

A novel social polymorphism in a primitively eusocial bee

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Halictine sweat bees (Hymenoptera, Halictidae) are model organisms for the evolution of altruism, reproductive castes, and eusocial colony organization. Halictine social behavior is not only extremely variable, but also ecologically and evolutionarily labile. Among social species, colony social organization ranges from communal societies of egalitarian females to eusocial and semisocial ones with reproductive queens and more or less sterile workers. A striking aspect of halictine social variation is the mutual exclusivity of communal and eusocial types of colony social organization within the same species, these two types of social behavior being characteristic of different genera and subgenera. We report a recently discovered exception to this rule in a population of *Halictus sexcinctus* (Fabricius) at Daimonia-Pyla in southern Greece, that contained both communal and eusocial colonies. Moreover, communal and eusocial females exhibit morphological differences that imply a preimaginal developmental switch, which could also underlie the two types of social behavior. That the communal and eusocial forms are not merely cryptic sister species with different social behavior is indicated by the comparison of mitochondrial DNA sequences of two sections of cytochrome oxidase I, which indicate that Greek specimens of both social types are more similar than they are to conspecifics from elsewhere in Europe. The phylogenetic position of *Halictus sexcinctus* suggests that this unusual communal/eusocial polymorphism may represent an unstable intermediate step in an evolutionary reversal from eusocial to solitary behavior.

sweat bee | Halictidae | social evolution | communal | social variation

Halictine sweat bees (Hymenoptera, Halictidae) exhibit an astonishing range of social behavior and are significant model organisms for studying the evolution of altruism and eusociality. Most species are solitary, with female bees constructing nests and raising their own brood, but there are also many types of colony social organization, ranging from more or less egalitarian communal societies, to semisocial and eusocial (1) types, in which more or less sterile workers aid queens that dominate reproduction. The differences between communal and eusocial halictine societies are fundamental. First, communal societies do not have queen and worker castes, being composed of multiple females that may or may not be related, and exhibiting a low degree of reproductive skew and little aggression among nestmates (1–3). Eusocial societies are characterized by queen and worker reproductive castes with a high degree of reproductive skew (queens lay all or most of the eggs) and substantial aggression, especially by queens toward workers (4). Second, communal societies are composed of adults of the same generation, whereas eusocial societies are formed when a queen's offspring become workers.

Halictine social behavior is not only variable, but extremely labile, both evolutionarily and ecologically. Recent phylogenetic studies of halictine bees (5–10) show that there have been multiple independent origins of both communal and eusocial colony organization, and even more numerous reversions to solitary existence. There are also many examples of “socially polymorphic” species that facultatively express solitary or social

behavior in different environments (11). Given their extreme social lability, a striking aspect of halictine social variation is the mutual exclusivity of communal and eusocial types of colony social organization within the same species, these two types of social behavior being characteristic of different genera and subgenera (12, 13). Although communality has been proposed as an intermediate evolutionary step between solitary bees and their eusocial descendants (14), the conspicuous, phylogenetic nonoverlap of communal and eusocial behavior suggests instead that these are evolutionary alternatives (3, 9).

In this paper, we report a definite exception to the rule that communality and eusociality are mutually exclusive types of colony social organization in halictine bees. We studied a population of *Halictus sexcinctus* (Fabricius) in southern Greece that contains both communal and eusocial colonies. Moreover, we found morphological differences between communal and eusocial females that imply a preimaginal developmental switch underlying the two types of social behavior. The phylogenetic position of *H. sexcinctus* (5, 7) suggests that this unusual communal/eusocial polymorphism may represent an unstable intermediate step in an evolutionary reversal from eusocial to solitary behavior.

Methods

Fieldwork. The field site was located on the Mediterranean coast at Daimonia-Pyla, in the southeastern peninsula of the Peloponnese in southern Greece. Field methods have been described (15). Eusocial colonies of *H. sexcinctus* at this site exhibit the typical eusocial colony cycle of temperate halictines (Fig. 1) (15).

About 500 eusocial nests and 25 communal nests were individually marked with numbered flags. Eusocial nests were excavated throughout the season, beginning just after eusocial foundresses provisioned their first few brood cells (15). Communal nests were not excavated because they were in the middle of a road. Queens, workers, and communal foundresses were individually marked with colored Testor's enamel on the mesosoma; this had no perceptible effect on either behavior or survival. Behavioral observations and collections of females at nest entrances were used to estimate numbers of foundresses per nest. Adult females were dissected within 3 days of collection, so assessment of reproductive status was straightforward. Females were considered to be mated if the spermatheca was opaque, indicating it to be filled with sperm, and unmated if it was transparent. Ovarian status of adult females was assessed by counting the number of developing oocytes and estimating their relative size (1/4, 1/2, 3/4, or fully developed). Note that adult female Hymenoptera can lay fertilized or unfertilized eggs, and therefore need not be mated to reproduce. Although many dissected females had pollen or nectar in their crops, we noticed no evidence of oophagy.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY177915–AY177924 and AY185601–AY185604).

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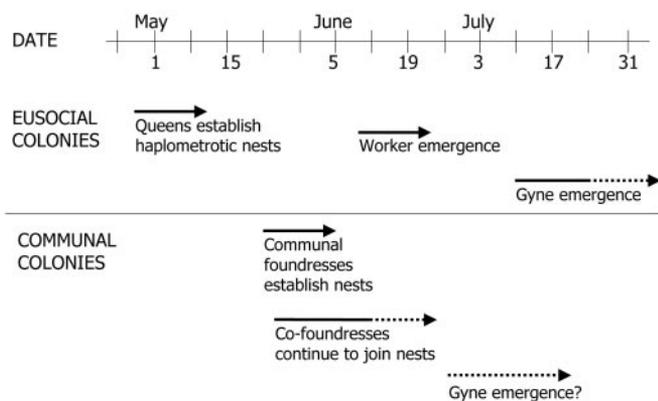


Fig. 1. Colony cycles of eusocial and communal *H. sexcinctus* in southern Greece. Eusocial colony cycle: in late April or early May, overwintered gynes (females destined to become queens) awaken from diapause, establish new nests, and begin raising their first brood, which consists of workers and varying numbers of males. When the adult workers emerge beginning around June 8–10, they take over the job of raising the second brood, which consists of gynes and more males. Adults of the second brood begin emerging around July 7. Gynes mate and enter overwintering diapause to become queen-foundresses the following year. Communal colony cycle: communal foundresses begin establishing nests \approx 3 weeks after the eusocial nests are founded, from about May 26 to June 7.

DNA Sequence Analysis. To ensure that communal and eusocial females were conspecifics and not members of two morphologically cryptic species, we sequenced portions of the mitochondrial gene cytochrome *c* oxidase I (COI), which has previously been used to distinguish two cryptic species, *Halictus ligatus* and *Halictus poeyi* (16, 17). DNA was isolated from 10 individuals from Daimonia, one eusocial queen, four eusocial workers, and four communal foundresses, as well as two gynes from Germany. Total genomic DNA was isolated from either thoraces or heads of individual specimens by using the DNEasy Tissue kit (Qiagen, Valencia, CA, kit no. 69504), after initially freezing and crushing the tissue in liquid nitrogen. We amplified portions of the mitochondrial gene COI, by using primers mtd-7, 8, 9, and 12 (18) (University of British Columbia Biotechnology Laboratory, Vancouver) in 20- μ l reactions with 2.5 mM MgCl₂, 200 μ M dNTPs, 2.0 μ M each primer, 0.25 units of *Taq*DNA polymerase (MBI Fermentas, Hanover, MD), and 2 μ l of *Taq* reaction buffer (supplied with the enzyme). Initially, PCRs were heated to 94°C for 5 min, followed by 30 repetitions of 94°C for 1 min, 52–54°C for 1 min, and 72°C for 1.5 min, followed by a further 94°C for 1 min, 50°C for 1 min, and 72°C for 5 min. Negative controls using sterile water as a template were carried out in all reactions. Amplification products were run out in 1.5% agarose gels, and purified by using the Qiaex Gel Extraction kit (Qiagen kit no. 20021). Double-stranded sequencing was carried out by the York University Core Molecular Biology Facility in Toronto, using the same primers. Sequences were edited and aligned by using BIOEDIT (19) and CLUSTAL (20). All sequences have been deposited in GenBank under the accession nos. AY177915–AY177924. Because different sections of the gene were sequenced in different individuals, we created two nonoverlapping alignments corresponding to mitochondrial nucleotide positions 2194–2533 (“front” section) and 2771–2993 (“back” section), numbered with reference to the honey bee complete mitochondrial genome sequence (GenBank accession no. NC.001566). For each alignment, we calculated all possible pairwise, uncorrected genetic divergences, as well as calculating a neighbor-joining tree of relationships based on outgroup comparison with *Halictus poeyi* (AF102841) and *H. scabiosae* (AY185601) with PHYLIP (21).

Morphometric Analyses. Measurements were limited to adult females with fully extended wings. Traits measured were head width (the widest part of the head including the compound eyes, HW); forewing: length of the forewing subcostal vein (SC), length of vannal vein, length of first medial cell (MEDIAL1), first cubital cell length, second submarginal cell length (MARG2); hind wing: length, cubital vein length, total wing length; and hind leg: basitarsus length, femur length (FEMUR), width of femoral-trochanter ridge. All measurements were carried out by A.R. to avoid interinvestigator error. A complete data set includes all 12 measurements for 10 queens, 36 workers, and 40 communal foundresses. Because old bees often have damaged wings, we also used stepwise discriminant analysis to select a second data set with fewer measurements (HW, SC, MEDIAL1, RHWTOT, FEMUR, LHWCU), but with 18 queens, 39 workers, and 44 communal foundresses, with almost exactly the same results as those reported in *Results*.

Statistical analyses were carried out by using SAS 8.0. Canonical discriminant analyses were done by using the STEPDISC, CANDISC, and DISCRIM procedures. In this technique, each observation has a classification variable such as caste (in this case, queen, worker, or communal foundress), and quantitative variables (morphometric measurements). The method derives linear combinations of the quantitative variables that summarize the variation among them, and which can be used to compare the variables among the different classes. Discriminant analysis in some ways resembles analysis of variance, in that measurement variables are compared among various groups.

Results and Discussion

Comparison of Eusocial and Communal Nests. Observations of nests during the spring and summer of 1998 show that at Daimonia, there were two distinct types of nesting colony (Fig. 1). During the last week of April and the first week of May, eusocial foundress-queens established >500 horizontal nests in two vertical, sandy cliff faces, beside a paved road. The nesting phenology and colony social organization of these eusocial colonies were described in detail by Richards (15), and the colony cycle was typical of temperate eusocial halictines. Eusocial nests were invariably haplometrotic (founded by a single female), and conspecific nest usurpers were met with strong aggression by resident foundresses, sometimes resulting in fights to the death (M.H.R. and E.J.v.W., unpublished observations). In eusocial nests, the first adult workers and males produced in the first brood began to emerge as adults no earlier than June 10. Adults of the second brood, which is comprised of gynes and more males, begin emerging around July 7.

By contrast, from about May 26 to June 7, a second set of \approx 25 nests was founded in the horizontal roadbeds within 2 m of the cliff face where eusocial nests were excavated, and only 6–7 m from the majority of eusocial nests in the other cliff face. The new nests were pleometrotic, containing 2–10 foundresses that expressed relatively little aggression toward each other. Throughout the month of June, the appearance of new unmarked, unworn females in these nests, often with large fat deposits, mated, and with developing ovaries (see below), indicated that new foundresses were continually joining these colonies. The earliest communal nests were established before the emergence of workers and males in brood 1 of the eusocial nests. Together with the fact that the vast majority were mated, this observation suggests that early communal foundresses had just emerged from overwintering diapause. However, foundresses that joined communal nests in mid-June could have been produced in the worker brood of eusocial nests. Adult females found in late June could conceivably have been brood produced in the communal nests themselves. Unfortunately, the location of the communal nests in the middle of the road prevented us from excavating them to solve these puzzles.

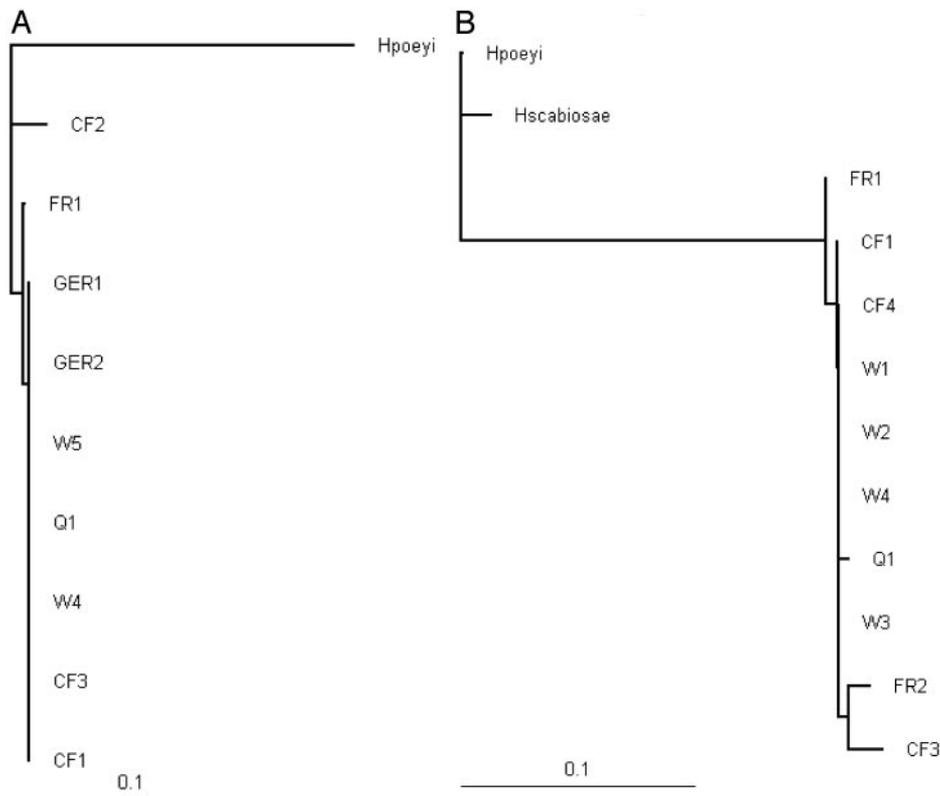


Fig. 2. Genetic relationships among eusocial and communal females. Neighbor-joining trees were constructed based on uncorrected pairwise divergences among communal foundresses (CF), eusocial queens (Q), and eusocial workers (W) from *Daimonia*, *H. sexcinctus* specimens collected in France (FR) and Germany (GER), and outgroup comparison with GenBank COI sequences for *H. poeyi* and *H. scabiosae*. Because different lengths of COI sequence were obtained for different individuals, we present separate trees for the front (A) and back (B) alignments described in the text.

Social Polymorphism or Cryptic Species? A mix of communal and eusocial nesting strategies is unprecedented within halictine species, and could well occur if two morphologically cryptic sister species with different social strategies were nesting in the same aggregation. To address this possibility, we compared mitochondrial COI DNA sequences of eusocial and communal females, as well as *H. sexcinctus* specimens from France and Germany. Based on both alignments, a total of 592 nt of sequence, the average uncorrected sequence divergence among pairs of eusocial and communal females captured in 1998 was 0.74%, and the maximum was 2.2%. Relationships among communal and eusocial females are illustrated in the form of neighbor-joining trees (Fig. 2 and Table 2, which is published as supporting information on the PNAS web site, www.pnas.org). If the communal and eusocial specimens were of different species, we would expect to see a “communal” clade and a “eusocial” clade. On the contrary, we observe that the communal and eusocial sequences are intermingled, indicating that there is no clear genetic differentiation between the two social forms. Although two different species could have identical DNA sequences, the high rate of nucleotide substitution in the COI gene makes this very unlikely (22). Moreover, there is no consistent pattern of geographic variation, so divergences among eusocial and communal females from Greece are not that different from divergences between Greek and French or German specimens. We conclude that the communal and eusocial females are members of the same species and population. This conclusion is further supported by the observation that when the eusocial nests were destroyed by construction work in the spring of 2000, the communal foundress nests also disappeared, though their nesting site remained undamaged.

Sequences of COI have been used to measure genetic variation within and among other species in the genus *Halictus*. *Halictus ligatus* and *Halictus poeyi* are a morphologically cryptic species pair of which both are eusocial (23, 24). They exhibit about 0.4% COI sequence variation within each species, and $\approx 4\text{--}5\%$ between species (16, 17, 25). In contrast, the socially polymorphic, holarctic species *H. rubicundus*, which exhibits both solitary and eusocial behavior (26, 27), exhibits an average of 1.21% COI sequence divergence among pairs of individuals within continents, the maximum difference being $\approx 3.8\%$ (28). Thus, the levels of sequence variation observed among Greek eusocial and communal *H. sexcinctus*, are well within the range of intraspecific variation noted for other members of the genus.

Differences Between Eusocial and Communal Females. Despite the necessity of addressing the issue of a cryptic species pair, we did in fact, detect morphometric differences between adult eusocial and communal females. As is true in the vast majority of eusocial halictines, *H. sexcinctus* queens and workers exhibit no morphological differences except in size. Although the size distributions of the two castes are overlapping, eusocial queens are always larger than the workers in their own nests (15). In marked contrast, we found that eusocial and communal females are morphologically distinct: based on initial measurements of head width and wing length (measured as length of the subcostal vein) of 141 adult females, we found that communal females have proportionately shorter wings than queens and workers (Fig. 3). We used discriminant functions analysis (DFA) on all 12 morphological traits to determine whether morphometrics can be used to discriminate the same three caste groups, eusocial queens, eusocial workers, and communal foundresses, that we

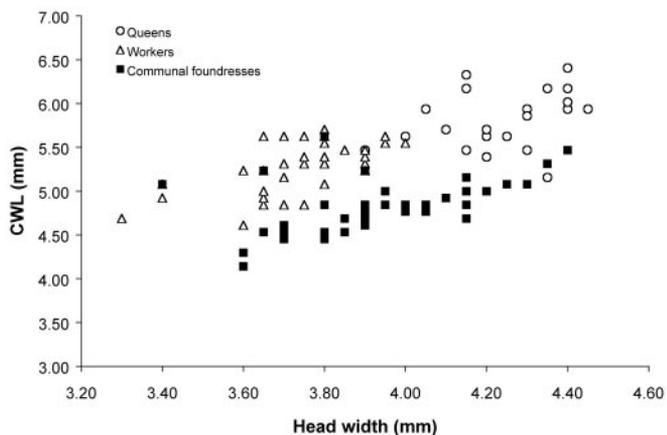


Fig. 3. Body size and shape differences among eusocial queens and workers and communal foundresses of *H. sexcinctus* nesting at Daimonia in 1998. Eusocial queens and workers exhibit similar relationships between head width and wing length (measured as length of the costal vein, CWL), although queens are clearly larger than workers. Communal foundresses exhibit proportionately shorter wings than eusocial females, but their overall size range in terms of head width (as well as other measures of overall body size, not shown) is about the same as queens and workers put together.

had previously identified based on behavior. DFA confirms the statistical significance of size and shape differences among the three castes (Fig. 4). When we tested whether the castes assigned on the basis of behavior are reflected in the sizes of bees, only two communal foundresses were misclassified as workers, an error rate of just 1.7% in caste assignment. Stepwise discriminant analysis shows that when only the six most important measurements are used (length of subcostal vein, length of femur, length of vannal vein, length of first medial cell, head width, and total hind wing length), the misclassification rate is only 3%. The two

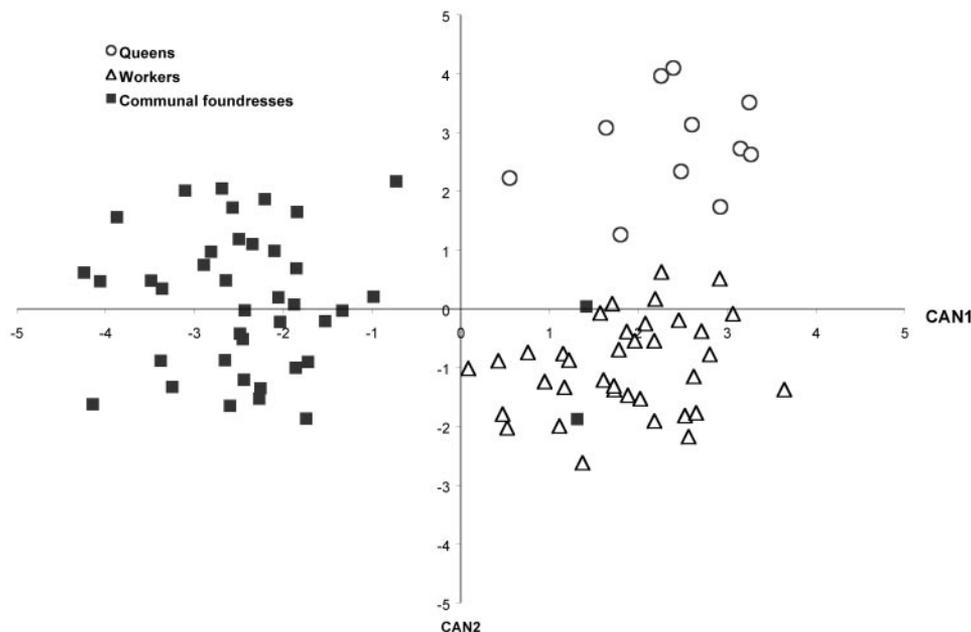


Fig. 4. Discriminant functions analysis of 12 measurements of body size of adult female *H. sexcinctus*. The first canonical discriminant axis (CAN1) represents wing length, and the second (CAN2) represents a general measure of body size. Total-sample standardized canonical coefficients for CAN1 and CAN2, respectively, are: head width = 1.010647 and 1.53280; length of the forewing subcostal vein = 2.01014 and 0.30294; length of vannal vein = 0.37967 and 0.25372; length of first medial cell = -1.05421 and -0.59198; hind wing length = -0.48228 and 0.02183; cubital vein length = 0.048778 and 0.81325; total hind wing length = -0.89673 and 0.67738; first cubital cell length = -0.21567 and 0.13924; second submarginal cell length = 0.20516 and -0.52179; basitarsus length = 0.08897 and 0.62650; femur length = 0.11631 and -0.13720; and width of femoral-trochanter ridge = 0.29124 and 0.04492.

communal foundresses that were misclassified as workers (Fig. 4) were discovered on June 16th and 19th, and so could have been eusocial workers that joined communal nests.

Dissections of communal foundresses indicate that almost all are potential reproductives, having at least one 1/4 developed oocyte in their ovaries. In *H. sexcinctus*, queens are more likely than workers to be mated and have higher levels of ovarian development (15, 29). Based on these same characteristics, communal foundresses resemble queens more than they resemble workers (Table 1). More than 90% of all communal and eusocial foundresses mate, and $\approx 43\%$ of those dissected have at least one fully developed oocyte ready to lay. Moreover, reproductive skew among cofoundresses from the same nest is lower than between queens and workers (Table 1). Of 44 cofoundresses collected from six nests with at least three simultaneously active cofoundresses, 57% had at least one fully or 3/4-developed oocyte, indicating that they were ready to lay at least one egg within a day or two. In fact, only two cofoundresses collected with their nestmates had undeveloped ovaries; one of these was a newly emerged adult (she exhibited no mandibular or wing wear) and the other's abdomen was being consumed by endoparasitic conopid larvae.

Social Polymorphism in *H. sexcinctus*. *H. sexcinctus* represents the first definite example of a eusocial/communal polymorphism in halictine bees. In fact, if central European populations are confirmed to be solitary (ref. 30 and M. Fellendorff and M. Ayasse, personal communication), then *H. sexcinctus* arguably represents the most extreme degree of intraspecific social polymorphism in insects (allodapine bees also exhibit marked social polymorphism, but it is most clearly expressed among species within genera; intraspecific variation as strong as that in *H. sexcinctus* has not yet been observed; refs. 31–33). Other examples of solitary/social polymorphism appear to be facultative reversion of eusocial species to solitary behavior in areas with short breeding seasons (26–28, 34, 35). Less dramatic social

Table 1. Frequency of mating and readiness to lay eggs in *H. sexcinctus* females nesting at Daimonia in 1998

Caste	Proportion mated, % (n)	Size of largest oocyte, n (%)					N
		Full-size, ready to lay	3/4	1/2	1/4	None	
Communal foundresses	91 (59/65)	19 (43)	6 (14)	9 (20)	8 (18)	2 (5)	44
Queens	93 (64/69)	33 (43)	14 (18)	4 (5)	5 (6)	21 (27)	77
Workers	61 (49/80)	25 (27)	16 (17)	14 (15)	4 (4)	33 (36)	92

Newly emerged females were not excluded, as some newly emerged communal foundresses had significantly developed ovaries. Whether a female had mated was determined by visual inspection of the spermatheca, which becomes opaque when filled with sperm. Females for which mating status could not be unequivocally determined were not included. For the communal foundresses, we show ovarian development only for females who at the time of capture, belonged to nests with at least three active cofoundresses. Communal foundresses resemble queens, but not workers, in the frequency of mating ($\chi^2 = 29.704$, $df = 2$, $P < 0.01$). Communal foundresses also resemble queens more than workers in their readiness to lay eggs ($\chi^2 = 28.569$, $df = 8$, $P < 0.01$). Note that the apparently higher frequency of queens exhibiting no ovarian development is not statistically significant, and is caused by the fact that we collected old, senescent queens but not old communal foundresses.

variation also occurs in some obligately social species that exhibit variation in colony phenology in response to local environmental variation (36). In some cases, marked differences in colony social organization have been observed in different populations. For instance, a population of *Lasioglossum umbripenne* in Damitas in western Costa Rica exhibited a typical annual, eusocial halictine colony cycle (37) with monogynously founded colonies, and discrete queen and worker castes. In another locality, Turrialba, a population of the same species exhibited perennial colonies that often had multiple queens, which were only statistically larger than workers (38). The social behavior of the cryptic species pair *H. ligatus* and *H. poeyi*, is also instructive. Obligate eusociality is characteristic of *H. ligatus* (23, 39, 40), but Caribbean and Central American populations that most likely are *H. poeyi* exhibit large colonies in which many females seem to be reproductive, and in which it is difficult to distinguish the queen and worker castes (41). The large, polygynous colonies of both *L. umbripenne* and *H. ligatus/poeyi* seem to be intermediate between eusocial and communal forms of colony social organization, in that the castes are difficult to distinguish but do not seem to have disappeared completely. Further investigation might reveal that this type of social variation occurs not only in different populations, but within the same population, as in *H. sexcinctus*.

The existence of mixed social strategies within apparently eusocial populations could have important ramifications for social evolution and devolution, perhaps shedding light on the sequence of steps by which populations evolve between the extremes of solitary univoltinism and eusocial bi- or multivoltinism. The phylogenetic position of *H. sexcinctus* implies that it had a social ancestor and that solitary populations in central Europe represent evolutionary reversions (7, 30). This in turn suggests that communal social organization could be an intermediate step in the transition from eusociality back to solitary existence, even though it probably is not a transitional step in the solitary-eusocial transition (3). Why then is the coexistence of communal and eusocial behavior in halictine bees so rare, especially given the large number of halictine reversions from eusocial to solitary behavior? A communal transition between solitary to eusocial colony cycles is likely to be unstable and should disappear rapidly. As in any type of society based on mutualism, communal societies are open to cheating by non-egalitarian members. If dominance hierarchies associated with reproductive skew are formed, the colony ceases to be communal. This means that attempts by some individuals to dominate reproduction will tend to promote either the evolution of caste-based societies (eusocial or semisocial) or the founding of solitary colonies in which females can simply avoid cheaters, so communal, casteless societies would tend to be transient.

Another reason why communal/eusocial polymorphism might seem rare is that it may often go undetected. When we first observed the establishment of communal nests, we mistakenly assumed them to be bees of a different species. Once we realized that they were *H. sexcinctus*, we still could have overlooked the body shape difference if we had followed the usual practice of comparing only head widths or wing lengths of adult females, especially because the communal nests comprised only $\approx 5\%$ of the total number of nests in the Daimonia aggregation. Behavioral observations of other halictine bees suggest that there may be other examples of eusocial/communal polymorphisms. In addition to the examples previously cited (*L. umbripenne* and *H. ligatus/poeyi*), it is possible that in *Lasioglossum duplex*, new nests, founded in midsummer, are not founded by absconding workers (42), but by a caste of communal foundresses.

The morphological differences between eusocial queens and workers and communal foundresses, together with their associated behavioral differences, suggest that the latter can be regarded as a novel third caste. In halictine bees and other primitively social insects, queen versus worker caste determination is traditionally considered to be postimaginal, influenced primarily by the timing of adult emergence, social interactions with nestmates, and whether young females mate (43, 44). Newly emerged adult females with the physical characteristics of the queen or worker castes, sometimes display the behavior of the other caste (36, 40). In marked contrast, communal and eusocial *H. sexcinctus* females exhibit morphological differences that must reflect preimaginal developmental differences (45), and this implies that their behavioral differences also have a preimaginal basis. The differences between communal and eusocial females could be due to a genetic difference (46, 47) between the communal and eusocial phenotypes, or could be a result of a developmental switch that is environmentally induced during the larval stages (48, 49), perhaps as a result of differential feeding by queens (50). The latter possibility seems more likely, as previous studies have shown that preimaginal developmental switches must underlie morphological polymorphism in both female (50) and male (51) halictines. Whether the strategies are genetically based or manipulated by queens and workers rearing female larvae, the proportions of communal and eusocial nests could represent either evolutionary or phenotypically plastic adjustments to local environmental conditions.

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