response was scored 0, the response was scored 1/3 when the sting extruded less than the half of its length, it was scored 2/3 when it extruded between half and all of its length without opening of the sting chamber, and it was scored 1 when the sting was fully extruded and the sting chamber was completely open. Number within parentheses is the corresponding percentage of the total number of responses at each stimulation.

types of	Successive stimulations			
Stinging responses	1	2	3	4
1	193 (29.24)	176 (26.67)	148 (22.42)	134 (20.30)
2/3	330 (50.00)	304 (46.06)	308 (46.67)	325 (49.24)
1/3	120 (18.18)	158 (23.94)	180 (27.27)	173 (26.21)
0	17 (2.58)	22 (3.33)	24 (3.64)	28 (4.24)

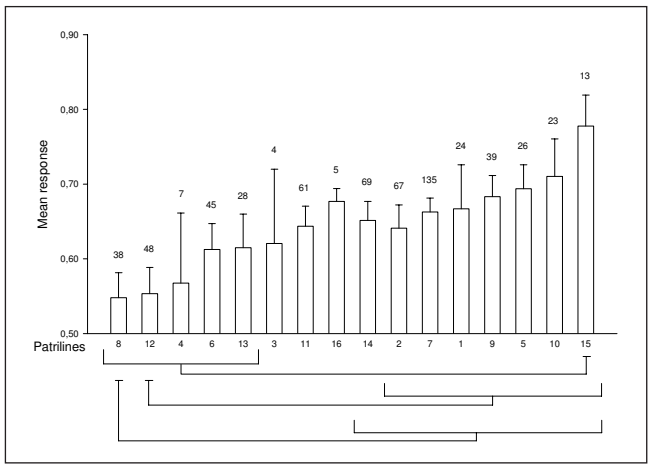

Figure 2. Mean (±SEM) of the sum of the responses to the four stimulations for bees in each patrilines. A Kruskal-Wallis test, performed on the sum of the responses of each individual, indicated significant differences in the responses of patrilines ($P = 0.0397$). Significant differences between patrilines (non-parametric pairwise comparisons) are shown on the figure as solid lines drawn between groups of patrilines. For example, the mean responses of the individuals belonging to patriline 15 differ from those of patrilines 8, 12, 4, 6 and 13. The number of bees in each patriline is indicated above the respective error bars.

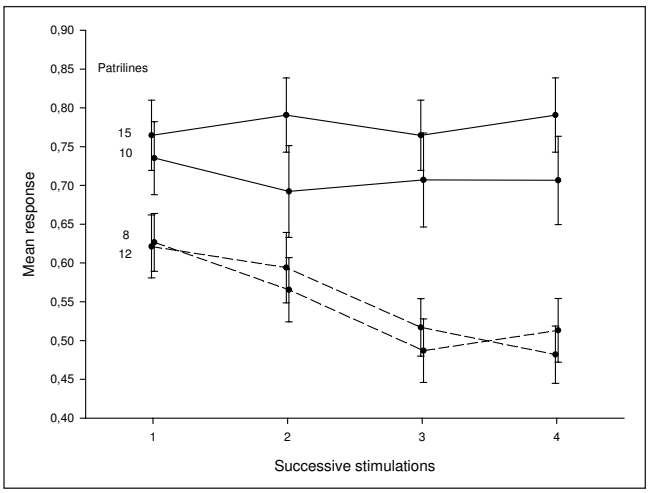

Figure 3. Four examples of the changes in the mean (± SEM) sting extension responses over the four successive stimulations. Solid lines: typical stable responses; dashed lines: typical decreasing responses. Patrilines 10 and 15 exhibited stable responses, while patrilines 8 and 12 exhibited significantly decreasing responses over the four stimulations (Friedman test).

significantly over successive stimulations while other patrilines showed a constant response. For example, typical decreasing and stable responses are shown in Fig. 3. We assume that the variation in the stability of the response might be rather graded across patrilines, some of these showing intermediate patterns between the extreme decreasing and stable ones. In any case, our results indicate that, within the variability of the sting extension response, genotype influences at least the stability of the response over repeated electric stimulations.

Discussion

Investigating the intra-colonial variation of the sting extension in response to a noxious stimulus, we found that the individual sting response varied according to patrilines. Such a result indicates a genetic influence for this behaviour. Compared to the previous works in which the response was binary scored (Balderrama et al., 2002; Nuñez et al., 1998, 1983), our results have shown that a great deal of variability exists in the responses which were below the maximal response. Under the binary scoring system, Nuñez et al. (1998) recorded 55 % of all responses as scoring 0, i. e., the sting was not fully exposed and the sting chamber was not open, when a 4-volt stimulation was applied. In the present study 70 % to 80 % of the bees responded at a level that would have been scored 0 using the previous system, whereas the number of bees showing no response at all was actually very low (<5 %). Our results are in agreement with studies on isolated abdominal preparations, which showed that most responses were partial sting extension (Burrell and Smith, 1994; 1995).

In a colony composed of 16 patrilines, we have shown that the responses of workers in the sting extension test differed according to their patriline. This patriline effect indicates that the variation in the sting response among workers in a colony has a significant genetic component. Many stud-

ies have shown that the inter-individual variability of honey bee behaviour can be explained by genetic predispositions, and that the genetic diversity due to polyandry is one of the determining factors of the specialisation (Calderone and Page, 1988; Nuñez et al., 1983; Robinson, 2002). The sting extension assay is generally considered to reproduce, under controlled conditions, the natural stinging response which is a major component of the alarm fanning display (Balderrama et al., 2002; Nuñez et al., 1983, 1998). This assay has not yet been directly correlated with the defensive behaviour of the colony, but the variability of this response fits well with the models of division of labour based on differences in response thresholds among workers of different subfamilies (Beshers and Fewell, 2001; Bonabeau et al., 1996; Robinson, 1992). If the stimulus exceeds the individual's internal threshold, the bee engages in stinging. Due to the high level of polyandry in colonies, genetically heterogeneous workers could have different response thresholds. As it has been argued for various aspects of the colonial life such as foraging behaviour (Cox and Myerscough, 2003) and thermoregulation (Jones et al., 2004), colony defence may benefit from genetically determined differences in response threshold among workers, since it allows the colony to respond adequately to a broader range of perturbations. Even if the hypothesis remains to be tested experimentally, it is possible that bees exhibiting different patterns of sting extension response contribute differently to guarding of nest entrance, typically performed by specialised guards (Arechavaleta-Velasco et al., 2003; Moore et al., 1987), or defending the colony in case of major aggression, a task typically performed by recruited defenders (Breed et al., 1990, 2004). These assumptions could be verified applying the sting extension test and comparing the responses as function of patrilines of a representative sample of the general nest population to those of guards. Additionally, the patrilines of defenders would be determined by analysing sting's DNA deposited on black leather targets during aggression tests.

Previous studies have associated genetic variance with several components of colony defence. Many of these studies, all based on colonies produced from a queen artificially inseminated with the semen of several drones, have shown that workers from the different patrilines were not represented equally in the guard and defender cohorts (Breed et al., 1990; Giray et al., 2000; Robinson and Page, 1988). Using backcrossed colonies produced from super-sister queens artificially inseminated with the semen of one "gentle" or "defensive" male, Hunt et al. (1998), Guzmán-Novoa et al. (2002) and Arechavaleta-Velasco et al. (2003), showed that quantitative trait loci can influence the expression of guarding or stinging behaviour of workers from the same patriline. In our study, using bees from a naturally mated colony and comparing them at the patriline level, we obtained evidence of a genetically-based specialisation of workers in the response to a noxious stimulus. As stinging behaviour is implicated in colony defence, one can suggest that workers could become specialised in one of the various tasks involved in the colony defence as a result of inter-individual variability in the responsiveness to alarm signals.

In the light of recent advances in molecular genetic analysis of behaviour in social insects (Robinson, 1999; 2002), in genomic resources such as microarrays (Kucharski and Maleszka, 2002) and linkage map (Solignac et al., 2004), there is a need for studies showing genotype effects on social behaviour. Our work on sting extension response, as well as other studies using highly standardised behavioural assays, might provide powerful measures for future studies investigating how genes and changes in gene expression might control behavioural specialisation in social insect colonies.

Acknowledgments

We wish to thank Bernard Roger and Mercedes Charreton for their considerable help in rearing the bees, Dimitri Antouchev and Noël Guitton for adapting and improving the experimental device and procedure, Dominique Vautrin and Véronique Auger who provided invaluable assistance in DNA analysis. Elizabeth Langridge and Hannah Reynolds reviewed the English of our text. The experiments were performed in accordance with the rules and regulations of France.

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