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Male reproductive investment and queen mating-frequency in fungus-growing ants

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Sperm number and male accessory gland compounds are often important determinants of male mating success but have been little studied in social insects. This is because mating in social insects is often difficult to manipulate experimentally, and first evidence for an explicit influence of accessory gland secretions on male mating success in social insects was obtained only recently. Here we perform a comparative analysis of male sexual organs across 11 species of attine fungus-growing ants, representing both genera with single- and multiple-queen mating. We found that the general morphology of the male sexual organs was very similar across all species, but the relative sizes of the accessory glands and the sperm-containing accessory testes vary significantly across species. Small testes and large accessory glands characterize species with singly mated queens, whereas the opposite is found in species with multiply mated queens. However, in the social parasite *Acromyrmex insinuator*, in which queens have secondarily reverted to single mating, males have accessory gland characteristics reminiscent of the lower attine ants, but without having significantly reduced their investment in sperm production. We hypothesize that the main function of accessory gland compounds in attine ants is to monopolize male paternity in similar ways as known from other social insects. This would imply that the evolution of polyandry in the terminal clade of the fungus-growing ants (the leafcutter ants) has resulted in selection for decreased investment by males in accessory gland secretions and increased investment in sperm number, in response to sperm competition for sperm storage. *Key words:* accessory glands, accessory testes, fungus-growing ants, multiple mating. *[Behav Ecol 15:426–432 (2004)]*

Sexual selection is a major evolutionary force and has been intensively studied in many species (Birkhead and Moller, 1998; Eberhard, 1996; Simmons, 2001), the social insects being one of the few notable exceptions (Boomsma and Ratnieks, 1996). This is surprising given that many aspects of sexual selection can be expected to act differently in social insects, allowing general principles to be tested in unusual contexts (Baer, 2003). For example, sex does not play a role in the daily life of social insects (Boomsma and Ratnieks, 1996). In single-queen societies of ants, bees, and wasps, pair formation is irreversibly achieved during a single short mating flight. Males die almost immediately after mating (Hölldobler and Wilson, 1990; Starr, 1984) and only "survive" (sometimes for decades!) as sperm stored in a queen's spermatheca (Hölldobler and Bartz, 1985; Pamilo, 1991). Even in societies that replace queens or adopt newly mated daughter queens back into existing colonies, mating normally takes place outside the nest and is not part of normal social life. In other words, social Hymenoptera are characterized by pair formation for life even in species in which queens mate with multiple males.

Where multiple mating by females (polyandry) is widespread among insects and vertebrates in general (Birkhead and Moller, 1998; Simmons, 2001; Strassmann, 2001) and life-long pair formation (monoandy) rare, this is opposite in the social insects in which monoandry is widespread and polyandry relatively rare (Boomsma and Ratnieks, 1996; Strassmann, 2001). Another remarkable idiosyncrasy of social insects is that reproductive success can only be realized after a phase of ergonomic colony growth during which only sterile workers are produced (Oster and Wilson, 1978). A male contributes to this female offspring (but not to the haploid queen sons, which have no father) but will not obtain any fitness until the colony has become mature and starts to produce female sexuals. This long time lag between mating and reproduction in combination with pair formation for life should effectively preclude the evolution of ejaculate traits that promote male fitness at the expense of survival or residual reproductive success of females (see below). These exceptional characteristics of social insect mating systems imply that males can be expected to have a number of key-traits that are likewise exceptional compared with those of solitary insects and vertebrates. In the paragraphs below, we summarize our current knowledge of these traits, starting with the well-documented lack of continuous sperm production in males of social Hymenoptera. Subsequently, we single out two specific male traits (accessory gland [AG] volume and sperm number) and formulate a conceptual framework for the way in which sexual selection may have affected the evolution of these traits. We then test formulated hypotheses by presenting comparative data on sperm number and AG size in fungus-growing ants.

The haploid, short-lived males of social Hymenoptera hatch with a fixed amount of clonal sperm (Heinze and Hölldobler, 1993; Heinze et al., 1998; Hölldobler and Bartz, 1985; Hölldobler and Wilson, 1990), so that sperm competition within a male's ejaculate does not occur (Baer, 2003). Mature males have inactive testes and have all their spermatozoa stored in the accessory testes (AT), ready to be ejaculated. Sexually mature social insect males often have enough sperm to fully inseminate at least a single female (Fjerdingstad and Boomsma, 1997; Franck et al., 2002; Reichardt and Wheeler, 1996; Röseler, 1973; Tasei et al., 1998), but they have no possibility to increase their lifetime amount of sperm as adults (Heinze and Hölldobler, 1993; Hölldobler and Wilson, 1990; Simmons, 2001). The only known exception to the rule of fixed sperm complements are *Cardiocondula* ants, in which a special second male morph is long-lived, mates within the nest, and has secondarily evolved continuous sperm production (Heinze and Hölldobler, 1993; Heinze et al., 1998).

The transfer of AG compounds during copulation is well known for many social insects (Baer et al., 2003; Baer, 2003). As

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far as data are available on AG function in social insect males, they all suggest that these secretions are meant to increase male reproductive success. For example, AG compounds form mating plugs in ants and bumblebees (Brown et al., 2002; Duvoisin et al., 1999; Monnin and Peeters, 1998). In the latter, mating plugs contain an antiaphrodisiac, reducing the willingness of the female to mate again within her short time window of receptivity (Baer et al., 2000, 2001; Sauter et al., 2001). Bumblebee males thus control the mating process against the interest of queens who would benefit from multiple mating as genetically heterogeneous offspring is less susceptible to disease (Baer and Schmid-Hempel, 1999, 2001, 2003). In *Melipona* stingless bees, AG compounds form mating signs (Da Silva et al., 1972) that may have a similar manipulative function, as this group of bees is known to have predominantly single queen mating as well (Strassmann, 2001). In *Apis* honeybees, queens mate with many males in a quick succession while each male leaves a mating sign (Koeniger, 1990; Woyciechowski et al., 1994). However, in this highly derived and (even for social insects) unusual mating system, the function of mating signs seems to be to prevent sperm from leaking out (Boomsma and Ratnieks, 1996; Woyciechowski et al., 1994) and to encourage further copulations by subsequent males (Koeniger, 1990). Finally, male AG compounds are known to be involved in the formation of spermatophores in the ants *Carebara vidua* (Robertson, 1995) and *Diacamma* sp. (Allard et al., 2002) and in the ichneumonid wasp *Diadegma semiclausum* (Madel et al., 1990). However, as already indicated above, AG compounds in social insect males are not expected to become agents of chemical warfare between the sexes. The life-long storage of sperm after a single mating event and postponed reproduction (i.e., production of sexual offspring after at least one but often many broods of sterile workers) should prevent the evolution of harmful traits of AG fluids, such as the induction of excess oviposition/oogenesis or a reduction of female survival, as has been found in several nonsocial insects (Chapman et al., 1995; Chen et al., 1988; Gillott, 1996; Simmons, 2001).

In social Hymenoptera, males typically leave the maternal colony for a single mating flight and die shortly afterwards (Hölldobler and Wilson, 1990; Starr, 1984; Weber, 1972). It therefore seems highly unlikely that males can replenish AG compounds in this short and energetically demanding time window, which implies that (1) the amount of AG compounds of males during the mating flight is fixed, and (2) larger AGs result in increased male control over paternity patterns. We thus have a fundamentally different situation than in solitary insects such as *Drosophila melanogaster*, in which sperm production is continuous, and AG and AT size are positively correlated, because larger males mate at higher frequencies, so that they need both more sperm and larger AGs (Bangham et al., 2002). Similar data were recorded from moths in which larger testes are associated with larger AGs (Morrow and Gage, 2000) or from bush crickets in which larger ejaculates are associated with larger AG secretions (spermatophylax size; Wedell, 1993). We therefore hypothesize that, apart from differential success in mate-finding, reproductive fitness of social insect males is determined by two factors: (1) their ability to monopolize paternity (monoandry) by decreasing the reproductive success of competing males—this strategy would necessitate maximal investment in AG compounds and minimal investment in sperm numbers as long as enough can be transferred to guarantee that availability of stored sperm will not limit the life-time reproductive success of a queen mated with—and, (2) their ability to maximize their share in paternity when they cannot prevent their mates from remating with additional males (polyandry). This strategy implies maximal investment in sperm number and minimal investment in AG secretion. In the present study, we investigate these hypotheses by correlating species-specific queen mating-frequency with accessory testis size and AG size of males across the fungus-growing ants.

The fungus-growing ants are highly suitable for such comparative study because queens express a large bimodal variation in mating frequency, whereas males perform a single mating flight and die on the same day throughout the entire tribe (Weber, 1972). Genus- and species-specific frequencies of single- and multiple-queen mating have been extensively studied with genetic markers and have indicated a single transition from exclusively single mating in the lower- and basal higher attine ants to obligatory multiple mating in the leaf-cutting ants (Villesen et al., 2002). Multiple-queen mating in the *Acromyrmex* and *Atta* leafcutter ants is correlated with larger and more long-lived colonies (Hölldobler and Wilson, 1990) and with larger body size of both queens and males. This implies that the absolute volumes of sperm and AG secretion per male are larger (Crozier and Page, 1985), but the relative investments in these organs can still be compared with those in the more basal genera, after removing the effect of body size.

METHODS

Males of 11 species of fungus-growing ants (tribe Attini) were collected in the surroundings of Gamboa, Panama. Colonies were excavated from mid April to mid May in 2001 and 2002, shortly before the rainy season when they contain sexually mature males. Species were chosen according to their availability in the field and cover the total attine phylogeny (Villesen et al., 2002). Males were kept alive for a maximum of 2 days. They were killed by decapitation, after which the sexual organs were immediately dissected and transferred to a microscope slide to inspect the general morphology of the sexual organs. Throughout the rest of this article, we will only use the term AT when referring to the morphological structures in which mature males store their sperm prior to ejaculation. Male maturation was investigated by dissecting young or freshly eclosed males from both laboartory and field colonies. Males were identified as being freshly hatched when they were of known age (laboratory colonies), or (in field colonies) when they were of light pigmentation typical for newly eclosed ants, when there were male larvae and male pupae present suggesting continuous hatching over a period of time, or when we resampled males from marked colonies over a period of time. Pictures of the AT and the AGs were taken with a digital camera (Canon EOS D30), connected to a Leica stereomicroscope, at magnifications of 12.5 to 64. These pictures were then transferred to a Macintosh computer, and the area of the left and right AT and AG was measured by using the public domain National Institutes of Health Image 1.62 program (developed at the US National Institute of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Because the AG and the AT are relatively simple tubular structures (Figure 1), the area measured correlates directly with the volume of these morphological structures. For every male, one AT was pierced, and a subsample of sperm was transferred to a microscope slide to check whether it was sexually mature and spermatogenesis had been completed. Males that still had functional testes were discarded as being sexually immature and likely having incompletely filled ATs. Head width (HW), that is, the maximal distance between the compound eyes, was used to estimate body size of the males. This measurement, taken with the ocular grid of a Leica dissecting microscope at magnifications of 7.8 to 62.5, has earlier been shown to be a reliable predicator of body size in leaf-cutting ants (Fjerdingstad and Boomsma, 1997). We dissected three to six males for each colony and sampled two to five colonies per species.

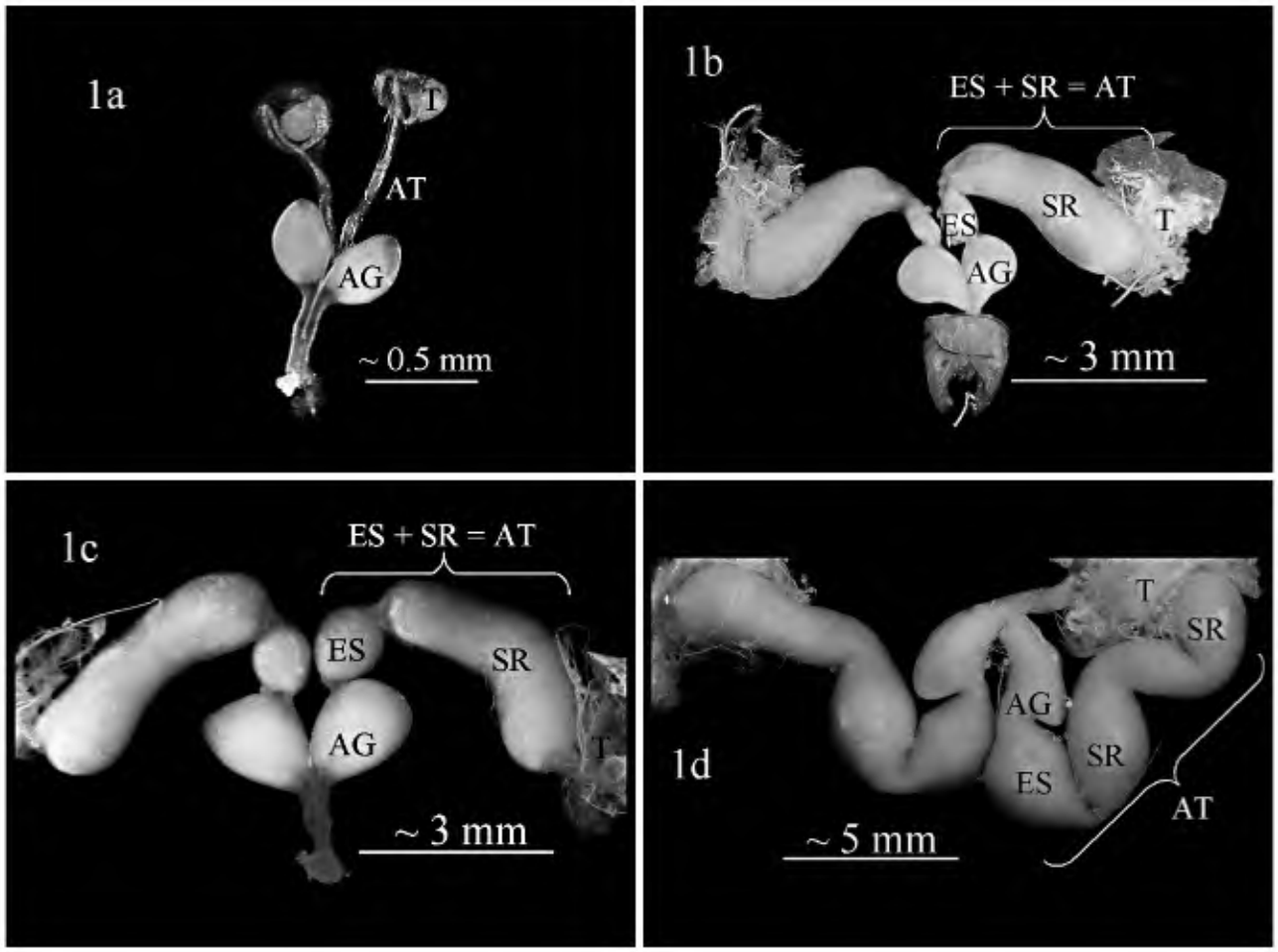

Figure 1
Digital pictures of male reproductive organs of four different fungus-growing ant species: *Trachymyrmex* cf. *zeteki* (a), *Acromyrmex echinatior* (b), *Acromyrmex insinuator* (c), and *Atta colombica* (d). T indicates testis; AT, accessory testis; and AG, accessory gland, which in *Acromyrmex* and *Atta* could be subdivided into an ejaculatory section (ES) and a sperm reservoir (SR).

For statistical analysis, we used the average of the left- and right-hand measurements of each male. In rare cases in which an AT or AG had been damaged during dissection, only data from the undamaged side were used. For the first series of analyses, AT and AG measurements were scaled for body size by expressing them as relative values, that is, relative AT (RAT) size = ($\sqrt{\text{AT area}}$)/HW, and relative AG (RAG) size = ($\sqrt{\text{AG area}}$)/HW. Data were subsequently analyzed by using ANOVA with colonies nested within species. All statistics were done by using SPSS 10 for Macintosh, and all tests are reported with two-tailed probabilities. Finally, RAT size and RAG size were evaluated as a function of the effective mating frequency of queens by using data from Strassmann (2001), Villesen et al. (2002), and Sumner et al. (2004b).

As comparative data can be confounded by different degrees of common ancestry, we also used independent contrasts, calculated with CAIC (Comparative Analysis by Independent Contrasts, version 2.6.9) for Macintosh (Purvis and Rambaut, 1995). This combined approach allowed us to unambiguously analyze whether queen mating-frequency had a direct or partial effect on RAT size and RAG size. The genus-level phylogeny used in the analyses was based on combined information from Schultz and Meier (1995) and Wetterer et al. (1998), as recently reviewed in Villesen et al., (2002). Data for more than two species per genus were treated as polytomies in CAIC, except for *Acromyrmex* and *Atta*, for which additional phylogenetic data were available, indicating that *A. insinuator* is more closely related to *A. echinatior* than to *A. octospinosus*, and that *A. colombica* is more closely related to *A. cephalotes* than to *A. sexdens* (Sumner et al., 2004a). However, as no data on queen mating-frequency are available for *A. cephalotes*, we removed this species from the final analysis.

RESULTS

The total data set consisted of 210 males from 37 colonies belonging to 11 species. Males of all species shared the same basic morphology of the sexual organs (Figure 1). As in other social insects (Duchateau and Marien, 1995; Hölldobler and Wilson, 1990), males were found to have empty ATs shortly after eclosion (Figure 1), but as males became older, mature sperm was increasingly detected in the ATs while the testes became smaller. We never found males possessing both completely filled ATs and large and active testes at the same time. We concluded from this pattern of development that all species investigated had the standard ontogeny of social insect males, so that the amount of sperm and (in all likelihood) the volume of available AG secretion is fixed at sexual maturity. This implies that the size measurements reliably reflect the total male investment in sperm and AG compounds.

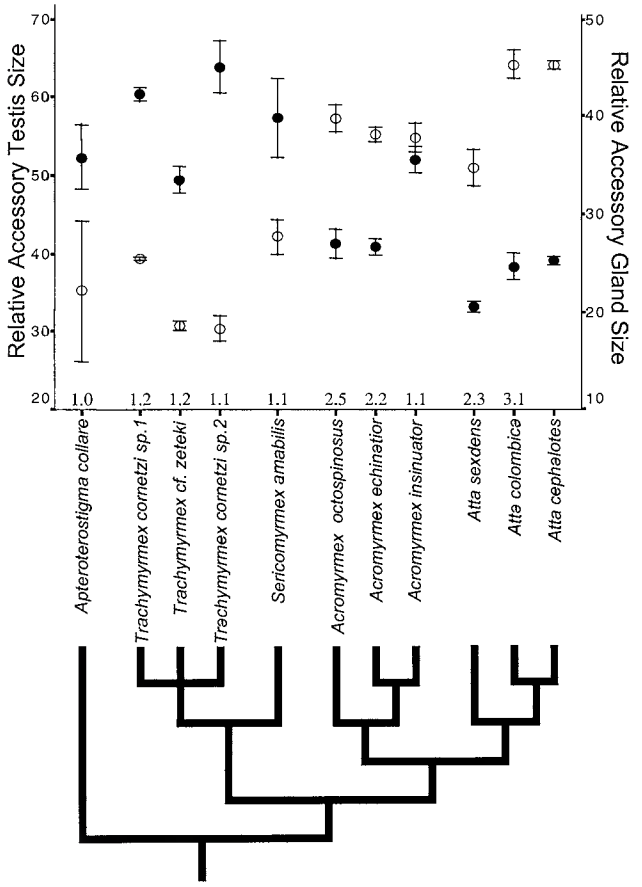

Figure 2
Relative AT size (RAT, open dots) and relative AG size (RAG, black dots) of the different species of fungus-growing ants investigated. Data are means per species ± 1 SE. Values above the x-axis refer to queen mating-frequencies in the literature (Strassmann, 2001; Villesen et al., 2002; Sumner et al., 2004b). No data on queen mating-frequency was available for *A. cephalotes*. Sample sizes were three to six males per colony and two to five colonies per species. Original data were square-root transformed as described in the text and adjusted for differences in body size. The phylogenetic tree under the graph is based on the method of Villesen et al. (2002) and Sumner et al. (2004a).

We found that the ATs in the obligatory multiply mating leaf-cutting ants (*Atta* and *Acromyrmex*) were greatly enlarged and consisted of two distinct structures (Figure 1b–d): an upper part, which we defined as the sperm reservoir, and a lower part close to where the AT and AG ducts join together, which we defined as the ejaculatory section. In *Atta* we were able to provoke ejaculations by decapitation of the male, which invariably resulted in all sperm from the ejaculatory sections being ejected. When dissecting such males immediately after ejaculation, new sperm was observed to be transferred from the sperm reservoirs into the sperm ejaculatory sections by contractions of the entire AT. The ejaculatory sections were thus refilled within a few minutes, and in some cases, we were able to observe a second unprovoked ejaculation.

Male body size was larger in *Atta* (HW = 2.5 to 3.1 mm) compared with *Acromyrmex* (HW = 1.63 to 1.83 mm), which in turn exceeded the typical male body size in the other genera of fungus-growing ants (HW = 0.70 to 0.99 mm). Male body size and queen mating-frequency were positively correlated (linear regression, $F_{1,10} = 11.45$, $p = .010$, HW = 0.926 queen mating-frequency + 0.243; $r^2 = .589$), and this remained significant when analyzing independent contrasts (linear regression, $F_{1,18} = 23.34$, $p < .001$, $r^2 = .58$). However, we stress that increased body size is likely to be an evolutionary consequence rather than a cause of multiple-queen mating, and we thus only used this correlation to arrive at accurate estimates of relative AG and AT sizes.

Table 1
Nested ANOVA testing for differences among species and colonies (nested within species) using body size corrected values of relative testes size (RAT) and relative AG size (RAG) as dependent variable (Dep. Variable). SS refers to the sums of squares of the ANOVA

Source	Dep. Variable	SS	df	*F*	*p*
Species	RAT	26519.81	10,26	177.70	<.001
	RAG	10355.64	10,26	108.68	<.001
Colony nested within species	RAT	1553.38	26,156	4.00	<.001
	RAG	1359.66	26,156	5.49	<.001

RAT and RAG size differed significantly among species and among colonies within species.

AGs were present in all species investigated, but their size differed across species as shown in Figure 1, which compares a representative selection of four species. RAT size and RAG size differed significantly among species (Figure 2) and even among colonies within species (Table 1).

Queen mating-frequency was found to be a significant predictor of both RAT size and RAG size. The regression of RAT size on queen mating-frequency was significantly positive (linear regression, $F_{1,9} = 10.643$, $p = .011$) (Figure 3a), whereas the regression of RAG size on queen mating-frequency was significantly negative (linear regression, $F_{1,9} = 13.68$, $p = .006$) (Figure 3b). An interesting exception is the social parasite *A. insinuator*, whose males have similar AT size as that of the males of the two other *Acromyrmex* species (Figure 2) but substantially larger AGs, of a relative size comparable to non-leaf-cutting ant species. The phylogenetic analysis (CAIC) of the independent contrasts of RAT and RAG gave the same results: a significantly positive regression between the independent contrasts of RAT and queen mating-frequency (linear regression, $F_{1,17} = 6.09$, $p = .02$, $r^2 = .26$) and a negative regression between RAG and queen mating-frequency (linear regression, $F_{1,17} = 6.26$, $p = .02$, $r^2 = .27$).

We found a negative relationship between RAT size and RAG size across species (Pearson correlation coefficient = −0.749, $p = .008$), but this could not be confirmed by CAIC because of insufficient independent contrasts (Pearson correlation coefficient = −0.017, p = ns)

DISCUSSION

Our results indicate that species in which females (queens) evolved multiple mating are characterized by males that have (1) larger body size, (2) reduced relative AG size, and (3) increased relative accessory testis size. Because our analysis of the original data and the independent contrasts (CAIC) gave the same results, evolutionary changes in these traits do not seem to be particularly constrained by phylogenetic inertia. Our data (Figure 3) imply that male investment into AGs may have been reduced after males lost the ability to control female multiple mating with compounds from the AGs. The position of the inquiline social parasite *A. insinuator* is highly interesting in this respect. This parasite has realized a secondary reduction of queen mating-frequency compared with that of its host *A. echinatior* (Sumner et al., 2004b). However, only the AG size

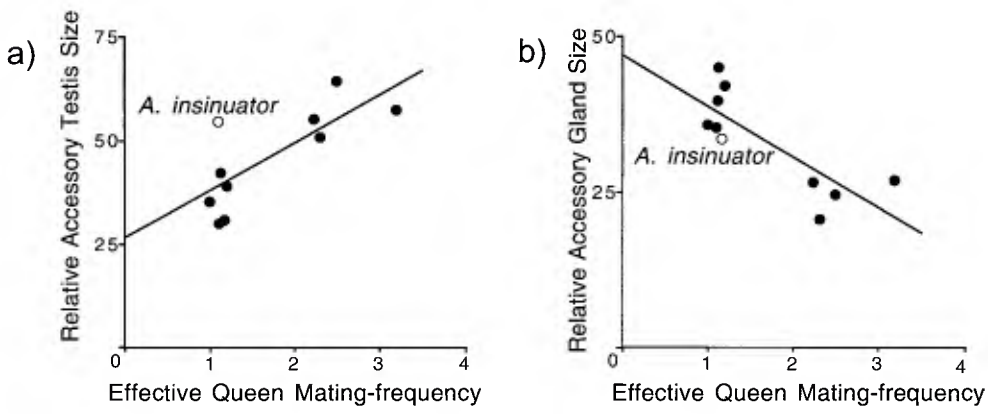

Figure 3
Relationship between effective mating frequency of queens (QMF) and relative AT size (RAT) (a) and relative AG size (RAG) (b). (a) Regression line in: RAT = 11.54 QMF + 26.38 ($r^2 = .571$). (b) Regression line in: RAG = −8.20 QMF + 46.97 ($r^2 = .631$). The social parasite *A. insinuator* is specifically marked with an open dot and was included in both regression analyses. When removing this species from the analysis, the regression fit improved in panel a ($F_{1,8} = 25.03$, $p = .002$; $r^2 = .750$) and remained virtually unchanged in panel b ($F_{1,8} = 12.40$, $p = .010$; $r^2 = .639$).

of the parasite males has increased significantly relative to the host, whereas AT size has remained similar (suggesting that sperm competition still occurs, consistent with the fact that some queens are still multiply mated; Sumner et al., 2004b).

Increased investment in sperm production as a result of sperm competition is predicted by general sexual selection theory (Parker, 1970) and has been shown in other insects such as dung flies (Hosken and Ward, 2001). Here we found support for the idea that sperm competition selects for increased male investment into sperm, but it is important to remember that the main issues concerning sperm competition in ants might differ from those in most other organisms. Both in leaf-cutting ants and in promiscuous nonsocial insects, males benefit from larger ejaculates when this results in an increased representation of their sperm in the female sperm storage organ. In ants, however, sperm competition between ejaculates for storage is fundamentally different from sperm competition for later egg fertilization. This is because the time lag between mating and reproduction implies that the former would favor sperm that reaches the spermatheca first, whereas the latter would favor sperm which is used relatively late, namely, after the yearly phases of ergonomic growth when only sterile workers and no sexual offspring is produced. During the long period of storage, sperm from different ejaculates is normally thoroughly mixed by the queens (Boomsma and Ratnieks, 1996; but see Sundstrom and Boomsma, 2000 for an exception). The fact that ant queens often just about store the amount of sperm that they will need to fertilize their life-time production of eggs (Tschinkel and Porter, 1988) implies that stored ejaculates may not gain anything from wasting or loosing stored sperm of rivals. It would most likely only reduce the number of worker cohorts that a queen is able to realize and, thus, the number of reproductive cycles that these workers can complete. The only competitive interaction after storage that would be unambiguously favorable would be the manipulation of the timing of use, namely, ejaculates biasing their use toward the periods in which fertilized eggs predominantly develop into new queens instead of workers. However, it seems hard to imagine a mechanism by which such bias could be achieved. As a consequence, sexual selection in social insects may have disfavored the evolution of any AG compounds that have negative interference effects on other ejaculates after storage. Insemination success in fungus-growing ants after sperm storage may thus simply follow the principle of a fair raffle, with the number of fertilizations being directly proportional to the number of spermatozoa stored (Simmons, 2001). Further work on these issues would be highly desirable to test these inferences, despite the technical difficulties of studying the dynamics of sperm storage in social insects.

We found that the ATs of leaf cutting ants are greatly enlarged structures, something that has been reported from other social Hymenoptera as well (Duvoisin et al., 1999; Baer, 2003). The morphological structures and our observations on ejaculations support the idea that males of *Acromyrmex* and *Atta* are able to mate with multiple queens under natural conditions, owing to the described sperm-reloading mechanism in the ejaculatory section of the ATs. This idea is consistent with earlier inferences for *Atta colombica* (Fjerdingstad and Boomsma, 1997) and *Acromyrmex versicolor* (Reichardt and Wheeler, 1996) and is supported by the fact that numerical sex ratios in attine ants are generally close to 0.5 (Mueller, 2002), implying that the observed multiple-queen matings in *Acromyrmex* and *Atta* are only possible when males mate multiply as well. An interesting and now almost classic question related to observations of this kind is why males are not fully inseminating a specific female, given that they would have enough sperm to do so (Crozier and Page, 1985). Possible selective forces promoting multiple-queen mating by queens have been extensively reviewed elsewhere (Boomsma and Ratnieks, 1996; Strassmann, 2001). However, males might also directly benefit from distributing their limited number of sperm over multiple females if they benefit from genetically variable offspring in the same way as queens (Arnquist and Nilsson, 2000; Jennions and Petrie, 2000; Schmid-Hempel, 1995), for example, because offspring raised together with less related or nonrelated individuals suffer less from parasitism (Baer and Schmid-Hempel, 1999, 2001, 2003).

Our finding of a decrease of investment in AG secretion would have been remarkable in a nonsocial insect (see Bangham et al., 2002), but it is consistent with the expectations based on the specific characteristics of social insect mating systems (see Introduction). Our analysis of RAT and RAG size detected differences not only between species but also between colonies within species (Table 1). These differences in male investment indicate that there may be a direct trade-off between relative investment in AT and AG, because the amount of resources allocated to the production of sperm and the production of AG compounds are both fixed after maturation (see Introduction). Our data provide some evidence for this as there is a negative relationship between RAT size and RAG size across species, although this is not confirmed by CAIC. However, even in the absence of decisive evidence document-

ing a direct trade-off between AG size and AT size, it is clear that there is no positive correlation between these two variables, as was recently found in moths (Morrow and Gage, 2000). Colony-level differences in the relative investment in AG and AT of males may also be related to sex allocation, if colonies specializing on males would face different constraints during the maturation of sexuals than do colonies specializing on the production of young queens. Unfortunately, we have no information on how colony differences in sex allocation and individual male traits translate into reproductive success, so that further work is needed to make the connection between sexual selection and kin selection in social Hymenoptera.

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