Mate guarding and alternative reproductive tactics in the ant *Hypoponera opacior*

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In many animals, males have evolved weapons, elaborate courtship displays, or costly ornaments to increase their reproductive success. Ants, in contrast, commonly mate during nuptial flights, in which males do not profit from fighting or attempting to monopolize females. However, where mating occurs in the nest, males can use other reproductive tactics. We found that wingless (apterous) males of *Hypoponera opacior* sat on top of queen cocoons, inserted their genitalia into the cocoons and remained in copula with cocooned queens for up to 40 h. These males were tolerant of each other; fighting was never recorded. Our observations therefore suggest that wingless males of *H. opacior* ensure reproduction by copulatory mate guarding. This strategy, although time consuming, presumably reduces the likelihood of subsequent inseminations by other males. Apterous *H. opacior* males have only a limited amount of sperm available: histological preparations showed that, in contrast to *Cardiocondyla* fighter males, the testes degenerate in early adult life. Males of *H. opacior* have relatively few mating opportunities. Although some wingless males were reproducitively active for more than 3 weeks, we observed a maximum of only six matings per male, with a mean slightly above one. Some *H. opacior* males used an alternative reproductive tactic of dispersal and outbreeding. We found colonies headed by single, dealate queens, which did not rear wingless sexuals but presumably reproduced through winged reproductives that mate in nuptial flights. The social structure of those colonies contrasted with nests containing wingless reproductives, which were highly polygynous and polydomous.

Within species, individuals can use a variety of strategies to secure mating opportunities. Most fundamentally, males differ from females. Furthermore, reproductive behaviour can vary within the sexes (Clutton-Brock 1988; Choe & Crespi 1997). Of particular interest are those cases in which alternative behaviour has coevolved with alternative morphologies (Gadgil 1972; Gross 1996). The majority of taxa with intrasexual polymorphism have variable males, reflecting the predominance of sexual selection on male traits (Howard 1978; Kuris et al. 1987; Shuster 1992). An important exception to this generalization are the Hymenopteran social insects. Ants, bees and wasps have polymorphic females that can be dichotomized into reproductive and nonreproductive castes (Hamilton 1964; Wilson 1971). The queen–worker distinction typically implies morphological and reproductive specialization within colonies. Furthermore, reproductive females themselves show considerable morphological variation within and between species. Indeed, a specialized vocabulary has been coined to describe this variation (Peeters 1991; Buschinger & Heinze 1992; Heinze 1998).

Morphological variation among fertile females is strongly associated with differences in reproductive strategies as well. A fundamental dichotomy divides females capable of founding their own colonies (independent colony foundation) from those that must become adopted into or invade already existing colonies (dependent colony foundation; Keller & Vargo 1993; Heinze & Keller 2000). Typically, the latter females have small body size and/or small energy reserves (Stille 1996). Different reproductive strategies associated with female morphology have profound consequences for the social organization of colonies, kin selection dynamics and life histories (Keller 1993).

By contrast, male polymorphism tends to be generally limited in Hymenopteran social insects. In the majority of ant species, males have short life spans, with limited opportunities for repeated mating. In species that mate in...
large nuptial flights, males cannot obtain or defend a harem easily. Thus for social insects, male–male competition for matings occurs primarily through a scramble mechanism. As a result, male morphology reflects strong competition for finding females (large eyes, sensitive olfactory capabilities) rather than adaptations for fighting, and selection for alternative reproductive strategies has been weak (Heinze et al. 1998).

Even so, in some social insects males vary in morphology and reproductive behaviour. In particular, winged and wingless (apterous) male morphs have been described for several species of social bees and ants (Michener 1974; Hamilton 1979; Kugler 1983). This male dimorphism is associated with different reproductive behaviour as well. Typically, wingless males remain in their natal nest and mate with nestmate females while winged males disperse to mate in swarms (Le Masne 1956; Kukuk & Schwarz 1988; Yamauchi et al. 1996, 2001; Heinze et al. 1998). In several species these wingless males have adaptations for fighting (e.g. large mandibles in Cardiocondyla), kill their rivals and monopolize all matings in the nest (Kinomura & Yamauchi 1987; Heinze & Hölldobler 1993; Yamauchi et al. 1996). Here, we show that apterous males in the ant species Hypoponera opacior (Forel 1893) do not fight over access to receptive females, but rather use a mate-guarding strategy evolved in the context of male–male competition.

Hypoponera opacior appears to have two kinds of colonies that we characterize below. Some colonies contain reproductive males and females with typical winged morphology, whereas others contain worker-like apterous queens and wingless males. We describe the social structure of these two kinds of colonies, explore the mating behaviour of the morphs, and discuss our results in terms of sexual selection theory.

METHODS

Hypoponera opacior is a small ponerine ant, with workers 3 mm in length. Nests are generally small with about a dozen workers and brood. We studied this species in an oak–juniper forest (elevation 1700 m) in the Chiricahua Mountains at the Southwestern Research Station near Portal, Arizona, U.S.A. in late summer 1992, 1997 and 1998. There, H. opacior is a common, although inconspicuous species, reaching densities of more than 40 nests/100 m². We collected 207 nests over the 3 study years. The largest collection was made in 1998 by two people, when 168 nests were collected within 2 weeks. The nests occur in the soil under stones. They reach depths of only 30 cm and are often connected to neighbouring nests via subterranean tunnels. We collected entire nests, with aspirators. In all 3 years we gathered nests at random, and in 1998 we also mapped nest locations and the position of connecting tunnels in five plots of 10 × 10 m. To do this we carefully lifted the leaf litter and stones in the study areas and followed tunnels that could lead to other nests. Small colonies (five or fewer workers, no brood) were frozen at the Southwestern Research Station and then stored in 70% ethanol. After the behavioural experiments or observations the colonies were kept in the laboratory in Fort Collins for 6 months to permit the production of sexuals. Thereafter, the ants were frozen at −70°C to allow genetic analysis.

We conducted simple aggression tests (N=5) to determine colony boundaries for colonies with dealate queens. We introduced individually marked workers from neighbouring nests, which were less than 2 m from the recipient nest that contained a dealate queen. We conducted these tests on artificial observation nest sites in the laboratory at the Southwestern Research station. All interactions were observed within 30 min of introduction. After 24 h we recorded the survival of introduced workers.

Ant nests were taken to the laboratory, censused, settled in artificial nest sites inside small boxes (10 × 10 cm area and 1.5 cm high) with moistened plaster floors (Buschinger 1974; Heinze & Ortius 1991) and placed in an incubator at 25°C and high humidity (>80% relative humidity). Ant colonies were fed twice weekly on artificial diet (Bhatkar & Whitcomb 1970) and frozen fruit flies. Once a week we provided ca. one or two live, wingless fruit flies per 10 workers, which the ants readily hunted and killed. We kept these colonies alive in the laboratory for 6 months until all sexuals had emerged and all reproductive activity of these sexuals had ceased.

Censusing H. opacior colonies posed special problems. In addition to wingless and winged males, four kinds of females were found: winged gynomorphic queens, dealate gynomorphic queens, apterous intermorphic (worker-like) queens and workers. Apterous queens resembled workers closely in external morphology differing from workers only in having slightly larger eyes. This character was useful only in recently eclosed (callow) individuals, in which the yellow cuticle contrasted with the dark eyes. In older individuals, the cuticle had darkened considerably, rendering the difference in eye size undetectable. In contrast to the small external differences, the internal morphology varied strongly between these two castes: apterous queens had fully functional ovaries consisting of 3+3 ovarioles and a spermatheca, while workers lacked all reproductive organs. However, we chose not to dissect individuals in these colonies, because intact colonies were required for behavioural observations. Rather, during our censuses we noted the number of dealate queens, worker-like individuals, winged and wingless males, pupae and larvae without differentiation between apterous queens and workers. To discriminate between individuals, we marked males and callow queens in nests containing more than one of each with quick-dry, metallic-ink pens with an extra-fine point (0.7 mm). These marks lasted up to 3 weeks, and we reapplied marks as necessary. We observed colonies directly through a microscope for a total of 1400 min. Observations were made from 2 to 9 September 1997 and 24 August to 5 October 1998. Each colony was observed one to four times a day for 5 min each. We recorded the frequency, duration and mating partner for each copulation. Special attention was paid to interactions between apterous males.

To verify sperm transfer, we dissected the ovaries of eight apterous queens and inspected their spermathecae. We disrupted the copulation of wingless males with
coconut queens. For dissection ants were killed by freezing at \(-20^\circ C\) for at least 2 h. Testis development was analysed in four males that had mated and thus were sexually mature. Males were fixed in ethanol, alcoholic Bouin fixative, embedded in Durcupan (Fluka), and serially sectioned at 1 \(\mu\)m with a glass knife and a Reichert microtome. The sagittal sections were attached to microscope slides and stained with 0.1% Toluidine Blue.

RESULTS

Mating Behaviour of Apterous Sexuals

While we did not observe the mating behaviour of winged sexuals directly, we had ample observations of copulatory behaviour for apterous reproductives. In 36 colonies, both apterous males and queens were present simultaneously. The first matings were observed in late August, shortly after males emerged from their cocoons. Apterous males were tolerant of each other: several males (up to 13, \(X \pm SD=3.7 \pm 3.1\)) co-occurred in the majority of nests and fighting between them was never observed in 1400 min of total observation time. In that time, we recorded mainly two behaviours of apterous males: copulation with very young apterous queens, which were still partially enclosed in the cocoon (Fig. 1), and inspection of cocoons at the rear end. We interpret the latter behaviour as a search for receptive females, because during the majority of intranidal matings males inserted their genitalia through an opening at the rear end of the cocoon through which the meconium (stored faeces) is expelled during pupation. Apterous males were never observed in the foraging arena or leaving the inner part of the nest.

We observed 96 matings in 23 nests, all between nestmates that had emerged from cocoons while kept in the laboratory. In 74 instances, we observed males clinging to cocoons of queens and inserting their genitalia through the opening of the cocoon (Fig. 1, Table 1). These apterous males were mating with very young virgin queens.

Nests with apterous males were observed one to four times per day at irregular intervals, but typically once in the morning, around noon, in the afternoon and in the evening. This allowed us to make lower estimates for the duration of matings. Of 59 copulations between apterous males and queen pupae that were observed without interruption, 21 (35.6%) were observed only during a single observation period of 5 min. The other copulations (64.4%), however, continued into the next observation period. On average, these copulations lasted for 12 h 21 min, and ranged from 2 h 20 min to at least 41 h 15 min. These durations, calculated from the time difference between observation periods when the copulation was first and last recorded, are minima. Matings between

<table>
<thead>
<tr>
<th>Year</th>
<th>Queens in cocoon</th>
<th>Emerged queens</th>
<th>Workers</th>
<th>Males</th>
<th>2 males/1 queen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1998</td>
<td>65</td>
<td>10</td>
<td>2</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>
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partially encased queens and apterous males generally ended when the young queens started to move and emerge from the cocoon. These callow queens tried to escape, sometimes dragging the males behind while still in copula.

We interrupted eight matings that had started since the last observation period and dissected the young callow queens; in all cases, sperm had already been transferred. Thus, males clearly succeeded in inseminating females over two observation periods. Because the majority of uninterrupted copulations lasted over several observation periods, sperm transfer cannot be the reason for long copulation times.

In 11 cases, we observed two males sitting simultaneously on a single cocoon. In all cases, only one male succeeded in copulating and we never saw the second male displace the successful male. These mating associations of two males and a single apterous queen were typically short, with only three (27%) continuing into a second observation period. Even so, no such mating association broke up within a 5-min observation period, and one lasted for at least 8 h 5 min, always with the same male in copula.

We saw only 10 copulations between males and fully emerged young queens. These never lasted very long. In two cases they broke up during the 5-min observation time, and no copulation persisted until the subsequent period. The matings were always initiated by males antennating the queen's head violently; the callow queens frequently attempted to escape, often dragging the males in copula.

We twice observed a male inserting his genitalia into a worker cocoon. One such 'copulation' lasted for 4 h 22 min, while the other terminated before the next observation period. We also observed 'pseudocopulations', in which two males tried to insert their genitalia into each other. In all cases both males were fully emerged from the cocoon. Pseudocopulations were observed six times (Table 1) and were relatively unstable, never lasting to a second observation period. Both males survived these interactions, apparently unharmed.

Apterous males showed a long duration of sexual activity for ant males. Our record was a male still alive and copulating 24 days after his first mating. For 62 males from 14 colonies our lower estimate for the mean number of matings ± SE was 1.12 ± 0.15, with a range of zero to six. Repeated matings included those with callow queens encased in cocoons as well as with older queens. Six males were observed mating with both cocooned and emerged queens.

Queens mated only once while encased in the cocoon. However, seven of these queens copulated again after their emergence from the cocoon, always with a different male. Although we determined that sperm transfer always occurred during matings with males and encased queens, we could not ascertain whether sperm transfer occurred in subsequent matings as adults as well.

In general, the mating behaviour of males was tolerated or ignored by workers, queens and other males. However, workers started to bite males after most or all individuals had emerged from cocoons. The presence of cocoons in the nest was a good predictor of worker aggression: as long as cocoons were present, males were allowed to stay in the nest and be reproductively active. In contrast, in 11 colonies we observed workers killing all apterous males within 24 h of the emergence of the last adult from the pupae. Thus, male life span appeared to be determined by the workers and was correlated with presence of pupae in the nest.

The histological preparations showed that none of the apterous males had developed testes with ongoing spermatogenesis (Fig. 2). All sperm had been transferred into the seminal vesicles and thus the sperm supply must be limited.

Nest Demography and Social Structure

We collected 207 nests over the 3 study years. The largest collection (N=168) was made in 1998, for which we report demographic data. Nests contained a mean ± SE of 9.9 ± 11.2 worker-like individuals (apterous queens+workers) and 18.1 ± 52.7 pupae; some nests contained up to 70 worker-like individuals and 400 pupae. The majority of nests (N=98) contained at least one apterous queen or wingless male. These nests did not have winged sexuals at collection or during the 6 months of laboratory culture. We found only five nests with dealate queens, three of which were colony foundations, containing one to two queens but no workers. These nests never reared apterous sexuals. No colony contained or reared both winged and apterous sexuals.

We found clear differences in social structure between nests with apterous and winged reproductives (Table 2). Of nests with apterous queens 85% were in high-density areas (>0.25 nests/m²). In these dense nest accumulations, nests were interconnected via tunnels, and the mean distance to the nearest neighbour ± SE was 1.02 ± 0.05 m. We frequently observed workers running along these subterranean tunnels in the field; they were apparently recognized by ants of both sites as nestmates. Nests often contained several apterous queens; we dissected only a small subsample of nests (N=6) containing between two and 15 mated apterous queens with developed ovaries. Aggressive interactions between apterous queens were observed in the autumn, shortly after the emergence of the last callow queen and before the first eggs were laid. In four colonies, which we observed in more detail, intensive antennal boxing between nestmate apterous queens occurred over several weeks. Aggression ceased in the late autumn, but interactions between queens clearly indicated the maintenance of a reproductively hierarchical structure.

All nests with dealate queens were found in low-density areas (<0.05/m²). Independent colony founding appeared to predominate, as more than half of these colonies were newly founded. The mature colonies contained just a single dealate queen and nests were never interconnected through tunnels. The aggression tests showed that workers in nests with dealate queens were highly aggressive towards all neighbouring nests: all five introduced workers were killed within 24 h of introduction, but none of the workers of the recipient colonies died.
DISCUSSION

The ant genus *Hypoponera* is known for its diversity of reproductive tactics in males and queens (Table 3). In some species (*H. nippona*, *H. sauteri*: Yamauchi et al. 2001) all sexuals are winged and engage in nuptial flights, while in others wingless males fight for access to females (*H. punctatissima*: Hamilton 1979; *H. bondroiti*: Yamauchi et al. 1996). In contrast, wingless males of other *Hypoponera* species (*H. eduardi*, *H. nubatama*) use a third tactic, mate guarding (Le Masne 1956; Yamauchi et al. 2001). We have shown that apterous males of the species *H. opacior* not only mate within their mother colony, but also mate-guard. Males were observed in copula for days with young queens encased in their cocoons, although sperm had clearly been transferred at the onset of the copulation. Copulation times such as those we observed are extremely unusual, especially for ants. Matings in ants usually last between 0.5 and 12.0 min (Rosengreen et al. 1986; Lenoir et al. 1988; Keller & Passera 1992), and the record was previously held by the Japanese species *H. nubatama* with 88 min (Yamauchi et al. 2001). Our record of 2480 min is not only the longest copulation ever recorded for any ant species, but it exceeds the previous record by a factor of more than 25. These long copulation times are doubtless facilitated by the immobility of the young queens, as copulations in other species are often terminated by queens biting the males (Marikovsky 1961; Nagel & Rettenmeyer 1973; Hölldobler 1976; Keller & Passera 1992). Males in *H. opacior* also showed a prolonged sexual activity; they were able to mate repeatedly over several weeks. As mate guarding is a time-consuming strategy during which rival males cannot be excluded from mating with other virgin queens, the mating success of *Hypoponera* males should be much lower than that of wingless *Cardiocondyla* males, which kill their rivals. While wingless males of the genus *Cardiocondyla* mate with more than 80 queens (Heinze & Hölldobler 1993; Yamauchi & Kinomura 1993; Heinze et al. 1998), we found a maximum of only six matings. In

Table 2. Overview of the two distinct social structures of *Hypoponera opacior* colonies in the Chiricahua mountains, Arizona

<table>
<thead>
<tr>
<th>Nests with winged reproductives</th>
<th>Nests with apterous reproductives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monogynous</td>
<td>Polygynous</td>
</tr>
<tr>
<td>Unidomous</td>
<td>Polydomous</td>
</tr>
<tr>
<td>Independent colony founding</td>
<td>Dependent colony founding</td>
</tr>
<tr>
<td>Presumably outbred, nuptial flights</td>
<td>Inbred, intranest mating</td>
</tr>
<tr>
<td>All nests at low density (&lt;0.05/m²)</td>
<td>85% of nests at high density (&gt;0.25m²)</td>
</tr>
</tbody>
</table>

Figure 2. Longitudinal section through the gaster of a reproductively active *Hypoponera opacior* male. Sperm production had clearly ceased in this male. All sperm has been transferred to the seminal vesicles (a), between the midgut (b) and the crop (c).
Table 3. Social structure and reproductives strategies found in different species of the ant genus *Hypoponera*

<table>
<thead>
<tr>
<th>Species</th>
<th>Colony origin</th>
<th>Colony structure</th>
<th>Queens</th>
<th>Males</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gyny</td>
<td>Domy</td>
<td>Winged</td>
<td>Apterous</td>
</tr>
<tr>
<td>H. nippona</td>
<td>Japan</td>
<td>Mono</td>
<td>Uni</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>H. sauteri</td>
<td>Japan</td>
<td>Mono</td>
<td>Uni</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>H. nubatama</td>
<td>Japan</td>
<td>Poly</td>
<td>Poly</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H. opaciceps</td>
<td>Japan</td>
<td>Poly</td>
<td>Poly</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H. bondroiti</td>
<td>Japan</td>
<td>Poly</td>
<td>Poly</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H. opacior</td>
<td>Arizona, U.S.A.</td>
<td>Mono</td>
<td>Uni</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The country of origin is given for colonies used in the behavioural observations. We show which type of sexuals are found in the different species and whether they co-occur in colonies. Gyny refers to the number of queens in the nest, domy to the number of nests per colony (NA=not applicable).
the majority of ant species males have only limited mating opportunities and thus the ancestral character in ant males is a degradation of testes in early adult life (Holldobler & Bartz 1985). Sexual selection, however, has led to a continuation of sperm production throughout the lifetime of wingless Cardiocondyla males (Heinze & Hölldobler 1993). In contrast to these species with fighting males, our histological analyses show that the testes of apterous, mate-guarding *H. opacior* males degenerate in early adult life. It remains to be seen whether sperm are produced continuously in *Hypoponera* species with fighting males (i.e. *H. punctatissima* or *H. bondroiti*; Hamilton 1979; Yamauchi et al. 1996; Table 3). The evolution of fighting behaviour in males should be associated with a high reproductive skew among males. This is possible only when ant males are able to mate repeatedly, creating strong selection pressures to prolong the sperm production period.

Mate guarding is often associated with sperm competition in species with multiple mating in females. If there is a last-male advantage, then mate guarding can pay off for a male (Birkhead & Hunter 1990; Dickinson 1995). Apterous queens of *H. opacior* are presumably incapable of controlling the number of their mating partners while they lay motionless in their cocoons. However, they may exert cryptic female choice by preferentially using sperm of certain males (Lewis & Austad 1994; Eberhard & Cordero 1995). As adults, however, they show little interest in copulations and appear to mate only reluctantly. Thus, by mating with a queen before she emerges from the cocoon, a male certainly increases the likelihood that he is her last mating partner.

Male mating behaviour should also be influenced by the operational sex ratio, which is the ratio of sexually receptive males to receptive females in a population at any given time (Emlen & Oring 1977). In birds, for example, guarding behaviour is more common when only few receptive females are available (Hasselquist & Bensch 1991). Because we could not ascertain the receptivity of virgin queens encased in their cocoons, we could not estimate the operational sex ratio and thus do not know whether males adjust mating durations to the competitive environment. However, in the Japanese *Hypoponera* species *H. nubatama* wingless males showed increased copulation times in the presence of other males (Yamauchi et al. 2001).

In our study population in the Chiricahua mountains two types of *Hypoponera* ants coexist with different social organization, reproductive and dispersal strategies, but indistinguishable worker morphology. As in *H. eduardi* (Le Masne 1956), some colonies contain winged sexuals, while others rear exclusively wingless queens and males; we never found both types of reproductives in the same colony or observed matings between them. Thus, without the usage of genetic markers, it remains unclear whether these two nest types belong to a single species or are sibling species. Colonies with apterous sexuals are polydomous and probably reproduce by budding as indicated by dense nest clusters and tunnels connecting different parts. Nests are highly polygynous, with young mated queens forming dominance hierarchies in the autumn. Intraspecific fighting appears to be the rule in this social form, because we regularly observed matings between nestmate queens and males. Lacking the nuptial flights that presumably promote outbreeding (Hölldobler & Wilson 1990), this social form may experience extensive inbreeding. Nevertheless, we cannot conclude inbreeding from intraspecific mating alone; intraspecific mating within nests of the highly polygynous ant *Linepithema humile* was not associated with detectable inbreeding (Krieger & Keller 2000). Even so, males of *L. humile* are winged and disperse on the wing, while apterous *H. opacior* males never leave the nest and appear to mate exclusively inside their mother colony. Thus, our observational data strongly suggest inbreeding in this social form, but genetic analyses are necessary to verify this assumption.

Colonies of the second social form with dealate queens are rare, as has been found in other species with both winged and apterous queens (Heinze & Tsuji 1995). All mature colonies with dealate queens were monogynous, and behavioural tests revealed that they were uniparental. These colonies occurred at exceptionally low density, suggesting that winged queens disperse over long distances. The majority of dealate queens occurred in recently founded colonies, which further suggests that independent colonies have a low success rate. A comparison of the ecological, genetic and social conditions under which the different types of *Hypoponera* colonies occur is needed to help us to understand the diversity of reproductive and dispersal strategies in this genus.

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