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Task specialization in a wild bee, *Apis florea* (Hymenoptera: Apidae), revealed by RFLP banding

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Abstract. Workers in a wild *in situ* colony of the dwarf honey bee, *Apis florea*, were observed undertaking the following behavior: liquid foraging, pollen foraging, guarding, stinging, fanning and wagging abdomen. Bees of each behavioral class were separately collected and frozen. Collections were made over a period of 10 days. Random samples of brood and workers were also collected. DNA was extracted from each bee and "fingerprinted" using a probe of unknown sequence obtained from an *A. mellifera* genomic library. Patterns of fingerprints (Fig. 1) were dissimilar among behavioral classes (Tables 1 and 2), strongly suggesting a genetic component to division of labor in this species. This result supports similar findings in *A. mellifera* in a species that is not troubled by many of the experimental difficulties inherent in *A. mellifera*.

Key words: Honey bee – Subfamily – RFLP – Task specialization – Dwarf honey bee – Thailand – Multiple mating

Introduction

Queens of the eusocial Hymenoptera often mate with several males. Multiple mating reduces the average genetic relatedness of workers below 0.5, thereby eliminating the kin selection advantage of haplodipliody proposed by Hamilton (1964). Multiple mating also increases the risk of predation while on mating flights (Moritz 1985). Since there is no physiological reason why a single drone could not produce enough spermatozoa to successfully inseminate a queen, evolutionary causes of polyandry have been sought and speculated upon (Crozier and Page 1985).

If a queen's mates are unrelated and heterogenous, then colonies of genotypically diverse workers result from the mating. Such colonies may be fitter than genetically uniform colonies for one or more of the following reasons.

First, genetically diverse colonies should be able to tolerate a wider range of environmental conditions (Crozier and Page 1985). This is particularly important in species which form perennial colonies but forage on seasonal sources of food. Since division of labor is thought to be a significant component in the ecological success of social insects (Oster and Wilson 1978), "task specialization" (Robinson and Page 1988), in which the highly related daughters of each haploid male have genetic predispositions to undertake certain tasks, may be a powerful selective force for polyandry. Colony-level selection may then maintain behavioral polymorphisms, and such selection was perhaps necessary for the evolution and maintenance of multiple mating and eusociality (Page and Robinson 1991; Oldroyd et al. 1992a, b, 1993).

Second, multiple mating eliminates the possibility that a queen will mate with a single drone carrying the same sex allele as herself (Shaskolsky 1976; Page 1980; Ratnieks 1990). Because of the method of sex determination in *Apoidea*, such matings result in 50% reduction in brood viability (Woyke 1963, 1986).

Third, genetic diversity resulting from multiple mating may increase resistance to parasites and pathogens (Sherman et al. 1988; Shykoff and Schmid-Hempel 1991).

None of these hypotheses is mutually exclusive, and all may contribute to colony fitness.

In *Apis mellifera*, the phenomenon of task specialization has been amply demonstrated. Bees of the same age often vary in the probability of performing a task despite identical stimuli (Calderone and Page 1988). Within-colony genetic variance was demonstrated by Frumhoff and Baker (1988) using the mutant *cordovan* to identify subfamilies, while Robinson and Page (1988) demonstrated the same phenomenon using biochemical markers. Robinson (1992) provides a complete review of more recent work on task specialisation in *Apis*. Moritz and Hillesheim (1989) found that groups of unrelated bees perform tasks with different efficiency from ho-

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mogeneous groups. Calderone and Page (1991) provided a theoretical framework which explained how interactions among genotypes could enhance the task specialization process. Finally, Oldroyd et al. (1992b) provided preliminary evidence for evolutionary advantages of genotypically mixed colonies over genotypically uniform colonies. They showed that *A. mellifera* colonies comprising three subfamilies had larger brood nests than colonies containing just one of the constituent genotypes.

These observations lend considerable support to the task specialization hypothesis. However, observations on domestic honey bees have a number of potentially serious difficulties, and may not reflect the situation in unselected wild populations, nor ancient evolutionary pressures. One potential problem with task specialization experiments in *A. mellifera* is the need to use genetic markers. These marker genes may cause variation in behavioural thresholds (Frumhoff 1991; Carlin and Frumhoff 1990). This is not a problem if the markers occur naturally in populations, but is problematic if they do not. For example, combining black and yellow bees (presumably of different ecotypes) in one experimental colony may generate artificially high genetic variability. Further, because the number of allozyme and other genetic markers available is low, experimental colonies need to contain unusually low numbers of subfamilies. The effects of this have not been investigated, although Hogendoorn and Velthuis (1988) suggested that artificially low genetic diversity could lead to the false impression of subfamily recognition. (Their approach has been criticised since their sample sizes and thus the power of their statistical tests varied with the numbers of subfamilies; Page et al. 1990). Calderone et al. (1989) demonstrated task specialization in colonies headed by naturally mated queens, avoided these problems to a large degree. But their bees were still domestic bees, and had presumably been subjected to at least some artificial selection. Finally, where data are collected by direct behavioral observations in which genotypes are phenotypically identified, there is the possibility of observer bias.

With these criticisms in mind, an ideal experiment to confirm the task specialization hypothesis in *Apis* would: (1) use a wild species that has not been subjected to artificial selection or combination of genotypes; (2) use a colony with a natural number of subfamilies; (3) use DNA "fingerprinting" or other biochemical techniques to infer genotypic classes of bees.

DNA fingerprinting is a technique in which an individual's DNA is extracted and purified and then cut into relatively small fragments with a restriction enzyme. These fragments then are separated according to size by gel electrophoresis. The fragments are then permanently bonded to a nylon membrane. A standard piece of DNA known as a probe is suitably labeled so that it can be visualized and is then allowed to hybridize with the DNA on the membrane. The probe will only hybridize with fragments with which it has a similar sequence. Thus the probe forms patterns on the nylon membrane from which family history of the individual can be inferred. Using the generalized probes M13, (Blanchetot 1991) or the oligonucleotide $(GATA)_4$ (Moritz et al. 1991) it has been demonstrated that super- and half-sisters can be readily distinguished in colonies of *A. mellifera* using DNA fingerprinting.

The advantages of DNA fingerprinting for testing the task specialization hypothesis are many. Determining genotype after determinations of behavior eliminates the possibility of observer bias. Since mutant or allozyme markers are not required, the marker cannot affect behavior, and the number of subfamilies is normal. The disadvantage of DNA fingerprinting techniques is that restriction fragment length polymorphism (RFLP) analysis is extremely time-consuming and expensive. Thus high levels of replication are not possible.

A. florea, a wild Asian species of honey bee, is an ideal candidate for an experiment to confirm the existence of task specialization in *Apis*. Not only has *A. florea* never been domesticated, propagated or transported by beekeepers, its behavioural ecology means that collection of bees performing various tasks is relatively easy. The bee builds a single comb in the open, and is not very defensive. Many activities occur on the dance platform and are easily observed (Ruttner 1988). Measurement of semen volume in newly mated queens suggests that they mate 1–3 times (Koeniger et al. 1989). Species that mate more often than this, such as *A. cerana* or *A. mellifera*, are less suitable for RFLP analysis, since the labor and expense involved in each fingerprint analysis is so high.

Methods

Collection of bees. We analyzed bees taken from two *Apis florea* colonies. Both colonies were located in the fronds of coconut palms. They had populations of about 10000 bees, so our sampling did not affect colony structure. Colony A was moved from Samut Songkhram to Chulalongkorn University in Bangkok Thailand. Bees were taken directly from colony B which remained *in situ* in Chanthaburi, Thailand. With forceps we grasped bees that we observed undertaking the following activities. (1) *Stingers*: bees that stung the collector. (2) *Guards*: in the region where an *A. florea* nest meets the substrate the bees maintain an area of sticky propolis as a defence against ants and other insects (Seeley et al. 1982). This area is maintained by a group of workers we called guards. (3) *Fanners*: bees which fanned their wings to cool the nest. (4) *Waggers*: bees which stood on the outer curtain of bees and vigorously waved their abdomen in the air. The function of this behavior is not known to us, but it does not seem to be related to communication of the location of food sources. Interestingly, almost all bees will exhibit this behavior when they come in contact with a queen. We only collected bees that appeared to undertake the behavior for no apparent reason. (5) *Liquid foragers*: bees which returned to the nest without pollen were grasped and their abdomens gently squeezed. Those which did not express liquid were discarded. Those that did so were collected. (7) *Pollen foragers*: bees that returned to the nest with pollen on their corbiculae.

For colony A, bees were collected from 30 January to 17 February 1992. For colony B, bees were collected from 18 to 28 February 1992. In addition, a sample of pupae was collected from this colony on 29 January 1992, and a sample of random workers was taken by passing a vial through the cluster on 28 February 1992. Unfortunately, we were unable to collect RFLP data from drones of either colony.

Bees were collected from 0600 to 1000 hours, stored separately on ice according to behavioral class during the daily collection

period, and then transferred to liquid nitrogen for transport to the laboratory where they were stored at −60° C until DNA was extracted.

DNA extraction and RFLP analysis. Total DNA was extracted from the head and thorax of each individual by the method of Sheppard and McPheron (1991), except that we used 0.15 times the volume of all reagents and incubated extracts for 1 h at 65° C instead of 15 min on ice. Briefly, the thorax of each bee was placed in 150 µl of solution A [0.01 M Tris-HCl (pH 8), 0.06 M NaCl, 0.01 M EDTA, 5% sucrose] in a microcentrifuge tube on ice and thoroughly chopped with dissecting scissors. Then 150 µl of solution B [0.3 M Tris-HCl (pH 8), 0.02 M EDTA, 1.5% SDS, 5% sucrose +2.5 µl DEPC] was added. The preparation was incubated at 65° C for 1 h, followed by one phenol extraction, one phenol-chloroform extraction, and one chloroform extraction. An equal volume of TE (0.01 M Tris HCl, 0.001 M EDTA pH 8) was then added to the aqueous layer. DNA was then twice precipitated on ice for 30 min with 0.5 volumes of 3 M sodium acetate and 2.5 volumes of cold absolute ethanol, and pelleted by centrifugation at 12000 rpm for 30 min at 4° C. The final pellet was rinsed with 100 µl of cold 80% ethanol, air-dried and resuspended in 25 µl of TE.

Twelve microliters of DNA extract were digested with EcoRI using buffer and incubation conditions specified by the manufacturer (Sigma Chemical Co. St. Louis, MO). Fragments were separated on 1% agarose gels run in TBE buffer at 20 V overnight which were then pressure blotted onto nylon membranes (Biosbrane, Bios Corp., New Haven, CT). DNA was bound onto the membranes using UV irradiation.

An *Apis mellifera* genomic library was constructed by ligation of approximately 15 kb *Sau*3A fragments into the *Bam*H1 site of lambda phage strain EMBL3. Clones were screened as probes on Southern blots of *Eco*RI digests of DNA extracted from individual workers of colony B until one was found which showed variation for several bands among workers.

The chosen probe (designated probe 24 and available from H.A.S) was labeled with digoxigenin using the Genius system according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). The membranes were hybridized overnight with the probe in 50% formamide at 37° C and the Genius phosphatase-linked colorimetric detection reaction was carried out according to the manufacturer's instructions under conditions of medium stringency.

Analyses. Restriction fragment length polymorphism (RFLP) genotypes were compared among behavioral groups (including base lines) with χ^2 tests of homogeneity. Where several expected frequencies in a contingency table analysis are less than five, the approximation to the χ^2 distribution is weakened (Steel and Torrie 1980, p. 474). To avoid this problem we used the χ^2 test of Smith (1986) for small numbers in which, on the null hypothesis, the expected values of the mean, m, and square root of the variance, v, of χ^2 are computed. Smith shows that the quantity:

$$z=(\sqrt{\chi^2}-m))/\sqrt{v}$$

is a standardised normal variate, and can be used as a test criterion for heterogeneity in contingency tables. Smith (1986) suggests that the test is robust provided that minimum expected values are at least 0.5. In addition to Smith's test, we also pooled the four rarest RFLP categories into a single category, and reanalyzed all the data with these collapsed tables.

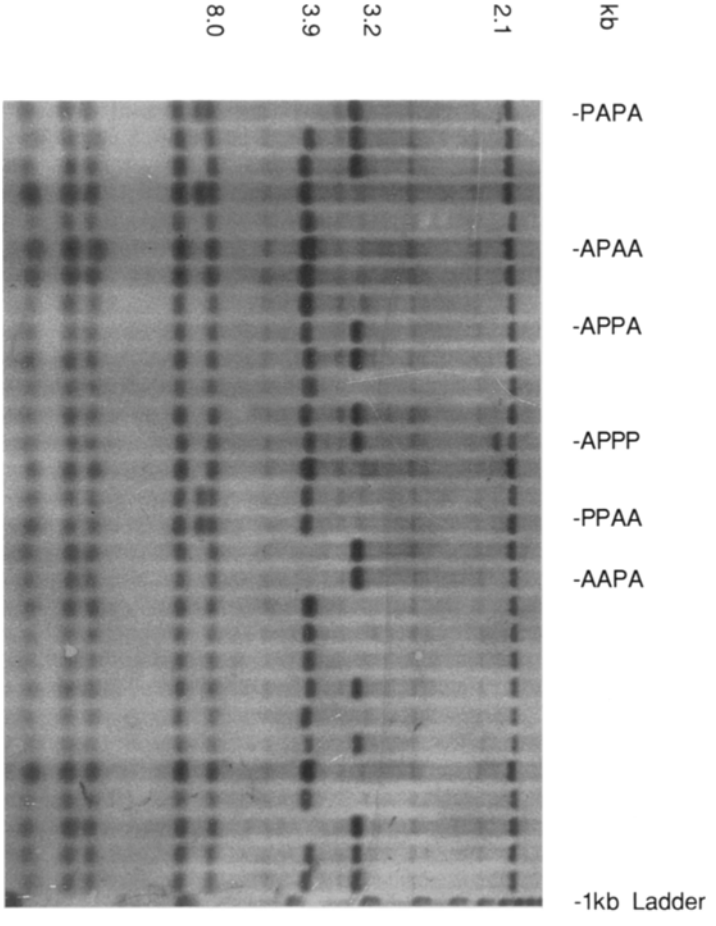

Fig. 1. Typical blot showing heritable variance in restriction fragment length polymorphism (RFLP) genotypes as revealed by probe 24, on random workers taken at the last sample. Four bands were variable among bees (at 2150, 3200, 3900, and 8000 bp). Presence (P) or absence (A) of these bands in a lane resulted in eight unique genotypes, respectively PAPA (lane 1); APAA (lane 6); APPA (lane 9); APPP (lane 13); PPAA (lane 16); AAPA (lane 18). Not shown on this gel are APAP and PPPA

Results

Regrettably, the chosen probe did not reveal heterogeneity of RFLP genotypes among workers of colony A, and we did not proceed beyond approximately 200 extractions. In colony B, a number of different RFLP genotypes were discernible (Fig. 1). A total of four bands (representing fragments of approximately 8.0, 3.9, 3.2, and 2.15 kb) were polymorphic in *Eco*RI digests of bees from this colony, and we observed a total of eight phenotypes or fingerprints (Fig. 1 shows six of the eight). There were no correlations among bands. That is, loss of one band did not lead to the automatic appearance of another. Thus it seems likely that the probe has several different homologies with unlinked sections of the *A. florea* genome.

Table 1. Classification of bees by behavioral class, including random samples and DNA restriction fragment length polymorphism (RFLP) class

	Restriction fragment length polymorphism								
Behavioral class	AAPA	APAA	APAP	APPA	APPP	PAPA	PPAA	PPPA	*n*
Fanners	0	21	1	25	1	1	2	0	51
Liquid foragers	3	47	4	71	6	10	9	0	150
Pollen foragers	1	30	3	59	8	3	4	1	109
Guards	1	19	11	27	7	1	1	1	68
Stingers	1	42	10	34	12	1	1	2	102
Waggers	0	8	2	14	2	1	0	1	28
Random samples									
Random pupae (29 January)	1	26	7	25	6	4	2	0	71
Random workers (28 February)	3	56	3	44	3	6	4	1	120

RFLP classes are defined in Fig. 1; behavioral classes are defined in the text

Table 2. Tests of heterogeneity among behavioral class and RFLP phenotype

Comparison	χ^2	*df*	*P*	*z*	*P*	Number of cells with expected value less than:	
						5	0.5
A. No pooling							
Brood vs. all tasks combined	4.9	7	0.7	0.5	0.3	6	0
Random workers vs. brood sample	10.0	7	0.2	0.9	0.2	9	1
Random workers vs. all task groups	14.4	7	0.04	1.8	0.04	5	0
Random workers + brood sample vs. all tasks	10.5	7	0.2	1.0	0.2	3	0
All task groups	60.3	35	0.005	2.7	0.004	28	2
Liquid foragers vs. random workers	9.0	7	0.2	0.7	0.2	7	1
Pollen foragers vs. random workers	13.8	7	0.05	1.8	0.04	10	0
Stingers vs. random workers	18.0	7	0.01	2.5	0.005	8	0
Fanners vs. random workers	4.2	7	0.7	0.8	0.2	12	1
Guards vs. random workers	22.2	7	0.002	3.3	0.0005	9	0
Waggers vs. random workers	8.6	7	0.3	0.5	0.3	11	1
Liquid vs. pollen foragers	6.6	7	0.5	0.007	0.5	6	0
B. AAPA, PAPA, PPAA, PPPA pooled							
Brood vs. all tasks combined	3.3	4	0.5	0.09	0.5	1	0
Random workers vs. brood sample	9.0	4	0.06	1.6	0.05	2	0
Random workers vs. all task groups	12.9	4	0.01	2.5	0.007	0	0
Random workers + brood sample vs. all tasks	8.7	4	0.07	1.5	0.06	0	0
All task groups	45.8	20	0.0009	3.3	0.0004	6	0
Liquid foragers vs. random workers	7.1	4	0.1	1.1	0.1	3	0
Pollen foragers vs. random workers	12.6	4	0.01	2.4	0.007	2	0
Stingers vs. random workers	14.3	4	0.006	2.7	0.003	0	0
Fanners vs. random workers	4.0	4	0.4	0.2	0.03	4	0
Guards vs. random workers	12.8	4	0.01	2.4	0.007	0	0
Waggers vs. random workers	1.9	4	0.7	0.7	0.2	4	0
Liquid vs. pollen foragers	4.4	4	0.4	0.3	0.4	2	0

Individuals of colony B were classified according to behavioral class and RFLP genotype (Table 1). RFLP genotypes did not differ significantly between the brood sample and the random sample of workers (Table 2). This indicates that (1) few if any bees had drifted in from other nests, and (2) subfamily ratio was stable with respect to time. When the table was collapsed by combining rare RFLP genotypes (AAPA, PAPA, PPAA and PPPA) into a new category, the χ^2 test remained non-significant (Table 2).

Estimates of RFLP genotype proportion did not show significant heterogeneity among unclassified bees. Neither brood and random worker samples combined, random workers alone or brood samples alone differed from all task groups combined (Table 2). Neither pooling of rare RFLP genotypes nor the use of the less conservative Smith (1986) test altered this conclusion (Table 2). However, when bees were grouped in contingency tables based on their observed behavior, striking differences in RFLP genotype relative frequency were discernible among behavioral groups. Overall, there was extreme heterogeneity among task groups ($P=0.0009$, χ^2 test of pooled data, Table 2). When RFLP genotype relative frequencies in individual behavioral class groups were compared with the random worker sample, pollen foragers ($P=0.01$), stingers ($P=0.006$) and guards ($P=0.01$) had significantly different RFLP frequencies. Liquid foragers ($P=0.1$), fanners ($P=0.4$) and waggers ($P=0.7$) did not differ from the random worker sample. Pollen foragers and liquid foragers did not show significant differences in RFLP profiles ($P=0.4$).

Discussion

The extreme heterogeneity in RFLP genotype relative frequencies among behavioral groups (Table 2) provides the first strong evidence for genetic heterogeneity among behavioral groups within a single wild colony of social insects. This heterogeneity may be due to: (1) changing patterns of sperm use by a single queen leading to changing subfamily proportions in age classes; (2) genotypically different queens in the colony; or (3) multiple mating and task specialization.

If, as in *A. mellifera*, *A. florea* behavior changes with worker age, then age polyethism could result in changing RFLP genotypes among contemporaneously sampled bees, if spermatozoa in the spermatheca are sampled non-randomly. However, it is extremely unlikely that age polyethism can explain all of the genetic heterogeneity among task groups in colony B. First, samples were not collected contemporaneously, but over a period of 10 days, thus reducing possible effects due to age polyethism. Second, RFLP genotype relative proportions were not significantly different among the brood and random worker samples, although they approached significance when rare genotypes were pooled (Table 2), perhaps indicating some population changes in RFLP genotype relative frequency. By contrast, overall heterogeneity among task groups was extremely high. In comparisons with random workers, three of five task groups showed significant heterogeneity in RFLP genotype relative proportions. These differences are much larger than those between brood and worker samples.

Since RFLP genotypes are inherited in a co-dominant manner, it is not possible that a single once-mated queen could have produced all the worker genotypes shown in Fig. 1. Either several queens contributed to the brood, or a single queen mated to several drones carrying different RFLP markers produced the brood. It is not possible to exclude either possibility from the data presented here. However, since monogyny is usual in *A. mellifera*, there is no reason to expect polygyny in *A. florea*.

Knowledge of the parentage of the different RFLP classes shown here is not crucial to a demonstration of genetically based behavioral polymorphisms in *A. florea*. For that, all that needs to be demonstrated is a covariance between behaviour and genotypic class, which these data convincingly do. Since polyandry is usual in *A. florea* (Koeniger et al. 1989), the most likely source of at least some of this covariance is multiple mating. However, if some variance was due to segregation from a heterozygous queen, this only emphasises the existence of genetically based within-colony behavioral polymorphisms in *Apis*.

Our study strongly supports the existence of genetically based task specialization in a hitherto unexplored social insect *A. florea*. We confirm similar observations in *A. mellifera* and an ant (Stuart and Page 1991) using techniques which are not troubled by some problems of technique and biology inherent in studies of *A. mellifera*.

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