

# Interrogating an insect society

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This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2006.

Contributed by Raghavendra Gadagkar, April 21, 2009 (sent for review March 26, 2009)

**Insect societies such as those of ants, bees, and wasps consist of 1 or a small number of fertile queens and a large number of sterile or nearly sterile workers. While the queens engage in laying eggs, workers perform all other tasks such as nest building, acquisition and processing of food, and brood care. How do such societies function in a coordinated and efficient manner? What are the rules that individuals follow? How are these rules made and enforced? These questions are of obvious interest to us as fellow social animals but how do we interrogate an insect society and seek answers to these questions? In this article I will describe my research that was designed to seek answers from an insect society to a series of questions of obvious interest to us. I have chosen the Indian paper wasp *Ropalidia marginata* for this purpose, a species that is abundantly distributed in peninsular India and serves as an excellent model system. An important feature of this species is that queens and workers are morphologically identical and physiologically nearly so. How then does an individual become a queen? How does the queen suppress worker reproduction? How does the queen regulate the nonreproductive activities of the workers? What is the function of aggression shown by different individuals? How and when is the queen's heir decided? I will show how such questions can indeed be investigated and will emphasize the need for a whole range of different techniques of observation and experimentation.**

division of labor | *Ropalidia marginata* | social evolution | social organization | social wasp

As social beings we are conscious of the enormous benefits we derive from cooperation and division of labor, but we are also justifiably obsessed with the problems that social life inevitably brings with it, the potential for selfishness and conflict. A reasonable way to reflect on these issues, and indeed to understand why we behave as we do, is to turn to other societies. Anthropologists can offer us a glimpse into the lives and mores of “primitive” and “exotic” human societies. Biologists can do much more; they can offer us insights from a whole range of animal societies with millions of years of evolutionary history. And those of us who study insect societies can hope to harness wisdom from an altogether different subkingdom of animal life. I certainly do not think we should imitate animal societies blindly but I do think that they can hold a mirror to us and offer us a means to reflect on our own society and learn more about ourselves (1).

## The Insect Societies

Many insect societies, such as those of ants, bees, wasps, and termites, parallel, if not better, human societies in the sophistication and complexity of their organization, communication, division of labor, and even their caste systems (2–6). There can be little doubt that they have much of interest for us fellow social creatures. In the so-called primitively eusocial species queens and workers are not morphologically differentiated, queens control worker reproduction by physical aggression, and colony labor is divided by a top-down control by the queens. In highly eusocial species, however, queens and workers are morphologically differentiated, queens regulate worker reproduction by pheromones, and colony labor is divided by bottom-up self-organization. Why do the members of a species organize themselves into colonies? What might have been

the evolutionary forces that brought about the transition from solitary to social life in the past and what evolutionary forces maintain social life today and prevent a reversal to the solitary mode of existence? In parallel with these evolutionary questions (or, sometimes called “ultimate” or “why” questions), one can and should also ask more “proximate” questions that address the physiological and other mechanisms that make it possible for members of the species to organize themselves into eusocial colonies. How do members of a colony recognize themselves as distinct from those of another colony, how do they differentiate themselves into reproductive and nonreproductives, what mechanisms ensure that workers do not begin to reproduce, how do colony members communicate with each other and divide colony labor efficiently among themselves, how do they respond when the queen dies and/or when their nest is attacked by a predator, and so on. Although the debate about the relative utility and mutual interdependence of why and how questions has waxed and waned, I believe that we need to simultaneously address both kinds of questions, ideally with the same study animals and by the same research group, and that is exactly what my students and I have attempted to do at all times. I summarized what we have learned about *Ropalidia marginata* by asking more evolutionary questions some years ago (7) and will therefore focus more on the proximate questions in this article.

## Natural History of the Primitively Eusocial Wasp *R. marginata*

More than 25 years ago I chose the Indian paper wasp *R. marginata* as an exemplar of a primitively eusocial society worthy of long-term study. At this point I will give some brief background information about *R. marginata*, which led to the first in the series of 9 questions and answers that I will describe here. *R. marginata* is an Old World, tropical, primitively eusocial, polistine wasp abundantly distributed in peninsular India. Apart from its easy and local availability, this wasp genus potentially holds important clues concerning insect social evolution, because it contains both primitively eusocial and highly eusocial species. *R. marginata* builds simple, open (without envelope) nests from paper cartons that they make from cellulose fibers scraped from plants (Fig. 1). New colonies may be founded either by a single female or a small group of female wasps. A solitary foundress builds a nest, lays eggs, forages to feed her growing larvae, guards them from predators and parasites, and brings them to adulthood all by herself, until her daughters eclose (emerge from their cocoons as adults) and begin to stay back and assist her in rearing subsequent batches of brood. In multifemale nests, only 1 individual becomes the egg layer or queen and does little more than egg laying, while the rest function as sterile workers and perform the tasks of nest building, maintenance, and brood care. All male wasps eclosing on nests of *R. marginata* disappear within ≈5–6 days of their eclosion and appear to lead a nomadic life, attempting to mate with female wasps that may be on foraging trips, and never return to their natal nests or any other nest. Female wasps, however, may

Author contributions: R.G. designed research, participated in performing research reviewed here, and wrote the paper.

The author declares no conflict of interest.

See Profile on page 10404.

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Fig. 1. A typical nest of the Indian paper wasp, *R. marginata* (Photo: Sujata Kardile).

remain on their natal nests all of their lives (1–160 days, mean  $\pm$  SD =  $27 \pm 23$  days). The wasps in a colony exhibit reproductive caste differentiation into fertile queens and sterile workers. At any given time only 1 individual monopolizes all egg laying while the rest of the individuals never lay eggs when the queen is alive. However, queens are often replaced by their nest mates, resulting in serial, rather than simultaneous, polygyny. Several features of the biology of *R. marginata* make it an ideal model system for addressing both ultimate and proximate questions. The open nests make it possible to observe all behaviors performed by all of the wasps. The relative small size of the colonies makes it possible to individually mark all wasps with unique spots of colored paints and document the lifetime behavioral profiles of known individuals. The absence of morphological differentiation between queens and workers makes it possible, at least in principle, for any or most adult wasps to take on either the role of the queen or that of a worker, depending on the opportunities available (7), which makes the society of even greater interest from a human perspective.

How then does one interrogate such an insect society? From the aphorism “Nature loves to hide” attributed to the Greek philosopher of 500 BC Heraclitus and the declaration by Sir Francis Bacon in the 16th century that “Nature does not reveal itself except under the torture of experiments,” there has been a long and fascinating discussion about the secrets of nature and how they might be unveiled (8). Ethology, the science of animal behavior, too, has had a somewhat uneasy relationship between (unobtrusive) observation and (manipulative) experimentation. Whereas Konrad Lorenz, 1 of the 3 founding fathers of ethology appears to have been in favor of observation without experiment and interference, Niko Tinbergen and Karl von Frisch, the other 2, might well be described as the founders of experimental ethology (9). I hope to show here that to unveil the secrets of an insect society we need to use every method available to us and as required by the question at hand. I also hope to show that every well-answered question leads to at least 1 new question. Let me therefore proceed with a description of a series of 9 questions and their possible answers, exactly as I proceeded in my research that was performed with the active collaboration of a large numbers of students who never failed to match my passion for interrogating this remarkable insect society.

#### Question 1: How Is the Queen Behaviorally Different from the Workers?

In highly eusocial insects where queens and workers are morphologically differentiated, one tends to take it for granted that queens ought to be behaviorally different from workers and in predictable ways, too. In primitively eusocial insects, however, where queens are morphologically identical, it is best to make no assumptions about how queens and workers ought to behave and especially how they

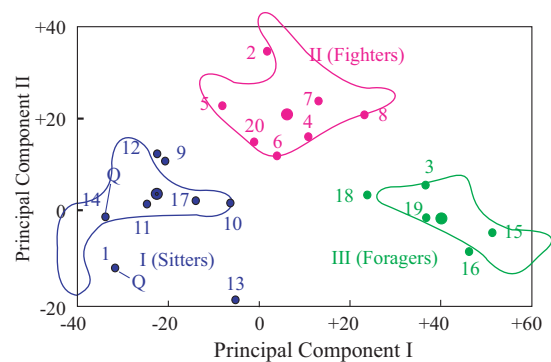


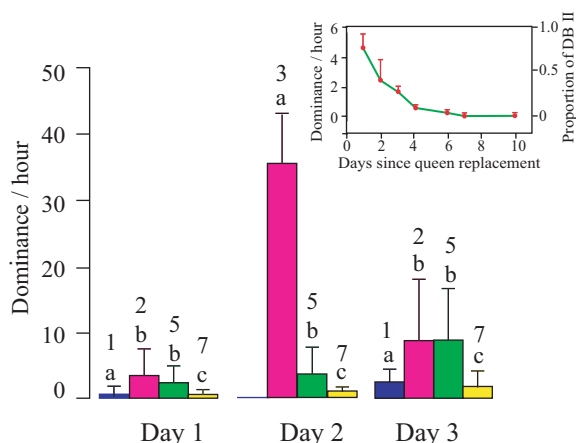
Fig. 2. Behavioral castes of *R. marginata*. Twenty wasps are shown as points in the coordinate space of the amplitudes associated with the first 2 principal components. The points fall into 3 clusters (or castes) by the criterion of nearest centroid. Dots indicate centroids. Q = queen. [Redrawn with permission from ref. 10 (Copyright 1983, Elsevier).]

ought to differ from each other. We therefore simply marked all of the wasps in a colony with unique spots of colored paints and recorded their behavior by using a battery of sampling methods. From this we chose the top 6 behaviors in which an average wasp spent most of its time and computed the proportion of time that each wasp spent in each of these 6 behaviors. We then subjected the resulting time-activity budgets to multivariate statistics and cluster analysis. To our surprise, we found that the members of a colony could be classified into 3 rather distinct clusters, which we named sitters, fighters, and foragers, based on their mean behavioral profiles (Fig. 2) (10).

Having deliberately avoided categorizing individual wasps as queens and workers before the behavioral study, we were now able to retrospectively examine the position of the queen in this system of behavioral caste differentiation of a colony into sitters, fighters, and foragers. Because queens of primitively eusocial wasps are known to be aggressive individuals and are believed to use physical aggression to suppress worker reproduction and enforce work schedules on their work force, we expected *R. marginata* queens to belong to the fighter caste. But this was not the case. In colony after colony we found that the queens of *R. marginata* belonged to the sitter caste (10). Does this mean that there was no aggression in *R. marginata* colonies? To check this out, we made a more explicit study of dominance/subordinate behaviors. Incidentally, I will interchangeably use aggression, dominance behavior, or dominance-subordinate behavior to mean the same thing. We found that, like in other primitively eusocial species, these wasps also showed reasonable rates of aggressive behaviors. Indeed, we were able to use the frequencies of dominance/subordinate behaviors to construct dominance hierarchies among the members of a colony, as can be done in any primitively eusocial species. The only difference, but a profound one at that, was that *R. marginata* queens were almost never at the top of the dominance hierarchies of their colonies; they were in the middle or at the bottom of the hierarchy (7, 11). Thus, the answer to question 1 is that queens of *R. marginata* are nonaggressive, noninteractive (I have not shown data on interaction rates because of lack of space), meek, and docile sitters, despite the fact that workers exhibit dominance-subordinate behaviors typical of primitively eusocial species.

#### Question 2: If the Queen Is Such a Meek and Docile Sitter How Does She Become a Queen in the First Place?

It is unlikely that we will find the answer to this question by mere observation. Clearly, manipulative experiments are called for. We therefore designed a “queen-removal” experiment to answer this question. It involved observing a normal, queen-right colony on day 1, experimentally removing the queen and observing the so-



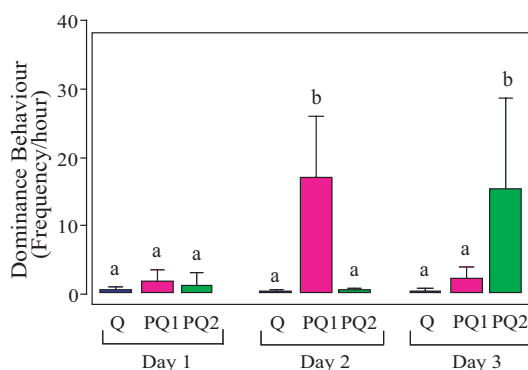
**Fig. 3.** A typical queen removal experiment showing the frequencies per hour of dominance behavior shown by the queen (blue bars), PQ (pink bars), maximum workers (green bars), and mean worker (yellow bars) on days 1–3. Bars that carry different letters are significantly different from each other ( $P < 0.05$  or less) within each day, bars that carry different numbers are significantly different from each other ( $P < 0.05$  or less) among the 3 days. Comparisons are by the 2-tailed Wilcoxon matched-pairs signed-rank test. [(Copyright 1995, Oxford University Press).] (Inset) Dominance acts per nest mate per hour shown by replacement queens from the day of takeover up to 10 days after queen replacement. Means and 1 SD are shown for 13 nests for days 1–7 and 6 nests on day 10. [Redrawn with permission from ref. 13 (Copyright 1996, Elsevier).]

manipulated queenless colony on day 2, returning the queen, and then observing the colony again on day 3. There were no surprises on day 1 but the behavior of the wasps on days 2 and 3 was most unexpected and interesting. Within minutes after queen removal, the reasonably peaceful *R. marginata* colony was transformed into a highly aggressive society. On day 2 the queenless colony showed a several-fold increase in dominance behavior compared with that of the queen-right colony on day 1. Interestingly, dominance behavior on day 3 after the queen was returned reduced to near day 1 levels. More surprisingly, all of the increased dominance behavior on day 2 was on account of a single worker who stepped up her levels of dominance behavior some 10-fold relative to her own levels on day 1 and indeed brought down her aggression on day 3 after the queen was returned, to levels significantly lower than on day 2 (Fig. 3). In experiments in which we did not return the queen on day 3, the worker who stepped up her aggression as soon as the queen was removed, gradually brought down her aggression (Fig. 3 Inset), developed her ovaries, and went on to become the next queen of the colony. We therefore call this hyperaggressive worker the potential queen (PQ) until she lays her first egg when she would, of course, qualify for the title queen (12–14). The answer to question 2 then is that a meek and docile individual manages to be the queen of *R. marginata* colonies by being a very aggressive individual at the beginning of her career and only later becoming meek and docile.

**Question 3: How Does the Queen Inhibit Worker Reproduction and Maintain Her Reproductive Monopoly?**

It is clear that the *R. marginata* queens could not possibly be using physical aggression to suppress reproduction by their workers. And yet they are remarkably successful in maintaining complete reproductive monopoly in their colonies. There are perhaps many ways in which the queens can achieve reproductive monopoly but we decided to first consider the hypothesis that *R. marginata* queens do so with the aid of pheromones. The reason for starting with this hypothesis is that queens of highly eusocial insects are well known to achieve reproductive monopoly by means of pheromones.

But how do we test such a hypothesis? For starters, it would be helpful to know whether the queen pheromone is volatile or nonvolatile? To answer this limited question we designed a new



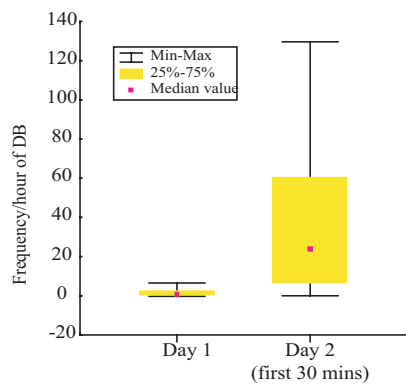
**Fig. 4.** A typical mesh experiment. Mean and SD of the frequency per hour of dominance behavior of the queen (Q), PQ1 and PQ2 on day 1 (unmanipulated colony), day 2 (after dividing the colony), and day 3 (after exchanging the queen) ( $n = 12$  colonies). Comparisons are by Wilcoxon matched-pairs signed-ranks test and significantly different values ( $P < \alpha$ , where  $\alpha$  is set to 0.0023 after Bonferroni correction) are indicated by different letters. [Redrawn with permission from ref. 15 (Copyright 2008, Springer).]

kind of experiment: the “mesh experiment.” After observing a normal colony on day 1 as before, we cut the nest in half and separated the 2 halves with a wire mesh in a closed cage on day 2. We then introduced the queen on 1 of the 2 sides by tossing a coin and similarly released a randomly chosen half of the workers on the queen-right side and the other half on the queenless side. On day 2 we made observations simultaneously on both sides. Our predictions were as follows. If the queen pheromone is volatile, workers on the queenless side should perceive the presence of the queen across the mesh and should behave just as they did before the nest was cut up and separated by a wire mesh. In other words, there should be no PQ on either side. However, if the queen pheromone is nonvolatile, workers on the queenless side should not perceive the queen across the wire mesh and should behave as they would if their queen had been lost. In other words, 1 of the workers should begin to behave like a PQ on the queenless side but there should be no PQ on the queen-right side.

We carried out 24 such mesh experiments, and in each one of them the hypothesis that the queen pheromone is nonvolatile was supported. In every experiment, there appeared a hyperaggressive PQ (we designated her as PQ1) on the queenless side within minutes of the workers on that side being separated from their queen and no such hyperaggressive individual appeared on the queen-right side in any experiment. As further proof, we exchanged the queen from the queen-right side to the queenless side, leaving the workers as they were, on day 3. Sure enough the PQ of day 2 now dropped nearly all her aggression and 1 of the workers on the new queenless side who was very nonaggressive on day 2 became hyperaggressive and was designated as PQ2. There was no significant difference between the levels of aggression of PQ1 on day 2 and PQ2 on day 3 (Fig. 4) (15). Our answer to question 3 is still preliminary because we cannot yet conclude that *R. marginata* queen maintains her reproductive monopoly by means of a pheromone. However, we can certainly conclude that the workers perceive the presence or absence of the queen by a pheromone that is nonvolatile. This is because of the certainty and rapidity with which we witnessed a PQ on the queenless side and, equally importantly, the certainty and rapidity with which the PQ dropped her aggression as soon as the queen was moved to her side. Recent work with another primitively eusocial wasp genus *Polistes* lends support to the idea of chemical signaling between queens and workers (16, 17).

**Question 4: How Does the Queen Signal Her Presence to Her Workers, or How Do the Workers Perceive the Nonvolatile Queen Pheromone?**

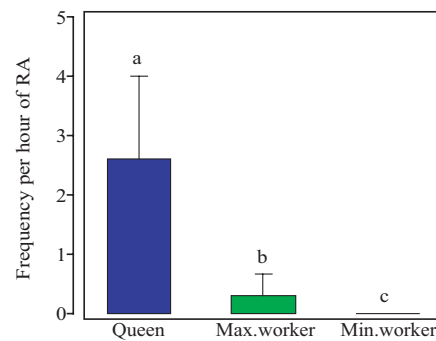
If the queen pheromone were volatile, there would be no great mystery about how it gets from the queen to the workers. But



**Fig. 5.** Frequency per hour of dominance behavior (DB) shown by the PQ within 30 min of queen removal as compared with her own levels of dominance in the queen right colony. Wilcoxon matched-pairs, signed-ranks test;  $n = 50$ ,  $P = 0.000$ . [Redrawn with permission from ref. 19 (Copyright 2007, Elsevier).]

because the pheromone of *R. marginata* queens is nonvolatile, the question of its transmission from the queen to the PQ is an open one. The most obvious hypothesis is that the transmission occurs through routine physical contact between the queen and the PQ. However, the rapidity with which the PQ realizes the absence of the queen and the relative noninteractive nature of the queen made us unsure of the validity of this otherwise reasonable hypothesis. We therefore decided to test it by using a simple model that relates the time taken by the PQ to realize the absence of the queen, the rates of interaction between the queen and PQ, and the rates of decay of the pheromone. We designated the time taken by the PQ to realize that the queen is missing as  $t_r$ , the time taken by the pheromone to decay as  $t_d$ , and the average age of the pheromone available with the PQ at the time that we remove the queen as  $t_a$ . Clearly  $t_a$  will depend on when the PQ last interacted with the queen, and that is how we bring rates of interaction between the queen and PQ into the model. The PQ should realize the loss of the queen as soon as the queen pheromone available with her decays completely. Therefore,  $t_r = t_d - t_a$ . If this equation is satisfied then physical interaction between the queen and PQ are adequate to explain the rapidity with which the PQ realizes the absence of the queen.

Hence we set out to measure all of the 3 parameters of the model namely,  $t_r$ ,  $t_d$ , and  $t_a$ . Measuring PQ's realization time is not straightforward. The response of the PQ to the loss of her queen is that she becomes very aggressive. This is so dramatic that the number of acts of aggression shown by the PQ in the first 30 min of queen removal (on day 2 of the queen removal experiment) itself is vastly and significantly greater than all of the aggression she shows in the presence of the queen during all day (8 h of observation on day 1 of the queen removal experiment) (Fig. 5). Hence she definitely has realized the absence of the queen in 30 min. Perhaps she realizes sooner than 30 min but we cannot be sure. The reason is that we need at least 30 min to get a statistically valid estimate of her new rate of aggression on day 2, to compare with that on day 1. Hence we conservatively (meaning that it could be even less) estimate  $t_r$  as 30 min. In 26 of 50 colonies observed, the PQs (identified retrospectively, after queen removal) did not interact with their queens even once in the 5 h they were observed. If they did interact with their queens that must have been  $>5$  h ago, before we began our observations. And yet they behaved as if the queen was present, until we actually removed the queen. Hence the pheromone with them had not decayed in 5 h. This logic allowed us to estimate  $t_r$  as 300 min or more. Pooling data from all 50 colonies, we calculated that PQs interacted with their queens on average once in 213 min. Because we might have removed the queen any time from, just after a queen-PQ interaction to just before the next such interaction, we estimate the average age of the pheromone with the

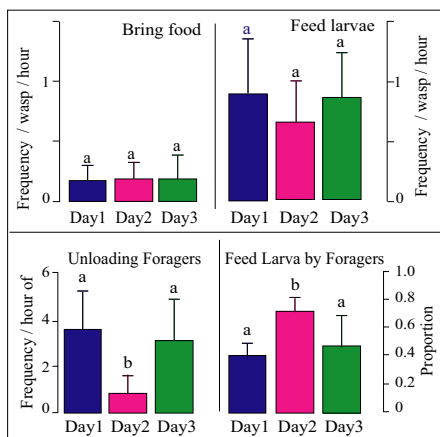


**Fig. 6.** Mean and SD of the frequency per hour of RA shown by the queen (blue bar), Max worker (the worker showing the highest value among workers in the colony; green bar), and Min worker (the worker showing the lowest value among workers in the colony; black bar).  $n = 8$  colonies. Comparisons are by Wilcoxon matched-pairs, signed-ranks test; bars with different letters are significantly different ( $P < 0.05$ ) from each other. [Redrawn with permission from ref. 19 (Copyright 2007, Elsevier).]

PQ at the time of queen removal,  $t_a$  as  $213/2 = 106.5$  min. Now if  $t_r = t_d - t_a$ , then  $t_r = 300 - 106.5 = 193.5$  min. But, of course, this does not match the experimentally determined  $t_r$  of 30 min or less. Thus, we concluded that the rates of direct physical interactions between the queen and PQ are inadequate to explain the rapidity with which the PQ realizes the absence of the queen.

It is, however, possible that the PQ does not need to directly interact with the queen to receive the queen pheromone; it may be adequate for her to interact with another worker who in turn has interacted with the queen. Thus, pheromone transfer between the queen and PQ may occur by a relay mechanism. We tested this hypothesis, too, in a similar way. For this we had to estimate the rates of relayed interaction (through other workers) between the queen and PQ. Here, we used a well-known procedure called the Dijkstra's algorithm (18) to determine the fastest possible path of interaction between the queen and the PQ, from data on rates of direct interactions between all possible pairs of wasps in each colony and thus determine  $t_a$  once again. This time we obtained a value of  $t_a = 102.9$  min. In testing the hypothesis of direct interactions between queen and PQ, we estimated  $t_d$  as 300 or more minutes because many of the PQs did not interact with their queens even once in 300 min. While testing the relayed interaction hypothesis, we found that all PQs interacted with their PQs through relay but the slowest interaction time was once in 340 min. So here we set  $t_d = 340$  min. Now if  $t_r = t_d - t_a$ , then  $t_r = 340 - 102.9 = 237.1$  min. Once again, this does not match the experimentally determined  $t_r$  of 30 min or less. Thus, we concluded that neither the rates of direct physical interactions nor the rates of relayed interaction between the queen and PQ are adequate to explain the rapidity with which the PQ realizes the absence of the queen.

Because physical interactions appeared inadequate we considered the hypothesis that the queen applies her pheromone to the nest surface. This hypothesis suggested itself because of the characteristic manner in which the queen (but not the workers) rubs the tip of her abdomen on the nest while walking on the nest. To test the plausibility of the hypothesis that the queen applies her pheromone to the nest surface using the rub abdomen behavior (RA), we undertook a detailed study of RA, with special attention to the rates at which this behavior is performed. We found that only the queen performs the RA to any significant extent and even if some of the workers do so in some of the colonies those rates are negligible. More importantly, the queen perform RA on average once in 23 min (Fig. 6). And if she indeed used this behavior to apply her pheromone to the nest surface, she must apply a fresh coat of pheromone every 23 min. Thus, if the queen is removed the PQ should realize the absence of the queen after 23 min. At last the



**Fig. 7.** Foraging, feeding larvae, and unloading in a typical queen removal experiment. (Upper) Frequencies per hour of food brought per individual per hour and feeding larvae per individual per hour are not significantly different on days 1–3, as can be seen from the identical letters on the bars. (Lower) Frequencies at which foragers were unloaded and the proportion of the feeding of larvae done by foragers on days 1–3. For each behavior, bars with different letters are significantly different from each other. All comparisons are by 2-tailed Wilcoxon matched-pairs signed-rank tests. [Redrawn with permission from ref. 12 (Copyright 1995, Oxford University Press).]

numbers match because we have determined that the PQ realizes the absence of the queen in 30 min. So our current answer to question 4 is that the queen signals her presence by applying her pheromone to the nest surface using RA. In other words, the workers perceive the queen’s presence or absence by the presence or absence of the queen pheromone on the nest surface, without having to necessarily physically interact with the queen either directly or through relay (19).

**Question 5: How Does the Queen Regulate the Nonreproductive Activities of the Workers?**

If *R. marginata* queens overcome their lack of aggression by using a pheromone to regulate worker reproduction and maintain reproductive monopoly, how do they overcome their lack of aggression when it comes to regulating the nonreproductive activities of their workers? How, for instance, do *R. marginata* queens ensure that their workers bring adequate quantities of food and feed the larvae? To answer this question we went back to the queen-removal experiment and measured the rates at which workers brought food back to the nest and fed larvae in the presence of the queen (day 1) and compared these rates to the corresponding rates in the absence of the queens (day 2). We expected a sharp decline in these rates after queen removal, based on the results of similar experiments in other primitively eusocial species (refs. 20 and 21 but see ref. 22). To our surprise however, we found no statistically significant differences in both the rates of bringing food to the nest and the rates of feeding the larvae, in the presence of the queen (day 1), in the absence of the queen (day 2), and again after the queen was returned (day 3). The workers did not seem to care whether their queen was present or not; they continued to bring food and feed the larvae regardless of the queen’s presence or absence (Fig. 7 Upper). Thus, *R. marginata* queens do not regulate the nonreproductive activities such as bringing food and feeding larvae by their workers (12).

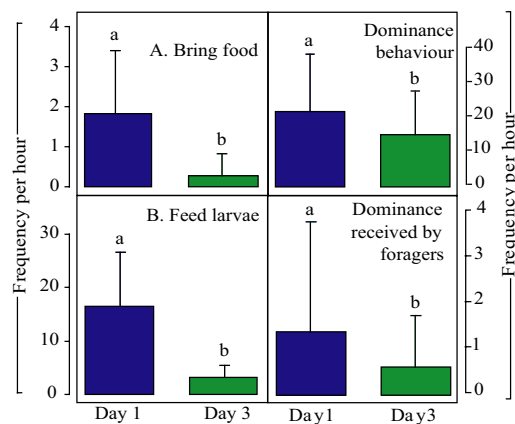
But who regulates the nonreproductive activities of the workers? A closer examination of another behavior that occurs in between bringing food and feeding the larvae provided a clue. When *R. marginata* foragers return to their nest with food they are usually mobbed by the intranidal workers (workers who work on the nest rather than outside) who take the food from them and feed the larvae. This behavior of unloading of the foragers by the intranidal

workers was significantly reduced in the absence of the queen on day 2 compared with the corresponding rate on day 1 in the presence of the queen and on day 3 with the queen returned. The reason for this reduction in unloading was rather unexpected and interesting. Because of the highly elevated levels of aggression of the PQ after queen removal, most of the intranidal workers were chased away from the nest at least temporarily. Consequently, there were few or no intranidal workers present on the nest to do the unloading. The response of the foragers to this failure to be unloaded was even more interesting; they went on to feed the larvae themselves before going off to fetch more food (Fig. 7 Lower). Our interpretation of these results is as follows. In a normal queen-right colony, intranidal workers unload the foragers and feed the larvae, and for this reason they have information about the state of hunger in the colony. They can therefore convey this information to the foragers during unloading. In the absence of unloaders, foragers themselves feed the larvae and gain first-hand information about the hunger levels and can therefore regulate their own rates of foraging (12). In summary, the answer to question 5 is that an *R. marginata* queen does not regulate the nonreproductive activities of her workers and the workers themselves do so in a decentralized, self-organized manner.

**Question 6: What Is the Function of the Dominance Behavior Shown by the Workers?**

Because we postulated above that intranidal workers convey information about hunger levels in the colony while unloading foragers, we were looking for a possible mechanism by which they may do so. Having found that *R. marginata* queens do not use dominance behavior to regulate worker reproduction or worker foraging, we had also been looking for a function for the dominance behavior shown by the workers toward each other. Putting the two together we hypothesized that dominance behavior that no longer has a role in modulating reproductive competition in the colony (see answers to questions 2 and 7) may have been co-opted in the course of evolution to serve a different function, namely, to convey colony hunger levels to foragers. This hypothesis also suggested itself because we had often seen intranidal workers aggress foragers who after delivering a load of food would sometimes become lazy and remain on the nest. And departures of foragers from the nest would often be in response to aggression from intranidal wasps. We now have 3 lines of evidence in support of this “hunger-signal” hypothesis for the function of worker dominance behavior. From observational data on normal colonies we find a weak, but statistically significant, positive correlation between a worker’s contribution to the total foraging effort of a colony and the amount of dominance behavior she received from other workers (12). Apart from the correlation being weak, meaning that only a small proportion of the worker-to-worker variation in foraging could potentially be explained by variation in dominance received, correlational evidence cannot be taken as proof of cause and effect. There could be a third factor that influences both foraging rates and dominance received, without there necessarily being any causal link between these 2 variables. To infer a causal link between dominance received and foraging performed, it is essential to use an experimental approach where factors other than foraging and dominance behavior can be held constant or nearly so. Ideally one would have liked to experimentally increase (or decrease) the dominance behavior received by selected foragers and see if they then foraged more (or less) in response. This is not easy to do. However, we have now been able to experimentally increase or decrease the demand for food (hunger level) in the colony and examine the effect of these treatments on the levels of dominance behavior received by the foragers in the colony.

To decrease the demand for food we learned how to hand-feed the wasps with excess food until they seemed to be entirely satiated. In this set of “excess feeding” experiments we observed a normal, unmanipulated colony on day 1 and spent day 2 in hand-feeding the

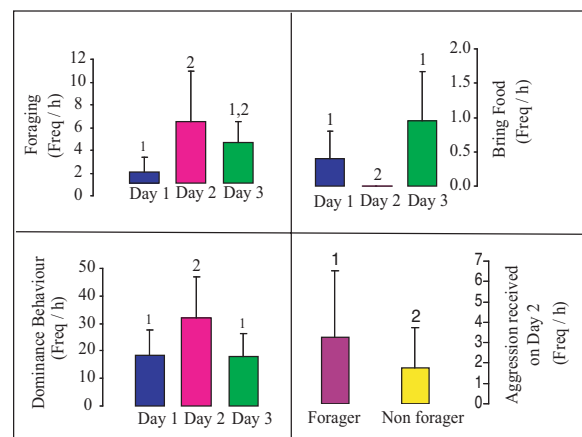


**Fig. 8.** Excess feeding experiment. Comparisons of bring food, feed larvae, and dominance behavior among workers and dominance received by foragers, on day 1 (normal colony) and day 3 (1 day after excess feeding). Bars shown are the means and SDs across 11 nests. For all variables, values on day 1 are significantly greater than the corresponding values on day 3 (two-tailed Wilcoxon matched-pairs, signed-rank tests;  $P < 0.05$ ). [Redrawn with permission from ref. 23 (Copyright 2006, Elsevier).]

wasps in the colony without making any observations. We then spent day 3 in observing the colony to examine the effects of excess feeding on day 2. The rates of bringing food to the nest and feeding larvae showed a significant decline on day 3 as compared with day 1. Concomitantly, the rates of dominance behavior among the workers and, more specifically, the rates of dominance received by the foragers also showed a significant decline on day 3 as compared with day 1 (Fig. 8) (23). This, of course, is a much stronger evidence for the hunger-signal hypothesis. Similarly strong evidence came from the converse “food deprivation” experiments where we increased the demand for food. In these experiments also we observed colonies on 3 consecutive days. On day 1 we observed a normal, unmanipulated colony and on day 2 we closed the doors of the cage, preventing any possibility of foraging. On day 3 we reopened the doors of the cage so that foraging could resume. Foraging attempts increased on day 2 (closed cage) relative to day 1 (open cage) although they were inevitably unsuccessful so that no food was brought to the nest. At the same time rates of dominance behavior rose significantly in relation to day 1. The foragers of day 1 were specifically targeted to receive dominance behavior on day 2 as if the intranidal workers knew who was more likely to bring back food to the nest. Once the cage doors were opened on day 3 food began to be brought to the nest and rates of dominance behavior returned to levels comparable to those on day 1 (Fig. 9) (24). Taken together, the initial qualitative information about intranidal workers targeting foragers who did not resume foraging after delivering some food, the evidence that bringing food and receiving dominance behavior were correlated, and the experimental evidence that decreased demand for food decreases dominance behavior directed toward foragers and that increased demand for food increases dominance behavior directed toward known foragers inspire confidence in the hunger-signal hypothesis. Hence our answer to question 6 is that dominance behavior among *R. marginata* workers functions to relay colony hunger signals from intranidal workers to foragers and makes possible the decentralized, self-organized regulation of foraging.

### Question 7: What Is the Function of the Temporarily-Elevated Aggression Shown by the PQ?

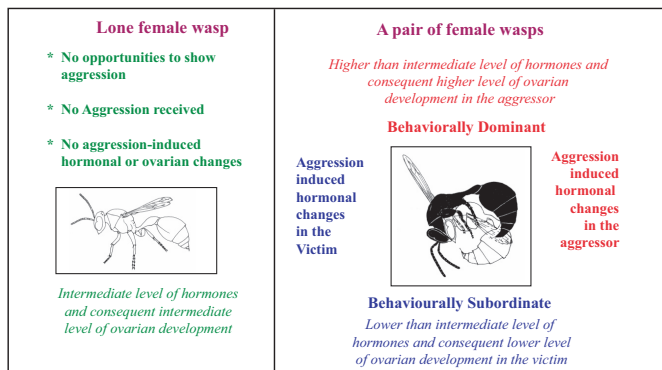
As I have described above, the PQ displays highly-elevated levels of aggression immediately after removal of the queen. What is the function of this hyper aggression? It cannot possibly function to signal hunger to foragers as we have postulated for the low levels



**Fig. 9.** Food deprivation experiment. Comparison of mean and SD of frequency per hour of dominance behavior, foraging attempts, and bring food on day 1 (normal colony), day 2 (food deprived by preventing foraging), and day 3 (foraging permitted). Different numbers indicate a significant difference between the bars (two-tailed, Wilcoxon matched-pairs test;  $n = 8$ ). [Redrawn with permission from ref. 24 (Copyright 2008, Springer).]

of aggression displayed by workers in an unmanipulated colony. In keeping with what is known in other primitively eusocial species we considered the hypothesis that PQ's aggression might function to suppress reproduction by other contenders to the position of the queen and permit the PQ to claim that position for herself. If the PQ does indeed use aggression as a mechanism to suppress workers posing a reproductive threat to her, then the amount of aggression shown by the PQ is expected to correlate with factors that contribute to this threat. The 3 such factors we considered are number of nest mates, dominance status, and ovarian condition of the nest mates. We expect 1 or more of these 3 variables to be positively correlated with the amount of aggression displayed by the PQ. Using data from a large number of queen removal experiments we failed to find evidence for any of these predictions. The rate of aggression shown by the PQ did not scale with the number of nest mates she had to contend with, the dominance ranks of the recipients of her aggression, or indeed the state of ovarian development of the recipients of her aggression. Thus, it does not appear that the PQ uses her hyper aggression to neutralize any threat from her nest mates to her chances of taking over the colony as its next queen (25).

We then considered a different, more radical hypothesis, which we initially thought was less likely to be upheld. This was that the amplified aggressive behavior shown by the PQ is necessary for the rapid development of her own ovaries. If this is true, we predict that a lone PQ, lacking the opportunity to display any aggression, should take longer to develop her ovaries and lay her first egg in comparison with a PQ who has the opportunity to aggress nest mates and gain whatever advantage she might from that aggression. Rather surprisingly this hypothesis was in fact supported; lone PQs indeed took significantly longer ( $\approx 8$  days) to lay their first eggs compared with PQs with nest mates who took only  $\approx 6$  days to do so (25). At the present time we do not know the physiological processes that might link the act of showing aggression with ovarian development but it seems reasonable to imagine that hormonal changes that result from the expression of aggressive behavior might trigger ovarian development in the aggressor. This seems like a reasonable hypothesis because, given that queens in most primitively eusocial species use aggression to suppress worker reproduction, we should also expect that hormonal changes in the recipients of aggression lead to converse physiological changes that suppress ovarian development. Thus, the answer to question 7 is that the temporarily elevated levels of aggression shown by the PQ functions to rapidly



**Fig. 10.** A schematic diagram postulating physiological effects of aggression in the aggressor and the victim (Right) and the absence of aggression in a lone wasp (Left).

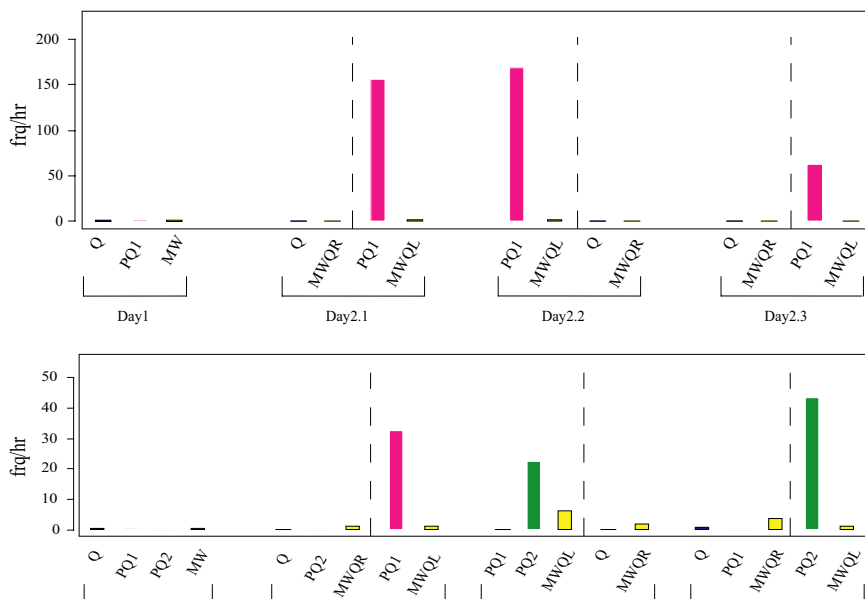
develop her own ovaries and take over the colony as its next queen. This somewhat unexpected result has now spurred in us great interest in studying the physiology of reproduction in *R. marginata*, both the physiology of ovarian development and ovarian suppression (Fig. 10).

**Question 8: How and When Is the Queen’s Heir Decided?**

We have already seen that as soon as the queen is experimentally removed one of the workers takes on the role of PQ and goes on to become the next queen. How is the queen’s successor chosen and when is she chosen? These questions are of obvious interest. In other primitively eusocial species dominance-subordinate interactions are an expression of reproductive competition in the colony. Therefore, the position of a worker in the dominance hierarchy is the position of that worker in the queue for becoming a future queen of the colony. In *R. marginata*, however, the queen seldom occupies rank 1 and the position of a worker in the dominance hierarchy has no bearing on the probability that she will become the next queen of the colony. Who then is the queen’s successor? Despite many experiments explicitly designed to predict the PQ even while the previous queen is still present on the nest we have so far failed. After making detailed observations on queen-right colonies, we have removed the queen, identified the PQ, and then went back to data collected before queen removal on the PQ and

other workers who did not become the PQ and have tried to understand what was unique about the PQ. There appears to be nothing unique about the PQ. She is not unique in her behavior, her dominance rank, her body size, her age, and not even the state of her ovarian development. Thus, we simply cannot predict the queen’s successor before removing the queen. Just to be sure that it is not our inadequacy in coming up with appropriate methods to predict the queen’s successor, we have carried out a comparative study of *Ropalidia cyathiformis* (26, 27), a congeneric, sympatric species that is in every way a typical primitively eusocial species. In this species we are always able to predict the queen’s successor although I do not have the space here to describe these experiments.

Although we cannot predict the identity of the PQ in *R. marginata*, because (i) only 1 individual steps up her aggression after queen removal, (ii) the swiftness with which she does so, and (iii) she alone is unanimously accepted by the rest of the workers led us to suspect that, just as in other primitively eusocial species, there may also be a designated successor to the queen in *R. marginata*, who may be “cryptic” to us in the presence of the queen. We therefore designed another experiment to test such a “cryptic successor hypothesis.” This experiment was a modification of the mesh experiment. The modification was that after a PQ became evident on the queenless side of the wire mesh, we exchanged the PQ and the queen from side to side, leaving the workers undisturbed. The logic of this queen–PQ exchange experiment is as follows. Because the workers are randomly distributed between the 2 sides, the cryptic successor, if there is indeed one, has a 50% chance of being on either the queen-right or the queenless fragment. In those experiments where the cryptic successor happened to be in the queenless side, she would become a PQ (we call her PQ1) and, being the true successor, she should be acceptable to the workers on both sides even when she is moved from side to side. And in those experiments where the cryptic successor happened to be on the queen-right side just by chance, a different individual should become the PQ1 on the queenless side because of the absence of the legitimate successor on her side. Hence, this PQ1 should be unacceptable to the workers on the opposite side when she is moved to that side. Instead, the real cryptic successor should now become the new PQ (we call her PQ2). Finally, the PQ2 should remain unchallenged on both sides if she is moved from side to side. Thus, the PQ1 should be acceptable to the workers on both sides in about half the experiments and the PQ2 but not the PQ1 should



**Fig. 11.** The queen (Q)–PQ exchange experiment. (Upper) A typical experiment in which the PQ1 was the cryptic successor. The frequency per hour of dominance behavior exhibited by the queen, PQ1, and Max worker (defined as the worker showing maximum aggression) on day 1 in the normal colony and on the queen-right and queenless fragments in the 3 sessions on day 2 are shown. (Lower) A typical experiment in which the PQ2 was the cryptic successor. The frequency per hour of dominance behavior exhibited by the queen, PQ1, PQ2, and Max worker on day 1 in the normal colony and on the queen-right and queenless fragments in the 3 sessions on day 2 are shown. [Redrawn with permission from ref. 28 (Copyright 2008, The Royal Society).]

be acceptable to workers on both sides in the remaining half of the experiments.

This is a very difficult experiment to perform but we have managed to perform it 8 times. In 3 of these experiments the first PQ was accepted on both sides and in the remaining 5 experiments, the second PQ was accepted on both sides (Fig. 11). We therefore concluded that there is indeed a designated successor to the queen in *R. marginata*, as in other species. But we refer to her as a cryptic successor because we cannot identify her in the presence of the queen by the same criteria that are adequate to identify the PQ in other primitively eusocial species such as *R. cyathiformis* (27). An important feature of our results was that neither PQ1 nor PQ2 ever received a single act of aggression from any individual, although they themselves showed high levels of aggression. Thus, when we say PQ1 was unacceptable when we moved her to the opposite side, we simply mean that she, on her own, stopped being aggressive and went back to work although she was never challenged by anybody, not even by the PQ2. Hence we argue that the cryptic successor is “known” to the wasps even though we cannot identify her in the presence of the original queen (28). Thus, our answer to question 8 is that the successor to the queen is decided even before the loss of the original queen although I must confess that we are at present ignorant about how this decision is made.

### Question 9: Why Is *R. marginata* Such an Unusual Primitively Eusocial Wasp?

I have so far described our efforts to ask 8 different questions about *R. marginata*. And every one of the answers we have obtained suggests that *R. marginata* is strikingly different from what is expected of a primitively eusocial species. This assessment of the contrast between *R. marginata* queens and those of other primitively eusocial wasps is based on what is known from the literature and our own study of the typical primitively eusocial species *R. cyathiformis* (27, 29–33). What does one make of these striking contrasts? We have argued that *R. marginata* is not as primitively eusocial as we thought at the beginning of our study and that it appears to have acquired some features of highly eusocial species. This argument demystifies in 1 stroke all of the eccentricities of *R. marginata* in the context of primitively eusocial species.

If our reading of the position of *R. marginata* in social evolution is correct, we can then begin to draw a number of other inferences. Our interrogation of *R. marginata* suggests that features such as (i) nonaggressive, noninteractive queens, (ii) pheromonal as opposed to aggression-based regulation of worker reproduction, (iii) decentralized, self-organized regulation of the nonreproductive activities of the workers as opposed to centralized, top-down control, and (iv) the function of queen pheromones as honest signals of queen fertility as opposed to physical intimidation of workers, can all appear in the course of evolution even before the evolution of large colony sizes ( $\gg 100$  individuals) and before the appearance of morphological caste differentiation between queens and workers. This conclusion needs to be tested with other evolutionary lineages among social bees and wasps at other points in the primitively-highly eusocial continuum. Our findings that the identity of the PQ appears to be known to the wasps and that aggression by the PQ serves to boost her own ovarian development are findings that can potentially spawn additional lines of research.

But in the meantime my students and I have by no means completed interrogating *R. marginata*. As I have already demonstrated, the answer to every question opens at least 1 other question. Presently we are engaged in trying to better understand *R. marginata*, including for example, to identify and characterize the queen pheromone, understand when, how, and why the queen loses her status and gives way to a successor, identify not merely the queen's next successor but also a series of other workers who might be in the queue to occupy the position of the queen, and so on. The number of questions is, as far as we can see, truly endless.

**ACKNOWLEDGMENTS.** The work I have described was done over a 30-year period and was only possible because of the friendship, collaboration, and shared passion for unlocking the mysteries of *R. marginata* of a large number of students and colleagues including Anindita Bhadra, Nadia Bruyndonckx, Krishnappa Chandrashekar, Kannepalli Chandrasekhar, Sujata Deshpande, Saubhik Ghosh, Priya Iyer, Niranjani Joshi, Yasmin Claire Kazi, Shakti Lamba, Meghana Natesh, Sudha Premnath, Anindya Sinha, Annagir Sumana and Martin Surbeck. I also thank Jae Choe, Robin Crewe, Helga Nowotny, and John Wenzel for kindly reading the manuscript and making many helpful suggestions. My research has been supported by the Centre for Ecological Sciences, Indian Institute of Science, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, and several agencies of the Government of India including the Department of Science and Technology, Department of Biotechnology, Ministry of Environment and Forests, and Council of Scientific and Industrial Research. The Wissenschaftskolleg zu Berlin provided an intellectually stimulating atmosphere for writing this essay.

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