Cape honeybees, *Apis mellifera capensis*, police worker-laid eggs despite the absence of relatedness benefits

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In the Cape honeybee, *Apis mellifera capensis*, workers lay diploid (female) eggs via thelytoky. In other *A. mellifera* subspecies, workers lay haploid (male) eggs via arrhenotoky. When thelytokous worker reproduction occurs, worker policing has no relatedness benefit because workers are equally related to their sister workers' clonal offspring and their mother queen's female offspring. We studied worker policing in *A. m. capensis* and in the arrhenotokous African honeybee *A. m. scutellata* by quantifying the removal rates of worker-laid and queen-laid eggs. Discriminator colonies of both subspecies policed worker-laid eggs of both their own and the other subspecies. The occurrence of worker policing, despite the lack of relatedness benefit, in *A. m. capensis* strongly suggests that worker reproduction is costly to the colony and that policing is maintained because it enhances colony efficiency. In addition, because both subspecies policed each others eggs, it is probable that the mechanism used in thelytokous *A. m. capensis* to discriminate between queen-laid and worker-laid eggs is the same as in arrhenotokous *A. m. scutellata*. *Key words: Apis mellifera*, egg removal, honeybee, thelytoky, worker policing, worker reproduction. *[Behav Ecol 14:347–352 (2003)]*

Inclusive fitness theory (Hamilton, 1964a,b) has been successful in explaining and predicting social behavior (Crozier and Pamilo, 1996; Jarvis, 1981; Ratnieks et al., 2001). Hamilton's rule, $Br > C$, shows the condition under which a social action is favored in terms of the benefit to the recipient, the cost to the actor, and their genetic relatedness. Genetic relatedness is a key parameter and can now be measured with comparative ease and accuracy (Pamilo et al., 1997; Ross, 2001). Many empirical studies confirm the importance of genetic relatedness in social evolution (e.g., Foster and Ratnieks, 2000; Sundström, 1994). However, it is unlikely that any comparable technological breakthrough will facilitate the measurement of costs and benefits (Ratnieks et al., 2001). One solution to investigating the importance of costs and benefits in social evolution is to chose study systems in which relatedness is held constant but costs and benefits vary. Worker reproduction and worker policing in the Cape honeybee, *Apis mellifera capensis*, is one such system.

In most eusocial Hymenoptera, workers cannot mate but retain ovaries and can lay eggs (Crozier and Pamilo, 1996). Unfertilized worker-laid eggs are normally haploid (arrhenotoky; Crozier and Pamilo, 1996) and develop into males if reared. However, in a few species (currently six ants and *A. m. capensis* are known; Wenseleers and Billen, 2000), workers lay unfertilized diploid eggs that develop into females (thelytoky; Crozier and Pamilo, 1996; for rare cases of thelytoky in other subspecies of *A. mellifera*, see Mackensen, 1943; Tucker, 1958). *A. m. capensis* is native to the fynbos region (a macchia-like biome) in the western and eastern Cape provinces of South Africa (Hepburn and Radloff, 2002). Thelytokous reproduction by Cape honeybee workers has been long known (Onions, 1912; reviewed in Hepburn and Radloff, 2002), and many of the genetic details are also understood (Greeff, 1996; Moritz and Haberl, 1994; Verma and Ruttner, 1983). Although the thelytoky is via automictic parthenogenesis after meiosis (Verma and Ruttner, 1983), recombination through crossing over is rare (Moritz and Haberl, 1994). Thus, a worker's offspring are almost clonal.

Workers' sons are rarely reared in queenright European honeybee colonies (Visscher, 1989, 1996). This is because few workers have active ovaries (Ratnieks, 1993) and because the eggs they lay are eliminated by worker policing (Ratnieks, 1988). Honeybee workers in queenright colonies eat eggs laid by other workers (Ratnieks, 1993; Ratnieks and Visscher, 1989; Visscher, 1996). Queen-laid and worker-laid eggs are probably discriminated by means of a queen-produced egg-marking pheromone (Ratnieks, 1992, 1995).

Worker policing is selected for in a population of arrhenotokous social Hymenoptera on relatedness grounds alone when each colony has a single queen mated to more than two males (Foster and Ratnieks, 2001; Ratnieks, 1988). Honeybee queens, *Apis mellifera*, are typically mated to 5–30 males (Estoup et al., 1994; Fuchs and Moritz, 1999; Neumann and Moritz, 2000; Palmer and Oldroyd, 2000). As a result, worker policing is beneficial on relatedness grounds because workers are less related to other workers' sons (nephews: $r = .15$, for an effective paternity of 10) than to the queen's sons (brothers: $r = .25$). However, in *A. m. capensis* the situation changes significantly. In particular, workers lay female eggs and are as related to other workers' daughters as to the queen's daughters. But an individual laying worker is still more related to her own offspring (clonal daughter: $r = 1$) than to the queen's offspring ($r = .3$, assuming an effective

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paternity frequency of 10). Therefore, on relatedness grounds alone, one might expect that *A. m. capensis* workers either would not police each other or would do so less effectively than in other *A. mellifera* subspecies (Greeff, 1996). Indeed, brood are frequently observed in hive-boxes located above the queen excluder (a grid too small for the queen, but not for the workers, to pass through, so that the queen cannot lay eggs in the upper boxes) in queenright Cape honeybee colonies (Hepburn and Radloff, 1998; Pettey, 1922; Neumann P, personal observations). Moritz et al. (1999) have shown that this brood is the female offspring of workers.

Hamilton's rule for worker policing in *A. m. capensis* can be written as follows:

$$Br_q > Cr_w \qquad B/C > r_w/r_q \qquad B/C > 1$$

where r_q and r_w are the relatedness of police workers to the queen's and other workers' female offspring, and B and C are the relative efficiencies (i.e., total reproduction) of colonies with and without worker policing. Clearly, worker policing is favored if it increases the efficiency of the colony. Colony efficiency could be lowered, e.g., if the laying of additional eggs in a cell reduces the overall efficiency of brood rearing. The existence of worker policing in *A. m. capensis* would support the hypothesis that worker reproduction is costly.

In the present study, we investigated whether worker policing occurs in *A. m. capensis* by quantifying the removal rates of worker-laid and queen-laid eggs of both *A. m. capensis* and of the neighboring arrhenotokous subspecies *A. m. scutellata* in queenright colonies of both subspecies. Our results show that both *A. m. capensis* and *A. m. scutellata* police their own and the other subspecies' worker-laid eggs.

METHODS

Sampling colonies and experimental design

Queenright study colonies of *A. m. capensis* were obtained near Port Elizabeth, within the native range of *A. m. capensis* in the Eastern Cape province in southern South Africa. Queenright *A. m. scutellata* colonies were from the Pretoria area, within their native range. These localities were chosen because morphometrically and physiologically pure *A. m. capensis* and *A. m. scutellata* occur there (Hepburn and Radloff, 1998, 2002). Hepburn and Radloff (1998) and Hepburn et al. (1998) review the distribution and biology of these two subspecies.

The colonies were placed in two study apiaries in Grahamstown, South Africa. The *A. m. scutellata* apiary was distant, greater than 1 km, from any other bee hives to minimize intersubspecific drifting and/or dispersing (Neumann et al., 2000b, 2001), which may result in social parasitism by *A. m. capensis* laying workers (Neumann and Hepburn, 2002; Neumann and Moritz, 2002). Both *A. m. capensis* and *A. m. scutellata* colonies were studied in order to compare African subspecies with arrhenotokous and thelytokous worker reproduction (Hepburn and Radloff, 2002; Neumann et al., 2000a). The experimental set-up followed standard methods for investigating worker policing via egg removal (Oldroyd and Ratnieks, 2000; Ratnieks, 1995; Ratnieks and Visscher, 1989). We used colonies of both subspecies as discriminator and egg-source colonies. Discriminator and egg-source colonies were different colonies. All discriminator colonies and the source colonies for queen-laid eggs retained their original queens during the time they were used in the study. The source colonies for worker-laid eggs were made queenless 2 weeks before egg-removal trials started. All colonies were housed in hives composed of two deep or medium

Langstroth boxes with a queen excluder between the boxes and the queen in the bottom box.

Quantifying egg-removal rates

Our primary aim was to compare the removal rates of queen-laid and worker-laid eggs within each subspecies. Worker-laid eggs are either haploid or diploid in the two subspecies (male in *A. m. scutellata* or female in *A. m. capensis*). Therefore, we compared these to queen-laid eggs of the same sex, and used haploid male eggs laid by *A. m. scutellata* queens and workers and diploid female eggs laid by *A. m. capensis* queens and workers. Because *A. mellifera* queens lay fertilized eggs in worker cells and unfertilized eggs in drone cells (Ratnieks and Keller, 1998), we were able to obtain unfertilized male eggs from drone cells in the queenright *A. m. scutellata* colonies and fertilized female eggs from worker cells in the queenright *A. m. capensis* colonies. For both the *A. m. capensis* and *A. m. scutellata* discriminator colonies, we used test frames with both drone and worker cells, because *A. m. capensis* workers naturally lay diploid female eggs mainly in worker cells (Neumann et al., 2000a), and workers of arrhenotokous subspecies lay male eggs mainly in drone cells (Page and Erickson, 1988; Ratnieks, 1993). The drone cells were used for male eggs laid by *A. m. scutellata* workers and *A. m. scutellata* queens. The worker cells were used for female eggs laid by *A. m. capensis* workers and *A. m. capensis* queens. Following standard procedures (Oldroyd and Ratnieks, 2000; Ratnieks, 1995; Ratnieks and Visscher, 1989), the test frames were placed above the queen excluder in each queenright discriminator colony and sandwiched between two frames containing brood of all ages (eggs, larvae, and pupae). The other frames in this upper box contained a mixture of empty cells, honey, and pollen. Queen-laid eggs were obtained from below the excluder in queenright source colonies ($N = 2$ for *A. m. scutellata* and $N = 2$ for *A. m. capensis*). Worker-laid eggs were obtained from the queenless source colonies ($N = 2$ for *A. m. scutellata* and $N = 3$ for *A. m. capensis*). For each discriminator colony ($N = 2$ for *A. m. scutellata* and $N = 3$ for *A. m. capensis*), we used a single test comb that was initially placed into the hive 2 days before egg removal trials began.

Twenty queen-laid and 20 worker-laid eggs of *A. m. capensis* were transferred from the source colonies into worker cells of the test frames. Likewise, 20 queen-laid and 20 worker-laid eggs of *A. m. scutellata* were transferred from the source colonies into the drone cells of the test frames. Eggs were arranged in batches of 20 eggs each on the test combs. The test combs were then reintroduced into their discriminator colonies. After 2 and 4 h, the test combs were briefly removed and inspected to determine which eggs were still present. After 24 h the remaining eggs were counted and removed. A new set of eggs was then transferred. Egg removal trials were made for five consecutive days for each discriminator colony. Moreover, removal rates of queen-laid and worker-laid eggs from *A. m. capensis* and *A. m. scutellata* source colonies ($N = 2$ queenright $+ 2$ queenless *A. m. capensis* and $N = 2$ queenright $+ 2$ queenless *A. m. scutellata*) in queenright discriminator colonies of the same subspecies ($N = 3$ *A. m. capensis* and $N = 3$ *A. m. scutellata*) were also evaluated on three sequential days using the same experimental approach with the exception of the cross tests. In this study a total of five *A. m. scutellata* and six *A. m. capensis* were used as discriminator colonies; another 17 colonies supplied the different types of eggs for the trials (four queenright *A. m. capensis* and *A. m. scutellata* each for queen-laid eggs, and four queenless *A. m. scutellata* and five queenless *A. m. capensis* as source for worker-laid eggs). We compared the removal rates of worker-laid and queen-laid eggs both within and between subspecies
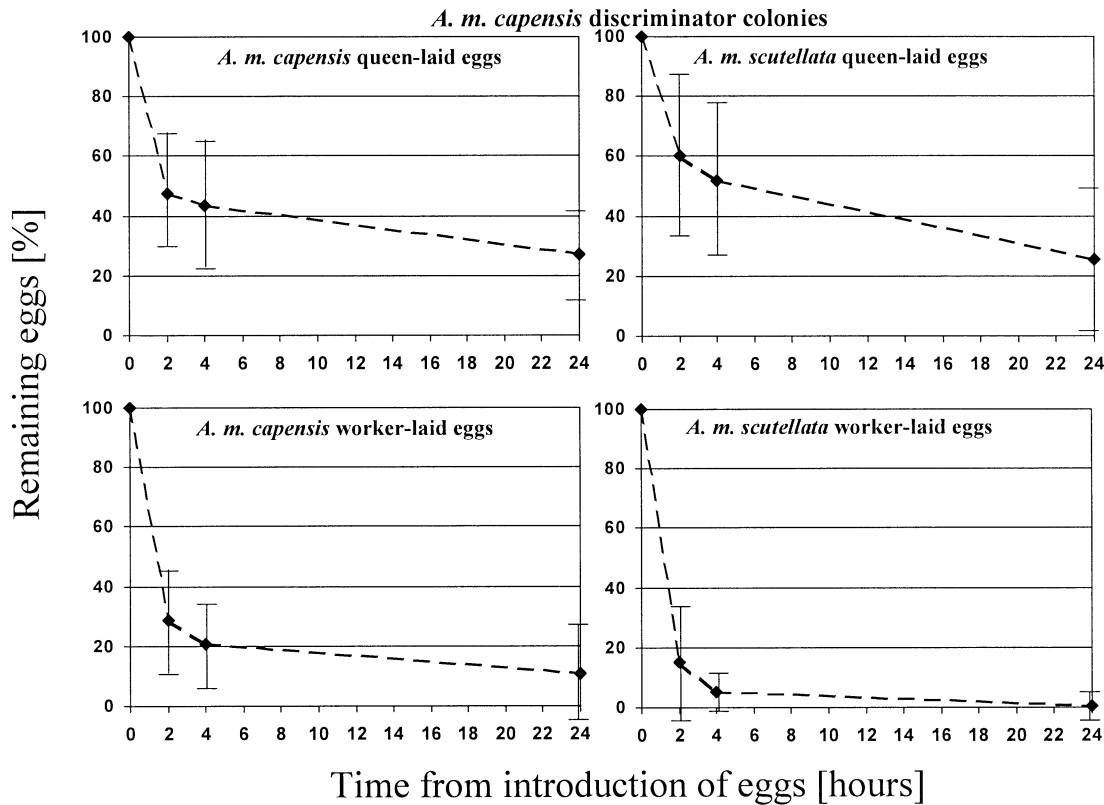
Figure 1
Numbers (mean ± SD) of queen-laid and worker-laid eggs of *Apis mellifera capensis* and *A. m. scutellata* remaining after 0, 2, 4, and 24 h in queenright *Apis mellifera capensis* discriminator colonies.

by using Kruskal-Wallis ANOVAs and Mann-Whitney U tests (with Bonferroni adjusted levels of significance) using Statistica.

RESULTS

Time to removal was determined for 2720 eggs. The percentages of remaining eggs per egg source in the two subspecies of discriminator colonies are shown in Figures 1 and 2. The results of the Mann-Whitney U tests are shown in Table 1. *A. m. capensis* and *A. m. scutellata* discriminator colonies removed worker-laid eggs of their own subspecies significantly faster than did queen-laid eggs of their own subspecies. Likewise, in both subspecies of discriminator colonies, worker-laid eggs of the other subspecies were removed significantly faster than were queen-laid eggs of the other subspecies. Worker-laid eggs of *A. m. scutellata* were removed significantly faster in *A. m. capensis* discriminator colonies than in *A. m. scutellata* colonies. However, *A. m. capensis* worker-laid eggs were not removed significantly faster in *A. m. scutellata* than in *A. m. capensis* discriminator colonies. Finally, there were no significant differences among the individual discriminator colonies of either subspecies in the removal rates of queen-laid and worker-laid eggs of their own subspecies after 24 h (*A. m. scutellata*: queen eggs, H = 7.56, p = ns; worker eggs, H = 6.99, p = ns; *A. m. capensis*: queen eggs, H = 5.61, p = ns; worker eggs, H = 5.72, p = ns).

DISCUSSION

The data clearly show that queenright *A. m. capensis* and *A. m. scutellata* colonies both police worker-laid eggs of their own and of the other subspecies but accept a much larger proportion of worker-laid eggs than do European honeybees. Although *A. m. capensis* discriminator colonies remove worker-laid eggs of *A. m. scutellata* faster than *A. m. scutellata* colonies, *A. m. capensis* worker-laid eggs were not removed faster in *A. m. scutellata* discriminator colonies. Within each subspecies, there were no significant differences among discriminator colonies.

Our data suggest that the two African subspecies are similar in their general pattern to European bees, i.e., worker-laid eggs are less acceptable than queen-laid eggs, but there are differences in the details. In the *A. m. scutellata* discriminator colonies, many more worker-laid eggs of both *A. m. scutellata* and *A. m. capensis* remained after 24 h (17% of *A. m. scutellata* worker-laid eggs and 14% of *A. m. capensis* worker-laid eggs) compared with those of colonies of European subspecies (1%; Ratnieks and Visscher, 1989) and even to *A. m. capensis* (1% of *A. m. scutellata* worker-laid eggs and 10% of *A. m. capensis* worker-laid eggs). Thus, it is possible that either policing is lower and/or worker-laid eggs are more acceptable in African than in European subspecies. The similar pattern in both subspecies suggests that the African origin of the tested subspecies may matter but not the genetics of worker reproduction. Moreover, fewer queen-laid eggs remained in the *A. m. capensis* colonies after 24 h (26%) compared with those in earlier studies on European honeybees (45%; Ratnieks and Visscher, 1989) and on *A. m. scutellata* (41%). This might be owing to nest-mate recognition for queen-laid eggs, which is well expressed in African honeybee subspecies (Pirk et al. 2001).

Our observations that worker policing in the thelytokous Cape honeybee is at least as effective as in the arrhenotokous *A. m. scutellata* (and possibly better) contrast to theory, which shows no benefit of worker policing in *A. m. capensis* (Greeff,
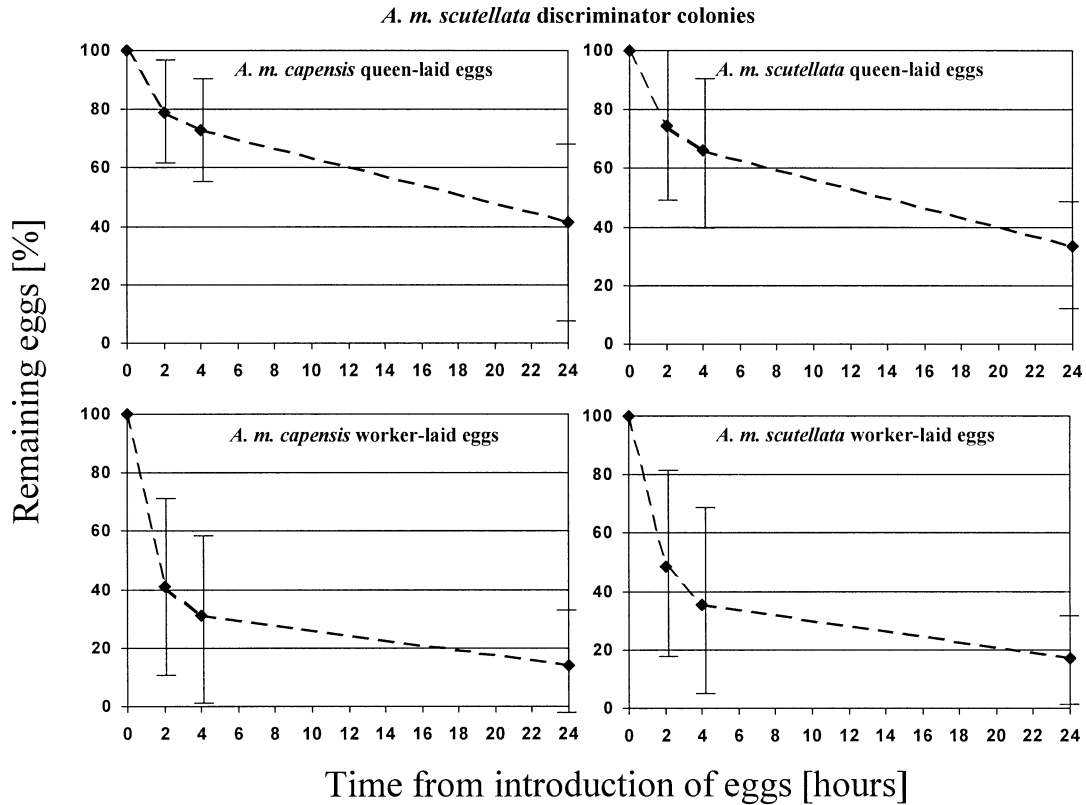
A. m. scutellata discriminator colonies

Figure 2
Numbers (mean ± SD) of queen-laid and worker-laid eggs of *Apis mellifera capensis* and *A. m. scutellata* remaining after 0, 2, 4, and 24 h in queenright *A. m. scutellata* discriminator colonies.

1996). In fact, the observed difference between the two African subspecies (*A. m. scutellata* with relatedness benefits and *A. m. capensis* without) is in the opposite direction to prediction (Greeff, 1996). But this prediction is based on relatedness grounds alone (Greeff, 1996). Our observation of worker policing in *A. m. capensis* is also partly in contrast to the other existing empirical study (Moritz et al., 1999), which showed that laying worker offspring does occur in queenright colonies. However, even if most worker-laid eggs had been removed, some may have remained, and it was these that were detected by using DNA microsatellites (Moritz et al., 1999). Thus, Moritz et al. (1999) showed that some workers' eggs are reared, but they do not show that worker policing is absent. The observed egg-removal rates in this study agree well with our casual observations of the study colonies, which showed that no larvae were being reared above the queen excluders in the test colonies immediately before or during the experimental period. Because the occurrence of brood above the queen excluder seems to be frequent in Cape honeybees (Pettey, 1922; Neumann P, personal observations) and in *A. m. scutellata* (Wossler T, personal communication), it was quite possible that worker policing would prove to be less effective than we actually observed. Thus, the combined results of Moritz et al. (1999) and this study indicate that worker policing does occur in *A. m. capensis* but is sometimes not fully effective in preventing worker reproduction.

Why does worker policing still occur in *A. m. capensis*? Earlier theoretical studies (Greeff, 1996) may have missed a critical piece in the cost-benefit analysis for policing in the Cape honeybee. The occurrence of policing should be dependent on a trade-off between the cost of policing and the cost of worker reproduction to overall colony efficiency and reproduction (Ratnieks, 1988). Given that policing

probably costs very little, because eggs are held in open cells that workers are regularly checking anyway, the costs derived from unhindered worker reproduction might easily be higher than the costs of policing. Thus, a large efficiency gain is not needed to favor worker policing. In the *A. m. capensis* situation, the gain need only be marginal. Even if a queen is single mated, so that policing of worker-laid eggs has a relatedness cost, policing is still favored if colony efficiency increases by 20% (Ratnieks, 1988). A recent theoretical study by Foster and Ratnieks (2001) on the European hornet, *Vespa crabro*, shows that worker policing can even more easily be selected for at a mating frequency of one as part of a sex allocation biasing strategy of workers. That is, workers want to eliminate males to cause a female-biased sex ratio, and the only eggs they know to be male are workers' sons.

What are the possible costs of worker reproduction in honeybees? There are probably two main potential costs: reduced brood rearing efficiency and a reduced work rate of reproductive dominant workers, when worker reproduction occurs in queenright colonies:

1. *Reduced brood rearing efficiency.* Honeybee nests have a limited brood rearing area, which constrains the number of eggs that can be laid by the queen, given that a queen typically will not lay an egg in a cell that already contains an egg (Ratnieks, 1990). Worker egg laying, when common, is characterized by multiple eggs per cell because workers will lay additional eggs in cells that already contain an egg (Gary, 2000). The earlier-laid eggs are often knocked down, squashed, and killed by the abdomen of the laying worker. Only one larva can be reared to adulthood in a single cell, and additional larvae are eaten by workers. This may lead to costs associated with cannibalism (Elgar and Crespi, 1992).

Table 1
Numbers of eggs from different subspecies, colony, and caste sources remaining after 24 h in *Apis mellifera capensis* and *A. m. scutellata* discriminator colonies

Type of comparison	Egg source	DC (No. of DC, trials)	Trend	Egg source	DC (No. of DC, trials)	U	p
Worker-laid versus queen-laid	C−	C (6,24)	Faster	C+	C (6,24)	107.5	<.0002
	S−	S (5,19)	Faster	S+	S (5,19)	78	<.003
	S+	C (3,15)	Slower	S−	C (3,15)	47.5	<.001
	C+	S (2,10)	Slower	C−	S (2,10)	13.5	.019
Worker-laid versus worker-laid	S−	S (2,10)	Slower	S−	C (3,15)	46	.003
	C−	C (3,15)	Faster	C−	S (2,10)	46	ns

Results of Mann-Whitney *U* tests are shown. The Bonferroni adjusted level of significance is *p* = .025. DC indicates discriminator colony; C, *A. m. capensis*; S, *A. m. scutellata*; +, queenright; and −, queenless.

Such cannibalism costs are likely to be small because when two larvae occur in one cell, one is removed within a few days of hatching. Moreover, the larvae are cannibalized rather than thrown out of the colony, suggesting that some energy can be recycled. However, when many workers are laying eggs, it may simply take longer for any cell to yield a worker, which is probably the main cost aspect. For example, if it took just one additional day to rear a worker per cell, this would lead to a 5% reduction in the maximal rate of colony build-up, given an egg-adult stage of approximately 19 days in workers of the Cape honeybee (Hepburn and Radloff, 1998).

2. *Work rate of laying workers.* Laying Cape honeybee workers in queenless and queenright groups do not participate as much in hive duties such as brood rearing compared with the participation of subordinate workers (Hillesheim et al., 1989; Moritz and Hillesheim, 1985). Thus, a high frequency of laying workers may also reduce colony productivity (Hillesheim et al., 1989). This cost may not be greatly reduced by egg eating, because worker policing via oophagy does not directly stop or penalize egg-laying workers. It may cause a reduction in worker egg-laying over evolutionary time, but for it to be selected for, there has to be an immediate benefit in the colony with policing.

Reproductive dominance seems to be strongly genetically determined (Moritz and Hillesheim, 1985, Moritz et al., 1996). Therefore, almost clonal (Moritz and Haberl, 1994) laying worker offspring are predisposed to develop into laying workers. Thus, worker policing via oophagy may limit the establishment of such laying worker matrilines in queenright colonies, constituting an immediate benefit for policing colonies. Alternatively, but not mutually exclusive, worker-worker aggression in queenright colonies, which is directed toward nest mates with activated ovaries (Visscher and Dukas, 1995), might also restrict the establishment of such laying worker matrilines.

Worker reproduction in the Cape honeybee, if kept to a low level, will probably have almost zero efficiency cost. Therefore, we can expect some worker reproduction, even if there is policing. Indeed, there is considerable variation for egg-laying behavior in Cape honeybee workers (Neumann and Hepburn, 2002). Although in some laying worker colonies, the brood nest is virtually indistinguishable from that of a queen, because there is only one egg per cell (Neumann and Hepburn, 2002), others show the typical pattern of a queenless colony with laying workers such as multiple eggs per cell, as did the colonies in our study (Neumann P et al., personal observations). This suggests that it is possible in Cape honeybees to have a low level of worker reproduction, which does not interfere with brood rearing. Thus, the costs need not be high for a colony if the amount of worker reproduction is low.

The Cape honeybee example is a particularly convincing example. Thelytoky causes a change in kin structure rendering relatedness neutral with respect to worker reproduction (see above). Thus, if worker policing were not beneficial in *A. m. capensis*, it should be evolutionarily lost. That is, workers should accept eggs laid by other workers. Loss of policing would be a simple adaptation. In fact, loss of policing already occurs in queenless *A. mellifera* colonies (Miller and Ratnieks, 2001), which have failed to rear an emergency replacement queen. Worker-laid eggs are accepted and reared into a final cohort of males before the colony dwindles in population and dies (Page and Erickson, 1988). The results also show that both races are able to police worker-laid eggs of the other subspecies, indicating that the same underlying mechanism is used for worker policing. Arrhenotoky (Crozier and Pamilo, 1996), multiple paternity (Neumann and Moritz, 2000, Palmer and Oldroyd, 2000), and worker policing (*A. mellifera*: Ratnieks and Visscher, 1989; *A. florea*: Halling et al., 2001; *A. cerana*: Oldroyd et al., 2001) appear to be ancestral in *Apis*, indicating that thelytoky is a derived condition in *A. m. capensis* and arose in a clade in which worker policing occurred.

In conclusion, we hypothesize that worker policing still exists in the Cape honeybee owing to colony efficiency grounds. As predicted by Hamiltons' rule, this illustrates that relatedness alone cannot predict the reproductive characteristics of insect societies. The application of the inclusive fitness theory requires knowledge of costs, benefits, and relatedness.

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