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Are multiple honeybee queen pheromones indicators for a queen-workers arms race?

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ABSTRACT

Honeybees are endowed with pheromones, many of which exhibit caste specific composition, one of which is Dufour's gland pheromone (QDP). The queen possesses long chain esters that are absent in queenright (QR) workers. However queenless (QL) workers that develop ovaries also express these queen substances. The ability of QL-workers to produce the queen-like compounds suggests that under normal conditions glandular expression is inhibited. This is corroborated by studies *in vitro* where Dufour's gland detached from its normal regulation can synthesize the queen-like substances even in QR workers, indicating a role in the queen-worker arms race for reproduction.

QDP is only one of multiple queen specific pheromones in honeybees. A major evolutionary enigma is why the queen uses multiple signals to announce its presence, when theoretically a single pheromone should be sufficient. We hypothesize that it is the result of a pheromonal and reproductive arms race between queens and workers. Queens were selected to develop means to inhibit workers from reproducing through the production of a queen specific pheromone. Workers have counteracted not only by retaining their ability to develop ovaries, but also by retaining the capacity to produce the queen-like pheromone and by that becoming "false queens". The next step in this evolutionary arms race was the development of a new pheromonal bouquet by the queen that was again counteracted by the worker's ability to produce these substances. This process is facilitated because the genetic makeup of queen and workers is identical, since female larvae can develop to either caste.

The result of this arms race is that queens possess multiple queen signals, only the combination of which seems to be effective. Whether this postulated arms race have reached stalemate, i.e., for each queen-pheromone there is a matched production potential in workers, or is it an ongoing arms race, i.e., a true queen-specific pheromone exists.

Keywords: *pheromones, social behavior, worker reproduction*

INTRODUCTION

Social hymenoptera are typified by a strong reproductive skew whereby a few members of a large society reproduce (queens) with the help of mostly sterile individuals (workers). The evolution of such extreme altruism is generally explained in terms of kin selection coupled with the typical hymenopteran haplodiploid sex determination system. According to this

hypothesis, workers maximize their inclusive fitness by helping their mother to raise future reproductives because by rearing sisters they gain better genetic fitness than rearing daughters (relatedness of 0.75 Vs 0.5, respectively). However, the peculiar sex determination creates a relatedness asymmetry in the colony. While sister-sister relatedness promotes cooperation with the queen in rearing future gynes, that between sisters and brothers promotes competition over the motherhood of males (average relatedness of 0.5 for sons Vs 0.25 for brothers) (Hamilton 1964; Trivers and Hare 1976; Bourke and Franks 1995). Indeed, workers in most social hymenoptera are not irreversibly sterile and under certain social conditions develop ovaries and lay unfertilized eggs from which males develop. A consequence of the genetic conflict between queens and workers is an arms race whereby queens are selected to inhibit worker reproduction directly or indirectly, whereas workers are selected to overcome this inhibition, i.e., attempt to reproduce as well as destroy the queen's male eggs.

A queen strategy that may have evolved to indirectly control worker reproduction is multiple inseminations. While in colonies headed by a monoandrous queen workers benefit more from rearing sons or nephews (average relatedness of 0.5 and 0.375, respectively) than brothers (average relatedness of 0.25), under polyandry the relatedness of worker produced males to a random workers falls below that of a queen produced males (average relatedness of 0.125 vs. 0.25, respectively). Consequently workers are predicted to selectively remove other worker-laid eggs but retain the queen's haploid eggs. This selective egg destruction was termed worker policing and is characteristic of honeybee colonies (Woyciechowski and Lomnicki 1987; Ratnieks 1988). Supporting this hypothesis are the findings that in normal QR honeybee colonies only a negligible fraction of the emerging males are worker-produced (Page and Erickson 1988; Visscher 1996). Moreover, worker-laid eggs that are experimentally introduced into a QR colony are rapidly removed while queen-laid haploid eggs are mostly retained (Ratnieks 1993; Katzav-Gozansky *et al.* 2001; Martin *et al.* 2002). This ability of workers to discriminate between the two types of haploid eggs is probably pheromonally based, albeit neither the chemistry nor the glandular source of this putative egg-marking pheromone is known (Katzav-Gozansky *et al.*, submitted).

Another strategy that may be employed by queens to prevent worker reproduction is by producing queen specific pheromones that inhibit oogenesis and/or oviposition in workers. Especially known with this respect is the queen mandibular gland pheromone (QMP) that for many years was considered to be the primer cause for worker sterility (Butler 1959), but this report was challenged in recent years (Willis *et al.* 1990). It follows, from an on going arms race point of view, that workers should be selected to bypass the effect of such pheromones. Although primer pheromones affecting worker sterility were postulated for many years, experimental proof of their existence is surprisingly scant or non-existent. This led to the formulation of a new hypothesis that queen specific pheromones act as signals rather than directly control worker physiology, and that workers behave in a signal-dependent manner so as to increase their own inclusive fitness (Keller and Nonacs 1993). Such signal should not only convey the presence of a queen in the colony, but also her quality, e.g., how fecund she is. While proof that the queen presence in the hive is pheromonally broadcasted, there is no evidence that these pheromones also signal her reproductive status.

Whether the queen pheromones act by direct inhibition of worker reproduction or through pheromone broadcasting, the question why honeybee queens have multiple pheromonal sources when a single pheromone should suffice remains open. In the following we shall attempt to address this evolutionary enigma.

QUEEN-SPECIFIC PHEROMONES IN THE HONEYBEE.

Honeybees are endowed with exocrine glands that produce many caste specific pheromones (Blum 1992). The queen mandibular glands produce a caste-specific bouquet of six components of which 9-oxo-2-decenoic acid is a major constituent (Slessor *et al.* 1988). The queen mandibular pheromone (QMP) is by far the best studied queen specific pheromone and was found to have major effects on worker behavior including primer and releaser effects (Winston and Slessor 1992). The most pronounced releaser effect of the pheromone is that of retinue behavior. Workers, primarily young nurses, constantly surround the queen, antennate and lick her extensively. The significance of this behavior is not only in inducing nurse bees to constantly groom the queen, but also provide an effective means for the dissemination of QMP to other workers in the hive. Studies with radioactive QMP components have shown that they disperse quickly in the hive, as a bouquet rather than as single compounds (Naumann 1991; Naumann *et al.* 1992). Other releaser effects of the pheromone include stimulating foraging, brood rearing, comb building, and food storing. Its primer activity includes the inhibition of emergency queen rearing (Winston *et al.* 1989), and perhaps it also contributes to the inhibition of worker ovarian development (Winston 1987).

The feces of virgin queen contain *o*-aminoacetophenone that acts as a worker repellent when tested in small worker-groups and seems to terminate agonistic interactions between the queen and the workers (Page *et al.* 1988). Queen feces contain also a series of long chain esters, dominated by decyl decanoate, that were also identified from sting apparatus extracts (Blum *et al.* 1983). These apparently modulate nestmate recognition since workers attack their nestmates that are smeared with these esters (Breed *et al.* 1992).

The abdominal tergal glands are fortified with oleic acid, long chain esters, and unsaturated hydrocarbons, the profile of which exhibit caste specificity (Wossler and Crewe 1999a). The queen's secretion is attractive to workers that exhibit a typical retinue behavior around the source, albeit less pronounced than QMP (Wossler and Crewe 1999b). It also inhibits ovarian development when tested in small groups of caged workers (Wossler and Crewe 1999c).

Dufour's gland that is associated with the sting apparatus is yet another abdominal gland that exhibits caste specific composition. The queen Dufour's pheromone (QDP) is composed of a complex mixture of hydrocarbons accompanied by a series of long chain esters (that are different from those of the tergal glands or the sting apparatus), whereas worker secretion contains only hydrocarbons (Katzav-Gozansky *et al.* 1997). The secretion is attractive to workers that form a retinue behavior around a surrogate queen smeared with the secretion. It is the esters constituents that are responsible for this activity, as revealed by attraction bioassays using a bouquet of synthetic esters (Katzav-Gozansky *et al.* 2001; Katzav-Gozansky *et al.* 2002).

QUEEN PHEROMONE PLASTICITY IN WORKERS

Queen pheromonal specificity does not seem to involve a "fixed caste-specific pheromone biosynthetic pathway" but rather show plasticity. At least for QMP and QDP it was demonstrated that under hopeless QL situation, i.e., in the absence of eggs or young brood from which replacement queens can develop, workers that initiate ovarian development concomitantly demonstrate queen-like secretions. This is not surprising considering that diploid larvae are totipotent to develop into queens or workers, and therefore both should possess the machinery for the queen pheromone production. Studies on the biosynthesis of both glandular secretions shed some light on this plasticity. This biosynthetic plasticity is also accompanied by behavioral changes, the effects of which render these workers to become "false queens".

The mandibular glands of queens are typified by carboxylic acids that have functionality at the ω -1 carbon including both enantiomers of 9-hydroxy-2-decenoic acid as well as 9-oxo-2-decenoic acid. Workers, in contrast, are typified by the occurrence of 10-hydroxy-2-decenoic acid: functionality being placed on the ω carbon. Biosynthesis studies revealed that the difference between workers and queens is at this initial step of functionalization from which the two biosynthesis pathways disparate, resulting the caste specific bouquets (Plettner *et al.* 1996). However, this functionalization step is not irreversible since egg-laying QL-workers show all the queen components, albeit in small quantities (Crewe and Velthuis 1980). These workers become attractive to other workers and can be considered as “false queens”. In fact, detailed observation in a QR colony have shown that also retinue bees become “false queens” for a short period after being in contact with the queen (Juska *et al.* 1981). It was further shown that these workers are highly contaminated by QMP after contacting the queen (Slessor *et al.* 1998), corroborating the above behavioral observation.

Pheromone plasticity in workers was also demonstrated with Dufour’s gland secretion. Using radioactive tracers, ester biosynthesis was studied both *in vivo* and *in vitro*. As predicted from its caste specific chemical composition, queens but not QR workers synthesize esters following an injection of radioactive acetate *in vivo*. Queenless workers on the other hand showed a fair amount of *de novo* ester biosynthesis (Katzav-Gozansky *et al.* 1997; Katzav-Gozansky *et al.* 2000). This implies a reversible inhibition of esters biosynthesis in workers that is exerted by the queen. It was further hypothesized that ester biosynthesis in the gland is the default situation and that workers that perceive the queen pheromone produce an inhibitor that shuts this specific biosynthetic pathway. It follows that if we detach the gland from its regulatory factors, i.e., by its incubation *in vitro*, it should produce the esters irrespective whether the workers contributing the gland were QR or QL. The results of such an *in vitro* incubation using radioactive acetate as a precursor were consistent with that hypothesis. Unlike the case *in vivo*, glands that originated from QR workers were able to synthesize the queen-like esters *in vitro*, albeit after a certain time-lag compared to glands that originated from QL workers. The time-lag observed by the glands of the QR workers was apparently needed for recruiting the biosynthesis machinery as evident by and early rise of the corresponding alcohols and their decline thereafter, concomitantly with the rise in the esters (Katzav-Gozansky *et al.* 2000). As in the case of QMP, workers that possess QDP become attractive to other worker (Katzav-Gozansky *et al.* 2003). Although workers may form retinue around such a “false queen”, whether QDP is transferred between workers during such acts, or whether it is dispersed between the hive bees under QR conditions remains to be investigated.

THE QUEEN PHEROMONES EFFECT ON WORKERS OVARIAN DEVELOPMENT

The most important primer effect of the queen, from an evolutionary point of view, is the inhibition of worker reproduction. Early studies with QMP have shown that application of the pheromone to QL group prevented worker reproduction (Butler 1959). The role of QMP was recently reevaluated giving contrasting results than the earlier report. QMP was applied to QL hives for 43 days at doses ranging from 10^{-3} to 10 queen equivalent per day, but none of these doses inhibited worker ovarian development (Willis *et al.* 1990).

A recent study may point to the fact that the tergal gland pheromone may have an inhibitory effect on worker reproduction. Bioassays in which glass dummy-queens that were applied with tergal gland secretion were introduced into QL groups showed that this secretion is effective in blocking worker reproduction (Wossler and Crewe 1999c).

The queen specific Dufour's gland secretion makes it another candidate for mediated queen control over worker reproduction. We tested this hypothesis using 4-combs mini-hives (22x27x24cm) including 5 different experimental groups. 1). A QR colony in which the queen was confined to one part of the hive by a queen excluder. 2). A QL colony that was left untreated. 3). A QL colony supplied with QMP. 4). A QL colony supplied with QDP. 5). A QL colony supplied with both QMP and QDP. The hives were checked daily for presence of eggs. When egg-laying was observed in the untreated QL nest, a sample of 15 workers from the QL part of each colony were collected every 2 days for a total of 5 samples. The bees were dissected, and their ovarian development was assessed according to (Velthuis 1970) (undeveloped ovaries, early stage of development and ovaries with full size egg).

The results are presented in Table 1. As expected, the overwhelming majority of the QR workers had undeveloped ovaries which was statistically different from that of all the QL colonies, treated or untreated (ANOVA, Fisher's LSD post hoc test $p < 0.001$). Among the QL colonies, those treated with QMP or QMP + QDP had higher percentage of inhibited workers when compared to either the untreated QL or the QL treated with QDP only. Colonies treated with QMP+ QDP were statistically different from QR colonies and from colonies treated with QDP (ANOVA, Fisher's LSD post hoc test $p = 0.044$ and $p = 0.00017$, respectively).

Table 1: Percentage of QR and QL workers with developed ovaries as a function of treatment either with QMP, or QDP, or their combination. Values for undeveloped ovaries that are accompanied by different letters are statistically different (ANOVA, Fisher's LSD post hoc test; n- number of replicates).

Ovarian development	Queenright (n=5)	Queenless (n=5)	QL+QMP (n=5)	QL+QDP (n=4)	QL+QMP+QDP (n=5)
Undeveloped	94.1+3.3 ^a	59.5+8.1 ^b	65.7+4.1 ^{bc}	47.6+3.8 ^c	67.5+4.2 ^{bc}
Early stage	1.5+0.9	23.1+4.1	21.0+3.7	32.0+3.1	17.7+4.0
Mature eggs	4.4+2.9	17.4+4.8	13.3+2.6	20.5+2.5	14.8+2.7

These results indicate that none of the queen pheromone alone is able to mimic the inhibitory effect the queen exerts on worker reproduction. QMP alone or in combination with QDP were a little more effective but still significantly inferior to the effect of a live queen.

THE EVOLUTION OF MULTIPLE QUEEN SIGNALS IN HONEYBEES

The vast knowledge on honeybee pheromones and especially the richness of queen-specific pheromones provide a good opportunity to hypothesize on the possible evolution of such pheromones. 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. For example three pheromone bouquets from disparate gland are very effective in eliciting retinue behavior. Two of these, mandibular and tergal pheromones, also act as primer pheromones implicated in the control of worker reproduction. In view of the fact that many of the queen-specific pheromones elicit multiple behavioral responses in workers, it will not be surprising if the other queen-specific pheromones encompass both releaser and primer effects. Nonetheless, none of the queen-specific pheromones when tested alone was able to mimic the living queen. This suggests that the perception of the complete set of the queen pheromones, or at least a large part of it, is necessary for the proper reaction of the workers (Keeling *et al.* 2003).

The question that arises is what were the selection pressures that have driven the evolution of such multiple pheromonal sources, when theoretically one set should have sufficed. We hypothesize that the evolution of multiple queen pheromone-sources is the result of an ongoing arms race between the queen and her workers - primarily over the production of males. Kin selection theory predicts that because the honeybee queen is multiply inseminated workers should coalesce with the queen in eliminating worker-laid eggs (Woyciechowski and Lomnicki 1987; Ratnieks 1988). One consequence of worker policing is the high cost for worker reproduction resulting in worker-reproductive self-restraint. This seemingly has resolved the queen-worker conflict, the queen winning the race. However the fact the workers have retained the ability to develop ovaries as well as conserved their ability to produce the queen-like pheromones suggests that the conflict is not fully resolved and that an arms race may be still in effect. The discovery of a mutant in which worker-laid eggs evade policing resulting in the majority of males being the progeny of workers (Oldroyd *et al.* 1994) lend credence to the arms race. Another bee race, *Apis mellifera capensis*, may have responded in a different way. Workers are able to produce diploid eggs from which workers or queens develop. Interestingly in the presence of a queen *A. m. capensis* workers refrain from reproduction but if introduced to an *A. m. mellifera* they rapidly dominate reproduction (Hepburn and Radloff 2002). Interestingly QR workers of *A. m. capensis* unusually possess also the queen characteristic pheromones, at least for the mandibular (Crewe and Velthuis 1980) and Dufour's glands (Sole *et al.* 2002). The coupling between worker reproduction the pheromonal mimicry may be another indication of the ongoing arms race. Another discovery supporting the arms race hypothesis is a line of bees that are less responsive to QMP (Pankiw *et al.* 1994; Pankiw *et al.* 2000). According to the arms race hypothesis the queen have evolved a pheromone that either acts as a signal or directly control worker reproduction, for example QMP. The workers in turn may have become less sensitive to the pheromone, exerting selection on the queen to evolve another pheromone from another glandular source. The end results is that the queen have multiple pheromonal sources, none of which is able to mimic a living queen, but their combination may act either additively or synergistically (Keeling *et al.* 2003).

The ability of egg-laying workers to mimic the queen pheromone also warrants an explanation. The advantage for egg laying worker under "queenless hopeless situation" is clear. There is a very small period between the loss of the queen and the social breakdown of the colony. Workers that try to reproduce too early may be antagonized by their nestmates, whereas the late reproducers may miss the opportunity due to the breakdown of the social organization of the nest (Page and Erickson 1988). 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 therefore can be argued that worker-mimicry of the queen pheromone is maintained by the above advantage. However, "queenless hopeless situation" are unlikely to occur, or a very rare, in nature. Generally even if the queen is lost there is ample young brood for requeening the colony. If queen mimicry is a part of the queen - worker arms race, why queen mimicry in these workers is extremely rare, if exists at all? There are reports that workers in the hive can detect egg-laying workers and aggress them. It is not known whether the possession of queen pheromones disclose the egg laying workers, but they are a likely candidate. Such punishment on such reproductive challenger was reported in the ant *Dinoponera quadricaps* (Monnin *et al.* 2002).

While many questions regarding pheromonal evolution remain unanswered, it emerges clearly that the honey bee queen represent an excellent model for studying a complex set of pheromones all of which, singly and in concert, direct the complicated as well as sophisticated social behavior.

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