CHAPTER 4

WORKER POLICING IN *A. dorsata*

In colonies of eusocial Hymenoptera, selection can act at different levels. In particular, the reproductive interests of individual workers differ from those of the workers and queen collectively, thus creating the potential for conflict over male production given that, because of arrhynotokous parthenogenesis, unmated workers can lay eggs that result in normal males. In species with colonies headed by a single queen mated to more than two males, such as honeybees (*Apis*) and wasps (*Vespula*), workers are more closely related to their own sons (r = 0.5), than to the sons of their half sisters (r = 0.125), or sons of the queen (r = 0.25) (Ratnieks, 1988). These relatedness values mean that selection acts in favor of alleles that foster repression of worker reproduction rendering the workers functionally sterile and leaving the production of male-producing eggs to the queen (Ratnieks, 1988). However, selection also acts to favor alleles that permit reproduction by individuals, creating an evolutionary tension between the genetic interests of individuals and the colony as a whole (Ratnieks, 1988; Visscher, 1996; Barron et al., in press).

This tension between individual and group interests is resolved in many species by mutual suppression of reproduction among workers. In queen-right colonies, pheromonal signals produced by the larvae (Arnold et al., 1994) and the queen (Wossler and Crewe, 1999) signal to workers that they should

refrain from ovary activation and repress the reproduction of all other workers (Barron et al., in press).

'Worker policing' is the term used for any behavior by workers that acts to reduce the reproductive success of other workers. Two mechanisms have been documented: selective oophagy of worker-laid eggs (Ratnieks and Visscher, 1989), and aggression towards individual workers with activated ovaries (Visscher and Dukas, 1995).

Worker policing via oophagy of worker-laid eggs is a widespread phenomenon within the eusocial Hymenoptera, and has independently evolved at least twice (in honey bees and vespine wasps) (Foster and Ratnieks, 2001). Analogous behavior is also present in some ant species (Kikuta and Tsuji, 1999; Monnin and Peeters, 1999; Tsuji, Egashira and Holldobler, 1999) and wasps with less pronounced reproductive skew (Arevalo, Strassmann and Queller, 1998).

Within *Apis*, policing via oophagy of eggs has been documented in the western hive bee *A. mellifera* (Ratnieks and Visscher, 1989), the dwarf honey bee *A. florea* (Halling et al., 2001), and the eastern hive bee *A. cerana* (Oldroyd et al., submitted). In *A. florea* (Halling et al., 2001) and *A. mellifera* (Ratnieks, 1993), worker ovary activation is extremely rare in queenright workers, but in *A. cerana* up to 5% of workers have activated ovaries. Despite this, no males arising from worker laid eggs were detected in a sample of 652 pupae in *A. cerana* (Oldroyd et al., submitted).

The giant honey bee, *A. dorsata,* differs from all other *Apis* in comb construction. Uniquely, the cells used for rearing worker brood and drone brood are identical. In all other species the cells used for drone rearing are about 30% larger in diameter than the cells used for workers, and can be readily distinguished from them (Ruttner, 1988). Moreover, the cells used for drone rearing in *A. mellifera, A. florea, A. andreniformis* and *A. cerana* are located in distinct areas, usually on the periphery of the comb (Seeley, 1985, Rinderer et al., 1996, Hadisoesilo and Otis, 1998). This means that drones are reared in patches of cells distinct from those used for rearing workers. In contrast, cells used for rearing *A. dorsata* drones are scattered throughout the brood area.

In *A. mellifera*, police workers use pheromonal cues placed on eggs by queens but not by workers to distinguish worker and queen laid eggs (Ratnieks, 1992; 1995). These cues may be produced by the Dufour's gland because the contents of the glands of laying workers and queens have several distinct elements (Katzav-Gozansky, Soroker and Hefetz, 1997), and because bioassays strongly indicate this link (Ratnieks, 1992; 1995). However, this system of differential pheromonal marking of eggs is subject to error, with some worker-laid eggs resembling queen-laid eggs (Oldroyd and Ratnieks, 2000). This means that workers may use additional cues such as cell morphology to distinguish worker from queen-laid eggs, and may concentrate their policing efforts on drone cells rather than worker cells.

I hypothesized that because cells of *A. dorsata* used for rearing of drones and workers are identical, and because they are scattered throughout

the comb, the potential for errors by police workers in distinguishing worker-laid and queen-laid eggs is far greater in this species than in other species of *Apis*. In *A. dorsata*, the capability of workers to discriminate between queen-laid eggs and worker-laid eggs must be based entirely on pheromonal cues alone and may therefore less effective than in those species where cell type gives additional information about egg sex, and possibly egg origin, since workers preferentially lay eggs in drone-sized cells (Oldroyd, Halling and Rinderer, 1999). That is, workers must police every cell, and could be removing both worker and male eggs laid by the queen. Although when an egg is consumed by a worker the nutrients contained within it are recycled within the nest, the capacity of queens to provide replacement eggs may be limited, and there is a delay even if queen can easily lay the eggs. Thus the fitness consequences for erroneously removing queen-laid eggs would seem to be substantial.

Given these consideration, I hypothesized that *A. dorsata* workers would have a more permissive acceptance threshold of worker-laid eggs than other species of *Apis*. To test this hypothesis, I dissected workers to determine the frequency of ovary activation among them. Additionally, I used microsatellite analysis to determine if the eggs that give rise to males are worker-laid or queen-laid. *A. dorsata* is extremely defensive of the nest site, and because of this I did not consider it feasible to directly bio-assay the survival of worker-laid and queen-laid eggs as has been done in other species (Ratnieks and Visscher, 1989; Halling et al., 2001; Oldroyd et al., submitted). In particular, dequeening a colony in order to obtain laying workers poses a formidable technical difficulty.

4.1 Materials and Methods

4.1.1 Maternity of Drones

4.1.1.1 Sample collection

To avoid erroneous sampling of bees drifted from other colonies, only pupae brood were used in this experiment. However, as mention earlier, drone cells in this species are scatted throughout the comb, and they are identical to worker cells. Hence, it not possible to distinguish between drone cells and worker cells. Several fresh *A. dorsata* combs were randomly cut from the wild or purchased from markets in Thailand in 1998-1999. Fresh combs were wrapped in aluminium foil and kept on ice during transportation to the Bee Biology Research Unit Chulalongkorn University. They were then temporarily stored in a freezer at-20°C. All samples were transported to the School of Biological Sciences University of Sydney, Australia and these sample were kept in a freezer (-70°C) until they were used for microsatellite analysis.

Sealed brood cells were then opened by using a pair of forceps to examine the cells for the presence of drone pupae. Male pupae have much larger eyes than worker pupae. Only 4 colonies with c.a. 200 drone pupae were found and they were used in this experiment.

4.1.1.2 DNA Extraction

Drone pupae were removed from cells and kept individually in Eppendorf tubes. DNA extractions of samples were conducted according to the protocol in Section 3.1.1.

4.1.1.3 Polymerase Chain Reaction (PCR)

Four polymorphic microsatellite primers (A14, A24, A88 identified by Estoup et al.,1994; 1995 and Ad3 identified by Parr et al, 2001) were used to determine the maternity of drones. The protocol for PCRs followed the procedure described in Section 3.1.2. DNA template dilution of each bee was mixed with PCR mixture and underwent a programme appropriate to each primer (Table 3.3). After cycling was completed, PCR products were kept in a refrigerater at 4 °C.

4.1.1.4 Electrophoresis

The PCR product of each bee was electrophoresed on an automated DNA fragment analyser (Corbett Research, Sydney). The procedures followed those described in Section 3.1.3.

4.1.1.5 Queen's Genotype

For each colony, the queen's genotype was inferred based on analysis of 24 worker genotypes, as the two alleles of which one was always present in a worker's genotype. This also allowed me to confirm monandry for the colony concerned. If any drone has a different maternal allele from the queen, it can be said that it was not a son of the queen and must therefore be a son of a worker in that colony (Section 3.2). Thus, any drone which carry an allele differs from queen's allele was considered as worker's son (Oldroyd et al., 1995).

4.1.2 Frequency of Ovary Activation

4.1.2.1 Sample Collection

Approximately 100-200 adult worker bees were sampled in Thailand from each of 8 queenright colonies in 1998-2000. Three samples were from isolated nests, and another 5 were from aggregations. The samples were collected during the dry season during a honey flow when drone rearing was very active. These samples were stored in 70% ethanol until dissection was conducted.

4.1.2.2 Dissection

Workers were first fixed to a wax board by an insect pin inserted through the thorax. Under a microscope, the third and fourth abdominal segments were separated using two pairs of forceps. The digestive tract was then removed to reveal the ovaries, which connect to the genital opening between the anus and sting (Dade, 1977). Ovaries were scored into 3 categories; 0) ovarioles not visible, 1) ovarioles clearly visible, 2) small eggs and/or full size eggs present.

4.2 Results

4.2.1 Maternity of Drones

None of the 660 drones examined carried alleles inconsistent with them being sons of the queen, indicating that worker reproduction is rare in *A. dorsata* (Table 4.1 and Table 4.2) (Figure 4.1).

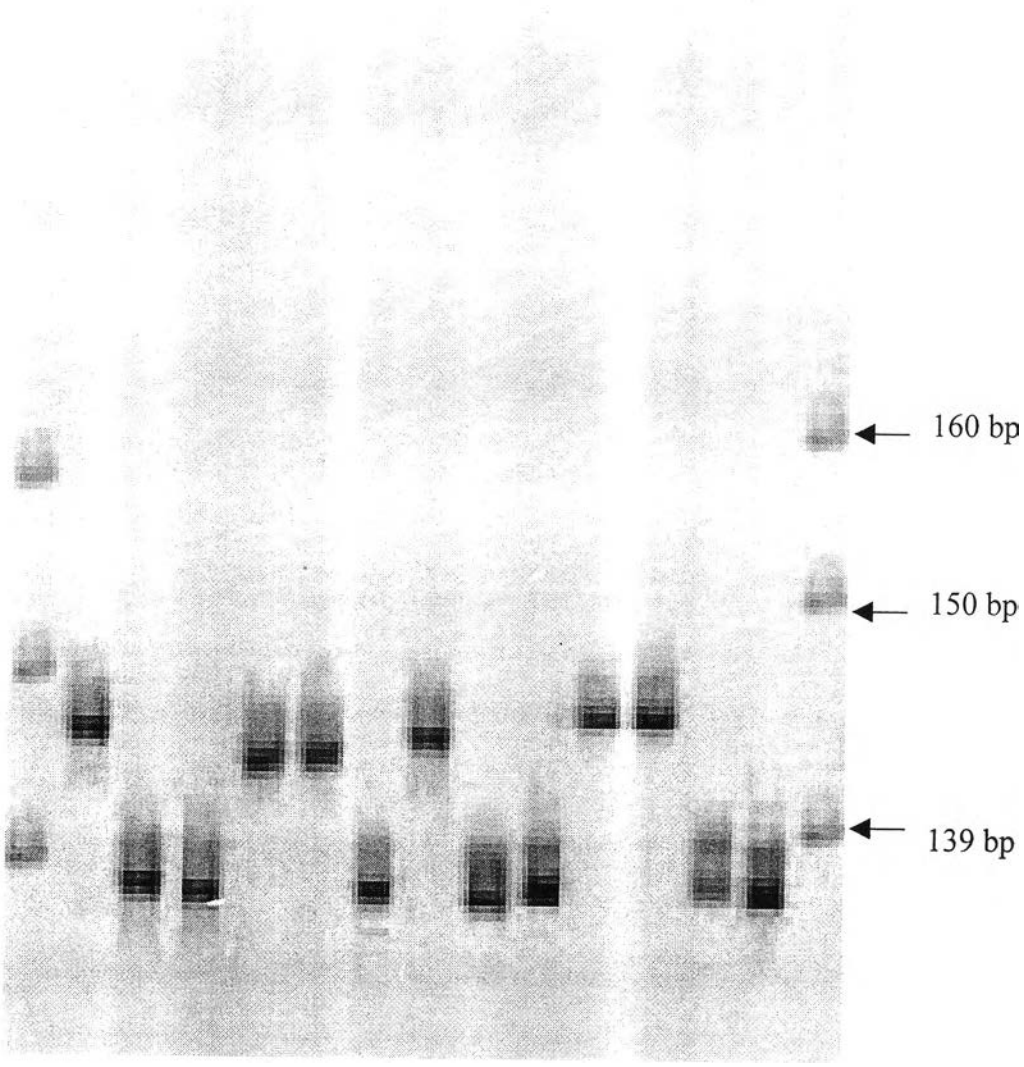

Figure 4.1 Image of microsatellite at A88 locus, resolved by using polyacrylamide gel electrophoresis.

However, by chance it is possible that any worker-laid drones would not be detected if a particular drone has queen alleles at all loci. This can occur in two ways. One possibility is that the drone may derive a queen allele from his worker-mother who must carry a queen allele at all loci. This situation error occurs at the rate of 50% at each locus examined. Another possibility is that the queen may mate with drone carrying allele identical to her own. Then the drone, which carried a queen-like allele, may be classified as the son of the queen in that colony. Thus the probability of miss-classification depends on the number of loci used, and the polymorphism of these loci, and this rate can be quantified. To estimate the rate at which errors occur, the non-detection error was calculated. In each colony at each locus, I calculated the proportion of the workers that their fathers possibly have the same allele as the queen (p_k). I then calculated the probability of non-detection error (P_{ND}) at each locus by using the estimation from Halling et al (2001):

$$P_{ND} = 0.5+0.5\ p_k$$

Across all loci studied, I calculated the over all probability of non-detection using the estimation:

$$P_{ND} = \Pi^{n}_{k=1} (0.5+0.5p_k)$$

Therefore, the probability of detection is:

$$P_D = 1 - P_{ND}$$

The average probability of detection all four colonies studied was calculated using:

$$\text{Average } P_D = \Sigma^{n}_{k=1} (\text{colony n x No. of drones in colony n}) / \text{ Total No. of drones from all colonies}$$

The average probability of detection of all 4 colonies examined was 0.79 and the average probability of non-detection over all 4 colonies examined was 0.21 (i.e. there was a 21% chance that any one worker-laid male would not be detectable as worker-laid because it had a genotype consistent with being queen-laid). Thus in a sample of 660 drones, I would expect to have detected at least one worker-laid male if the actual frequency of worker-laid males was as low as 1 in 521. As no worker-laid males were detected, this indicates that worker reproduction is extremely rare in *A. dorsata.*

To test whether the queen was the sole mother of the colony offspring, the expected drone frequencies was determined. These frequencies were compare with the observed drone frequencies using a χ^2test (Table 4.2). The statistical analysis confirmed the assumption that all drones examined derived from the queen head of the colony. Given the complete absence of males

whose genotype was consistent with being worker-laid, we conclude that males arising from worker-laid eggs are extremely rare in *A. dorsata*.

Table 4.1. Queen genotypes of 4 colonies inferred from workers alleles of each colony.

Colony	Queen Genotypes				
	Microsatellite Loci	A14	A24	A88	Ad3
1		206/212	106/108	135/145	168/192
2		211/214	104	133/138	170/226
3		208/216	102/106	142/144	164
4		207/212	104	133/135	166

Table 4.2. Frequencies of drones genotypes and testing of deviation.

Colony	Queen genotype				Observed drone genotype				Observed frequencies of drone genotype	Expected frequencies of drone genotype	χ^2	*df*	*p*
	A14	A24	A88	Ad3	A14	A24	A88	Ad3					
1	206/212	106/108	135/145	168/192	206	106	135	168	5	6.5	4.62	15	0.99
					206	106	135	192	8	6.5			
					206	106	145	168	7	6.5			
					206	106	145	192	4	6.5			
					206	108	135	168	8	6.5			
					206	108	135	192	7	6.5			
					206	108	145	168	8	6.5			
					206	108	145	192	8	6.5			
					212	106	135	168	6	6.5			
					212	106	135	192	6	6.5			
					212	106	145	168	4	6.5			
					212	106	145	192	5	6.5			
					212	108	135	168	8	6.5			
					212	108	135	192	6	6.5			
					212	108	145	168	7	6.5			
					212	108	145	192	7	6.5			

Colony	Queen genotype				Observed drone genotype				Observed frequencies of drone genotype	Expected frequencies of drone genotype	χ^2	*df*	*p*
	A14	A24	A88	Ad3	A14	A24	A88	Ad3					
2	211/214	104/104	133/138	170/226	211	104	133	170	17	16.5	6.79	7	0.45
					211	104	133	226	12	16.5			
					211	104	138	170	20	16.5			
					211	104	138	226	16	16.5			
					214	104	133	170	11	16.5			
					214	104	133	226	14	16.5			
					214	104	138	170	20	16.5			
					214	104	138	226	22	16.5			
3	208/216	102/106	142/144	164	208	102	142	164	26	27	3.70	7	0.81
					208	102	144	164	30	27			
					208	106	142	164	22	27			
					208	106	144	164	31	27			
					216	102	142	164	21	27			
					216	102	144	164	29	27			
					216	106	142	164	30	27			
					216	106	144	164	27	27			

Colony	Queen genotype				Observed drone genotype				Observed frequencies of drone genotype	Expected frequencies of drone genotype	χ^2	*df*	*p*
	A14	A24	A88	Ad3	A14	A24	A88	Ad3					
4	207/212	104	133/135	166	207	104	133	166	55	52	0.27	3	0.97
					207	104	135	166	50	52			
					212	104	133	166	52	52			
					212	104	135	166	51	52			

4.2.2. Ovary Development

None of 1,902 workers, from both single colonies and aggregations, had fully development of ovary or any developing eggs in the ovarioles. However, 34.12% of workers examined had a sign of ovary development, i.e. visible ovarioles (Table 4.3) (Figure 4.2).

Table 4.3. Ovary activation in *A. dorsata* workers.

Colony	Ovary activation			Total number of bees
	Undeveloped	Visible ovarioles	Fully developed	
1	134	66	0	200
2	149	56	0	205
3	179	71	0	250
1	164	74	0	238
2	131	78	0	209
3	135	115	0	250
4	168	82	0	250
5	193	107	0	300
Total	1253	649	0	1902
Percentage	65.88	34.12	0	100

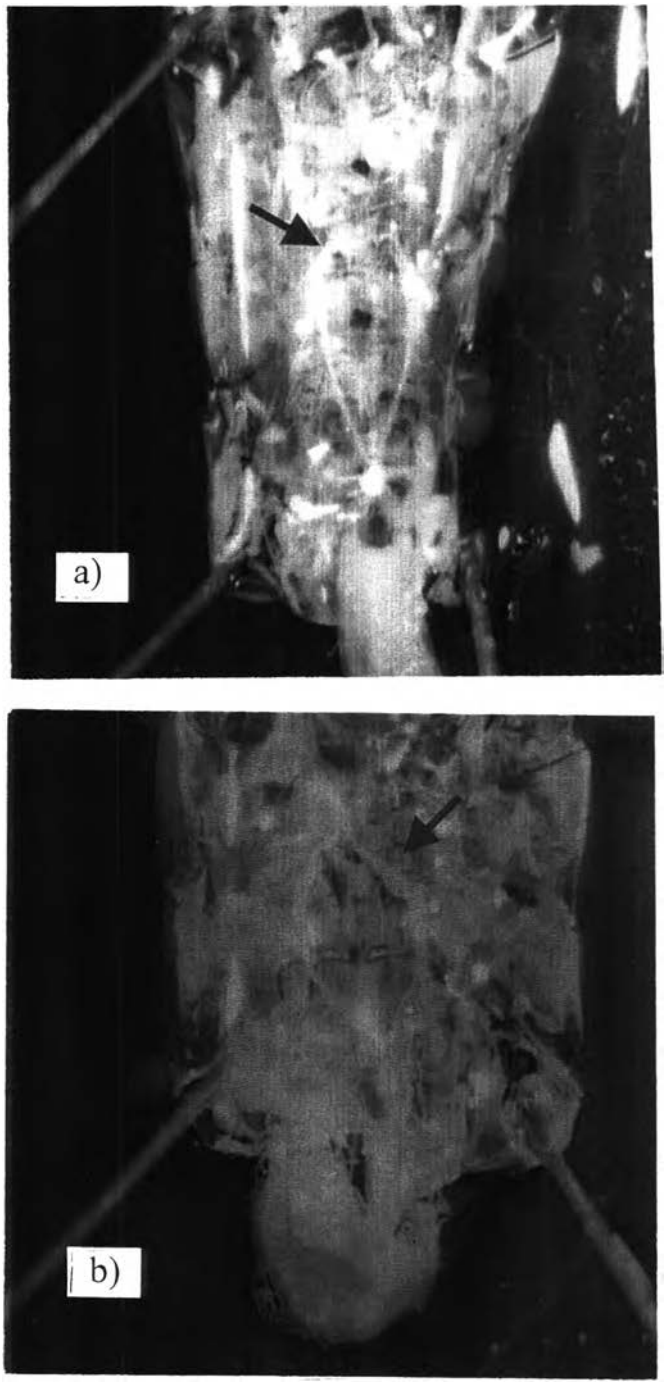

Figure 4.2 Ovary of *A. dorsata* workers.

a) Abdomen of a dissected worker with no sign of ovary development (category 0). Green arrow indicates undeveloped ovary.

b) Abdomen of a dissected worker with distinguished ovary development (category 1). Blue arrow indicated swollen ovary.

4.3 Discussion

No evidence of worker reproduction in *A. dorsata* was found. From the genetic investigation, there was no evidence that any male was derived from a worker-laid egg. Also no individual workers had activated ovaries. This suggests that worker reproduction is rare in *A. dorsata*.

A. dorsata is unusual in this genus in that the cells used for rearing drones are seemingly identical to those used for rearing workers, and are scattered throughout the brood nest, rather than being sequestered in distinct areas. This species-specific behavior led me to speculate that worker policing might be less well developed in *A. dorsata* than in the rest of the genus. Because this behavior is expected to increase the costs of policing and higher retention of worker-laid eggs is predicted. However, the hypothesis that worker policing might be less well developed in *A. dorsata* than in other species of its genus is not supported by the results presented in this thesis.

The findings in this thesis indicate that most, if not all *A. dorsata* drones are sons of queens. The absence of workers with developed ovaries, as shown here, may mean that policing is directed at workers with developed ovaries (Visscher and Dukas, 1995), reducing the need for policing via oophagy, and special cells for the rearing of males. Therefore, we might conclude that in *A.dorsata* worker sterility is strict. The evidence of worker sterility in *A. dorsata* was clearly shown here and it was also reported in the other species (i.e. *A. mellifera* (Ratnieks and Visscher, 1989), *A. florea* (Halling et al., 2001). So I suggest that worker policing is likely to occur across the genus. The occurrence

of worker policing in *A. florea* indicates that this behaviour may be evolved prior to the divergence of the genus *Apis*. This is because *A. florea* is believed to be the first species arose from the basal group within this genus. The results presented in this thesis confirmed that worker policing was not arise after the divergence of the genus *Apis*.