Emergence of cooperation and division of labor in the primitively eusocial wasp *Ropalidia marginata*

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In most primitively eusocial wasps new nests are initiated by a single female or by small groups of females. To study the emergence of division of labor (DOL) among the nest foundresses and to determine its possible effect on nest productivity we maintained newly eclosed females of *Ropalidia marginata* in small boxes with one, two, or three nestmate wasps of the same age per box. Only one wasp developed her ovaries and laid eggs in each box, while the other wasp(s) built the nest, brought food, and fed larvae, demonstrating the spontaneous emergence of reproductive DOL in the presence of more than one wasp. In nests with three wasps there was also a strong negative correlation between intranidal and extranidal work performed by the two nonreproductive workers, suggesting the spontaneous emergence of nonreproductive DOL; such nonreproductive DOL was absent in nests with two wasps. Both reproductive and nonreproductive DOL were modulated by dominance behavior (DB). In nests with two wasps the egg layer showed significantly more DB than the non-egg layer before nest initiation; in nests with three wasps queens showed significantly more DB than intranidal workers, which in turn showed significantly more DB than extranidal workers. Productivities of nests (as measured by total brood on the day of eclosion of the first adult) initiated by one or two wasps were not different from each other but were significantly lower than that of three wasps. Thus, nonreproductive DOL, and not merely reproductive DOL, is necessary for increase in productivity.

division of labor | primitively eusocial wasp | *Ropalidia marginata* | cooperation | dominance behavior

Social insects such as ants, bees, wasps, and termites are among the most evolutionarily successful and ecologically dominant animals, accounting for nearly half of the insect biomass in some terrestrial environments (1). This has been attributed to their extreme levels of intracolony cooperation and division of labor (DOL) (2, 3). DOL is generally defined as any temporary or permanent behavioral pattern which results in some individuals in the colony performing different functions from the rest (4–6). Reproductive DOL resulting in one or a small number of individuals in the colony becoming fertile reproductives (queens/kings) and the remaining large fraction of the colony functioning as sterile nonreproductive workers is an essential feature of these so-called eusocial insects (2). Because nonreproducing workers perform all of the tasks required to build and maintain nests, forage and feed the brood, and defend the colony from predators, insect societies provide the most extreme examples of cooperation in the animal kingdom (3, 7, 8). In addition to reproductive DOL, in many species workers further divide nonreproductive labor among themselves so that some may perform intranidal tasks and others extranidal tasks; some may feed the larvae while others guard the nest, some may forage for pollen while others for nectar, some may transport leaves or seeds to the nest while others guard the trail of food-laden foragers, and so on. Such "nonreproductive" DOL is expected and known to further enhance the success of eusocial insect societies (1). In eusocial insect societies nonreproductive DOL may be based on age of the workers, so that the tasks performed by individuals change along with their absolute or relative age in the colony, a phenomenon referred to as age polyethism (9–11). Nonreproductive DOL may also be based on morphologically specialized subgroups of workers performing specific tasks, or on a combination of morphology and age (3, 8, 12–14). Age- or morphology-based nonreproductive DOL has been reported mostly in colonies of eusocial species and very rarely in other forms of social organizations (15). However, parental manipulation of resources provided to offspring (16) and "maternal vibrational signals" by antennal drumming have been reported to increase the probability of larvae developing into subordinate (worker) adults (17–19). Communal species, which are considered to be a transitional step in the evolution of eusocial insects from their solitary counterparts, do not display reproductive or nonreproductive DOL (2, 20). Although division of nonreproductive labor has been reported for natural or forced foundress associations of communal species, there is no evidence that such association contributes to the success of the newly initiated nests (21, 22). The increased success of communal species arises from their "social dynamics" emerging as a result of the interactions among the cooperative individuals. This has been recently demonstrated in an interesting way by studying artificially constituted pairs consisting of a normally solitary queen and a normally cooperative queen of *Pogonomyrmex californicus* (23).

While many ant, bee, wasp, and termite societies have been studied to document cooperation and DOL in action (5, 13, 24–26), large natural colonies are less suited to investigate the emergence of cooperation and DOL. To understand the number of individuals required for the emergence of cooperation and DOL, the proximate mechanisms that give rise to these phenomena, and their effects on the colony fitness, here we have isolated and monitored one (solitary), two (pairs), or three (triplets) individual females of the primitively eusocial wasp *Ropalidia marginata*, under controlled laboratory conditions. *R. marginata* is ideally suited for such an investigation: it is a tropical wasp and nests are initiated throughout the year by one or a small group (up to 21) of female wasps. Postemergence colonies can occasionally have up to 100 female

Significance

Cooperation and division of labor are two important features of eusocial insects such as ants, bees, wasps, and termites. Using the primitively eusocial wasp *Ropalidia marginata*, we investigated the minimum requirements for the emergence of cooperation and division of labor, both reproductive and nonreproductive, and their effect on productivity (i.e., total brood of a colony). We show that (*i*) two wasps are adequate for the emergence of reproductive division of labor and cooperation and that three wasps are both necessary and sufficient for the additional emergence of nonreproductive division of labor and (*ii*) reproductive division of labor and cooperation are inadequate for increasing productivity, which comes about only with the addition of nonreproductive division of labor.

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wasps, although the average colony size is 21.9 ± 22.3 females (27). While preemergence nests are either solitary or semisocial, the postemergence nests satisfy the criteria of being eusocial. Colonies can be readily maintained in the laboratory and the behavior of all adult wasps can be readily observed because of their open, unenveloped nests. An additional advantage is that such experiments can be carried out using newly eclosed virgin wasps. Mating is not essential for a female wasp to build a nest, develop her ovaries, lay unfertilized eggs (28), and even become the sole egg layer of a colony despite the presence of other mated individuals (at least temporarily) (29). Virgin and mated females have an equal chance of becoming the sole egg layer when they are paired under laboratory conditions (30).

Results

When virgin female wasps of *R. marginata* were isolated in small acrylic boxes, nests were initiated in 51%, 67%, and 67% of the boxes for solitary, pairs, and triplets, respectively. The proportions of boxes in which nests were initiated were not significantly different between boxes containing one, two, or three wasps (Fig. 1*A*; to avoid clutter, here and elsewhere, we describe the results in words and refer the reader to the corresponding figures or tables for details of the statistical tests supporting the verbal statements). Of the initiated nests 51%, 61%, and 70% were successful for solitary, pairs, and triplets, respectively; these proportions were not significantly different from each other (Fig. 1*C*). Solitary wasps took 33–48 d to initiate nests, pairs took 14–19 d, and triplets took only 13–15 d. Such latency to nest initiation was significantly higher in solitary wasps compared with that in pairs and triplets, the latter two not being significantly different from each other (Fig. 1*B*). Successful nests took from 31 to 63 d (mean ± SD = 47.39 ± 8.77) for eclosion of the first adult offspring; these durations were not significantly different between nests made by one, two, or three wasps (Fig. 1*D*).

Reproductive DOL. In each box with a nest initiation event there was only one nest (with at least one egg in it) irrespective of the number of wasps present. All solitary foundresses (whether or not we actually observed egg-laying behavior) had at least one mature egg in their ovaries and we therefore designated these wasps as Q_1. In each nest with two wasps, only one wasp contained at least one mature egg in its ovary, while the other wasp had undeveloped, thread-like ovaries or partially developed ovaries but without any mature oocytes. We designated the wasps with mature eggs as queens (Q_2) and wasps without mature eggs as workers (W_2). In each nest with three wasps, once again only one wasp had at least one mature egg in her ovary while the other two wasps always had undeveloped, thread-like ovaries. We designated the individual with mature eggs as Q_3 (see below for the designations for the two individuals without mature eggs). There was significant variation in ovarian development (as measured by the ovarian index; *Materials and Methods*) based on the number of wasps in a nest; Q_2 and Q_3 had significantly better-developed ovaries compared with Q_1, while Q_2 and Q_3 were indistinguishable (Fig. 2*A*). When queens were compared with workers, queens had significantly better-developed ovaries compared with their workers in both pairs and triplets (Fig. 2*A*); in triplets the ovaries of the two non-egg layers were indistinguishable from each other (Fig. 2*A*).

Nonreproductive DOL. In nests with three wasps there was a significant negative correlation between intranidal and extranidal work performed by the two workers (Spearmann $\rho = -0.61$, $P = 0.004$). We designated workers with the higher frequency of intranidal work as the intranidal worker (IW_3) and the workers with higher frequency of extranidal work as the extranidal worker (EW_3). It should be noted that in nests with two wasps there was no significant correlation (positive or negative) between intranidal and extranidal work done by the queens (Q_2) and workers (W_2) (Spearmann $\rho = 0.09$, $P = 0.72$).

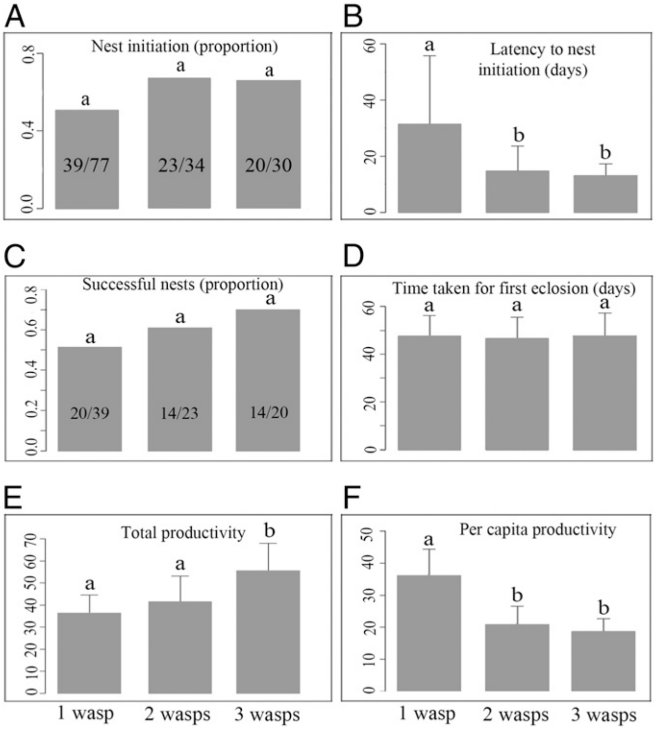

Fig. 1. Comparison between boxes with one (solitary), two (pairs), and three (triplets) wasps: bars carrying different letters are significantly different from each other. (*A*) Test of equal proportions; $\chi^2 = 3.93$, $P = 0.14$. (*B*) One vs. two wasps (Mann–Whitney *U* test; U = 229.5, $P = 0.01$, $n = 39$ and 23), one vs. three wasps (Mann–Whitney *U* test; U = 251, $P < 0.01$, $n = 39$ and 20), and two vs. three wasps (Mann–Whitney *U* test; U = 100, $P = 0.946$, $n = 23$ and 20). (*C*) Test of equal proportions; $\chi^2 = 1.98$, $P = 0.37$. (*D*) Kruskal–Wallis test; $\chi^2 = 0.12$, $P = 0.94$, $n = 20$, 14, and 14. (*E*) One vs. two wasps (Mann–Whitney *U* test; U = 184.5, $P = 0.12$, $n = 20$ and 14), one vs. three wasps (Mann–Whitney *U* test; U = 254.5, $P < 0.01$, $n = 20$ and 14), and two vs. three wasps (Mann–Whitney *U* test; U = 155.5, $P = 0.008$, $n = 14$ and 14). (*F*) One vs. two wasps (Mann–Whitney *U* test; U = 274, $P < 0.01$, $n = 20$ and 14), one vs. three wasps (Mann–Whitney *U* test; U = 280, $P < 0.01$, $n = 20$ and 14), and two vs. three wasps (Mann–Whitney *U* test; U = 128, $P = 0.178$, $n = 14$ and 14). We computed productivity as the number of eggs multiplied by 1.0, the number of larvae multiplied by 2.0, 3.0, and 4.0 depending on the stage of development, and the number of pupae multiplied by 5.0 (60).

Role of Dominance Behavior in DOL. We compared the frequency per hour of dominance behavior (DB) exhibited by wasps in pairs and triplets before nest initiation, excluding Q_1 from the analysis (as there was no possibility of any behavioral interaction in the case of solitary wasps). In pairs, Q_2 showed significantly higher DB than W_2, before nest initiation. Similarly, in triplets Q_3 showed significantly higher DB compared with both IW_3 and EW_3, and IW_3 showed significantly greater DB than EW_3 (Fig. 3*A* and Table S3).

We similarly compared the frequency per hour of DB exhibited by wasps in pairs and triplets after nest initiation. In pairs, Q_2 showed significantly higher DB than W_2 (Fig. 3*B* and Table S4) after nest initiation, while in triplets IW_3 showed significantly higher DB compared with both Q_3 and EW_3, and there was no difference in the DB shown by Q_3 and EW_3 (Fig. 3*B* and Table S4).

Work Organization. The caste of a wasp (i.e., whether it was a queen or a worker) had a significant effect on its intranidal (Fig. 2*B* and Table S1) and extranidal (Fig. 2*C* and Table S2) work pattern. In nests with two wasps, Q_2 performed more intranidal work but performed a similar amount of extranidal work compared with W_2 (Fig. 2 *B* and *C* and Tables S1 and S2). In nests with three wasps, Q_3 performed more intranidal work than IW_3 but was indistinguishable

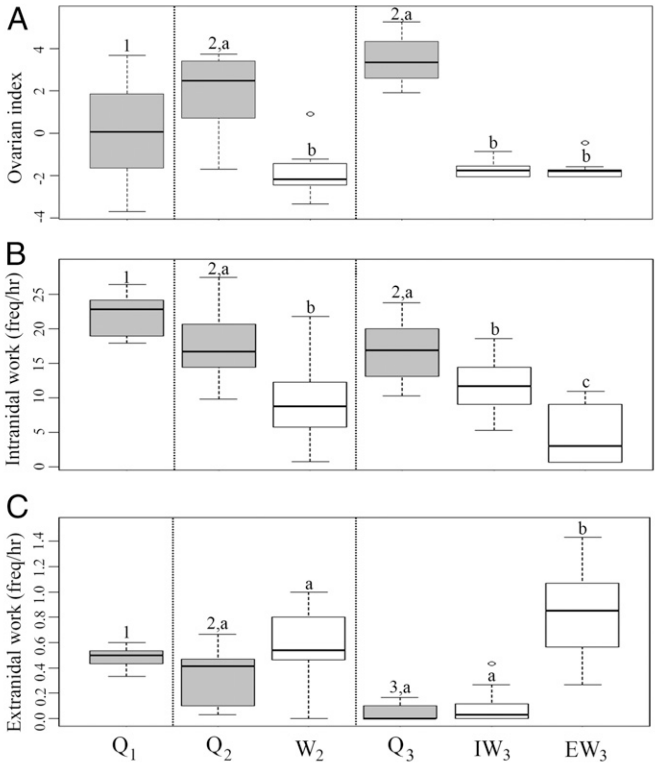

Fig. 2. Comparisons between queens in solitary, pairs, and triplets and between queens and workers in pairs and triplets; shaded bars show queens and unshaded bars show workers. Numbers above the bars represent comparisons between the queens across panels and letters above bars represent comparisons between queens and workers in the same panel; bars carrying different numbers or different letters are significantly different from each other. (*A*) Q_1 vs. Q_2 (linear model; estimate = 1.939, $t = 2.764$, $P = 0.009$; $n = 20$ and 10), Q_1 vs. Q_3 (linear model; estimate = 3.443, $t = 4.908$, $P < 0.0001$; $n = 20$ and 10), Q_2 vs. Q_3 (linear model; estimate = 1.504, $t = 1.856$, $P = 0.07$; $n = 10$ and 10), Q_2 vs. W_2 (linear model; estimate = −3.879, $t = -5.627$, $P < 0.0001$; $n = 10$ and 10), Q_3 vs. IW_3 (linear model; estimate = −5.171, $t = -15.69$, $P < 0.0001$; $n = 10$ and 10), Q_3 vs. EW_3 (linear model; estimate = −5.158, $t = -15.65$, $P < 0.0001$; $n = 10$ and 10); IW_3 vs. EW_3 (linear model; estimate = 0.013, $t = 0.072$, $P = 0.944$; $n = 10$ and 10). (*B*) Q_1 vs. Q_2 (linear mixed model, estimate = −4.42, $t = -2.31$, $P = 0.021$; $n = 10$ and 10), Q_1 vs. Q_3 (linear mixed model, estimate = −5.59, $t = -2.95$, $P = 0.003$; $n = 10$ and 10), Q_2 vs. Q_3 (linear mixed model, estimate = −1.17, $t = -0.62$, $P = 0.54$; $n = 10$ and 10), Q_2 vs. W_2 (linear mixed model, estimate = −8.46, $t = -5.87$, $P < 0.0001$; $n = 10$ and 10), Q_3 vs. IW_3 (linear mixed model, estimate = −5.01, $t = -2.78$, $P = 0.005$; $n = 10$ and 10), Q_3 vs. EW_3 (linear mixed model, estimate = −12.27, $t = -6.79$, $P < 0.0001$; $n = 10$ and 10), and IW_3 vs. EW_3 (linear mixed model, estimate = −7.26, $t = -4.21$, $P < 0.001$; $n = 10$ and 10). (*C*) Q_1 vs. Q_2 (linear model, estimate = −0.276, $t = -4.373$, $P < 0.001$; $n = 10$ and 10), Q_1 vs. Q_3 (linear model, estimate = −0.445, $t = -7.058$, $P < 0.0001$; $n = 10$ and 10), Q_2 vs. Q_3 (linear model, estimate = −0.169, $t = -2.685$, $P = 0.012$; $n = 10$ and 10), Q_2 vs. W_2 (linear model, estimate = 0.214, $t = 0.127$, $P = 0.11$; $n = 10$ and 10), Q_3 vs. IW_3 (linear model, estimate = 0.057, $t = 0.577$, $P = 0.569$; $n = 10$ and 10), Q_3 vs. EW_3 (linear model, estimate = 0.795, $t = 8.093$, $P < 0.0001$; $n = 10$ and 10), and IW_3 vs. EW_3 (linear model, estimate = 0.739, $t = 6.222$, $P < 0.0001$; $n = 10$ and 10).

from it in the extent of extranidal work (Fig. 2 *B* and *C* and Tables S1 and S2). Solitary queens (Q_1) did more intranidal work compared with Q_2 and Q_3; the latter two were not distinguishable from each other (Fig. 2*B* and Table S1). Q_1 performed more extranidal work compared with Q_2, which in turn performed more extranidal work compared with Q_3 (Fig. 2*C* and Table S2).

Nest Productivity. Productivity on the day of eclosion of the first adult wasp was not significantly different between nests with one or two wasps (Fig. 1*E*); however, nests with three wasps had significantly greater productivity compared with nests with one or two wasps (Fig. 1*E*). As expected, the per capita productivity decreased from one to two wasps but did not decrease further for three wasps' nests (Fig. 1*F*). We also compared daily changes in productivity using a generalized additive mixed model (*Materials and Methods*) with appropriate smoothers for nests with one, two, or three wasps. Nests with three wasps had significantly greater change in productivity over days compared with both nests with one or two wasps; however, there was no significant difference between nests with one or two wasps (Fig. 4).

We have confirmed that there were no significant differences in body size among Q_1, Q_2, W_2, Q_3, IW_3, and EW_3 (Fig. S1).

Discussion

Irrespective of the number of wasps in a box, we observed nests with eggs in more than 50% of the boxes set up, although solitary wasps took longer to initiate nests. The number of wasps in a box neither influenced the success rate (probability of producing at least one adult offspring) nor the time taken to produce the first offspring. If anything was different, it was only the total amount of brood at the time of eclosion of the first adult offspring. This suggests that when there were fewer wasps per nest they sacrificed productivity to produce at least some offspring as fast as nests with more wasps. This is not surprising because founding of nests by different numbers of wasps including solitary nest founding is a natural phenomenon in this species (27). Our results are somewhat different from a study with *Polistes fuscatus*, in which it has been found that about 60%, 91%, and 100% of nests were successful with one, two, or three wasps, respectively (31). However, this study used overwintered wasps (gynes) of *P. fuscatus*, a temperate-zone species with an annual colony cycle,

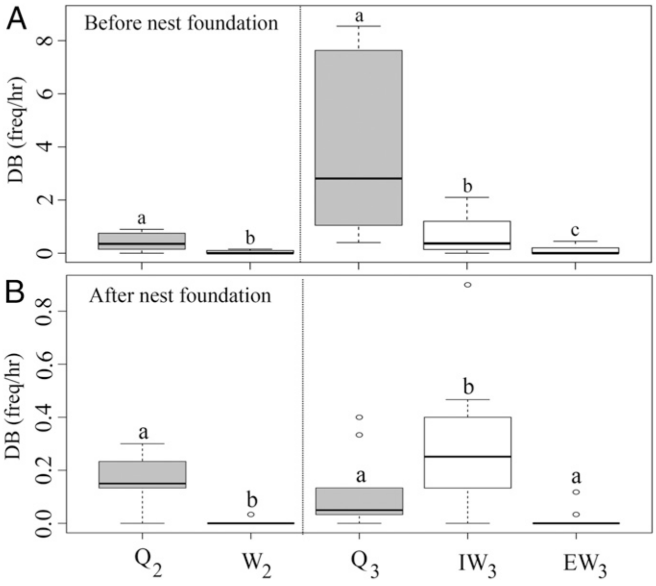

Fig. 3. Comparison of frequency per hour of DB for pairs and triplets; shaded bars show queens and unshaded bars show workers. Letters above bars represent comparisons between queens and workers in the same panel; bars carrying different letters are significantly different from each other. (*A*) Q_2 vs. W_2 (linear model; estimate = −0.38, $t = -3.78$, $P = 0.013$; $n = 10$ and 10), Q_3 vs. IW_3 (linear model; estimate = −3.45, $t = -4.05$, $P = 0.0003$; $n = 10$ and 10), Q_3 vs. EW_3 (linear model; estimate = −3.99, $t = -4.66$, $P < 0.0001$; $n = 10$ and 10), and IW_3 vs. EW_3 (linear model; estimate = −0.53, $t = -2.35$, $P = 0.019$; $n = 10$ and 10). (*B*) Q_2 vs. W_2 (linear model; estimate = −0.17, $t = -5.79$, $P < 0.001$; $n = 10$ and 10), Q_3 vs. IW_3 (linear model; estimate = 0.19, $t = 2.54$, $P = 0.02$; $n = 10$ and 10), Q_3 vs. EW_3 (linear model; estimate = −0.09, $t = -1.31$, $P = 0.20$; $n = 10$ and 10), and IW_3 vs. EW_3 (linear model; estimate = −0.29, $t = -3.60$, $P = 0.002$; $n = 10$ and 10).

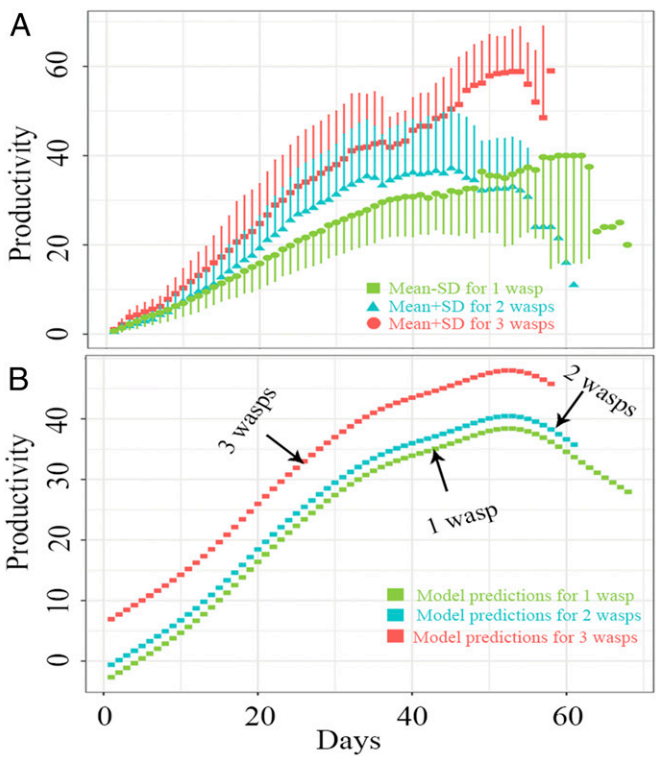

Fig. 4. Comparison of change in productivity with days among nests with one, two and three wasps. (*A*) Mean and SD of productivity. (*B*) Model predictions for productivity of nests with one, two, or three wasps. There was no significant difference between nests with one or two wasps (GAMM; estimate = 2.058, $t = 0.818$, $P = 0.414$; $n = 20$ and 14). Nests with three wasps had significantly higher change in productivity over days compared with nests with one wasp (GAMM; estimate = 9.581, $t = 3.81$, $P = 0.0001$; $n = 14$ and 20) or two wasps (GAMM; estimate = 7.523, $t = 2.756$, $P = 0.006$; $n = 14$ and 14).

where overwintered gynes routinely initiate new nests at the onset of spring (31, 32). We speculate that this difference between *R. marginata* and *P. fuscatus* for optimizing between success and productivity may have to do with the fact that the former species follows a perennial, indeterminate colony cycle in a tropical environment (33), whereas the latter follows an annual colony cycle in a temperate environment (32). In *R. marginata* there is no distinction between workers and gynes, and every adult female wasp has a chance of becoming a reproductive. Hence, sacrificing productivity for the sake of success may be a useful strategy; producing even a few adult offspring that can reproduce in the future is better than failing to produce any. In *P. fuscatus*, however, foundresses have to first produce workers that cannot mate and become reproductives and only later with the help of these workers produce gynes that will reproduce the following year. In this situation nests may fail anyway before producing gynes if they do not have a sufficient number of workers. Hence, sacrificing productivity for the sake of success (producing at least one worker) may not be a useful strategy in *P. fuscatus*.

Reproductive DOL, such that only one wasp developed her ovaries and laid eggs in each box, was invariably seen in all of the 43 boxes with two or three wasps (whether or not the nests were eventually successful). There was no evidence of any attempt to build more than one nest in any of the boxes, although this was possible in principle because individuals of primitively eusocial wasps are known not to be reproductively constrained (34) and each *R. marginata* female has an option to initiate its own nest (27). In all pairs and triplets (where there was a nest) there was only one egg layer as judged by egg laying and/or ovarian measurements; reproductive DOL is therefore an invariant property of any group of wasps with two or more individuals. In all cases the non-egg layer also participated in building the nest, feeding larvae, and other activities, both intranidal and extranidal. Thus, cooperation is also an invariant property of any group of wasps with two or more individuals.

As there were two non-egg layers in all boxes with three wasps, here was an opportunity to see whether nonreproductive DOL had emerged in groups of three wasps. Since demonstrating nonreproductive DOL required us to compare the two non-egg-laying workers across all boxes with three wasps, we had to find a way to distinguish between the two non-egg layers in a manner that is consistent across all boxes. As the distinction between intranidal work and extranidal work is the most primary form of nonreproductive DOL, we examined the correlation between intranidal and extranidal work performed by all pairs of non-egg layers. The non-egg layers indeed showed significant negative correlation in their work patterns so that we could identify non-egg-laying workers in each box as predominantly intranidal workers (labeled as IW$_3$) or predominantly extranidal workers (labeled as EW$_3$). We then confirmed that intranidal and extranidal workers performed significantly different amounts of intranidal as well as extranidal work. We conclude therefore that nonreproductive DOL in the form of intranidal and extranidal workers has already emerged with three wasps in the box. It should be noted that there was no such negative correlation between intranidal and extranidal work when the egg layers and non-egg layers in boxes with two wasps were subjected to similar analysis. We conclude therefore that nonreproductive DOL did not emerge when there were only two wasps per box. Hence, in boxes with three wasps reproductive DOL and cooperation as well as nonreproductive DOL were seen.

While there is evidence for the absence of nonreproductive DOL among the workers in small colonies of the ponerine ant *Rhytidoponera metallica* (35), both reproductive and nonreproductive DOL may sometimes emerge in artificially constituted pairs of normally solitary or communal species of bees (15, 21, 22) and normally solitary founding queens of some ants (36). We do not know the reason for this difference between *R. marginata* and these other species. In the case of artificially constituted pairs of the solitary carpenter bees *Ceratina japonica* and *Ceratina flavipes* the experimenters deliberately created an asymmetry in body size while constituting the pairs, and this may have been the reason for the emergence of nonreproductive DOL even with two individuals. In the case of the solitary and communal halictine bees *Lasioglossum* (*Ctenonomia*) NDA-1 and *Lasioglossum hemichalceum*, respectively, the observed nonreproductive DOL was in the absence of reproductive DOL and therefore probably a somewhat different phenomenon. The observed nonreproductive DOL in pairs of the normally solitary queens of the myrmicine ant *Pogonomyrmex barbatus* was also probably unrelated to reproductive DOL as no evidence of the latter is available. Both reproductive and nonreproductive DOL in social insects have been widely reported to be modulated by age and body size (3, 5, 8, 12, 25, 37–43). In our study, however, we obtained both reproductive and nonreproductive DOL without differences in body size and despite controlling for age.

Patterns of work organization changed with an increase in the number of wasps in a nest. A solitary nest foundress (Q$_1$) performed all of the work by herself. When the nest acquired a worker (boxes with two wasps), the non-egg layer (W$_2$) began to share the workload of the queen; Q$_2$ still did more intranidal work than W$_2$ but extranidal work was distributed equally between them. When the nest acquired a third wasp (boxes with three wasps), the queen (Q$_3$) still performed more intranidal work compared with the worker that we designated as the intranidal worker (IW$_3$), but the latter did more intranidal work compared with the worker that we designated as the extranidal worker (EW$_3$). In the case of extranidal work, Q$_3$ and IW$_3$ equally shared a small proportion of it but the bulk of the extranidal work was done by EW_3. Comparing queens across the three types of boxes one can see that Q_2 and Q_3 did a similar amount of intranidal work but both of them managed to do less of it compared with Q_1. In the case of extranidal work there was a reduction of the workload of the queen from Q_1 to Q_2 and also from Q_2 to Q_3. Thus, we witnessed a gradual increase in DOL with the addition of more wasps. One can therefore expect that such DOL, especially when it involves the more risky task of extranidal work, will get more skewed with the addition of even more wasps.

As nonreproductive DOL is known to be based on age polyethism in *R. marginata* (44) we deliberately used wasps of the same age for each box with pairs and triplets to check whether DOL can emerge even after the elimination of age as a proximate cue. Behavioral observations of nests with one, two, or three wasps also yielded some insights about the proximate mechanism by which reproductive as well as nonreproductive DOL emerged. As expected, dominance–subordinate behavior appeared to bring about both reproductive and nonreproductive DOL. Our comparison of dominance–subordinate interactions shown by the wasps to each other before nest initiation is more likely to capture the effect of dominance–subordinate interactions on DOL rather than vice versa. In primitively eusocial wasps it is well known that subordinate females forage for food in both preemergence and postemergence stages of the colony while the more dominant ones stay back and either reproduce or perform intranidal work (25, 45–51). This is not surprising because foraging is a risky task associated with worker mortality (52), less suitable for dominant individuals with higher probabilities of gaining direct fitness. Similarly, in the semisocial sweat bee *Megalopta genalis* the queens suppress the ovarian development of foragers by DB, and foragers develop ovaries and lay eggs when the queens are removed (53). Our results, however, show that dominance–subordinate relationships shape DOL among wasps even before nest initiation. Our interpretation is that dominance–subordinate interactions before nest initiation modulate DOL such that in the case of pairs the ability to show high DB makes individuals into queens (Q_2) and the inability to do so makes individuals into workers (W_2). In the case of triplets such modulation is more finely tuned such that very high DB leads to queens (Q_3), intermediate DB leads to intranidal workers (IW_3), and lowest DB leads to extranidal workers (EW_3). Dominance–subordinate interactions shown after nest initiation may be both the cause and the effect of DOL. In the case of pairs the pattern is the same as before nest initiation; DB of Q_2 is greater than DB of W_2. In the case of triplets IW_3 shows maximum DB and directs it mostly to EW_3, suggesting that this is how nonreproductive DOL is stabilized and maintained and leads to increased productivity.

In our experiment the singletons, pairs, and triplets were chosen randomly, unlike in nature where the wasps choose whether to be solitary or in pairs or triplets. We know from previous work that wasps choose their nesting strategies based on their abilities such that if those who choose to be workers are forced to be solitary foundresses their productivity is much lower compared with voluntary solitary foundresses (54). Such self-sorting by the wasps based on their abilities probably contributes to the emergence of DOL, both reproductive and nonreproductive, and the consequent changes in productivity. We show here that, as in the case of age, both reproductive and nonreproductive DOL as well as the consequent changes in productivity occur even when the wasps are denied the opportunity to sort themselves based on their differential abilities. Despite all of them being of same age we find “joiners” taking on worker tasks. Nonreproductive DOL may be even more pronounced in natural colonies, but the important point is that we find it in experimental colonies where age is held constant and self-sorting is absent. One might perhaps argue there may even be nonreproductive DOL in pairs in nature, something that we do not find in our colonies. In other words, self-sorting by the wasps may bring about nonreproductive DOL even in pairs. However, our conclusion is that nonreproductive DOL does not automatically emerge in pairs if the pairs are randomly chosen and, in contrast, it does emerge with three wasps even when they are randomly chosen. There is thus a clear and important difference between pairs and triplets.

While it has been sometimes claimed that “large colony size is a prerequisite for behavioral specialization”, “individually founded, small, short-lived colonies . . . [rely] on more generalists in the work force” (55) and that “there is only theoretical evidence that division of labour enhances colony performance” (56), this study provides clear empirical evidence both for the emergence of nonreproductive DOL and its ability to enhance productivity in groups of three wasps. However, perhaps our most interesting result is that productivity did not increase significantly with the addition of one wasp (solitary vs. pairs) but did so upon the addition of the third wasp (solitary and pairs vs. triplets). The additional emergence of nonreproductive DOL is essential for there to be an increase in productivity on account of DOL. We speculate that this may be because the subordinate wasp in a pair (W_2) and the more dominant of the two workers (IW_3) in a triplet have a higher chance of future direct reproduction and therefore a lesser incentive to work (especially the more risky extranidal work) compared with the most subordinate extranidal worker (EW_3) in a triplet. Genuinely altruistic and risky work is perhaps first done when there are at least three wasps.

Both pairs and triplets may, however, be thought of as having an advantage over solitary wasps because they initiate nests and start brood production significantly earlier than solitary wasps (Fig. 1*B*). Thus, when we compare daily changes in productivity using a generalized additive mixed model for nests with one, two, or three wasps considering the start of the experiment (rather than date of nest initiation) till day 60 (we chose day 60 because after that sample sizes dropped rapidly due to eclosion of adults from most nests), both pairs and triplets have significantly greater change in productivity over days compared with solitary nests and there is no significant difference between pairs and triplets (Fig. S2). The advantage for pairs and triplets observed when we count days from the start of the experiment rather than date of nest initiation is, however, an advantage unrelated to DOL because the advantage accrues due to early nest initiation. However, why should pairs initiate nests sooner than solitary wasps? This may be attributed to social interactions other than DOL. We have previously provided evidence that in natural nests potential queens devoid of nestmates, to whom they can show DB, take longer to develop their ovaries and lay eggs compared with potential queens who had the opportunity of showing DB toward nestmates. From this we have interpreted that the opportunity of showing DB helps to boost ovarian development of the aggressor (57). We now suggest that it is such advantage that helps pairs to start nest building and egg laying sooner than solitary wasps.

Materials and Methods

We collected nests of *R. marginata*, removed adults, eggs, and larvae, and kept the nests containing only pupae in aerated plastic boxes inside an air-conditioned room set at 30 °C. We removed female wasps on the day of eclosion and arbitrarily assigned them to one of the three following categories: solitary (one wasp in a box), pairs (two wasps in a box), and triplets (three wasps in a box). For the second and the third categories, the wasps kept together were always nestmates and were of same age or had an age difference of no more than 1 d. For solitary, pairs, and triplets we set up 77, 34, and 30 boxes, respectively, at the start of the experiment. We discarded a box if one of the wasps died before the eclosion of the first adult individual from that particular nest. We declared a nest to be successful if at least one adult wasp eclosed from it.

To collect data on the behavioral interactions between wasps we recorded 10 and 30 h of video before and after nest initiation, respectively. Following nest initiation in a box we maintained a nest map (count of the total number of cells and contents of each cell) every day till the eclosion of the first adult; we collected and stored the foundress(es) in a −20 °C refrigerator for ovarian measurements.

We conducted all statistical analyses using the software RStudio, version 3.3.2 (58). Names of statistical tests and levels of significance used are given in the appropriate figure legends. We used a linear mixed-effect model to investigate the effect of being an egg layer or a non-egg layer and being in boxes with one, two, or three wasps on the frequency per hour of intranidal work and a linear model with similar response variables for extranidal work performed by the wasps. We compared intranidal and extranidal work performed by egg layers in solitary, pairs, and triplets, egg layers and non-egg layers in pairs and triplets, and by the two non-egg layers in triplets.

Using similar models we studied the effect of DB shown before and after nest initiation (separately in two different models), by the wasps in boxes with two or three wasps, on the probability of an individual becoming an egg layer or a non-egg layer. We fitted a generalized additive mixed model (GAMM) (59) for comparing the time series containing the change in productivity with days. For datasets and detailed materials and methods see *Supporting Information*.

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