

Collective cues as a basis for nestmate recognition in polygynous leptothoracine ants

(kin recognition/aggression/social insects/*Leptothorax ambiguus*/*Leptothorax longispinosus*)

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Communicated by E. O. Wilson, February 8, 1988

ABSTRACT Three alternative hypotheses have been advanced to explain the dynamics of individually produced nestmate-recognition cues in colonies of social insects: (i) that there is no effective transfer of cues among nestmates (individual hypothesis); (ii) that cues are shared reciprocally among nestmates (collective hypothesis); and (iii) that cues derived from the queen are transferred to all colony members and dominate all other cues in nestmate recognition (queen hypothesis). In the present study, a bioassay based on aggression by laboratory colonies toward workers introduced into their nests was used in conjunction with isolation and interspecific-adoption experiments to test these hypotheses for colonies of two closely related, polygynous, and polydomous ant species, *Leptothorax ambiguus* and *Leptothorax longispinosus*. The results provide strong evidence for the collective hypothesis. A collective system has long been postulated as one of the primary modes of nestmate discrimination among social insects but to my knowledge has never before been clearly demonstrated.

Social insects are typically very aggressive in defending their nests, territories, trails, and food sources against intruding members of their own and other species. The ability to discriminate nestmates from non-nestmates enables colonies to cope with a wide range of predators and of competitors and tends to ensure that workers assist only their rightful queens (their relatives) in rearing offspring. Nestmate discrimination generally involves chemical cues (or odors) located on the surface of the body and learned by colony members. These cues may be produced or acquired by adult individuals, and they may have both genetic and environmental components. However, the ultimate origin, chemical nature, functional dynamics, and relative significance of these cues are not well understood and could vary extensively among species (1-12).

In species in which adult colony members produce nestmate-recognition cues, the dynamics of these cues may correspond to one of three alternative hypotheses (1-6, 11). (i) All colony members might retain their own recognition cues, with no effective transfer of these cues among nestmates (individual hypothesis). Under this system, colony members must effectively learn the full range of recognition cues that characterize their nestmates or must learn some generalized or common component of these cues. Depending on the nature of the cues (i.e., their ultimate origin, complexity, and variability) and the relevant learning and decision-making processes, this system might only be suitable for species with relatively small colonies or in which nestmates are extremely closely related. An individual system has been demonstrated in the primitively social sweat bee *Lasioglossum zephyrum* (2) and may occur in the acacia ant *Pseudomyrmex ferruginea* (4).

(ii) Recognition cues might be produced individually by colony members and transferred reciprocally among nestmates such that each bears a mixed-recognition odor (collective hypothesis). In the extreme case, this sharing of cues might be so extensive that all individual variability is effectively eliminated and a truly uniform colony odor (i.e., a colony-odor "gestalt"; ref. 11) is produced. However, the sharing of cues need not necessarily be this extensive; discriminating individuals could show fairly broad tolerances regarding acceptable mixtures of cues (e.g., a cue-similarity threshold model may apply; ref. 8) and even a limited amount of odor sharing could reduce individual variability and simplify the discrimination process. In comparison to the individual system, a collective system might facilitate increased colony size and be especially appropriate for colonies in which individually produced cues within colonies are highly variable, as might occur with multiple queens (polygyny) or with multiply mated queens (polyandry). The collective hypothesis has long been prominent in the empirical and theoretical literature, but evidence for this system has remained fragmentary and conflicting (1-6, 11-14).

(iii) Queens might function as primary sources for nestmate-recognition cues that are transferred to all colony members and effectively mask their individually produced cues (queen hypothesis). This system would also generate a uniform colony odor and might facilitate increased colony size, but it may be limited to species with single-queen colonies (monogyny). Certain carpenter ants in the genus *Camponotus* utilize this system (5).

The present study examines the production and dynamics of nestmate-recognition cues in laboratory colonies of two closely related ant species, *Leptothorax ambiguus* Emery and *Leptothorax longispinosus* Roger. These species occupy broadly overlapping ranges in eastern North America and typically nest in preformed cavities, such as hollow acorns, hickory nuts, stems, and twigs. Colonies often have multiple queens (facultative polygyny), adopt young queens (secondary polygyny), and inhabit multiple nests (polydomy) (15). Both species display intercolonial aggression within and between species and serve as hosts for the obligatory slave-making social parasites *Harpagoxenus americanus* and *Leptothorax duloticus*. These slave makers attack nests of their host species, kill or drive away the adults, and rear slave workers from the captured brood (16). Both host species also function as facultative intra- and interspecific slave makers and sometimes rear the brood captured during apparent territorial battles or transferred experimentally into their nests (17, 18).

METHODS AND RESULTS

Colonies (or colony fragments) were collected in early spring from individual nests in nature, maintained in plastic nests

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and in culture dishes in the laboratory, and fed uniformly on a synthetic diet (9). Colonies used varied extensively in size and consisted of queenless, monogynous, and polygynous nests. Nestmate acceptability was tested by introducing workers between nests (9, 10, 18). For each introduction, a test worker was arbitrarily chosen from within a donor nest, etherized, marked by tying a fine polyester fiber around its alitrunk (19), and isolated for ≈ 24 hr. At the time of introduction, all of the recipient colony's foragers were picked up and dropped into their nest entrance by using a camel's hair brush. Finally, the test worker was similarly introduced. The nest entrance was then blocked with plastocene, so that the initial interactions were confined to the nest chamber. Five observations were conducted at 15- to 30-min intervals, beginning 30–60 min after introduction. Twenty-four hours after introduction, two further observations were made that were separated by a 2-hr interval. The nest entrance was then unblocked and fresh food was provided in the foraging dish. Forty-eight hours after introduction, one further observation was made and the test ant, regardless of its condition, was removed but it was not returned to its own nest or used in any subsequent introductions. The test worker was considered "attacked" if it was observed being bitten or held in the mandibles of one or more recipient colony adults (with or without stinging) during any observation, "adopted" if it was alive inside the recipient nest with no current attackers during the final observation, or "killed" if it was dead by the final observation. The results were very similar for the two species and approximately equal numbers of replicates for each are pooled in the present analysis (18).

To test for the individual production of nestmate-recognition cues, worker pupae were removed from their parental colonies and allowed to eclose and age in isolation for 36–69 days ($\bar{x} = 49.2$, where \bar{x} is the mean; SD = 10.83) in separate plastic dishes (9). Eclosing workers were generally successful in shedding their pupal cuticle, but some failed to do so and died in the process. Each isolate was introduced either into its parental colony or into an alien conspecific colony collected at least 2 km from its parental nest. Colonies consisted of 1.3 ± 1.26 queens ($\bar{x} \pm$ SD) and 41.4 ± 21.54 workers. In parental nests, isolates were rarely attacked, frequently adopted, and never killed (Table 1). A single test

worker was observed being attacked in its parental nest, but by only one attacker and during a single observation. Isolates were significantly less acceptable in alien nests by all three measures of acceptability (Table 1). These results indicate that individual workers produce persistent and highly colony-specific nestmate-recognition cues (9). These cues might be genetically based, acquired prior to pupation, or both (7, 9, 18, 21).

The transferability of nestmate-recognition cues was tested by using artificially created mixed-species colonies that were produced by transferring a variable number of worker pupae between paired heterospecific nests. Two types of experiments were conducted: one in which pupae were transferred unilaterally between nests and a second in which pupae were transferred mutually between nests. The acceptance of workers eclosing from transferred pupae requires either the learning or the acquisition of recognition cues by either the fosterlings or the resident workers, and a combination of these processes might be involved. Nestmate acceptability between paired nests was tested by conducting introduction experiments 3 months after the transfer of pupae. At that time, pure colonies in the unilateral adoption experiment contained 1.8 ± 2.06 queens ($\bar{x} \pm$ SD) and 30.9 ± 12.19 workers; and mixed colonies had 1.3 ± 1.19 queens, 24.7 ± 11.57 resident workers, and 13.0 ± 10.04 fosterlings. Colonies in the mutual adoption experiment had 1.0 ± 0.69 queens, 27.6 ± 15.82 resident workers, and 19.0 ± 14.90 fosterlings.

When fosterlings in the unilateral adoption experiment were introduced from mixed colonies into their pure parental nests, they received a variable response. The number of replicates in which fosterlings were attacked was not significantly less than for control workers taken from pure nests of the same species (Table 1). However, fosterlings were adopted significantly more often and killed significantly less often than control workers. Detailed analysis indicated that the composition of the mixed colonies influenced these results. The absolute number of fosterlings and the ratio of fosterlings to resident workers were significantly less in replicates in which fosterlings were attacked than in replicates in which they were not attacked, but there was no

Table 1. Individual production and transferability of nestmate-recognition cues

Worker introduction	Replicates, no.	Attacked		Adopted		Killed	
		%	<i>P</i>	%	<i>P</i>	%	<i>P</i>
Isolate experiment							
Isolate to parental nest	44	2.3		77.3		0.0	
Isolate to alien nest	44	84.1	<0.001	27.3	<0.001	56.8	<0.001
Unilateral adoption experiment							
Mixed nest to pure nest							
Fosterling	23	56.5	>0.05	60.9	<0.01	8.7	<0.001
Control	23	78.3		21.7		69.6	
Resident	24	100.0	>0.05	0.0	>0.05	87.5	>0.05
Control	24	100.0		0.0		95.8	
Pure nest to mixed nest	29	62.1	<0.001	34.5	<0.01	27.6	<0.01
Control	29	96.6		6.9		70.0	
Mutual adoption experiment							
Within nests	104	1.0	<0.001	82.7	>0.05	0.0	>0.05
Between nests	104	13.5	<0.001	89.4	<0.001	0.0	<0.001
Control	104	92.3		10.6		62.5	

Controls in each experiment refer to workers taken from pure colonies of the same species as the corresponding test workers. The unilateral adoption experiment consisted of three separate paired-sample studies in which each recipient colony received each type of test worker once. The mutual adoption experiment utilized 26 pairs of colonies and a repeated-measures design in which each colony received six types of introduced workers: a member of each species from both paired nests and a control of each species. For the latter experiment, the results for fosterlings and resident workers were extremely similar and were pooled for this analysis; the statistics for only some of the resulting paired comparisons are shown. The probabilities given were derived from statistical analyses with 2×2 contingency tables and the log-likelihood ratio test with Williams' correction (20). A *P* value >0.05 is not significant.

Table 2. Comparison of the composition of mixed colonies in the unilateral adoption experiment

Attack	Replicate, no.	Workers, no.	Fosterlings, no.	Fosterling/resident ratio
Aggressive	13	34.85 (14.88)	8.85 (8.69)	0.392 (0.328)
Nonaggressive	10	41.30 (13.98)	18.40 (9.40)	0.984 (0.624)
<i>P</i>		>0.05	<0.01	<0.01

Adopted workers were either attacked (aggressive) or not attacked (nonaggressive) when introduced into their pure parental nests. The total number of workers consists of the total number of fosterlings plus residents. Data are reported as means (SEM) and were compared by the one-tailed Mann-Whitney tests. A *P* value >0.05 is not significant.

significant difference in the total number of workers in the mixed colonies in the two groups (Table 2).

The sharp contrast between the response of pure parental colonies to isolates (first experiment) and fosterlings (second experiment) indicates that recognition-odor transfer occurs in mixed colonies. The composition effect is consistent with an odor-transfer interpretation and indicates that it was the degree to which an individual was contaminated with alien recognition cues that determined its acceptability in its pure parental nest. It is noteworthy that fosterlings were often attacked by numerous recipient colony workers simultaneously. The maximum number of simultaneous attackers observed in the 13 aggressive replicates ranged from 1 to 9 ($\bar{x} = 3.0$; $SD = 2.12$), and two of the introduced workers were apparently killed as a result of the severity of these attacks. These observations support the odor-transfer interpretation, since intensive concerted attacks are unlikely to be elicited by any form of aversive behavior (e.g., flight or attacks) exhibited by the introduced ant in response to the recipient colony but are easily explained as a result of odor transfer in mixed colonies. Social facilitation might be a factor in concerted attacks but some means of distinguishing alien from nestmate would still be required and, hence, such a phenomenon is not damaging to this interpretation.

Pure parental colonies in the unilateral adoption experiment exhibited a consistently negative response toward resident workers from mixed colonies that contained their putative offspring, and this response was not significantly different from that displayed toward control workers (Table 1). This response contrasts with that shown toward fosterlings and indicates that fosterlings and resident workers in mixed colonies did not always possess identical recognition cues. Thus, the sharing of recognition cues in mixed colonies may not have been extensive enough to produce a completely uniform colony odor (i.e., a gestalt). However, the marking and introduction procedures may have effectively removed some of the shared-odor cues and contributed to the asymmetry of the results, and the absence of a gestalt, therefore, was not conclusively proven. Workers introduced from pure parental nests into mixed colonies also received a variable response and were frequently attacked by both fosterlings and resident workers. Nonetheless, overall, they were more acceptable than control workers (Table 1). No aspect of colony composition appeared to account for this variable response.

In the mutual adoption experiment, fosterlings and resident workers were treated similarly and were attacked more frequently when introduced between paired nests (Table 1) than when introduced back into the nests in which they had eclosed (Table 1). However, the attacks that occurred during between-nest introductions were rare (14 of 104 replicates) and involved a maximum of only one ($n = 13$) or two ($n = 1$) attackers, and test workers in between-nest and within-nest introductions were adopted with equal frequency and were never killed. By comparison, control workers from pure nests were much less acceptable (Table 1). Thus, the results of the mutual adoption experiment were vastly different from those of the unilateral adoption experiment and involved a

dramatic reduction in aggression between paired nests. One or more queens were often present in one (19.2%) or both (69.2%) of the 26 pairs of nests in the mutual adoption study, but their presence did not prevent the reduction in aggression. The variable composition of the mixed colonies also had no apparent influence on the results.

The general lack of aggression between paired nests following mutual adoption was confirmed by the ease with which four additional pairs of mixed colonies, produced in a similar manner, fused to form single colonies when placed together in a common culture dish. These colonies consisted of 1.0 ± 0.53 queens ($\bar{x} \pm SD$), 17.5 ± 10.94 resident workers, and 16.1 ± 10.20 fosterlings. Some mild fighting was observed between workers, but it was generally very brief and never resulted in any apparent injuries. However, queens were sometimes attacked intensively by workers from the other nest (sometimes their own putative offspring) and killed; and no more than one queen was ever accepted into a fused colony. These species are secondarily polygynous (15) and queens in field-collected nests might be mothers, sisters, or perhaps even unrelated to the associated adults and brood. Thus, genetic differences (perhaps between generations) may explain the differential treatment of queens. It is also possible that some form of queen number-regulation mechanism was involved (22) and that the cues that mediated the acceptability of queens were not directly related to the cues that mediated the acceptability of workers. In a control experiment, 10 pairs of pure colonies [1.0 ± 0.92 queens ($\bar{x} \pm SD$); 22.5 ± 7.40 workers], which had not exchanged brood, engaged in intensive and prolonged battles that usually resulted in the complete elimination of queens and workers from one colony. Victorious colonies often appropriated the brood from the vanquished colonies and accepted some of the eclosing workers as colony members, and one adult worker that survived the carnage was also finally adopted.

The mutual adoption experiments demonstrated that workers from colonies with similar mixtures of adults (and hence of cues) are highly acceptable in one another's nests. Thus, the transfer of recognition cues that occurs within mixed colonies is not unilateral and does not simply involve the original residents or their queen labeling eclosing workers. Rather, there appears to be a more general mixing of recognition odors among nestmates.

DISCUSSION

The results of this study demonstrate that a collective nestmate-recognition system is operating in artificially created mixed-species colonies of these ants. The isolation experiment showed that adult workers produce recognition cues that are highly specific to the pure parental colony of the individual ant. The unilateral adoption experiment demonstrated that recognition cues are readily exchanged among nestmates and that shared cues are capable of disrupting the acceptability of an individual ant in its pure parental nest. The mutual adoption experiment showed that queens are not major proximate sources for recognition cues shared among

adults and that similar mixtures of workers (and cues) result in high levels of intercolonial acceptability. These results confirm the collective hypothesis and rule out both the individual and the queen hypotheses stated above.

By inference, these results can be extended to naturally occurring colonies of these ants, but, in nature, other factors (e.g., transient environmentally based cues; refs. 10 and 18) may also be involved. The present results may be most applicable to the natural mixed colonies these species form with one another and with their obligatory social parasites (16–18). However, social parasites may have adaptations associated with their nestmate-recognition systems that further promote the formation and stability of mixed colonies. Most importantly, these results may also apply to pure colonies of these species. These species are extremely closely related, share an array of biological characteristics, and are even parasitized by the same social parasites (15–18). Nestmate-recognition systems may vary extensively among species but they are much less likely to vary among such similar species. In the present study, experiments were conducted in reciprocal fashion for both species in an effort to detect species differences in the dynamics of recognition cues, but none was found (18). Both species clearly demonstrated the capacity to donate and receive mutually effective nestmate-recognition cues. Moreover, it was evident that mixed colonies in this study discriminated nestmates in a colony-specific not a species-specific manner and, therefore, that the transferred cues were colony specific not merely species specific. Although this study cannot rule out minor differences in the recognition systems of these species (e.g., certain qualitative or quantitative differences in recognition cues), the dynamics and specificity of the cues detected here indicate that both species utilize collective nestmate-recognition systems. This conclusion could be further tested by using intraspecific-mixed colonies, but such colonies also differ from natural pure colonies in the degree of genetic diversity among nestmates and in the consistency of long-term environmental influences, and, again, inference would be required to extend such results to natural pure colonies. Indeed, because of these differences, it would remain possible that odor sharing in natural pure colonies could produce a true colony-odor gestalt even though it might never be achieved in mixed colonies. Thus, although mixed colonies are useful to test for odor sharing, they may not adequately test the gestalt hypothesis *per se*.

Colonies of these ants are polygynous and highly susceptible to social parasitism (15–17), two characteristics that are often closely associated in social insects (23). If nestmate-recognition cues in these species have an important genetic component, then the adaptive significance of collective nestmate-recognition systems might be that they enable colonies to cope with the relatively broad range of variation in individually produced recognition cues that are likely to arise in polygynous colonies, while still enabling colonies to maintain effective nestmate discrimination (1, 11, 18). A potential consequence of this system is the increased susceptibility to social parasitism that results from providing a preadapted mechanism that facilitates the incorporation of unrelated individuals into colonies. Thus, since polygyny may require a fairly flexible and open nestmate-recognition system, polygynous species may typically be much more susceptible to social parasitism than comparable monogynous species.

Numerous authors have suggested that nestmate-recognition cues might be learned through imprinting, but the

evidence is inconclusive (e.g., refs. 1 and 7). The present results indicate that workers learned new or additional nestmate-recognition cues when transferred heterospecific brood enclosed in their nests and that they used these new standards for subsequent nestmate discrimination. Thus, in these species, no critical period appears to be associated with the learning of nestmate-recognition cues and the relevant learning process is not an example of imprinting. Rather, the production, transfer, and learning of nestmate-recognition cues appear to be very dynamic and ongoing processes that occur throughout the lifetime of the colony. By virtue of being secondarily polygynous (15), colonies of these ants have a potentially limitless life expectancy, and changes in a colony's spectrum of individually produced recognition cues and, hence, a colony's collective recognition odor, could be quite dramatic over time. A collective nestmate-recognition system might involve genetically based cues exclusively, but it could also provide an excellent framework for the incorporation of relatively stable and more transient environmentally based recognition cues or cue components, all of which may function in these particular species (9, 10, 18).

The author thanks M. D. Breed, N. F. Carlin, R. H. Crozier, G. J. Gamboa, W. M. Getz, J. M. Herbers, B. Hölldobler, P. F. Kukuk, R. E. Page, E. O. Wilson, and anonymous reviewers for comments on this and previous versions of the manuscript. This research formed part of the author's doctoral dissertation at the University of Toronto and was supported by Ontario Graduate Scholarships to the author and grants from the Natural Sciences and Engineering Research Council of Canada to T. M. Alloway.

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