

THE EVOLUTION OF HONEYBEE MULTIPLE QUEEN-PHEROMONES – A CONSEQUENCE OF A QUEEN-WORKER ARMS RACE?

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ABSTRACT

Queen-worker interactions in the honeybee colony are mostly pheromone-mediated, and include both cooperative and conflictual interactions. The perceived presence of the queen, presumably through pheromone emission, induces harmony in the colony that promotes rapid ergonomic growth and eventually reproduction by swarming. Queens, however, are not portrayed by a single pheromone, but characteristically have a multipheromone-multiglandular bouquet. Some of these pheromones, despite being produced by disparate glands, appear to have the same behavioral and physiological effects on workers. Why should such redundancy have evolved? I suggest that this evolution is linked to the conflict between queen and workers over male production. Although multiple inseminations in the honeybee queen have resulted in worker policing, and seemingly a resolution to the conflict, results suggest that worker reproductive and pheromonal plasticity indicates an ongoing arms race between queen and workers. Queens are selected to inhibit worker reproduction (by producing inhibitory pheromones) whereas workers are selected to bypass this inhibition (through developing insensitivity to the pheromone). The arms race is thus expressed in the continuous evolution of new queen pheromones for inhibiting worker reproduction, while workers constantly evolve to resist them. To support the arms-race hypothesis, I provide here some examples from pheromone chemistry and biosynthesis as well as from mutant bee-lines in which worker reproduction exists.

Key words: Arms race, Dufour's gland, evolution, honeybee, mandibular gland, queen pheromone

The role of pheromones in queen-worker competition

Queen-worker conflict over reproduction has constituted a main thread throughout the evolution of sociality. The most important component of the colony is the existence of worker castes, which comprise females that are subservient to the needs of their mother and that give up their own reproductive option. This enigmatic altruistic behavior has played a major role in the study of animal behavior from Darwin to Hamilton's kin selection theory [16]. Members of the Hymenoptera are especially interesting since they inherit sex by haplo-diploidy. It is predicted that under normal (and presumably also primordial) conditions, i.e., monogyny and monandry, workers gain more by rearing sons than either nephews or brothers (average relatedness of $0.5 > 0.375 > 0.25$). Queens, on the other hand, under all

circumstances gain more by rearing sons rather than grandsons. A consequence of this genetic conflict between queens and workers is an arms race whereby queens are selected to inhibit worker reproduction directly or indirectly, while workers are selected to overcome this inhibition, i.e., attempt to reproduce (indeed workers are not irreversibly sterile) [57].

The intriguing phenomena of cooperation and conflict have led to research on the mechanisms and origins of social behavior. Conflicts arise when the within-group variability increases and the reproductive interests of the group members are not aligned. When conflict can not be prevented *a priori* in species with fertile workers, social control may ensure continued group stability. When the interests of the majority of group members are aligned, they suppress selfish individuals that threaten the group's performance and, therefore, the mean inclusive fitness of the majority of individuals [29]. These two opposing reproductive trends shape to a large extent the behavior of each member of the society.

Selection forces are apparently determined by whether each unit gains more from its participation

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in a harmonious and presumably more efficiently adapted whole, or from pursuit of individual reproduction with its attendant cost to group efficiency [54]. Selection for cooperation in the honeybee depends upon two variables: the relative relatedness of workers to queen- and worker-produced males (relatedness hypothesis) and the colony-level cost of workers reproducing (efficiency hypothesis) [17]. The relatedness hypothesis explicitly predicts that the parentage of males is dependent upon colony kin structure. Cooperation and harmony can be adaptive to both the queen and workers because it enhances colony competitive ability through ergonomic growth and ultimately the rearing of new female reproductives. In such kin-selected societies workers generally gain more by rearing sister-reproductives rather than direct descendants, and therefore assist the queen in rearing her daughters. On the other hand, according to the efficiency hypothesis, it is assumed that costs associated with worker reproduction are likely to be significant and that variation in these costs is the main factor underlying differences across species regarding the origin of males [17]. Under many circumstances a worker's investment in the queen's offspring probably yields more reproductive individuals than would investment in rearing her own offspring, due to elevation of overall colony productivity (elevated foraging force [8] and brood production [12]), and therefore increases the average fitness of the colony members [13]. Moreover, worker reproduction can cause a "tragedy of the commons" [56], with individual exploitation (worker reproduction) potentially causing a cost to the whole group (reduced foraging and lowered colony productivity).

The strong reproductive skew exhibited by social insects raises a number of proximate and ultimate questions. While the theoretical questions have been extensively treated, the mechanisms employed by social insects to regulate this skew are still elusive. The general opinion has been that the queen manipulatively prevents workers from reproducing by either physical or chemical means (queen control, [1,28]). In small colonies a single individual may have the power to dominate male production completely by intimidation. Such reproduction dominance becomes less likely however as colony size increases. It has long been assumed that queen pheromones, which are by far the major mediators of both cooperation and conflict, are involved in inhibiting worker

reproduction [7,18], but none have been conclusively proven to do so. On the other hand, kin-selection considerations suggest that worker reproduction is self-regulated and is social-context-dependent (worker-control). This latter hypothesis postulates that the primer effects of a queen pheromone may in fact not exist, and that the queen pheromones constitute signals that provide the workers with the necessary information for them to determine their behavior in the way that will maximize their fitness [28]. Both possibilities involve a considerable effect of the queen pheromones on worker physiology and behaviour.

The problem encountered in the identification of queen primer pheromones that regulate worker reproduction lies in the fact that, while in the presence of the queen, worker sterility is mostly maintained; all experimental attempts to completely substitute her presence by pheromones have failed [18,22,63]. It can thus be postulated that in order to properly mimic the queen all her pheromones must be present. Here I discuss the honeybee queen pheromones as a model for understanding the evolution of the queen multiple pheromone system. Understanding the evolution of this system would enhance our comprehension of how these complex interactions were selected for. Three basic problems are addressed. 1). The occurrence of multiple pheromones emanating from multiple sources with overlapping activity. 2). Queen pheromone appearance in workers. 3). Why none of the individual pheromone bouquets is as effective as a living queen in controlling worker behavior?

Honeybee queen pheromonal bouquet

Establishment of dominance hierarchies is a common phenomenon in social hymenoptera, and it can be mediated through behavioral interactions and/or pheromones [10,30]. Honeybees are especially rich in exocrine glands that produce many caste-specific signals [4]. Empirical investigations of queen pheromones initially focused on revealing the glandular source and its chemical composition, and the **mandibular gland** was the first to be demonstrated as such a source. Queen glandular secretion is composed of a blend of nine components of which 9-oxo-2-decenoic acid is a major constituent [6,27,50]. The queen mandibular pheromone (QMP, composed of only five of the components) was found to have major effects on worker behavior, including primer and releaser effects [58]. The most pronounced

releaser effect of the pheromone is that of retinue behavior around the queen within colonies, as well as externally to form swarm clusters [14,27,50,51,53,59]. Its other releaser effects include stimulating foraging, brood rearing, comb building, and food storing. Its primer activity includes the inhibition of ovarian development [6,18] and inhibition of emergency queen rearing [60]. However, in all cases the queen herself is much more effective than QMP alone in eliciting the expected response. Workers, on the other hand, produce 10-hydroxy-(E)-2-decenoic acid and this compound is the main component of the brood food fed to larvae [4].

Dufour's gland, which is associated with the sting apparatus, is another gland that exhibits caste-specific composition. The queen Dufour's pheromone is composed of a complex mixture of hydrocarbons ranging from C_{21} to C_{33} accompanied by a series of long-chain esters, whereas worker secretion contains only hydrocarbons [26]. The secretion, specifically its ester components, is attractive to workers, which display retinue behavior around a surrogate queen smeared with the glandular extracts [23,25]. Evidence suggests a tight coupling between the occurrence of queen characteristic Dufour's esters and ovary activation [11,21]. However, although ester quantity was not found to be correlated to the hierarchy status of bees kept in pairs, correlative evidence suggests that it acts as a fertility signal [11].

Another abdominal source is that of the **tergal gland** secretions, which in queens and workers of *Apis mellifera capensis* and *Apis mellifera scutellata* are composed of long-chain fatty acids, long-chain esters and a linear series of unsaturated hydrocarbons [61]. In *A. mellifera capensis*, queen abdominal secretion is attractive to workers, which exhibit typical retinue behavior around the source, albeit less pronounced than for QMP [62]. It also inhibited ovarian development when tested in small groups of caged workers [63]. In *Apis mellifera ligustica* the glandular production was suggested to be associated with natural mating of the queen [52]. An additional source of pheromones is that of **feces** of a virgin queen, which contain *o*-aminoacetophenone that acts as a worker repellent and terminates agonistic interactions between the queen and workers [3,39].

The richness of honeybee queen-specific pheromones provides good grounds for constructing hypotheses regarding their possible evolution. While it is less surprising that the queen-specific pheromones were found to play an important role

in queen-worker interactions, the fact that many of them possess overlapping functions is more enigmatic. As mentioned above, three different pheromone bouquets from disparate glands are effective in eliciting retinue behavior. Two of these, mandibular and tergal pheromones, also act as primer pheromones implicated in the control of worker reproduction (ovary inhibition). In view of the fact that generally queen-specific pheromones elicit multiple behavioral responses in workers, it would not be surprising to find that the other queen-specific pheromones too encompass both releaser and primer effects. Considering that pheromone production is costly, the question that arises is: what were the selection pressures behind the evolution of multiple pheromonal sources, when theoretically one set should have sufficed?

Pheromonal plasticity in workers

Another interesting aspect of honeybee pheromones is that the queen pheromonal specificity described above is not rigid but is plastic under changing environmental and social circumstances. For QMP and queen Dufour's pheromone, at least, it was demonstrated that under hopeless queenless (QL) conditions, workers that initiate ovarian development concomitantly produce queen-like secretions [9,26,45,49]. This is not surprising considering that honeybee diploid larvae are totipotent to develop into queens or workers, and therefore both can possess the machinery for queen pheromone production. Studies on the biosynthesis of Dufour's components confirmed this plasticity at the glandular level. *In vivo* studies, using radioactive precursor, have shown that the biosynthetic activity of the gland was constrained by the social environment of the bee. Queenright (QR) workers did not produce queen-like esters whereas *de novo* ester biosynthesis was evident in QL workers. On the other hand, *in vitro* studies revealed substantial *de novo* ester biosynthesis irrespective of the workers' social environment, i.e., QL or QR [24], thus demonstrating a glandular biosynthetic ability that is controlled by social factors. Biosynthesis studies of the mandibular gland pheromone have shown that at least for 9ODA the difference between workers and queens lies in this initial step of functionalization from which the two biosynthesis pathways separate, resulting in caste-specific bouquets [46]. Although the process in QL workers' mandibular glands has not

been investigated, from the data regarding Dufour's gland we can infer such biosynthetic plasticity and that the specificity of the functionalization step in workers is socially regulated.

This pheromonal plasticity is also accompanied by behavioral changes towards these workers that become "false queens" [23,45,48]. In fact, detailed observation in QR colonies have shown that retinue bees too become "false queens" for a short period after being in contact with the queen [20]. Recent studies have shown that QL workers housed in pairs establish a dominance-subordinance relationship with respect to the production of queen-like pheromones: 9ODA, the queen mandibular gland component for *A. m. capensis* (one worker will suppress the production of the queen substance in the other [33,34]).

Why should multiple queen signals have evolved?

Despite the extensive study of chemical communication in social insects some questions still remain unanswered. We have yet to understand why there are so many glandular sources; why such a complex blend evolved in the first place and which worker activities are influenced by which components? The evolution of signals suggests a paradox: senders are expected to try to manipulate the receivers for their own interests, because the interests of senders and receivers will often conflict [15]; while at the same time, in order to elicit the desired response from the receiver, the senders' signals must, on average, be reliable or honest. Moreover, in the struggle between senders and receivers, signals may be mimicked and their perception modulated.

In the honeybee the answer probably involves an evolutionary "arms-race", in which queen signals and workers overcoming such signals have evolved in tandem along evolution. Evidence that the queen pheromone is widely broadcasted is provided from studies of QMP distribution on the queen's body and its dispersion throughout the honeybee colony [35,36]. The attractiveness of both Dufour's gland and tergal glands secretion suggests a similar broadcasting mechanism, but this awaits experimental demonstration. The competition between the queen and her workers is primarily over male production. While worker policing exerts a high reproductive cost on workers, favoring reproductive self-restraint ([47,64], but see also [44], providing alternative explanation for worker policing), workers still possess the ability to develop ovaries and to produce the queen-like pheromones.

This suggests that the conflict has not yet been fully resolved and the arms race may still be in effect. Total sterility in workers among Hymenoptera is indeed rare and limited to a few ant genera [5], suggesting that it is generally counter-selected.

Several honeybee lines exhibit behaviors that support the arms race hypothesis. These findings provide evidence indicating on evolution towards more complex blends and more pronounced worker-queen differences in honeybee pheromonal systems. In the Australian "anarchistic bee" mutant workers lay eggs that evade policing, resulting in the majority of males being the progeny of workers [38]. This indicates an escalation in the arms race whereby the workers have become not only insensitive to the queen pheromones [19] but are also somehow able to camouflage their eggs in such a way that they are not recognized as worker eggs by their worker nestmates [2]. The discovery of a bee line that was poorly responsive to QMP in a retinue bioassay [41,42] lends credence to the hypothesis that workers are selected to bypass queen inhibition. Moreover, workers that have no response to QMP will nevertheless form a normal-appearing retinue around the queen, and their colonies show no apparent differences from colonies composed of high-responding workers [43], thus supporting the arms race hypothesis because other queen signals probably compensate for the sensitivity loss to QMP. Worker reproduction by thelytoky, producing either workers or queens, in the South African *A. m. capensis*, may represent yet another facet of the arms race. These workers are accepted as queens in colonies of *A. m. scutellata*, where they rapidly dominate reproduction, but interestingly refrain from reproduction in their own nest in the presence of an *A. m. capensis* queen [37]. This indicates a possible step in the arms race: these workers have developed insensitivity to the queen pheromone (along with the ability to reproduce by thelytoky); and as a countermeasure the *A. m. capensis* queen has developed a new means (pheromones) for controlling worker reproduction (such as the tergal glands, which evidently affect worker reproduction in that species, [63]). However, *scutellata* queens, which may represent an early evolutionary stage, do not appear to have changed as a result of the workers' pressure, since parasitism by *capensis* is a rare event. Evolving pheromone insensitivity coupled with thelytoky may have enabled *capensis* workers to successfully usurp *scutellata* nests by escaping

from the existing honeybee queen pheromone. It can be hypothesized that in their natal *capensis* nest a new queen-pheromone evolved that maintained the reproductive skew. According to their reproductive status, QR workers of *A. m. capensis* unusually also possess the queen characteristic pheromones, at least for the mandibular glands [9], and pheromonal mimicry may be another indication of the ongoing arms race.

According to the arms race hypothesis (Fig. 1) the honeybee queen first evolved a pheromone (pheromone a) that either acts as a signal or controls worker reproduction, for example the QMP. Workers then counteracted by becoming both less sensitive to the pheromone and also producing a queen-like pheromone (pheromone a'), exerting selection on the queen to evolve another pheromone from another glandular source (pheromone b) and so forth. The end result is that the queen developed multiple pheromonal sources (a, b, c, d), none of which is individually sufficient to mimic a living queen, but whose combination may act either additively or synergistically ([24,27]; but see also [18]). The threshold model of suppression suggested by Moritz and Crewe [32] fits well the arms race hypothesis. According to this model, workers are suppressed by exposure to levels of queen pheromone higher

than specific threshold. Unsuppressed workers will increase their own production of queen pheromone up to their genotypically set physiological limit. The suppression threshold is however not constant but depends on the amount of self-produced 9-ODA. The reaction of the worker is expressed in the change of the individual suppression threshold to the queen substance concentration. Thus, when a mutation reducing the threshold occurred the workers could compete with the queen over production of these components. The queens nonetheless keep producing pheromones to which workers have developed a resistance since the sensitivity of different workers towards the pheromones differs and the pheromones also affect many other colonial activities, not just reproduction. Thus any tendency for such pheromone disappearance will be counter-selected.

It should be noted that the asymmetric reproduction in social insect societies forces differences in the level of selection on queen and workers. Since queens are the main reproductives, worker mutation spreads across the population at a much lower rate (only via males and under certain circumstances). The queen advantage is thus indeed apparent in the present balance of power between the castes. However, the heritability of chemical communication pathways remains to be explored.

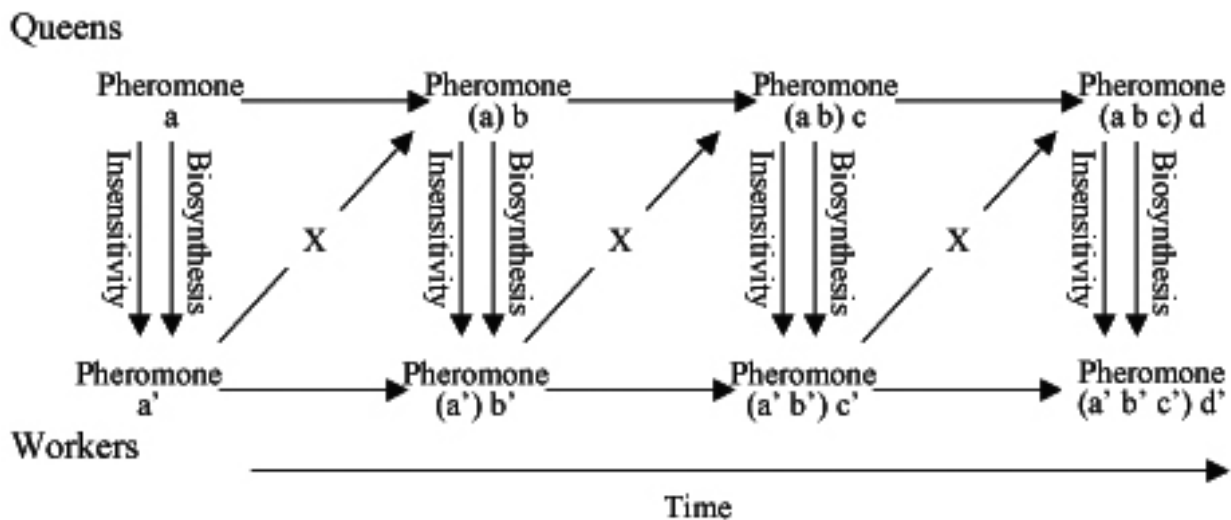


Figure 1. A stepwise model for the evolution of honeybee queen multiple pheromones as the result of an arms race between queen and workers over reproduction. The queen first evolved a pheromone (pheromone a) that either acts as a signal or directly controls worker reproduction. Workers then counteracted by becoming both less sensitive to the pheromone and also producing a queen-like pheromone, (pheromone a') exerting selection on the queen to evolve another pheromone from another glandular source (pheromone b). The end result is that the queen developed multiple pheromonal sources (pheromone abcd), none of which is sufficient individually to mimic a living queen, but whose combination may act either additively or synergistically.

The ability of egg-laying workers to mimic the queen pheromone also warrants an explanation. Worker reproduction may occasionally reflect a balance between selection pressures operating in two different contexts, a QR colony and one that has lost its queen. The advantage for egg-laying workers under “queenless hopeless situation” is clear. It is advantageous for a worker to quickly lay eggs in the event of queen loss, since it is principally the earliest worker eggs laid after queen loss that are successfully reared in an orphan colony [40]. The presence of the queen pheromones in these workers may encourage nestmates to help them rear the brood in order to increase their inclusive fitness. It could therefore be argued that worker-mimicry of the queen pheromone has been maintained for the above advantage. However, in nature a “queenless hopeless situation” is unlikely to occur, or is very rare. Generally, even if the queen is lost there is ample young brood available for requeening the colony. If queen mimicry is a part of the queen–worker arms race, why is queen mimicry in QR workers extremely rare, if indeed it exists at all? I suggest that its rearing reflects the cost of punishment by other workers. At least one report indicates that other workers in the hive can detect egg-laying workers and aggress them [55]. It is not known whether it is the possession of queen pheromones that exposes the egg-laying workers, but this is a possibility. Such punishment of reproductive challengers was reported in the queenless ant *Dinoponera quadriciceps* [31]. In honeybees the queen-like Dufour’s gland secretion in workers is invariably associated with ovarian development [11,21], suggesting that it is a reliable fertility signal, eliciting punishment in a QR colony or giving a head start in the reproductive race under QL conditions.

In conclusion, it is suggested here that, at least in the honeybee, the arms race is expressed as a continuous evolution of new queen pheromones for inhibiting worker reproduction, while workers constantly evolve to resist them. While many questions regarding pheromonal evolution remain unanswered, it is clear that the honeybee queen represents an excellent model for studying a complex set of pheromones; all of which, singly and in concert, direct the complex and sophisticated social behavior.

ACKNOWLEDGMENTS

This research was supported by THE ISRAEL SCIENCE FOUNDATION founded by the Israel Academy of Sciences (ISF grant # 720/04 to Abraham Hefetz). I thank A. Hefetz and V. Soroker for their helpful comments and N. Paz for editorial assistance.

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Received: April 26, 2006

Accepted: August 16, 2006