

Review Article

Queen Pheromone and Monopoly of Reproduction by the Queen in the Social Wasp *Ropalidia marginata*

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Ropalidia marginata is a primitively eusocial (truly social) wasp found in peninsular India. It is different from the typical primitively eusocial species in having docile queens that cannot use aggression to maintain reproductive monopoly. Recent studies using chemical analysis and bioassays indicate that Dufour's gland is a source of the queen pheromone in this species. Queens appear to signal their presence to workers through their Dufour's gland compounds, possibly by applying them on the nest surface, and this results in suppression of reproduction by workers, resulting in reproductive monopoly by the queen. The Dufour's gland was found to contain saturated long chain hydrocarbons, which have recently been suggested to be the ancestral state of fertility signals in Hymenoptera. The Dufour's gland composition differed significantly between queens and workers, and was also correlated with the state of ovarian development, varying continuously as a function of ovarian development, thereby advocating the honesty of the queen pheromone. This elucidates the mechanism of maintenance of eusociality through pheromonal queen signalling by the Dufour's gland compounds.

Key Words: Eusocial; Reproductive Monopoly; Queen Pheromone; Dufour's Gland; *Ropalidia marginata*; Honest Signal

Social insects like bees, ants and wasps have aroused the interest and imagination of humans since ages. They captivate our attention by the ways in which they organise and regulate their complex societies, employing their social design to solve various ecological problems. They have been held among the greatest achievements of evolution, illustrating the entire range of levels of organisation, from the molecular to the societal level (Wilson, 1971). The functioning of a social insect colony, involving hundreds, or even thousands of individuals simultaneously has been compared to the functioning of a single organism, or "superorganism" (Hölldobler and Wilson, 1990). How and why such complex altruistic societies evolved from solitary, selfish individuals remained unexplained for a long time. Even Charles Darwin was baffled by social insects and remarked "...one special difficulty, which at first appeared to me insuperable, and actually fatal to my

whole theory. I allude to the neuters or sterile females in insect communities: for these neuters often differ widely in instinct and in structure from both the male and fertile females, and yet from being sterile they cannot propagate their kind." (Darwin, 1859). This paradox was formally solved by W. D. Hamilton in 1964 in two seminal papers where he gave a formal theory stating evolution of altruism (Hamilton, 1964a; 1964b). Stated simply, it implies that even if an individual does not reproduce at all in its lifetime, it can still have some fitness by helping another individual to which it is genetically related, to reproduce. Thus an individual can give up reproduction and still pass on its genes to the next generation by helping its relatives to reproduce, as it always shares some genes with its relatives. This is now famously known as "Hamilton's rule" or "kin selection theory", and has subsequently stimulated entire fields of research resulting in the betterment

of our knowledge and understanding as to why animals should help each other.

Social insects are perhaps the best example of altruistic animals, as here altruism can be said to have reached its zenith, resulting in most individuals of a colony (workers) permanently sacrificing their ability to reproduce. They work for the benefit of the colony instead, with a few individuals specialising in reproduction alone (queens), literally functioning as egg-laying machines. This is why social insects are considered as “eusocial” or “truly social” organisms, characterised by cooperative brood care, differentiation of colony members into fertile reproductives and sterile non-reproductives, and overlap of generations (Batra, 1966; Michener, 1969; Wilson, 1971). Eusocial insects are further subdivided into primitively eusocial and highly eusocial species. Species where the reproductive caste or queen is morphologically distinguishable from the non-reproductive caste or worker are called as highly eusocial, while the species where there is no morphological difference between queen and worker are called as primitively eusocial. However, irrespective of the existence of morphological caste differentiation, the main feature of queens is that they either monopolise reproduction, or carry out a disproportionately large fraction of reproduction in the colony, with workers generally functioning as sterile individuals.

Amongst insects, eusociality has been observed mostly in Hymenoptera (all ants, some bees and wasps) and Isoptera (all termites), with a few examples being observed in Hemiptera (some aphids), Thysanoptera (a few thrips) and Coleoptera (one species of beetle) (Hölldobler and Wilson, 1990; Michener, 1974; Michener, 2000; Ross and Mathews, 1991; Aoki, 1977; Crespi, 1992; Kent and Simpson, 1992). Amongst all social insects, hymenopterans have received the majority of attention.

Study of eusociality can be broadly divided into two categories: (a) evolution of eusociality and (b) proximate mechanisms involved in maintenance of eusociality. The first part was addressed successfully by kin selection theory, which remains as the predominantly accepted paradigm till date (Hamilton

1964 a, b). It specifies certain conditions under which eusociality should spread in the population, simultaneously explaining conditions under which altruism is expected to evolve and those under which selfishness is expected to evolve.

Hamilton’s rule can be stated in a simplified version by the algebraic inequality:

$$\frac{b}{c} > \frac{1}{r}$$

where “b” is the benefit to the recipient of altruism, “c” is the cost to the altruist, and “r” is the coefficient of genetic relatedness between the recipient and the altruist, i.e. the probability of any gene present in the altruist also being present in the recipient by common descent. Benefit and cost are measured in fitness terms, i.e. number of surviving offspring. The equation can be rewritten as $r.b > c$, meaning the benefit to the recipient devalued by the genetic relatedness should be greater than the cost of the altruist. Thus it can be understood as to why workers remain sterile for their whole lives, working to help raise the queen’s offspring. They gain indirectly, increasing their inclusive fitness by helping a relative (the queen, who is usually their mother), that far outweighs any direct fitness they might have gained by attempting to reproduce themselves. Hamilton’s rule has pioneered a new era in the study of social behaviour, and social insects have been at the forefront of this new field (Wilson 1971, Hölldobler and Wilson 1990, Crozier and Pamilo 1996).

Maintenance of Eusociality by Queens of Social Hymenoptera

Maintenance of eusociality refers to maintenance of reproductive monopoly by the queen, and there are generally two modes by which this occurs in social insects – aggression and pheromone. Aggression refers to direct physical confrontation or dominance behaviour, where the dominant individual (queen) directly attacks, bites, attempts to sting or shows other acts of aggression towards the subordinate individuals, who are thereby socially dominated into taking up the role of workers. Pheromone refers to a chemical signal or cue released by the queen, which

when perceived by the workers results in inhibition of reproduction by workers resulting in reproductive monopoly by the queen. Hence aggression or dominance behaviour is also called as a physical method of queen control over worker reproduction, while pheromone is considered as a chemical method of the queen signalling her presence to the workers (Keller and Nonacs, 1993).

In most primitively eusocial species (queens and workers morphologically similar), the queen usually occupies the top-rank in the dominance hierarchy and is generally believed to use aggression to maintain her status. The queen is believed to subdue other females into restraining them as reproductively sterile workers in the colony (Pardi, 1948; West-Eberhard, 1969; Röseler, 1981). The queen stays on the nest while workers go out for foraging, and the queen often acts as the central pacemaker or work regulator of the colony. Since queens occupy the top of the dominance hierarchy, the aggression shown by them is often believed to play a dual role of suppressing worker reproduction and regulating worker activity (West-Eberhard, 1969; Brothers and Michener, 1974; Breed and Gamboa, 1977; Dew, 1983; Reeve and Gamboa, 1983, 1987; Litte, 1981; Gamboa *et al.*, 1990; Reeve, 1991). In some species like *Ropalidia cyathiformis* the queen (or alpha) directs most of her aggression towards the one who is next in dominance hierarchy, or beta, who in turn shows aggression to all other colony members (Kardile, 2006; Bhadra, 2008). In hover wasps (Stenogastrinae), in addition to being the most dominant, the queen is also the oldest individual of the colony, as age and dominance have a strong correlation (Field *et al.*, 1999; Field *et al.*, 2006). In addition to aggression or dominance behaviour, differential oophagy or policing is also employed, where the queen destroys and eats eggs laid by the workers, thereby ensuring that only the queen's eggs give rise to the next generation (Itô, 1993). In the paper wasps *Polistes dominulus* and *Polistes satan*, facial markings differ between dominants and subordinates, and hence between queens and workers, which hint at a possible visual queen recognition system based on facial markings (Tibbetts and Dale, 2004; Tannure-Nascimento *et al.*, 2008). Sweat bees (Halictidae) are primitively

eusocial and have behaviourally dominant queens who do not participate in or show very low rates of foraging, and can behaviourally regulate colony activity. Queens are mated and have larger ovaries while workers are mostly unmated have slender ovaries. Queens are believed to use aggression to suppress worker reproduction (Michener, 1990a). In some ants of subfamilies Amblyoponinae, Ectatomminae and Ponerinae, there is no morphological difference between queens and workers, and the morphologically distinct queen caste is believed to have been lost secondarily and replaced by mated workers, called "gamergates" (Peeters, 1993, 1997; Peeters and Crewe, 1984). Although such ants are perhaps not equivalent of other typical primitively eusocial species, like wasps, the absence of morphologically distinct caste qualifies them as primitively eusocial and they also present other situations similar to other primitively eusocial groups. In some species, reproduction is regulated by a dominance hierarchy, and the individual occupying the top-most rank in hierarchy (alpha individual) attains the status of gamergate, and workers can show aggression to periodically overthrow senile gamergates. In some species of *Diacamma*, the gamergate clips off the wing buds or gemmae of all newly eclosed workers of the colony, rendering them unable to mate, and thereby ensuring reproductive monopoly by enforcing them to lead sterile lives (Peeters and Higashi, 1989; Peeters *et al.*, 1992). It should be remembered however that these Ponerine ants are derived from other highly eusocial ancestors and can be considered as an example of reverse evolution, wherein they have secondarily lost the characteristics of highly eusocial species that were present in their ancestral lineage. Therefore, any means of chemical control of worker reproduction by the queen pheromone that might have existed in their ancestors may have been lost secondarily in them resulting in evolving alternate strategies of achieving reproductive monopoly by the queen by dominance hierarchy, or by mutilating the gemma.

The external body surface of insects is called the cuticle. It is a hard exoskeleton and is covered by a waxy layer of hydrocarbons whose primary function is to prevent desiccation and protecting the insect

from injury and pathogens. The cuticular hydrocarbons have secondarily acquired a role in chemical communication in many insects. Cuticular hydrocarbons have been shown to vary between foundresses and workers in different species of paper wasps, and have been also shown to be correlated with the fecundity of the individuals (Bonavita-Cougourdan *et al.*, 1991; Sedge *et al.*, 2001; Tannure-Nascimento *et al.*, 2008; Izzo *et al.*, 2010). Chemical difference of queens and workers in secretion of the van der Vecht's gland has also been shown in wasps (Dapporto *et al.*, 2007). In queen-less ants as well, differences in cuticular hydrocarbon profiles of gamergates and workers have been found, and the profiles have been shown to be correlated with fertility (Monnin *et al.*, 1998; Peeters *et al.*, 1999; Liebig *et al.*, 2000; Cuvillier-Hot *et al.*, 2001). Since queens in primitively eusocial species are generally believed to use dominance to maintain their status, this growing evidence for chemical differences suggest that chemical cues or pheromones may be used along with dominance to signal the presence of a queen to her workers. Queen control by aggression is generally believed to be restricted to species that have comparatively smaller colonies, and thus, achieving such control through dominance should become difficult and ineffective in larger colonies owing to the sheer numbers of individuals that would have to be dominated (Keller and Nonacs, 1993; Seeley, 1995).

In highly eusocial species that have much larger colony sizes, the queen maintains her status through chemical means by producing a pheromone that results in suppression of worker reproduction (Free, 1987; Winston and Slessor, 1993; Monnin, 2006). Highly eusocial species are characterised by morphologically distinct queens and workers. Since the queen is morphologically different, and body size in insects cannot change during the adult stage, caste determination in such species usually takes place during larval stage, and the fate of an adult individual that ecloses from the pupa is already fixed. Pheromonal signalling by the queen is the hallmark of highly eusocial hymenopterans. The honey bee queen pheromone has been extensively studied, and although multiple sources have been identified, the

main component is the mandibular gland pheromone which is believed to be directly responsible for inhibition of ovarian development in workers (Free, 1987; Winston and Slessor, 1993). When the quality of the queen pheromone becomes poor due to reduction or loss of fertility of the queen, the workers can kill the queen and rear a new queen (Engels and Imperatriz-Fonseca, 1990). The Dufour's gland has also been shown to be a source of fertility signal in honey bees (Dor *et al.*, 2005; Katzav-Gozansky *et al.*, 2002a, b), and bumble bee queens differ from workers in their cuticular chemicals that may act as queen signal (Sramkova *et al.*, 2008). Queens in vespine wasps are also believed to use pheromones, which seem to be transferred by trophallaxis or other direct contact (Landolt *et al.*, 1977; Akre and Reed, 1983; Greene, 1991). In ants, cuticular compounds are believed to be the main perpetrators involved in queen signalling, and most of the evidence of cuticular hydrocarbons signalling reproductive status come from ants. Cuticular hydrocarbons have been shown to be correlated with reproduction in several major ant subfamilies – Myrmeciinae, Myrmicinae, Formicinae and Dolichoderinae, in addition to the archaic subfamily Ponerinae, where queen-less species are placed (reviewed in Monnin, 2006). It has also been shown by electrophysiological studies in conjunction with bioassays that workers actually perceive and react to compounds characteristic of the queen cuticle (D'Ettorre *et al.*, 2004). Since queens in highly eusocial species are docile, they are expected to rely heavily on pheromones for communicating with workers, and the maintenance of reproductive monopoly depends on chemical signalling. The pheromone has an effect on the physiology of workers resulting in the prevention of ovarian development. Both aggression and pheromone seem to serve their purpose well in maintenance of eusociality in the species concerned.

Queen Control Versus Queen Signal

In most primitively eusocial species, the queen dominates other individuals behaviourally and is generally believed to suppress reproduction by workers by sheer aggression. Workers have no choice but to be dominated by the queen and hence are forced

to either stay on the nest as sterile individuals, or to leave the colony. This is easy to understand, because in general, receiving aggression can have an adverse effect on the physiology of an organism by increasing stress, thereby rendering the individual sterile. Regarding pheromone however, it would be relatively easier to escape from its effect, if the need arises, by biochemically neutralising the effect of the pheromone. To explain the maintenance of queen status by using pheromone and contrast it with aggression, in a landmark paper, Keller and Nonacs (1993) put forth the idea of honest pheromonal signalling. The queen pheromone should be an honest signal of fertility of the queen, and on perceiving it workers should refrain from reproduction by themselves (sometimes called as “self policing”). Here the workers are not being forced to abstain from reproduction directly as in case of aggression, and they are free to evolve some strategy to evade the effect of the pheromone. Yet, workers do not reproduce in presence of the queen, who is several folds more fecund than the workers and advertises this through her pheromone. Workers would gain more inclusive fitness by allowing such a fertile queen to reproduce, rather than reproduce themselves. The signal should be honest, otherwise it will not be able to evolve, as both signaller and receiver will not benefit from such a signal. Also, if it is not honest, the system will be prone to invasion by cheaters. This idea finds support in the fact that the queen can be overthrown and even killed by the workers when her fertility starts to decline (Engels and Imperatriz-Fonseca, 1990). The quality or strength of the pheromone can be a useful proxy by which the workers assess the fertility condition of their queen. It has also been argued that a signal should be costly in order to be honest, as that would be an efficient way of advertising the quality of the signaller thereby increasing the chances of the receiver responding to it, and this would also automatically make it resistant to invasion by cheaters. In absence of high cost associated with a signal, a reliable communication system using that signal should be difficult to evolve (Zahavi and Zahavi, 1997; Zahavi 2008). However, studies that specifically look at the cost of the queen signal are yet to be conducted. Thus in general, in

primitively eusocial societies, the queen is believed to actively suppresses workers by aggression, while in highly eusocial societies, she signals her presence by pheromone, and the onus is on workers to abstain from reproduction in presence of the queen pheromone. Such contrast between queen control by aggression and queen signal by pheromone has implications on the level of conflict over reproduction in a colony. Conflict is expected to be higher in species where the queen uses aggression, and lower in species where she uses pheromone. This probably results in higher colony productivity in highly eusocial species like honey bees and ants as compared to primitively eusocial species like paper wasps.

Ropalidia marginata

Ropalidia marginata is a tropical paper wasp found in peninsular India, Sri Lanka and South-East Asia (Vecht, 1962; Das and Gupta, 1983) (Fig. 1). It belongs to subfamily Polistinae under family Vespidae of order Hymenoptera. They construct open nests (not covered by envelope) with paper which they prepare by masticating cellulose material collected from plants and mixing it with their saliva; the nests have one or more pedicels (stalks). Nest initiation occurs throughout the year with absence of



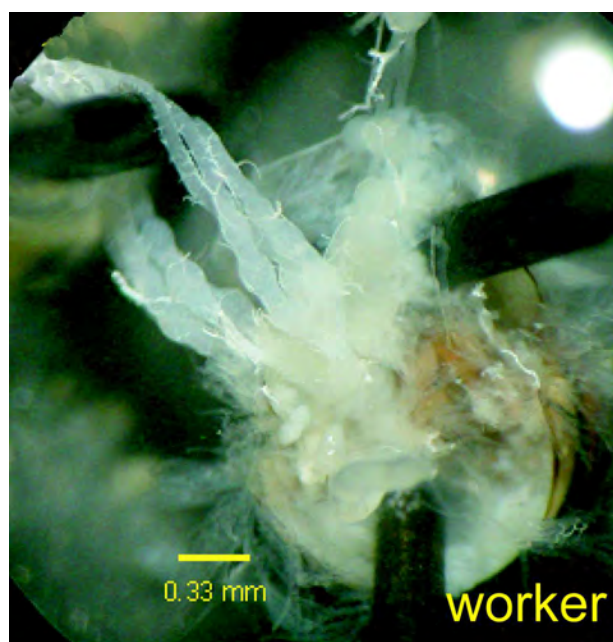
Fig. 1: Photograph showing a colony of *Ropalidia marginata* with individuals marked with non-toxic, quick-drying enamel paint to enable identification of individual wasps. The queen is indicated with an arrow and is morphologically similar to workers

any well-defined seasonal colony cycle, and nests also get abandoned throughout the year (Gadagkar *et al.*, 1982). Nests occasionally get preyed upon by hornets (*Vespa tropica*), and the wasps can get parasitised by solitary parasitic wasps (families Ichneumonidae and Torymidae), flies (family Tachinidae) and strepsipterans (order Strepsiptera) (Gadagkar, 2001). Colony sizes have been found to range from 1 to 200 females (Gadagkar *et al.*, 1982), and the approximate colony sizes found are generally between 10 to 30 females (personal observation). Males are produced throughout the year, and they rarely participate in any colony maintenance task. After eclosion, males stay on the nest for about a week and then leave the nest to lead a nomadic solitary life, and mating does not occur on the nest (Gadagkar, 2001; Sen, 2007). Each nest has a sole egg layer at any point of time, the queen, who lays single eggs in each cell, which then develop into larvae, are fed by workers and grow into pupae from which they eclose out as adults. The approximate time taken for brood development from egg to adult is around 62 days (Gadagkar *et al.*, 1993). Although there is only one queen on a nest at a given point of time, throughout its existence, a nest can experience several queens, temporally separated from each other, a phenomenon called serial polygyny (Gadagkar *et al.*, 1993). Both queens and workers can be mated, and queens have been found to be multiply mated as well (Muralidharan *et al.*, 1986). Nests can be founded by solitary or multiple foundresses and nest joining, usurpation and adoption have also been observed (Shakarad and Gadagkar, 1995). A wasp eclosing on a nest has several options available. She can (i) leave her natal nest and start her own solitary foundress colony, (ii) leave her natal nest and initiate a multiple foundress colony along with her nestmates or with wasps from other nests, (iii) leave her natal nest and join another colony, (iv) stay in her natal nest as a worker for her entire life, or (v) stay in her natal nest as a worker for some time and then become the queen of her colony, by overthrowing the existing queen or when the existing queen dies (Gadagkar, 2001). This variety available in options, makes *R. marginata* an interesting species to study with respect to evolution of eusociality, and the fact that the queen maintains reproductive

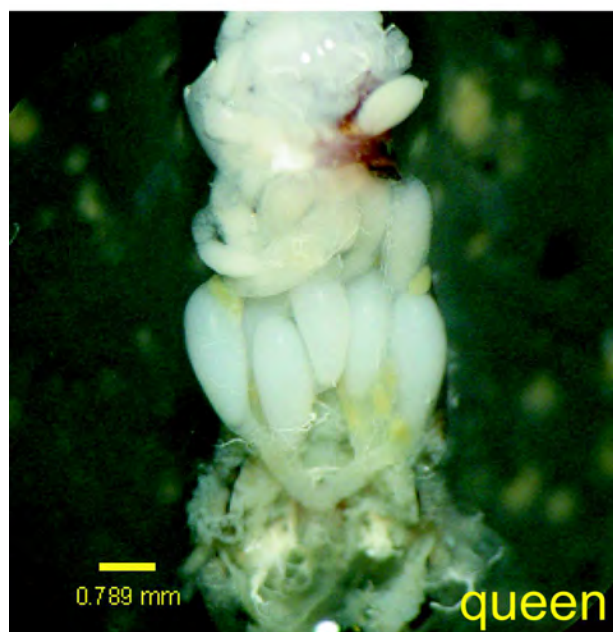
dominance over the workers despite being docile and non-interactive creates interest in studying the proximate mechanisms by which eusociality is maintained.

The Queen in *R. marginata*

The queen in *R. marginata* is morphologically similar to workers, and is the sole egg layer of a colony, confirmed from observations as well as ovarian dissections (Chandrashekara and Gadagkar, 1991 a; Gadagkar, 2001) (Figs. 1 and 2). Queens are also generally mated, as are some of the workers, but unmated queens have also been observed occasionally (Chandrashekara and Gadagkar, 1991 b; Mitra, 2011). Queens usually do not participate in any dominant subordinate interactions, rarely occupy top-ranks in the dominance hierarchy, and behaviourally belong to the docile “sitter” caste, amongst the three behavioural castes – sitters, fighters and foragers (Chandrashekara and Gadagkar, 1991a; Gadagkar and Joshi, 1983). Since the queen is a docile and non-interactive individual, unlike other primitively eusocial species, she is not the central pacemaker of the colony, and the workers themselves regulate colony activities by dominance- subordinate interactions (Bruyndonckx *et al.*, 2006; Lamba *et al.*, 2008). Queens can have longer life span than workers, life span of workers being 27 ± 23 days (mean \pm standard deviation) while that of queens being 103.5 ± 65.3 days (Gadagkar *et al.*, 1982; Gadagkar *et al.*, 1993). When a new queen establishes herself in the colony, it has been found that the individual who becomes the potential queen and subsequently the new queen, does not differ from other workers with respect to general behaviour, rank in dominance hierarchy, age, body size or ovarian development status, in presence of the existing queen (Chandrashekara and Gadagkar, 1992; Gadagkar *et al.*, 1993; Deshpande *et al.*, 2006). Thus the individual who becomes the potential queen has been called as an “unspecialised intermediate”. It has been found that there is a queen succession hierarchy in the colony and the wasps themselves seem to know the identity of at least the first successor (Bhadra and Gadagkar, 2008; Bang and Gadagkar, 2012). How the hierarchy is determined and how the wasps



(A)



(B)

Fig. 2: Photographs showing the ovaries of a worker and a queen of *Ropalidia marginata*. The queen ovary can be seen to have many mature eggs, while the worker ovary is undeveloped

themselves identify the next successor remains unknown.

As the queen is a docile individual and hence cannot be using aggression to maintain her status, it was speculated that she might be using a pheromone

to do so (Sumana and Gadagkar, 2003). Workers constantly perceive the presence of their queen, as they realise her absence within approximately half an hour of queen removal, and it was found that the rates of interaction of workers with the queen are not frequent enough to explain the rapidity with which the queen's absence is perceived (Bhadra *et al.*, 2007). It was also found that workers do not perceive their queen when separated from her by a wire mesh partition, suggesting that if a pheromone is indeed involved, it is likely to be non-volatile (Sumana *et al.*, 2008). In a bioassay it was found that a crude macerate of the queen's Dufour's gland can act as a proxy for the queen herself, suggesting that the Dufour's gland is probably one of the sources of the queen pheromone in this species (Bhadra, 2008; Bhadra *et al.*, 2010).

Thus *R. marginata* queens possess characters of both primitive and highly eusocial species. Their morphological similarity with workers places them in primitively eusocial category, but features like non-dominant behavioural profile, non-interactive nature, not featuring as the central pacemaker of the colony, and presence of pheromone bring them closer to highly eusocial species. Although the queen is docile, when she establishes herself as a new queen of her colony, she undergoes a phase of intense dominance behaviour, and can be said to be similar to queens of other primitively eusocial species in this phase of her life. This makes *R. marginata* an interesting system to study concerning the proximate mechanisms by which the queen maintains reproductive monopoly.

The Dufour's Gland as a Source of the Queen Pheromone in *R. marginata*:

It had been speculated that the queen in *R. marginata* might be using pheromones to communicate her presence to the workers (Sumana and Gadagkar, 2003). If the queen is lost or experimentally removed from the colony, one of the workers becomes highly aggressive within a few minutes, and if the queen is not returned, this individual develops her ovaries, gradually loses almost all her aggression and becomes the new queen of the colony after a few days. This individual has been designated as the potential queen

(PQ) (Chandrashekara and Gadagkar, 1992; Premnath *et al.*, 1996; Deshpande *et al.*, 2006). If the queen is returned to the colony within a day of her removal, the PQ decreases her aggression and goes back to being a worker in the colony (Sumana and Gadagkar, 2003). The fact that the PQ becomes very aggressive soon after queen removal suggests that at least the PQ perceives the absence of the queen within a few minutes. It has been shown that the rates of interaction of the PQ with the queen (both direct as well as relayed interactions through other workers) are not frequent enough to explain the rapidity with which the queen's absence is perceived (Bhadra *et al.*, 2007).

So, if the queen indeed uses a pheromone to signal her presence, how the signal reaches the workers remained poorly understood till the possibility was explored that queens may apply pheromone onto the nest surface. It was observed that the queen (but not the workers) frequently rubs her abdomen on the nest surface leading to the speculation that this might be a method of depositing pheromone on the nest surface (Bhadra *et al.*, 2007). Since the queen rubs the ventral side of the tip of her abdomen, another study focused on the Dufour's gland, which opens into the tip of the abdomen (Fig. 3), and has been shown to be one of the sources of the queen signal in honey bees (Katzav-Gozansky *et al.*, 1997; Katzav-Gozansky *et al.*, 2002a, b; Dor *et al.*, 2005), and considered the possibility that the Dufour's gland is a source of queen pheromone in *R. marginata*. Using the fact that the PQ loses her aggression immediately on return of the queen, a

bioassay was developed in which it was found that a crude macerate of the queen's Dufour's gland (Dufour's gland crushed in Ringer's solution) can act as a proxy for the queen herself in making the PQ drop her aggression, while the worker's Dufour's gland macerate had no effect on PQ's aggression (Bhadra, 2007; Bhadra *et al.*, 2010). This suggests that the Dufour's gland is a source of the queen pheromone in this species.

Dufour's Gland Morphology of Queens and Workers

Because the queen alone applies contents of her Dufour's gland onto the nest, it was expected that the activity level of queen gland should be more than that of worker gland, as the gland contents would have to get replenished periodically for queens but not for workers. The activity of a gland is often correlated with the size of the gland, and this has been found in vertebrates, as well as insects, and even in Dufour's gland of other hymenopterans (Herrera, 1992; Elmèr and Ohlin, 1969; Ravi Ram and Ramesh, 2002; Hassani *et al.*, 2010; Tobe and Stay, 1985; Roseler *et al.*, 1980; Ali *et al.*, 1988; Abdalla *et al.*, 1999a, b). Hence, because the queen is expected to have a more active Dufour's gland than the workers, the queen's Dufour's gland can also be larger than that of the workers. Size difference between Dufour's glands of queens and workers was investigated by dissecting out the glands in insect saline, photographing them and then taking measurements from the images with respect to length of the gland, maximum width of the gland, perimeter of the gland and area covered by the image of the gland. However, no difference was found between queens and workers for any of the parameters, or for all the parameters combined together in a multivariate analysis (Mitra and Gadagkar, 2012 a). This suggests that Dufour's gland activity and size are not correlated in this species. It also suggests that the total quantity of chemicals present in the gland should be similar for queens and workers. Thus, if the Dufour's gland indeed conveys the queen signal, and if there is no difference in quantity of chemicals between the Dufour's glands of queens and workers, one way in which the queen gland can be different from the

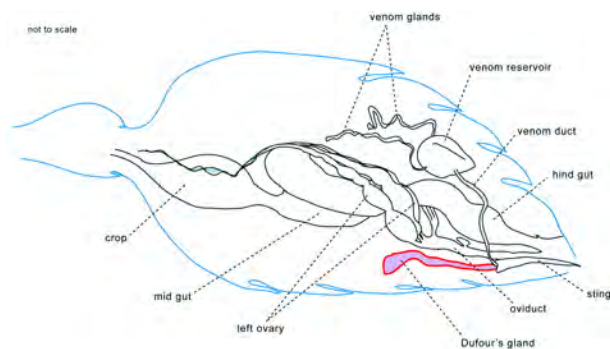


Fig. 3: Diagram showing location of the Dufour's gland in *Ropalidia marginata* among other abdominal organs (not to scale, reproduced from Mitra, 2013; copyright: author)

worker gland, is the composition of gland chemicals.

Chemical Composition of Dufour's Gland of Queens Versus Workers

Chemical analysis of the Dufour's gland was performed by dissecting out the glands and performing gas chromatography and gas chromatography coupled with mass spectrometry (Bhadra *et al.*, 2010). Dufour's glands were found to contain a series of linear, monomethyl and dimethyl branched alkanes with 21 to 33 carbon atoms in their main chains. Unsaturated compounds were not found. Amongst the branched alkanes, some compounds were represented by a mixture of two or more positional isomers. About 30 different compounds could be identified by mass spectrometry (Table 1). Any compound or set of compounds present exclusively in queens or in workers was not found. The total quantity of all compounds taken together was similar for queens and workers as had been predicted from the morphology of the Dufour's gland (Mitra and Gadagkar, 2012 a). Queens and workers differed in the composition of their Dufour's gland and could be separated into clear non-overlapping clusters in multivariate analysis done using relative proportions of all Dufour's gland compounds (Fig. 4). They also differed for categories of compounds: queens were higher than workers for monomethyl alkanes and conversely, lower than workers for dimethyl alkanes, while they did not differ for linear alkanes (Mitra and Gadagkar, 2011). This is interesting as it has been suggested that linear alkanes do not have any role in communication and are not perceived by polistine wasps, while methyl branched alkanes are discriminated by wasps and are good candidates for fertility signalling (Dani *et al.*, 2001). Queens and workers also differed for single compounds: queens were consistently lower than workers for four compounds (Heneicosane, 11-methyl heneicosane, Tricosane and 11-methyl tricosane). All these results suggest that the overall composition of the Dufour's gland plays a role in defining the queen signal, as queens did not have any compound that was absent in workers. Absence or decrease of certain compounds, and the automatic increase of relative abundance of other compounds contributes to a

Table 1: Compounds identified by gas chromatographic-mass spectrometric analyses of Dufour's glands of *Ropalidia marginata*.

Peak #	Identity of compound
1	Heneicosane
2	11-methylheneicosane
3	Tricosane
4	11-methyltricosane
5	Pentacosane
6	Mixture of 11- and 13-methylpentacosane
7	Heptacosane
8	Mixture of 11- and 13-methylheptacosane
9	3-methylheptacosane
9a	Octacosane
10	Mixture of 12-, 14-, 16- and 18-methyloctacosane
11	4-methyloctacosane
12	Nonacosane
13	Mixture of 11-, 13- and 15-methylnonacosane
14	5-methylnonacosane
15	11-, 15-dimethylnonacosane
16	3-methylnonacosane
17	2-methylnonacosane
18	8-methyltriacontane
19	14-, 16-dimethyltriacontane
19a	6-methyltriacontane
20	2-methyltriacontane
20a	Hentriacontane
21	Mixture of 11-, 13- and 15-methylhentriacontane
22	Mixture of 7- and 9-methylhentriacontane
23	Mixture of 11-, 17- and 13-, 17-dimethylhentriacontane
24	3-methylhentriacontane
25	Mixture of 5-, 21-, 5-, 19- and 5-, 17-dimethyldotriacontane
26	14-methyldotriacontane
27	8-methyldotriacontane
28	14-, 18-dimethyldotriacontane
28a	6-, 14-dimethyldotriacontane
29	Mixture of 13-, 15- and 17-methyltrtriacontane
30	13-, 19-dimethyltrtriacontane

queen-like signal, and the converse gives rise to a worker-like signal. This suggests that the Dufour's gland compounds can be having a bouquet effect, with overall chemical composition being more important than single compounds in defining the queen signal.

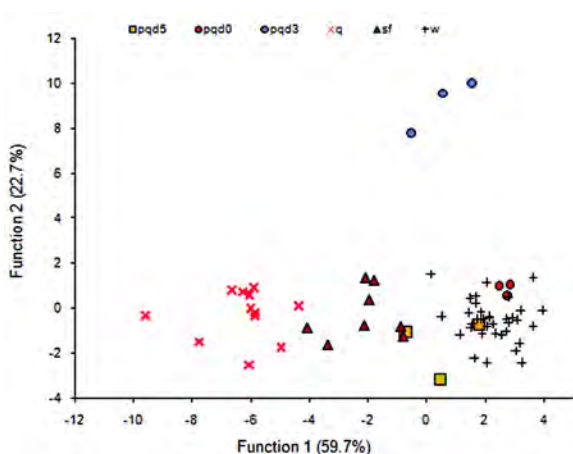


Fig. 4: Queens (q), workers (w), solitary foundresses (sf), potential queens immediately after queen removal (pqd0), potential queens 3 days after queen removal (pqd3), and potential queens 5 days after queen removal (pqd5) plotted on 2 discriminant functions that differentiate them based on the relative proportions of their Dufour's gland hydrocarbons. Figures in parenthesis on each axis denote the percent variance explained by the respective discriminant function. It can be seen that queens and workers are well separated, with solitary foundresses intermediate between them, and potential queens are similar to workers immediately after queen removal and move away from workers and come closer to queens by 5 days after queen removal (graph produced by combining data reported in Mitra and Gadagkar, 2011; Mitra and Gadagkar, 2012 b, c)

It is interesting to note that only non-volatile hydrocarbons were found from the Dufour's gland. Earlier it has been found that workers do not perceive the presence of their queen across a wire mesh partition, advocating the non-volatility of the queen pheromone (Sumana *et al.*, 2008).

Correlation of Dufour's Gland Profile of Queens with Ovary Development

The Dufour's gland composition of the queens was also found to be correlated with their state of ovarian development. The ovaries of the queens were dissected out and various measurements were done to construct an index of ovarian development that serves as a proxy for the fertility of the queen. Scores of queens on the ovarian index were found to be negatively correlated with those four compounds for which queens were found to have a consistently lower relative proportion than workers (Heneicosane, 11-

methyl heneicosane, Tricosane and 11-methyl tricosane) (Mitra and Gadagkar, 2011). This implies that queens with better developed ovaries tend to have a lower relative proportion of the four compounds than queens with less developed ovaries. Conversely, ovarian index of queens was positively correlated with the relative proportion of the remaining compounds. Since queens with better-activated ovaries have lower percent areas for these four peaks, this shows that the reduction or absence of these peaks, which automatically causes increase in relative proportion of the remaining peaks, might comprise the effectiveness of the queen signal.

Previous studies on other species that have investigated the link between fertility and queen pheromone have mostly concentrated on differences between queens and workers (Bonavita-Cougourdan *et al.*, 2001; Winston and Slessor, 1993; Peeters *et al.*, 1999; Sledge *et al.*, 2001; Katzav-Gozansky *et al.*, 2002 b; D'Ettoire *et al.*, 2004; Monnin, 2006) where the information on fertility gets reduced to categories. However, the state of ovarian activation is not a categorical but a continuous variable, and test for correlation between pheromone composition and ovaries (both treated as continuous variables) should provide clues regarding the honesty of the queen signal at a finer scale, as queens with better-activated ovaries are expected to have pheromones that are more differentiated from workers than queens having less activated ovaries. Chemical difference among established queens with respect to their variation in fecundity has generally not been investigated. The study on *R. marginata* looks at the ovarian development of the queen on a continuous scale and thereby shows that the composition of the queen signal can vary continuously with the ovarian activation status of the queen. Workers might perceive the fertility status of their queen through her Dufour's gland compounds that she applies to the nest surface, and thereby react accordingly (Bhadra *et al.*, 2010). This could have important implications in cases where queen turnover occurs (when a queen gets overthrown by workers), presumably due to decline in fertility of the queen, perceived through change in queen pheromone. It is possible that factors involved in the ovarian activation pathway influence the

biosynthesis of Dufour's gland compounds. It can also be concluded that the Dufour's gland profile of a queen may honestly signal the fertility status of the queen, as it is inevitably constrained by the state of ovarian development of the queen. Thus queens may maintain reproductive monopoly by advertising their fertility status to workers through their Dufour's gland compounds.

Solitary Foundress

A solitary foundress is an individual female who has left her natal nest and has started building her own nest. She will maintain the nest, start laying eggs in it and will forage and take care of her brood alone, without any worker to help her. Solitary foundresses provide an interesting system to study the honesty of queen signalling. Since solitary foundresses do not have any nestmates, expressing a queen signal is redundant, as there is no one to receive the signal. But, if the pheromone is an honest signal arising as an inevitable by-product of the state of fertility, i.e. inextricably linked to the fertility of the individual, expression of pheromone and pheromone composition should necessarily be correlated with the degree of fertility of the individual (Keller and Nonacs, 1993; Heinze and D'Ettorre, 2009; van Zweden, 2010). Such a signal should be produced by any fertile individual, irrespective of the presence or absence or receivers of the signal (nest-mates), by virtue of being physiologically linked to fertility (Maynard Smith and Harper, 1995; Searcy and Nowicki, 2005). A fertile individual cannot help but produce the signal due to this physiological constraint.

It was found that solitary foundresses were intermediate between queens and workers with respect to both their Dufour's gland hydrocarbon composition (Fig. 4) and their ovaries, suggesting that queen pheromone composition in *R. marginata* can be a by-product of the state of fertility and hence may honestly signal fertility (Mitra and Gadagkar, 2012 b). Although all solitary foundresses had laid eggs, they had lower fecundity than queens. Their state of ovarian activation was intermediate between queens and workers, and this could be a consequence of

energy consuming tasks like foraging and nest maintenance that they have to perform. Queens of established post-emergence nests never go out to forage and do almost no work for colony maintenance, and this could be a reason as to why their ovaries are better activated than those of solitary foundresses. This adds to the evidence for the link between fertility of individuals and chemicals likely to be involved in fertility signalling (Sledge *et al.*, 2001; Monnin, 2006; Izzo *et al.*, 2010; Mitra and Gadagkar, 2011), and reinforces the idea that a signal that arises as a by-product of fertility is bound to be honest and hence can be easily incorporated in a communication system for fertility signalling.

Potential Queens

As mentioned earlier, when the *R. marginata* queen is removed from her colony, one of the workers becomes extremely aggressive and if the queen is not returned, she gradually loses her aggression, develops her ovaries and becomes the new queen of the colony within a few days; this individual is called the potential queen (PQ) (Chandrashekara and Gadagkar, 1992; Premnath *et al.*, 1996; Deshpande *et al.*, 2006). The overt aggression shown by the PQ is reminiscent of the pattern observed in other primitively eusocial species where the beta (second most dominant individual) steps up her aggression on removing the alpha (most dominant individual) (Hughes and Strassmann, 1988; Miyano, 1991; Sledge *et al.*, 2001; Deshpande *et al.*, 2006). In such species, the original beta and now the new alpha continues to be the most dominant individual in the colony and therefore may use dominance for regulation of reproduction of other colony members (Hughes and Strassmann, 1988; Miyano, 1991; Sledge *et al.*, 2001; Deshpande *et al.*, 2006). In *R. marginata* however, the aggression of PQ is expected to drop down over time, as eventually the PQ will transform into a docile queen. It is also expected that the ovaries of the PQ will develop during this stage from a poorly developed state (similar to workers) to a highly developed state (similar to queens). The PQ presents an interesting opportunity to look at the development of the pheromone profile in conjunction with ovarian development and change in aggressive behaviour. As

the Dufour's gland hydrocarbon composition of queens is known to be different from workers (Bhadra *et al.*, 2010; Mitra *et al.*, 2011; Mitra and Gadagkar, 2011, 2012 a, 2012 b), PQs can be expected to be similar to queens by the time they have developed their ovaries. It would be interesting to find out whether PQs are initially similar to workers in their Dufour's gland hydrocarbons, on account of having undeveloped ovaries, or whether they can be differentiated from workers even at an initial stage. Therefore tracing the road to royalty in *R. marginata*, that is following the transition of the PQ from a worker-like state to a queen-like state, in terms of her ovaries, aggression, and Dufour's gland hydrocarbons should shed further light on the link between ovaries and pheromone and also show how these two factors change along with change in behaviour during the queen re-establishment phase after queen removal.

It was found that the PQ shows extremely high levels of aggression immediately after queen removal, and the aggression of the PQ decreased as a function of time since queen removal, coming down to normal queen-right level by four days after queen removal (Mitra and Gadagkar, 2012 c). At the same time, the ovaries of the PQ increased gradually, becoming significantly larger than workers by three days after queen removal, and becoming significantly similar to queens by four days after queen removal. With respect to Dufour's gland composition, it was found that PQs were similar to workers immediately after queen removal, and came closer to queens by five days after queen removal (Fig. 4). This shows that as PQs lose their aggression and develop their ovaries, they also start developing the queen pheromone, once again showing a strong link between ovarian development or fertility and queen pheromone.

The aggression shown by the PQ might be considered analogous to the situation in typical primitively eusocial species, where the queen is aggressive and occupies the top-rank in dominance hierarchy (Pardi, 1948; West-Eberhard, 1969; Gadagkar and Joshi, 1984; Deshpande *et al.*, 2006). The changes in aggression, ovaries, and Dufour's gland composition of PQs with time since queen

removal may suggest that the PQ could be using aggression to maintain her status in the initial queenless phase, and later, as her ovaries activate, she loses her aggression and uses pheromone to maintain reproductive monopoly. This may hint at an interesting transition in maintenance of eusociality from 'queen control' (aggression) to 'queen signal' (pheromone) (Keller and Nonacs, 1993). It is interesting to note that virgin honey bee queens have also been observed to engage in dominance interactions with workers (Weaver and Weaver, 1980), and they may express low quantities of queen pheromone at this stage (Wossler *et al.*, 2006), suggesting a possible mixture of dominance and pheromone as strategies used to maintain reproductive monopoly. Hence it may also be possible that the *R. marginata* PQ is using both aggression and pheromone to maintain her status in the initial queenless phase. However, earlier work has showed that the overt aggression of the PQ may be needed for her own ovarian activation, rather than be used for suppressing ovarian activation of workers (Lamba *et al.*, 2007). Thus, it is possible that the aggression that is used for mediating reproductive conflict during the queen establishment phase in other species may have a different function in *R. marginata*.

Universality of the Queen Pheromone

The Dufour's gland has been shown to be involved in nestmate discrimination, i.e. differentiating members of one's own nest from those of another nest, in the European paper wasp *Polistes dominulus* (Dani *et al.*, 1996a, b). Also in statistical analysis done on the chemical composition of the Dufour's gland of *R. marginata* it was found that individuals from different colonies can be differentiated into clear non-overlapping clusters based on their colony membership (Mitra *et al.*, 2011). Thus, it is interesting to find out if the queen pheromone from Dufour's gland of the queen from one colony is recognised and accepted as the queen signal in another colony as well. Earlier, a bioassay had been developed using the observation that the PQ immediately reduces her aggression when the queen is returned to the colony. In this bioassay, it was shown that crude macerates of the Dufour's gland of the queen (but not that of

the worker) mimics the return of the queen by a similar reduction in aggression of the PQ (Bhadra *et al.*, 2010). In a similar bioassay, after queen removal, the Dufour's gland macerate of a queen from another colony was applied on the nest to test the effect of a foreign queen's gland contents on the PQ, thereby testing whether the queen signal is perceived across colonies and whether the PQ can discriminate between gland macerates of nestmate and non-nestmate queens. Live queens are known to be dominated, attacked and even killed when presented to a foreign colony (Venkataraman and Gadagkar, 1993). Thus it can be expected that if Dufour's gland conveys only the queen signal and does not carry colony signal, the queen signal should get perceived across colonies and a foreign queen's gland macerate should cause reduction in aggression of the PQ, similar to the reduction in aggression known to occur in response to her own queen's gland macerate. On the other hand, in addition to conveying the queen signal, if the Dufour's gland is also involved in nestmate recognition, it can be expected that the PQ should not reduce her aggression in response to a foreign queen's gland macerate. Thereby, how both queen signal and colony signal can be transmitted through the Dufour's gland chemicals, was tested in this bioassay.

It was found that the PQ reduced her aggression significantly in response to the Dufour's gland macerate of a foreign queen (Mitra *et al.*, 2011). The degree of reduction of aggression was similar to what had been observed in response to Dufour's gland macerate of own queen (Bhadra *et al.*, 2010). This suggests that the queen signal in *R. marginata* is perceived across colonies, and also shows that the PQ does not differentiate between nestmate and non-nestmate queens on the basis of their Dufour's gland macerates. Earlier, when it was found that PQ reduced her aggression in response to her own queen's Dufour's gland macerate but not to Dufour's gland macerate of a worker, it led to the expectation that queens and workers should be differentiated in a statistical analysis using the composition of their Dufour's glands. This expectation had been found to hold true (Bhadra *et al.*, 2010). Similarly, since the PQ reduced her aggression in response a foreign

queen's Dufour's gland macerate, this leads to the expectation that classification of individuals on the basis of colony membership should not be possible from their Dufour's gland composition. However, all individuals can be correctly classified according to their colony membership in a multivariate discriminant analysis done on the chemical composition of their Dufour's glands. This leads to the important precautionary conclusion that our ability to correctly classify individuals in a statistical analysis does not necessarily imply that the animals themselves can make similar classification with the same information. The proof of whether animals make discrimination is therefore best sought through bioassays.

Cuticular Hydrocarbons

The major focus of studies investigating chemical communication of fertility status in primitively eusocial species has been on differences in cuticular hydrocarbons (CHCs) between reproductive and non-reproductive classes (Bonavita-Cougourdan *et al.*, 1991; Peeters *et al.*, 1999; Liebig *et al.*, 2000; Sledge *et al.*, 2001; Dapporto *et al.*, 2005). Analysis of CHCs in *R. marginata* showed that the cuticle and Dufour's glands contained the same set of hydrocarbons in similar proportions (Mitra and Gadagkar, 2014a, b). The same compounds were found to occur in the haemolymph as well (in similar proportions) suggesting that these compounds may be synthesised in the oenocytes of fat bodies (as it occurs in general in insects) and is then transported in the haemolymph from where they are sequestered into the Dufour's gland or into the cuticle (Mitra and Gadagkar, 2014b). Also, patterns related to fertility signalling found in the Dufour's gland hydrocarbons were found to occur in the CHCs as well. Queens and workers could be differentiated using their CHC profiles. Solitary foundresses were intermediate between queens and workers in their CHC profiles. The CHCs of PQs showed similar dynamics, as seen in the DG hydrocarbons. The CHC profile of PQs were similar to workers immediately after queen removal, but gradually appeared to come closer to that of queens with the passage of time. Thus not only the Dufour's gland, but also the CHC profile of *R. marginata* can

be linked to the state of ovarian development.

Since the *R. marginata* queen is remarkably non-interactive, it should not be possible to convey the queen signal from queen to workers through direct or indirect interactions with the queen (Bhadra *et al.*, 2007). Hence, CHCs are apparently unlikely to be candidates involved in conveying the queen signal, as it should not be possible to perceive any signals carried by CHCs without physically interacting with the queen. However, the patterns of fertility signalling found in the DG hydrocarbons are being found in the CHCs as well, and the hydrocarbon profile of the DG and cuticle are correlated with each other and also correlated with the haemolymph hydrocarbon profile, suggesting a common source of hydrocarbons for the DG and cuticle. Thus, for any change in the hydrocarbon profile related to fertility status and fertility signalling between individuals, the change is likely to occur at the hydrocarbon production site itself, and the physiological factors that affect fertility signalling by hydrocarbons are thereby likely to act on the site of hydrocarbon synthesis itself (oenocytes), with the cuticle and DG acting as portals for conveying information present within the haemolymph. It is not clear at this stage whether the wasps themselves can perceive the queen signal through the CHCs. Future studies using bioassays should be able to solve this question.

Concluding Remarks

The *R. marginata* queen is docile and non-interactive and thus, cannot use aggression or other behavioural interactions to suppress reproduction by workers. Still she maintains reproductive monopoly and is always the sole egg layer in a colony. How the queen conveys her presence to workers had remained an enigma for many years. Recent investigations reviewed herein, involving chemical analysis and behavioural assays show the role of Dufour's gland in conveying the queen signal. Now there is sufficient evidence to identify the Dufour's gland as at least one source of the queen pheromone in *R. marginata*. Queens and workers have been found to differ in the chemical compositions of their Dufour's glands, and this difference in chemical composition has also been

linked to the state of ovarian development, advocating the honesty of queen pheromone. Two ways by which a *R. marginata* worker can become a queen and hence can achieve reproductive monopoly have been elucidated. Although most workers remain as workers in their natal nests for their entire lives, some leave their natal nests and make their own solitary foundress colonies, successfully raising brood to the adult stage, and become the queens of post-emergence colonies. Another way by which a worker can become a queen is through becoming a potential queen. A worker can replace the existing queen of a colony when the old queen dies, by first becoming a potential queen, and then eventually becoming the new queen of the colony. In both of these options, as a worker goes on to become a queen, her ovaries gradually develop, and correlated with ovarian development, her Dufour's gland profile shifts from being worker-like to queen-like (Fig. 5).

With respect to maintenance of eusociality, it was found that during the queen establishment phase, potential queen had poor ovaries, had Dufour's gland profile similar to workers and was highly aggressive, showing more aggression towards the individuals who had better developed ovaries than her. This suggests that the potential queen may use aggression to suppress reproduction by workers. The condition is reminiscent of typical primitively eusocial societies, where the queen is generally the most dominant individual of a colony (Pardi, 1948; Gadagkar and Joshi, 1984; West-Eberhard, 1986). Gradually, as the aggression of the potential queen

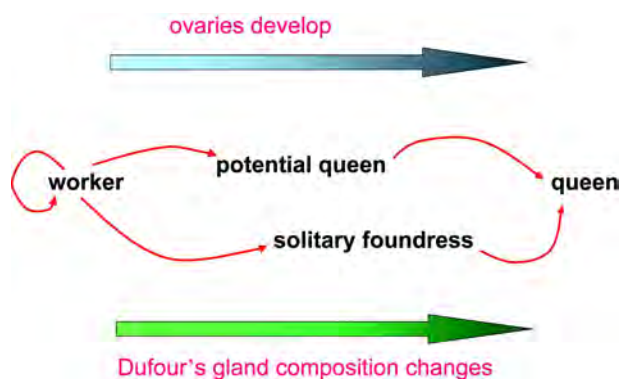


Fig. 5: Schematic representation of 2 pathways of achieving queen status by a worker of *Ropalidia marginata*

decreases, her ovaries develop, and her Dufour's gland profile becomes more and more queen-like. Finally, when she establishes herself as the new queen, she does not need to show any aggression, as she has well-developed ovaries and can advertise her presence to the workers through a pheromone, which is a universal status badge (perceived across colonies), and is an honest signal of her fertility (correlated with her state of ovarian development). This condition is similar to highly eusocial species, where maintenance of reproductive monopoly through the queen pheromone is well known (Free, 1987; Winston and Slessor, 1993). Although it has been suggested that the dominance shown by the potential queen may be needed for her own ovarian activation, rather than be used for suppressing ovarian activation of workers (Lamba *et al.*, 2007), in a recent study it was found that dominance behaviour shown by a wasp is not only correlated with her increase in ovaries, but also causes suppression of ovarian development in the wasp that is being dominated by the dominant individual (Shukla *et al.*, 2014). Therefore it is possible that the aggression of the PQ can suppress the ovarian development of workers, and it is likely that the PQ may be using a mixture of aggression and pheromone to maintain reproductive monopoly in the early stages of queen establishment, which later shifts to pheromonal queen signalling alone. This suggests that a transition in maintenance of eusociality could be occurring from queen control by aggression to queen signal by pheromone, not only

in the same species, but also within the lifetime of an individual, as the potential queen undergoes transition to become the queen of her colony (Fig. 6). Thus, *Ropalidia marginata* can be said to be at a higher position on the continuum of social complexity, higher than other typical primitively eusocial species who have aggressive queens acting as central pacemakers of their colonies. This throws light on the evolution of complex societies from simple and primitive societies.

Maintenance of reproductive monopoly in social insects has generally been found to take place through pheromones (Holman, 2010; Richard and Hunt, 2013). This is always the case in highly eusocial insects, but there is growing evidence for a similar phenomenon in primitively eusocial Hymenoptera as well (Monnin 2006). In *R. marginata* it can be inferred that aggression can also possibly play a role in queen establishment, which may be similar to the situation during colony founding in other primitively eusocial wasps (Hughes and Strassmann, 1988; Miyano, 1991; Sledge *et al.*, 2001; Deshpande *et al.*, 2006). However it definitely shifts to pheromonal queen signalling at a later stage, as *R. marginata* queens are known to be non-interactive and non-aggressive, and this could again be reminiscent of the situation found in other primitively eusocial polistine wasps (Sledge *et al.*, 2001). In a recent meta-analysis, it has been suggested that non-volatile saturated hydrocarbons may be the ancestral state of queen pheromones in Hymenoptera (Van Oystaeyen *et al.*, 2014). It is interesting to note that the compounds that are potentially involved in comprising the queen pheromone in *R. marginata* also belong to this class of compounds. This adds to the growing evidence of hydrocarbons as semiochemicals involved in chemical communication between individuals. In species where the queen pheromone has been identified, usually one or a few compounds have been singled out as components of the pheromone (Van Oystaeyen *et al.*, 2014). This does not appear to be the case in *R. marginata*, where the entire Dufour's gland composition seems to be important. The only consistent difference found between queens and workers in their Dufour's gland compounds is that queens have lower relative proportion than workers for four compounds. Since

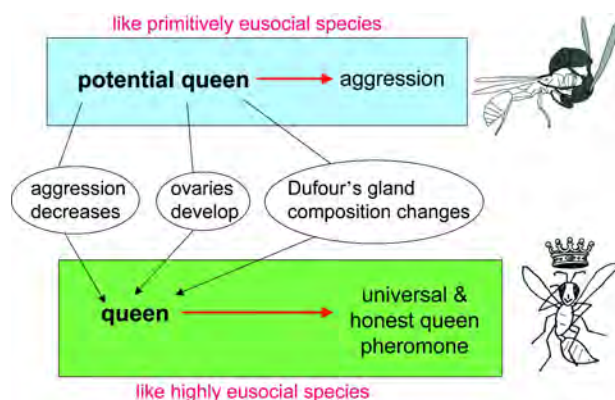


Fig. 6: Schematic representation of transition in maintenance of reproductive monopoly during transition of potential queen to queen during queen re-establishment phase after queen removal in *Ropalidia marginata*

the absence of a chemical cannot give rise to a signal unless the change in entire blend is taken into consideration, it is likely that the Dufour's gland compounds have a "bouquet effect" with the entire blend being important in differentiating queens from workers. Also, in addition to Dufour's gland compounds, whether CHCs can also be involved in reproductive monopoly, is not clear at this stage. Future studies should be able to address these issues.

It can be concluded that queen pheromone from

the Dufour's gland plays a primary role in the maintenance of reproductive monopoly by the queen in *R. marginata*.

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