Research

Sex allocation in fungus-growing ants: worker or queen control without symbiont-induced female bias

Michiel B. Dijkstra and Jacobus J. Boomsma

M. B. Dijkstra (michielbendert.dijkstra@unil.ch) and J. J. Boomsma, Centre for Social Evolution, Dept of Biology, Universitetsparken 15, DK–2100 Copenhagen, Denmark. MBD also at: Dept of Ecology and Evolution, Quartier Sorge, "Le Biophore", Univ. of Lausanne, CH–1015 Lausanne, Switzerland.

The fungal cultivars of fungus-growing ants are vertically transmitted by queens but not males. Selection would therefore favor cultivars that bias the ants' sex ratio towards gynes, beyond the gyne bias that is optimal for workers and queens. We measured sex allocation in 190 colonies of six sympatric fungus-growing ant species. As predicted from relatedness, female bias was greater in four singly mated *Sericomyrmex* and *Trachymyrmex* species than in two multiply mated *Acromyrmex* species. Colonies tended to raise mainly a single sex, which could be partly explained by variation in queen number, colony fecundity, and fungal garden volume for *Acromyrmex* and *Sericomyrmex*, but not for *Trachymyrmex*. Year of collection, worker number and mating frequency of *Acromyrmex* queens did not affect the colony sex ratios. We used a novel sensitivity analysis to compare the population sex allocation ratios with the theoretical queen and worker optima for a range of values of k, the correction factor for sex differences in metabolic rate and fat content. The results were consistent with either worker or queen control, but never with fungal control for any realistic value of k. We conclude that the fungal symbiont does not distort the ants' sex ratio in these species.

The fungus-growing ants (Formicidae: Myrmicinae: Attini), which include the famous leafcutter ants, have an obligate mutualistic association with specialized fungi (Basidiomycota: Lepiotaceae and Pterulaceae; Mueller 2002, Munkacsi et al. 2004), which they cultivate for food in underground gardens (Weber 1972, Currie 2001, Mueller 2002). These fungi are clonally and vertically transmitted by gynes (i.e. virgin queens) that disperse on mating flights, although horizontal transmission is known to occur occasionally (Weber 1972, Adams et al. 2000, Bot et al. 2001, Green et al. 2002, Mueller 2002, Fernández-Marín et al. 2003, Mikheyev et al. 2006, 2007).

Because the fungus is commonly transmitted by gynes but never by males, natural selection would favor fungal strains that increase the bias towards gynes in the ants' sex allocation ratio. For example, the fungus might secrete toxins to selectively kill developing males (Mueller 2002, Mehdiabadi et al. 2006), analogous to the 'male-killing' phenotypes of intracellular bacteria that distort the sex ratio of their arthropod hosts (Werren 1997, Bordenstein and Reznikoff 2005, Tinsley and Majerus 2006). The fungus might also produce agonists of Juvenile Hormone to stimulate the development of female larvae into gynes (cf. de Menten et al. 2005), or neuroactive chemicals that act on nurse workers to stimulate them to raise gynes (cf. 'parasitic manipulation': Thomas et al. 2005, Biron et al. 2006, Yanoviak et al. 2008). The potential (Ratnieks et al. 2006) ant-fungus conflict over the sex ratio is tightly linked to the

well-understood potential queen-worker conflict over sex allocation in eusocial Hymenoptera (wasps, ants and bees) (Bourke 2005). The latter conflict is a consequence of the hymenopteran sex determination system, in which fertilized eggs develop into diploid females and unfertilized eggs into haploid males. It has been shown that the expression of this queen-worker conflict can lead to a two-way evolutionary tug-of-war over the population sex allocation ratio (Boomsma et al. 1995, Bourke 2005, Trivers and Hare 1976, Nonacs 1986, Boomsma and Grafen 1991, Pamilo 1991, Chapuisat and Keller 1999, Mehdiabadi et al. 2003, Bourke 2005, Ratnieks et al. 2006). The stable equilibrium population sex allocation ratio is predicted to be gyne-biased (at the 'worker optimum') when workers have exclusive control over the colony sex ratio, near parity (at the 'queen optimum') under queen control, and intermediate between the queen and worker optima under joint queen and worker control (Pamilo 1991, Chapuisat and Keller 1999, Bourke 2005). The species-specific values of the queen and worker optimum can be exactly predicted from the average kin structure of colonies and the genetic population structure (Pamilo 1991). Many species have been shown to have worker control (reviewed by Chapuisat and Keller 1999, Mehdiabadi et al. 2003, Bourke 2005), but queen control has also been well documented in some species (Helms 1999, Passera et al. 2001, Passera et al. 2001, Cremer and Heinze 2002, Jemielity and Keller 2003, Duchateau et al. 2004, Strätz and Heinze 2004,

de Menten et al. 2005, Rosset and Chapuisat 2006). By extension, the population sex allocation ratio of fungus-growing ants might be subject to a unique three-way evolutionary tug-of-war between the fungus, workers, and queens, but this requires that all three parties have at some power (Beekman and Ratnieks 2003, Beekman et al. 2003) over the colony sex ratio (Mueller 2002).

In a recent experimental study, Mehdiabadi et al. (2006) found no evidence for a cryptic lethal effect on adult males of the fungal cultivars of *Cyphomyrmex* fungus-growing ants. However, this negative result is inconclusive as it is unlikely that the fungus would ever benefit from killing males after the pupal stage, because ant males steadily become lighter and consume little food after eclosion (Boomsma and Isaaks 1985, Dijkstra and Boomsma 2007). Two comparative studies (Mueller 2002, Ichinose et al. 2007) measured sex allocation in a total of 23 fungus-growing ant species. Both studies tentatively concluded that there was no evidence for sex ratio distortion by the fungus, but lacked the statistical power to distinguish between queen and worker control.

The aim of the present study was to perform a large-scale comparative test of the relative power of the fungus, workers, and queens over the colony sex ratio in six sympatric *Trachymyrmex*, *Sericomyrmex* and *Acromyrmex* species from Panama. These three genera form (together with the genus *Atta*) the monophyletic clade of the so-called 'higher' fungus-growing ants, which have a longer and more exclusive history of coevolution with their fungal symbionts (the monophyletic 'G1' clade of Lepiotaceae) than the 'lower' fungus-growing ants (Chapela 1994, Currie 2001, Mueller 2002, Currie et al. 2003, Munkacsi et al. 2004). We measured the numerical sex ratio in a total of 190 unmanipulated reproductive field colonies, estimated the gyne- to-male cost ratio, the operational population sex allocation ratio, and the predicted worker and queen optima with standard methods (Boomsma 1989, Pamilo 1991), and applied an extended and improved version of the logistic regression method proposed by Boomsma and Nachman (2002) to infer the likelihoods of fungal, worker and queen control. Our comparative approach is powerful because of unusually pronounced differences in the worker optimum between the species in our data set, due to an abrupt evolutionary transition from singly mated queens in *Trachymyrmex* and *Sericomyrmex* to obligately multiply mated queens in *Acromyrmex* (Villesen et al. 2002).

Material and methods

Study area and species

We collected complete colonies from the following fungus-growing ant species in the vicinity of Gamboa, Panama: 1. *Trachymyrmex cornetzi* (Fernández-Marín et al. 2004; called 'T. cornetzi sp. 1' in Villesen and Boomsma 2003, Dijkstra and Boomsma 2003); 2. *Trachymyrmex* cf. *zeteki* (Villesen et al. 2002, Villesen and Boomsma 2003, Fernández-Marín et al. 2004); 3. *Trachymyrmex* sp. 3 (called 'T. zeteki sp. 3' in Fernández-Marín et al. 2004); 4. *Sericomyrmex* cf. *amabilis* (Villesen and Boomsma 2002, 2003); 5. *Acromyrmex echinatior* (Sumner et al. 2004, Dijkstra et al. 2005);

and 6. *Acromyrmex octospinosus* (Fernández-Marín et al. 2003, Ortius-Lechner et al. 2003, Dijkstra et al. 2005). Like all 'higher' fungus-growing ants, these species exclusively cultivate 'G1' fungi, which provide their ant partners with food in the form of unique, morphologically derived hyphae called gongylidia (Schultz and Meier 1995, Wetterer et al. 1998, Mueller 2002). We deposited voucher specimens of each species in the Zoological Museum of the Univ. of Copenhagen.

We collected *Acromyrmex* colonies in 1998 and 2000–2004 (always in May–June) and *Trachymyrmex* and *Sericomyrmex* colonies in 2000–2004 and 2007 (in March–July in 2002, and May–June in other years). We exclusively collected *T.* cf. *zeteki* from the banks of a single creek in primary rainforest (Quebrada Juan Grande, 2 km from the entrance of Pipeline Road, 7 km northnortheast from Gamboa), and all colonies from the other five species from road verges, grassy slopes and secondary forest patches in Gamboa village. The study area has an approximately constant temperature throughout the year, with a pronounced rainy season from the end of April through November/December. All six species produce dispersing sexuals at the beginning of the rainy season, but mating flights are more synchronized between colonies in *Acromyrmex* than in *Trachymyrmex* and *Sericomyrmex* (Dijkstra unpubl.). Except for colonies that produce only one sex, colonies simultaneously produce virgin queens and males. Newly mated queens of all six species found new colonies independently and non-claustrally (i.e. not accompanied by worker sisters, and foraging until their first worker daughters eclose), either alone (always in *T. cornetzi*) or sometimes with 1–2 other queens (Fernández-Marín et al. 2003, 2004). In *A. echinatior*, multiple queens in the same colony are related (Dijkstra and Boomsma unpubl.) and share in the production of workers and sexuals (Bekkevold et al. 1999, Dijkstra and Boomsma 2007), but it is not known whether this is also the case in the other species. Queens are always singly mated in *T. cornetzi*, *T.* cf. *zeteki* and *S.* cf. *amabilis* (Villesen et al. 2002), and we assumed that they are likewise singly mated in *T.* sp. 3, because mating systems of congener species with similar life-style tend to be the same (Boomsma and Ratnieks 1996, Boomsma et al. 2009). In contrast, queens are always highly multiply mated in *Acromyrmex* (mean $\pm$ SE of effective mating frequency: 5.3 ± 0.6 in *A. echinatior*: Sumner et al. 2004; 6.1 ± 0.8 in *A. octospinosus*: Ortius-Lechner et al. 2003). Workers of all six species can produce sons after queen loss (Villesen and Boomsma 2003, Dijkstra and Boomsma 2007), but worker sons are rarely produced in the presence of the queen (0–5% of males, depending on the species: Villesen and Boomsma 2003, Dijkstra and Boomsma 2007, Dijkstra et al. unpubl.). The caste fate (worker or queen) of female fungus-growing ants appears to be determined both by nutritional status during late larval development (Dijkstra and Boomsma 2007) and genetic factors (Hughes et al. 2003, Hughes and Boomsma 2008). Both *Acromyrmex* species have two worker castes (large and small workers) with 2500–15 000 workers per mature colony, while the three *Trachymyrmex* species and *S.* cf. *amabilis* have a single worker caste with approximately 25–300 and 100–1000 workers per mature colony, respectively (Villesen et al. 2002, 2003, Dijkstra and Boomsma 2007).

Field collections

We located colonies by following returning foragers and by searching for mud entrance tubes (*T.* cf. *zeteki* only) or sand heaps near nest entrances. We dug a hole next to the entrance, opened each nest chamber sideways, and collected the fungus garden(s) with spoons of various sizes. We scored the number of fungus gardens, and collected (by hand or with an aspirator) all mother queens and sexuals, and most of the adult workers and worker brood. We transported the colonies indoors, where we counted mother queens, which we immediately returned to their colony, and gynes and males (adults and pupae), which we preserved in 95% ethanol. For *T. cornetzi* in 2002, we also counted the number of workers, but it was not feasible to do this for the other species. If no queen was found, we normally assumed that a queen had been present in the colony but had been missed during the excavation. In rare cases we scored a colony as 'orphaned' when it only contained darkly pigmented (old) workers and no female brood. We neither removed nor counted sexual larvae, but installed the colonies in the laboratory, where we maintained them for at least one week (frequently much longer, after transporting them to Copenhagen for research), thus allowing sexual larvae to pupate and be included in the counts. However, we excluded all sexuals that pupated later than June in the year of collection, as these likely represented different sexual cohorts. Preserved sexuals were stored at −20°C after transport to Copenhagen. We approximated the volume (in liters) of each *Acromyrmex* fungus garden with the formula $\frac{4}{3}\pi hlw$, where h, l and w are its maximum height, length and width respectively (in dm; estimated to the nearest 0.25 dm). If an *Acromyrmex* colony had multiple fungus gardens, we added the estimated volumes of all fungus gardens and rounded off to the nearest liter.

Expected worker optimum (Ŵ) and queen optimum (Q̂) for sex allocation

We calculated the predicted worker optimum for the population sex allocation ratio (Ŵ; expressed as the proportion of energy invested in gynes relative to the energy invested in gynes plus males) as:

$$\hat{W} = \frac{\bar{g} + 2}{2\bar{g} + 2} \tag{1}$$

in which $\bar{g}$ is the average effective mating frequency of queens. The predicted queen optimum for the population sex allocation ratio (Q̂; as above) is:

$$\hat{Q} = 0.50 \tag{2}$$

Equation 1–2 were derived by Pamilo 1991 for the simplest colony and population structures, i.e. random mating, one queen per colony (or multiple unrelated queens with equal effective mating frequencies, and which share equally in reproduction), no worker reproduction in the presence of the queen or in orphaned colonies, and no local mate competition, local resource competition, or local resource enhancement (Forsyth 1981, Taylor 1981, Pamilo 1991, Chapuisat and Keller 1999). These assumptions are all

likely to be valid for the species in the present study (Appendix 1). We calculated the expected variances (i.e. uncertainties for the estimates) for Ŵ and Q̂ as:

$$SD^2(\hat{W}) = SE^2(\bar{g}) \frac{4}{(2\bar{g} + 2)^4} \tag{3}$$

$$SD^2(\hat{Q}) = 0 \tag{4}$$

Derivations for Eq. 3 and subsequent equations are given in Appendix 2.

The cost ratio (c) and the metabolic parameter (k)

To convert counts of sexuals into sex allocation ratios, it is necessary to estimate the species-specific cost ratio of producing and maintaining an average adult gyne versus an average adult male until maturity (hereafter: 'cost ratio' c). We first sampled mature adult gynes and males (previously stored in 95% ethanol) from field and laboratory colonies, let them dry for 72 h at 56°C, and weighed them with 0.1 mg precision. The sample sizes were 18 ± 4 (mean $\pm$ SD) individuals per sex for each of 6 ± 2 (mean $\pm$ SD) colonies of *Trachymyrmex* and *Sericomyrmex*, and ten individuals per sex for each of three colonies of both *Acromyrmex* species. We then approximated c as (Boomsma 1989, Boomsma et al. 1995, Bourke 2005):

$$c_k = \left(\frac{\bar{f}}{\bar{m}}\right)^k \tag{5}$$

And its expected variance as:

$$SD^2(c_k) \approx SE^2(\bar{f}) \frac{k^2 \bar{f}^{2(k-1)}}{\bar{m}^{2k}} + SE^2(\bar{m}) \frac{k^2 \bar{f}^{2k}}{\bar{m}^{2(k+1)}} \tag{6}$$

In which $\bar{f}$ and $\bar{m}$ are the average dry masses of adult gynes and males, respectively, and k is the 'metabolic parameter', which is normally set at 0.7 to give an approximate overall correction for the lower metabolic rate and higher fat content of gynes (Boomsma 1989, Boomsma et al. 1995). Sexuals of fungus-growing ants undergo similar mass changes during development as other ants (Dijkstra and Boomsma 2007), justifying the use of this method for approximating cost ratios.

Estimating the population numerical sex ratio (N)

We excluded one colony from further analyses if at least one of the following applied: 1) it did not contain any sexuals; 2) we were unsure whether all fungus gardens had been excavated; 3) workers and/or sexuals of the inquiline social parasite *Acromyrmex insinuator* were present (exclusively in *A. echinatior*: Sumner et al. 2004); 4) it had been scored as 'orphaned'. Following the method by Boomsma and Nachman (2002), we performed maximum likelihood logistic regression on the colony numerical sex ratios (i.e. the number of gyne pupae plus the number adult gynes, divided by the total number of sexual adults and pupae), weighting the contribution of each colony to the regression by the number of sexuals that it contained. The purpose of this analysis was twofold: to identify the colony traits that were significantly associated with a gyne-biased versus a

male-biased numerical sex ratio, and to estimate the operational population numerical sex ratio (N; expressed as the simple proportion of gynes among all the counted sexuals) as the weighted average of the colony numerical sex ratios. In all analyses, we ignored the occasional and negligible contribution of workers to the male brood and assumed that all males were queen sons (Appendix 1). Colony sex ratios of social Hymenoptera are frequently overdispersed (i.e. each gyne that is being raised raising the probability that the next sexual in the colony will likewise be a gyne rather than a male), for example because colonies or individual nurse workers within colonies tend to specialize in raising a single sex (Boomsma and Nachman 2002). We corrected for overdispersion by dividing all F-values by an empirical scaling parameter equal to the ratio of the deviance and the residual degrees of freedom (Wilson and Hardy 2002). Depending on the species, the explanatory variables of the full model included some or all of the factors and covariates 'year of collection', 'number of fungus gardens', 'total fungus garden volume' (in l), 'number of mother queens', 'number of workers' and 'sexual production', as well as appropriate interaction terms (Table 1). The 'sexual production' of a colony was calculated by adding the number of males to the product of the number of gynes and c, assuming that $k = 0.7$ (above). We transformed each covariate by subtracting the mean value, to simplify estimating N in case of significant effects. We sequentially identified the least significant term with a type II F-test and deleted it, until we obtained the minimum adequate model. Because we found strong between-genus differences in the average sexual production, while our logistic regression method weights the contribution of colonies by the number of sexuals, we were obliged to perform a separate analysis for each of the three genera. In the analyses on *Trachymyrmex* (including three species) and *Acromyrmex* (two species), we also tested for the effect of the factor 'species' (Table 1). We performed an additional, separate analysis on a subset of *T. cornetzi* colonies (exclusively from 2002; above) for which worker counts were available, including the covariate 'number of workers' but excluding 'number of fungus gardens' because none of the *T. cornetzi* colonies had more than one garden (Table 1).

We calculated cumulative pseudo-r^2 values for each regression step with the formula (Boomsma and Nachman 2002):

$$r^2_{pseudo} = 1 - \frac{D_a}{D_0} \qquad (7)$$

in which D_a is the residual deviance in step a and D_0 the residual deviance of the null model. We estimated N from the intercept (all covariates set at the average value; above) of the minimum adequate model.

Estimating the population sex allocation ratio (I)

We estimated the operational population sex allocation ratio (I) – i.e. the proportion of energy invested in gynes relative to the total energy invested in sexuals – with the

Table 1. GLM tables for the colony numerical sex ratio.

	Correlation with gyne bias	Deviance	Cumulative r^2_{pseudo}	DF	Residual DF	Scaled F	p
Trachymyrmex (3 species):							
Intercept		2568.4	0	1	101	–	–
Species		2098.1	0.18	2	99	11.10	**<0.0001**
Year of collection		1918.0	0.25	5	94	1.77	0.128
Sexual production	positive	1873.6	0.27	1	93	2.20	0.141
Species × Sexual production		1826.9	0.29	2	91	1.16	0.317
Number of fungus gardens	negative	1801.3	0.30	1	83	1.18	0.280
Species × Number of fungus gardens		1800.1	0.30	1	82	0.05	0.818
Trachymyrmex cornetzi							
Intercept		897.0	0	1	41	—	—
Number of workers	negative	862.5	0.04	1	40	1.60	0.213
Sexual production	positive	842.4	0.06	1	39	0.93	0.340
Number of workers × Sexual production		840.1	0.06	1	38	0.10	0.748
Sericomyrmex cf. *amabilis*:							
Intercept		181.0	0	1	8	–	–
Number of fungus gardens	negative			1		23.61	**0.003**
		34.4	0.81		6		
Sexual production	positive			1		25.36	<0.001
Number of mother queens	negative	31.0	0.83	1	5	0.55	0.800
Year of collection		28.6	0.84	2	3	0.12	0.888
Acromyrmex (2 species)							
Intercept		8065.5	0	–	78	–	–
Number of mother queens	positive	7562.1	0.06	1	77	5.13	**0.026**
Species		7312.6	0.09	1	76	2.59	0.112
Year of collection		6654.9	0.17	5	71	1.40	0.234
Sexual production	positive	6522.4	0.19	1	70	1.42	0.237
Species × Sexual production		6402.7	0.21	1	69	1.29	0.260
Number of fungus gardens	positive	6308.0	0.22	1	68	1.02	0.316
Species × Number of fungus gardens		6156.8	0.24	1	67	1.65	0.204
Number of mother queens × Species		6120.6	0.24	1	66	0.39	0.534
Total fungus garden volume	zero	6093.8	0.24	1	65	0.29	0.594
Species × Total fungus garden volume		7233.7	0.24	1	73	1.51	0.223

following equation:

$$I_k = \frac{c_k N}{(c_k - 1)N + 1} \qquad (8)$$

And the corresponding expected variance as:

$$SD^2(I_k) = \left(SD^2(c_k)\left(\frac{1}{c_k^4}\right)\left(1 - \frac{1}{N}\right)^2 \right.$$
$$\left. + SD^2(N)\left(\frac{1}{c_k^2 N^4}\right) \right) I_k^4 \qquad (9)$$

In which N is the operational population numerical sex ratio estimated from the minimum adequate model (plus the factor 'species' for *Acromyrmex*; below), assuming that the metabolic parameter k = 0.7. We then used z-tests to compare I to $\hat{W}$ and $\hat{Q}$ (see Appendix 2 for details).

Between-colony variation in queen mating frequency

Part of the within-species variation in colony numerical sex ratio might be explained by variation in effective queen mating frequency for the highly multiply mated *Acromyrmex* species, but not for the obligately singly mated *Trachymyrmex* and *Sericomyrmex* species. *Acromyrmex* workers would benefit from raising exclusively gynes or males, depending on whether the mating frequency of their mother queen is lower or higher than the population average, respectively ('split sex ratios'; Boomsma and Grafen 1991, Sundström 1994, reviewed by Meunier et al. 2008). We therefore performed an additional analysis on eight reproductive *A. echinatior* field colonies with a single queen (part of the larger dataset used above) for which complete patriline data were available from previous studies (Sumner et al. 2004, Hughes and Boomsma 2008). Analogously to the previous analyses, we performed a weighted logistic regression on the colony numerical sex ratio with the covariate 'effective queen mating frequency' as the sole explanatory variable. We also derived an algorithm (Eq. 10–12) to estimate the fitness gain $\bar{R}$ (in %) of hypothetical workers that would perfectly adapt the sex ratio in their colony to the mating frequency of their mother queen, relative to the fitness they would have if the colony sex ratio was equal to the population sex ratio:

$$\bar{R} = -100 + \frac{100}{n} \sum_i^n \frac{\lambda_i}{I(A_i - 1) + 1} \qquad (10)$$

$$A_i = \frac{(1 - N)(g_i + 2)}{cNg_i} \qquad (11)$$

$$\lambda_i = \begin{cases} A_i & \text{for } g_i \leq \bar{g} \\ 1 & \text{for } g_i > \bar{g} \end{cases} \qquad (12)$$

In which λ_i is the inclusive fitness of workers per mg sexuals under perfectly split sex ratios, A_i the ratio of inclusive fitness pay-offs to workers from raising 1 mg of adult gynes versus 1 mg of adult males, c the cost ratio (estimated for k = 0.7), n the number of colonies in the sample, N the operational population numerical sex ratio, I the operational population sex allocation ratio, $\bar{g}$ the average effective queen mating frequency, and g_i the effective mating frequency of the queen in colony i. We applied this algorithm to 14 *A. echinatior*

colonies for which the effective mating frequency was known (the eight reproductive field colonies above plus six non-reproductive laboratory colonies; Sumner et al. 2004, Hughes and Boomsma unpubl.). Equations 10–12 over-estimate the potential benefit of split sex ratios to workers because it makes three unrealistic assumptions: 1) workers can perfectly assess the mating frequency of their mother queen relative to the population mean; 2) there are no other constraints on the sex ratios that can be achieved; and 3) sex ratio biasing does not carry any costs. Equations 10–12 thus yield the maximum inclusive fitness increment that could be gained from the most precise sex ratio adjustment possible.

Additional information on statistical analyses

We performed logistic regressions in SAS 9.1.3 for Windows XP Professional, and all other statistical analyses in SPSS 11 for Macintosh OS X. Unless specified otherwise, p-values are two-tailed and based on type III analyses, and results are given as means $\pm$ SE.

Supplementary material

Appendix 1 validates the assumptions behind the calculations for $\hat{Q}$ and $\hat{W}$, Appendix 2 contains derivations for the equations used in this paper, and the Appendix 3 contains the raw data for all 190 reproductive colonies. Appendix 3 can be accessed online at < www.oikos.ekol.lu.se/appendix > as Appendix O16822.

Results

Table 2 summarizes the numbers and masses of sexuals, various measures of colony size (number of workers or fungus gardens, fungus volume), and the numbers of queens in the six species. Sexual size dimorphism was smallest in *Acromyrmex*, intermediate in *Trachymyrmex*, and greatest in *Sericomyrmex*. Colony composition was least variable in *T. cornetzi*, which always had a single queen and a single fungus garden. The number of fungus gardens per colony varied in the other species, and a second queen was present in some colonies of *S.* cf. *amabilis* (11% of reproductive colonies), *A. echinatior* (11%), and *A. octospinosus* (3%). The number of fungus gardens per reproductive colony did not differ between the two *Acromyrmex* species (Kolmogorov–Smirnov z = 0.679, p = 0.746), but the total fungus garden volume was significantly higher for *A. octospinosus* than for *A. echinatior* (Mann–Whitney U test, p < 0.001). The number of fungus gardens did not differ between *T.* cf. *zeteki*, *T.* sp. 3, and *S.* cf. *amabilis* (Kruskal–Wallis, χ^2 = 4.4, DF = 2, p = 0.112).

Larger colonies produce more sexuals

As expected, 'sexual production' increased significantly with measures of colony size for all species in the present study. In *T. cornetzi*, there was a significant and positive relationship between 'sexual production' and 'number of workers' (linear regression after [10]log-transforming both variables; r^2 =

Table 2. Summary statistics[1] for the six study species.

	T. cornetzi	T. cf. zeteki	T. sp. 3	S. cf. amabilis	A. echinatior	A. octospinosus
Colonies collected	62	25	15	9	45	34
Total gynes counted	1449	848	150	981	8059	4940
Total males counted	1578	821	896	3048	7168	7159
Queens per colony	1	1	1	1.11±0.11 (1–2)	1.11±0.04 (1–2)	1.03±0.03 (1–2)
Sexual production per colony (male equivalents)	84±9 (1–259)	114±18 (7–338)	90±18 (2–252)	734±516 (1–4816)	421±72 (1–1845)	515±109 (1–2308)
Adult gyne mass (mg)	1.1±0.01	2.4±0.1	1.5±0.1	1.9±0.1	12.6±0.4	20.7±0.3
Adult male mass (mg)	0.3±0.02	0.7±0.02	0.4±0.02	0.3±0.03	7.4±0.1	7.8±0.1
Cost ratio $c_{k=0.7}$	2.6±0.1	2.4±0.1	2.6±0.2	3.6±0.3	1.5±0.04	2.0±0.03
Fungus gardens per colony	1	2.8±0.3 (1–5)	1.7±0.3 (1–7)	5.4±1.4 (1–14)	1.8±0.2 (1–5)	1.5±0.2 (1–5)
Fungus per colony (l)					1.7±0.3 (0.1–12)	4.0±0.3 (2–8)
Workers per colony	96±8 (25–225)					

[1]Given as mean±SE (range).

0.153; intercept: $-0.69±0.85$, $t=0.81$, $p=0.421$; slope: $1.18±0.44$, $t=2.69$, $p=0.010$). 'Sexual production' increased significantly with increasing 'total fungus garden volume' (in l) in both A. echinatior (as above; $r^2=0.33$; intercept: $2.257±0.08$, $t=27.5$, $p<0.001$; slope: $0.84±0.18$, $t=4.62$, $p<0.001$), and A. octospinosus ($r^2=0.39$; intercept: $0.95±0.33$, $t=-3.22$, $p=0.008$; slope: $2.44±0.56$, $t=4.41$, $p<0.001$). The vertical intercept was significantly higher for A. echinatior than for A. octospinosus (z-test, $z=36.5$, $p<0.001$), indicating that A. octospinosus colonies need to attain a higher fungus garden volume than A. echinatior colonies before they can reproduce, a difference that persists in the laboratory (Dijkstra and Boomsma 2007). The average 'sexual production' did not differ between A. echinatior and A. octospinosus (Mann–Whitney U test, $p=0.716$), with similar unimodal distributions across colonies in both species (data not shown). In T. cf. zeteki, T. sp. 3, and S. cf. amabilis, 'sexual production' increased significantly with increasing 'number of fungus gardens' (linear regression after [10]log-transforming both variables; $r^2=0.21$; slope: $1.19±0.41$, $F_{1,36}=8.56$, $p=0.006$). The slope of this increase was similar for the three species ($F_{2,36}=0.46$, $p=0.633$), indicating that S. cf. amabilis can occasionally achieve a much higher 'Sexual production' than T. cf. zeteki and T. sp. 3 (Table 2), but only in the largest (and presumably oldest) colonies.

Variation in colony numerical sex ratio

The distributions of the colony numerical sex ratios differed strongly between and within genera (Fig. 1), being rather even in A. octospinosus and T. cf. zeteki, but highly skewed towards all-male broods in T. sp. 3 and S. cf. amabilis, and moderately skewed towards all-gyne broods in A. echinatior. In T. cornetzi, the distribution was skewed towards both extremes, but all-male broods typically consisted of fewer (<10) sexuals than all-gyne broods (25–50 sexuals). We included the sex ratio data from eight colonies of S. cf. amabilis (collected from Gamboa and nearby Barro Colorado Island, Panama) from a previous study (Murakami et al. 2000) in Fig. 1, but we never used these additional data in any statistical analysis as the authors did not specify the number of sexuals per colony.

In the first analysis covering the three Trachymyrmex species, only the factor 'species' was significant ($r^2_{pseudo}=$

0.18, $F=11.0$, $p<0.0001$; Table 1), indicating that N (the operational population numerical sex ratio) was considerably more male-biased in T. sp. 3 than in T. cornetzi ($F_{1,95}=18.3$, $p<0.0001$) and T. cf. zeteki ($F_{1,95}=18.0$, $p<0.0001$). N did not differ between T. cornetzi and T. cf. zeteki ($F_{1,101}=0.18$, $p=0.681$). None of the other explanatory variables ('year of collection', 'sexual production', 'number of fungus gardens') explained any significant proportion of the variation in colony numerical sex ratios for Trachymyrmex (Table 1). A separate analysis on T. cornetzi, which included the additional explanatory variable 'number of workers' did not yield any significant effects either (Table 1). However, both 'number of fungus gardens' and 'sexual production' were highly significant in the analysis for S. cf. amabilis ($F_{1,6}=23.6$, $p=0.003$ and $F_{1,6}=25.4$, $p<0.001$, respectively; joint cumulative $r^2_{pseudo}=0.81$), which indicates that the colony numerical sex ratios became more male-biased with increasing numbers of fungus gardens and decreasing total investment in sexuals. Neither the 'number of mother queens' nor the 'year of collection' had any significant effect on the colony numerical sex ratio in S. cf. amabilis (Table 1).

In the analysis for Acromyrmex, the 'number of mother queens' was significant ($r^2_{pseudo}=0.06$, $p=0.026$; Table 1), indicating that colonies with two queens had more gyne-biased numerical sex ratios than colonies with one queen. The interaction 'number of mother queens' × 'species' was not significant ($F_{1,66}=1.65$, $p=0.204$), which indicates that the relationship between queen number and colony numerical sex ratio is similar in both Acromyrmex species, although colonies with two queens were more rare in A. octospinosus than in A. echinatior. The effect of 'species' was not significant for Acromyrmex ($F_{1,76}=2.59$, $p=0.112$), indicating that N was similar in A. echinatior and A. octospinosus, and none of the other tested variables were significant either (Table 1).

Two additional results suggest that split sex ratios (Boomsma and Grafen 1991) are weakly developed or absent in A. echinatior. First, there was no significant association between the effective queen mating frequency and the colony numerical sex ratio in the separate analysis for A. echinatior ($n=8$ colonies, $r^2_{pseudo}=0.032$, scaled $F_{1,6}=0.20$, $p=0.670$). Second, we estimated that the maximum inclusive fitness gain $\bar{R}$ to A. echinatior workers under perfect sex ratio splitting was only $6±1\%$ ($n=14$
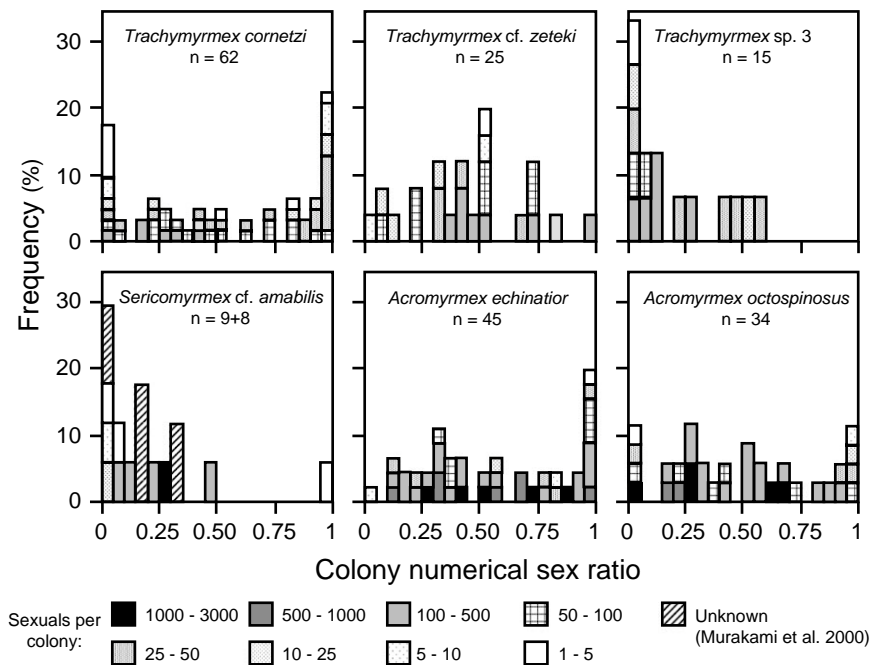
Figure 1. Histograms for the colony numerical sex ratios (expressed as the proportion of gynes) in the six study species. Different shades of grey and fill patterns indicate the number of sexuals per colony. Sample sizes (n = number of colonies) are given inside each panel. The histogram for *S.* cf. *amabilis* includes eight colonies (which contained a total of 397 sexuals) from Murakami et al. (2000), for which the number of sexuals per colony is not known. These eight additional colonies were not used in any statistical analyses and served only as an independent validation of our results.

colonies, range 3–14%). It is therefore unlikely that variation in effective queen mating frequency could ever explain more than a negligible proportion of the high remaining deviance for *A. echinatior* sex ratios (Table 1).

Testing for fungal, worker, and queen control over sex allocation

The minimum adequate model for *Acromyrmex* yielded estimates for N that were too gyne-biased for *A. octospinosus* and too male-biased for *A. echinatior* relative to the simple overall proportions of gynes among all collected sexuals (Fig. 2a). We therefore used a model that included the significant variable 'number of mother queens' and the non-significant factor 'species' to estimate N and the corresponding population sex allocation ratio I for both *Acromyrmex* species, whereas we estimated N and I for all other species from the minimum adequate models (Fig. 2a). In the following section, we assumed that the metabolic parameter k was equal to the across-species average of 0.7 (Boomsma 1989), but we relaxed this assumption in subsequent sections (below).

There was no significant difference between I and Ŵ (the worker optimum) for *T. cornetzi* (z = −1.36, p = 0.173), *T.* cf. *zeteki* (z = −0.84, p = 0.404), *A. echinatior* (z = 0.46, p = 0.648) or *A. octospinosus* (z = 0.03, p = 0.975) (Fig. 2b). I was significantly less gyne-biased than 0.75 for *A. echinatior* (z = 3.20, p = 0.001) and *A. octospinosus* (z = −3.94, p < 0.001), which was as expected because their queens are highly multiply mated (Fig. 2b). I was significantly more gyne-biased than Q̂ (the queen optimum) for *T. cornetzi* (z = 5.50, p < 0.0001), *T.*

cf. *zeteki* (z = 4.53, p < 0.0001), and *A. echinatior* (z = 2.17, p = 0.030), but not for *A. octospinosus* (z = 1.61, p = 0.109) (Fig. 2b). There was also no significant difference between I and Ŵ for either *A. echinatior* (z = −0.40, p = 0.693) or *A. octospinosus* (z = 1.63, p = 0.102) when the non-significant factor 'species' was excluded from the model (above). For *S.* cf. *amabilis*, I was significantly less gyne-biased than Ŵ (z = −6.31, p < 0.0001) and also more male-biased than Q̂ (z = −2.59, p = 0.010) (Fig. 2b). For *T.* sp. 3, I was also significantly less gyne-biased than Ŵ (z = −5.21, p < 0.0001) and more male-biased than Q̂ (z = −2.32, p = 0.020). The former test assumes that queens of *T.* sp. 3 are singly mated, but I would remain significantly less gyne-biased than Ŵ (z = −3.13, p = 0.002) in the highly improbable case that *T.* sp. 3 queens mate with as many males as *Acromyrmex* queens.

The sensitivity of I to differential choices of the metabolic parameter k (e.g. resulting from the uncertainty in estimating k across species) can be tested explicitly, provided that the variances of the estimates for the population numerical sex ratio N, the cost ratio c, and the worker optimum Ŵ can be quantified. Here we use our estimates for these variances to evaluate the robustness of our conclusions on worker versus queen control over sex allocation (Fig. 3). We found that our results for *T. cornetzi* and *T.* cf. *zeteki* were highly insensitive to the choice of k, whereas for *T.* sp. 3 and *S.* cf. *amabilis*, k would only need to increase from 0.7 to 0.8 to make I consistent with queen control (Fig. 2b). I was consistent with worker control for a wide range of k values for both *Acromyrmex* species, but only significantly more gyne-biased than for Q̂ k ≥ 0.63 (*A. echinatior*) and k ≥ 0.75 (*A. octospinosus*) (Fig. 2b). This means that *A. echinatior* is likely to have worker control,

while *A. octospinosus* might either have joint queen–worker control or worker control. The lowest value of the metabolic parameter k that would result in an estimate I that was significantly more gyne-biased than $\hat{W}$ was 1.13 for *T. cornetzi*, 1.18 for *T.* cf. *zeteki*, 1.95 for *T.* sp. 3, 2.03 for *S.* cf. *amabilis*, 1.23 for *A. echinatior*, and 1.05 for *A. octospinosus*. Because gynes attain their maximum mass as mature adults and males as pre-pupae (Dijkstra and Boomsma 2007), the simple ratio of the dry masses of adult gynes and males overestimates the degree of sexual dimorphism, so that k must be lower than 1. This implies that the estimate for the operational population sex allocation ratio was not significantly higher than the worker optimum for any realistic value of the metabolic parameter for any of the six species studied.

Discussion

Our study is the most detailed and statistically powerful comparative analysis of fungus-growing ant sex ratios published so far. Because the queen mating frequency and the proportion of workers sons (which determine $\hat{Q}$ and $\hat{W}$) were precisely known for five out of the six studied species, we were able to determine the relative likelihoods of queen, worker, and fungal control from the observed population sex ratios. We also used a novel type of sensitivity analysis to show that most results (exceptions are discussed below) are insensitive to the choice of the metabolic parameter k, which affects the estimates for the cost ratio c and the operational population sex allocation ratio I.

Variation in colony numerical sex ratio

The high residual deviances in all analyses (Table 1) imply that the probability of raising a gyne rather than a male differs significantly between colonies of the same species. Mating frequency-induced split sex ratios (Boomsma and Grafen 1991) cannot be responsible for this variation in the obligately singly mated *Sericomyrmex* and *Trachymyrmex* species, and our results indicate that variation in effective queen mating frequency cannot explain the remaining unexplained variation in *A. echinatior* colony sex ratios either. As expected, larger colonies (measured in fungus gardens, fungus volume or workers) had a greater sexual production in all six species. We found no evidence for the hypothesis that only large colonies can afford to produce gynes (Nonacs 1986, Bono and Herbers 2003), as larger colonies did not produce more gyne-biased sex ratios in any of our six study species. In *S.* cf. *amabilis*, we actually found both a negative correlation between gyne bias and fungus garden number and a positive correlation between gyne bias and sexual production (Table 1). Because there was also a positive association between fungus garden number and sexual production, this means that gyne bias was highest in medium-sized *S.* cf. *amabilis* colonies.

In the two *Acromyrmex* species, the numerical sex ratios were unexpectedly more gyne-biased in colonies with two queens than in colonies with a single queen. Because the worker-to-sister versus worker-to-brother relatedness asym-
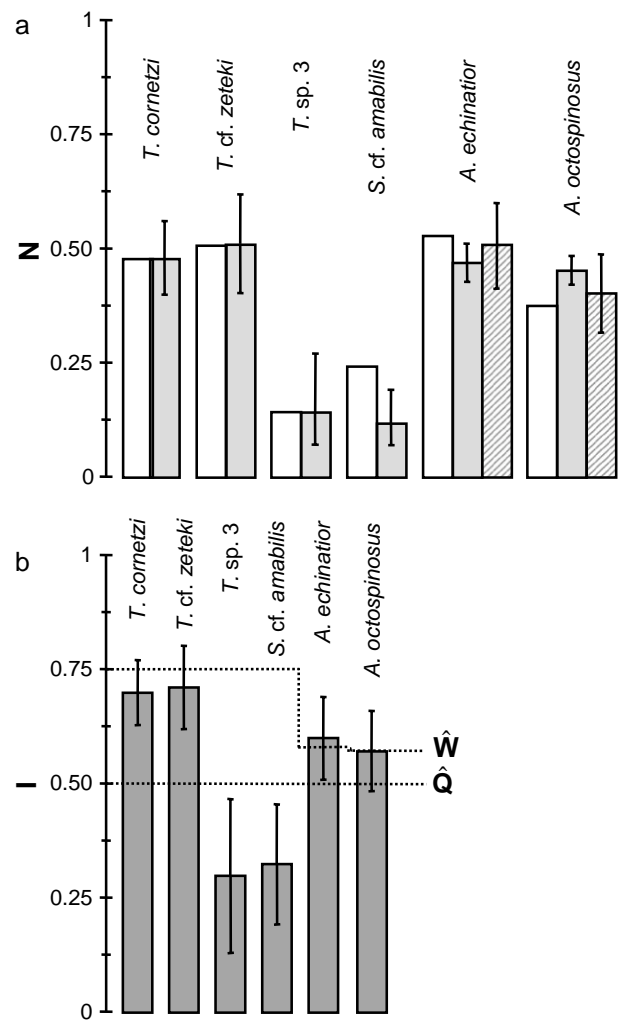

Figure 2. Estimates of the population numerical sex ratios (N; expressed as the simple numerical proportion of gynes) (Fig. 2a) and the population sex allocation ratios (I; expressed as the proportion of energy invested in gynes) (Fig. 2b). Error bars denote 95% CI. In Fig. 2a, white bars indicate the overall unweighted proportions of gynes among all collected sexuals (i.e. before doing any statistical analyses), light grey bars indicate the population numerical sex ratios estimated from the minimum adequate models, and diagonally shaded bars (*A. echinatior* and *A. octospinosus* only) indicate the population numerical sex ratios estimated from the minimum adequate models plus the non-significant factor 'species'. The population sex allocation ratios were estimated (using the standard empirical value k = 0.7 for the metabolic parameter) from the minimum adequate model plus 'species' for *A. echinatior* and *A. octospinosus*, and from the minimum adequate model for the other species. The species-specific estimates for the worker optimum $\hat{W}$ and the queen optimum $\hat{Q}$ are indicated by dotted horizontal lines in Fig. 2b.

metry is lower in colonies with multiple related queens (Material and methods) than in colonies with a single queen, colonies with two queens were expected to produce more males (Pamilo 1991). Fournier et al. (2003) and Helms et al. (2004) also reported a positive association between gyne bias and queen number in the ant *Pheidole pallidula*, and argued that this could result from competition between queens over their shares in the diploid brood.
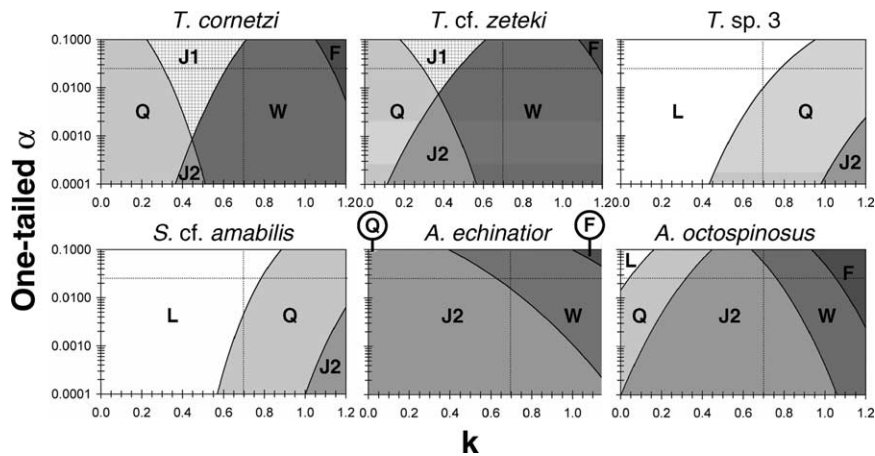
Figure 3. Sensitivity of the estimates for the population sex allocation ratio to the metabolic parameter k, which corrects for the higher metabolic rate and lower fat content of males compared to gynes. Different fill colors and patterns denote the values of k and α for which the observed population sex allocation ratio are consistent in one-tailed z-tests with: local resource competition (L), i.e. significantly more male-biased than the queen optimum; queen control (Q), i.e. not significantly different from the queen optimum but significantly more male-biased than the worker optimum; joint queen-worker control (J1 and J2), i.e. intermediate between the queen and worker optima and significantly different from both optima (J1) or not significantly different from either optimum (J2); worker control (W), i.e. significantly more gyne-biased than the queen optimum but not significantly different from the worker optimum; and partial or complete fungal control (F), i.e. significantly more gyne-biased than the worker optimum. Because only two-tailed significances were relevant for our comparisons, the critical value (indicated by horizontal dotted lines) is 0.025 rather than 0.05. Vertical dotted lines indicate the standard empirical value of the metabolic parameter k = 0.7 (see text).

The remaining variation in colony sex ratios may be due to environmentally and genetically induced differences in the physiology of larvae (e.g. variation in thresholds for developing into different female castes; Hughes and Boomsma 2008), workers (e.g. receptiveness to larval food begging: Lopes et al. 2005), and queens (e.g. in the rates at which they lay unfertilized eggs, Passera et al. 2001, Rosset and Chapuisat 2006). Our finding that I was approximately equal to Ŵ in *T. cornetzi*, *T.* cf. *zeteki*, *A. echinatior*, and *A. octospinosus* implies that worker traits that affect the colony-level sex ratio are probably selectively neutral in these species. Analogously, queen traits that affect the colony sex ratio may well be selectively neutral in *T.* sp. 3 and *S.* cf. *amabilis*, if the observed slight bias towards males could be reconciled with queen control (below).

Male-biased sex allocation: queen control or local resource competition?

Our finding that I was significantly more male-biased than Q̂ for *T.* sp. 3 and *S.* cf. *amabilis* (Fig. 2b) may have several explanations. Murakami et al. (2000) also reported a male-biased sex ratio for *S.* cf. *amabilis* (Fig. 1), indicating that our low estimate for I is not a mere artifact of the low number of collected colonies. Under worker control, a strong bias towards males is only expected if there is strong local resource competition (LRC) between colonies, due to severe constraints on female dispersal (Chapuisat and Keller 1999, Pearcy and Aron 2006). In ants, strong LRC is typically the result of 'colony budding' (i.e. colony founding by mated queens and workers that jointly disperse on foot) (Bourke and Franks 1995). However, colony budding cannot explain the observed male bias in *T.* sp. 3 and *S.* cf. *amabilis*, as Fernández-Marín et al. (2004) exclusively observed independent colony founding in these two species

at the same site in Gamboa. Weak LRC, such as might result from gynes having a shorter flight range than males, could explain the observed patterns when there is queen control over sex allocation in *T.* sp. 3 and *S.* cf. *amabilis*. However, an increase in k from 0.7 to 0.8 would be sufficient to make I consistent with queen control without LRC for these two species, and would hardly affect the relative likelihoods of worker versus fungal control in the other four species (Fig. 3). Because the standard value of k = 0.7 is itself an estimate based on comparative data and thus has its own uncertainty (Boomsma and Isaaks 1985, Boomsma 1989, Boomsma et al. 1995), we can at present only conclude that the sex ratios of *T.* sp. 3 and *S.* cf. *amabilis* are not consistent with worker control, unlike those of their sympatric relatives *T. cornetzi* and *T.* cf. *zeteki*. Similar pronounced differences in the relative power of workers and queens over the sex allocation may even exist between populations of the same species of fungus-growing ant, as *Trachymyrmex septentrionalis* is reported to have male- and gyne-biased sex allocation ratios in the northern and the southern USA, respectively (Table 4 in Mueller 2002). We conclude that the outcome of the queen-worker conflict over sex allocation is evolutionarily labile in fungus-growing ants, as there can be pronounced differences in I between related and ecologically similar species.

Possible mechanisms for optimizing sex allocation

Our finding that sex allocation is apparently controlled by workers in *T. cornetzi* sp. 1, *T.* cf. *zeteki*, *A. echinatior* and *A. octospinosus*, and possibly by queens in *T.* sp. 3 and *S.* cf. *amabilis*, raises the question how workers and queens can optimize the sex allocation in their colony. Workers of fungus-growing ants are known to police worker-laid eggs (Dijkstra 2005) and cannibalize male larvae (Goetsch 1939,

Dijkstra and Boomsma 2007), two mechanisms that can help to increase gyne bias (Chapuisat et al. 1997, Foster and Ratnieks 2001, Beekman and Ratnieks 2003). Additionally, workers can almost certainly optimize gyne bias by adjusting the feeding rate of larvae to control the proportion of females that develop into workers (Chapuisat et al. 1997, Hammond et al. 2002, Beekman and Ratnieks 2003, Dijkstra and Boomsma 2007, Hughes and Boomsma 2008). Queens of fungus-growing ants may even be able to reduce gyne bias by producing a gyne-inhibiting primer pheromone (Dijkstra and Boomsma 2007), and might also reduce gyne bias by laying an excess of unfertilized eggs during the reproductive season (Passera et al. 2001, de Menten et al. 2005, Rosset and Chapuisat 2006). However, it is unlikely that queens could optimize gyne bias by laying specialized worker- or gyne-destined eggs, as the caste fate of females appears to be determined late in development (Dijkstra and Boomsma 2007).

Why does the fungal symbiont lack power over the ants' sex allocation?

Our result indicate worker control and no queen control over the sex ratio in *T. cornetzi*, *T.* cf. *zeteki* and *A. echinatior*, worker control or joint worker-queen control in *A. octospinosus*, and queen control (possibly with some LRC) in *S.* cf. *amabilis* and *T.* sp. 3. None of our estimates for I were consistent with fungal control for any realistic choice of k. The most parsimonious explanation is, therefore, that the fungus does not affect the sex allocation in any of the six species. We cannot rule out that the fungus does have a biasing effect, but that workers fully compensate for this by raising more males, but this would still imply that the fungus has no effective power over the sex ratio of their ant partners. Our results are thus in agreement with earlier studies (Mueller 2002, Mehdiabadi et al. 2006, Ichinose et al. 2007), which also indicated (with varying levels of confidence) that the fungal symbiont does not bias the sex allocation of fungus-growing ants.

Now that three specific tests (Mueller 2002, Mehdiabadi et al. 2006, this study) have concluded that the cultivars of fungus-growing ants lack the power to distort the ants' sex ratio, it is legitimate to speculate on the reasons why this might be the case. The first reason might be that the fungus is an ectosymbiont, whereas all evidence for sex ratio distortion that has accumulated over the last two decades comes from intracellular bacterial endosymbionts (Werren 1997, Bordenstein and Reznikoff 2005, Tinsley and Majerus 2006). This obviously gives the host possibilities to protect their brood from being manipulated. Although the eggs and larvae of fungus-growing ants lie embedded in the fungus gardens (Weber 1972, Dijkstra et al. 2005, Mehdiabadi et al. 2006) and the larvae are fully dependent on the fungus for food, it is the ant workers that actually do the feeding and nursing. The workers are thus in an excellent position to remove any manipulating fungal secretions or to compensate their effects. Second, the fungus occurs as a single clone per colony (Poulsen and Boomsma 2005) and grows in a modular fashion, so that any sex ratio distorting mutants need to arise as somatic mutations.

It may be relatively easy for workers to detect and discard these mutant mycelia, in the same way as they remove incipient fruiting bodies, alien cultivar clones, and fungus garden fragments that are infected with pathogens (Bot et al. 2001, Currie 2001, Currie and Stuart 2001, Mueller 2002). Third, even when the fungus could produce agonists of ant hormones to induce the development of female larvae into gynes, workers might be able to compensate this by underfeeding the affected larvae and force them back onto the worker developmental pathway. The behavioral flexibility of hosts of ectosymbionts, relative to endosymbionts, may thus provide hosts with a 'power of veto', to which any power of the fungal symbiont must always yield.

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Appendix 1

Validation of assumptions about colony and population structure.

Assumption	Effect when assumption is not valid	Validation	References
Only one queen per reproductive colony	LRC or LRE are possible when colonies contain multiple related queens (see below). Different maternal lineages in the same colony may have different values for $\hat{W}^1$, depending on queen mating frequency	Reproductive colonies with multiple queens were not observed in *T. cornetzi*, *T.* cf. *zeteki*, and *T.* sp. 3 and were rare in *S.* cf. *amabilis, A. echinatior, A. octospinosus*	This study
No local resource competition (LRC)	Eq. 1–2 overestimate $\hat{W}^1$ and $\hat{Q}^2$	Colonies of the six study species have been exclusively observed to be founded independently[3] by newly mated queens	Fernandez-Marin et al. 2003, 2004
No local resource enhancement (LRE)	Eq. 1–2 underestimate $\hat{W}^1$ and $\hat{Q}^2$	Reproductive colonies with multiple queens were only (occasionally) observed in *S.* cf. *amabilis, A. echinatior, A. octospinosus*, and these did not have a greater sexual production than colonies with a single queen	This study
No local mate competition (LMC)	Eq. 1–2 underestimate $\hat{W}^1$ and $\hat{Q}^2$	Sexuals of all six species mate in mating swarms in the field, and brothers and sisters have never been observed to mate in laboratory colonies	Dijkstra and Boomsma unpubl.
No worker reproduction in queenright[4] colonies	Eq. 1 overestimates $\hat{W}^1$ in singly mated species and underestimates $\hat{W}$ in multiply mated species. Eq. 2 underestimates $\hat{Q}^2$ in singly and multiply mated species	Microsatellite data indicate only queen sons in queenright[4] colonies of *T. cornetzi, T.* cf. *zeteki,* and *S.* cf. *amabilis,* and <5% worker sons in queenright[4] colonies of *A. echinatior*. Ovary dissections indicate no worker laying in queenright[4] colonies of *A. octospinosus* and *T.* sp. 3	Villesen and Boomsma 2003, Dijkstra et al. 2005, Dijkstra unpubl., Dijkstra et al. unpubl.
No worker reproduction in orphaned colonies	Eq. 1 and 4 underestimate $\hat{W}^1$ and $\hat{Q}^2$	Orphaned field colonies are probably short-lived in all six study species. Laboratory colonies of *A. echinatior* and *A. octospinosus* produce approximately equal numbers of gynes and males in first six months after queen loss	This study, Dijkstra and Boomsma 2007

[1]The worker optimum for the population sex allocation ratio. [2]The queen optimum for the population sex allocation ratio. [3]Colony budding or fission has never been observed in these six species. [4]i.e. colonies containing a mother queen.

Appendix 2

Equations

The symbols used in this study are (in alphabetical order):

A_i	Ratio of inclusive fitness pay-offs to workers of from raising 1 mg of gynes vs 1 mg of males
c	Cost ratio of raising an average gyne versus an average male to maturity
$\bar{f}$	Average dry mass of adult gynes
g_i	Effective mating frequency of the queen in colony i
$\bar{g}$	Average effective mating frequency of queens across colonies
I	Operational population sex allocation ratio, expressed as the proportion of energy invested in gynes relative to the total energy invested in sexuals
k	Metabolic parameter (normally set at 0.7)
λ_i	Inclusive fitness of workers per mg sexuals under perfectly split sex ratios
$\bar{m}$	Average dry mass of adult males
N	Operational population numerical sex ratio (expressed as the simple proportion of gynes among the sexuals)
n	Number of colonies in the sample
$\hat{Q}$	Queen optimum for the population sex allocation ratio
$\bar{R}$	Average extra inclusive fitness gained (%) by workers under perfect sex ratio splitting based on sister-to-brother relatedness asymmetry, relative to the inclusive fitness gained from producing the average sex ratio
$r_{i,f}$	Average life-for-life relatedness of a random worker in colony i to a sister
$r_{i,m}$	Average life-for-life relatedness of a random worker in colony i to a brother
v	Inverse of I
$\hat{W}$	Worker optimum for the population sex allocation ratio
$\bar{x}_f$	Average mating success of gynes in the population
$\bar{x}_M$	Average mating success of males in the population
Y_i	Sex allocation ratio in colony i, calculated as the proportion of energy invested in gynes relative to all the energy invested in sexuals in the colony
ω_i	Inclusive fitness of workers per mg sexuals in colony i

Worker and queen optima

Consider a species with a single queen per colony, in which sexuals mate randomly without local resource competition, local resource enhancement, or local mate competition, in which workers do not reproduce in queenright colonies, and the contribution of orphaned colonies to mating swarms can be ignored (either because orphaned colonies produce a negligible number of sexuals, or because they have approximately the same sex ratio as queenright colonies). These assumptions are valid for the six species in the present study (see Appendix 1 for validations). The predicted worker optimum (see text) is calculated as (Pamilo 1991):

$$\hat{W} = \frac{\bar{g} + 2}{2\bar{g} + 2} \tag{S1}$$

Assuming that the errors in estimating $\bar{g}$ are approximately normally distributed, the variance of $\hat{W}$ can be calculated exactly with the standard formula for error propagation in a function with a single variable:

$$SD^2(\hat{W}) = SE^2(\bar{g})\left|\frac{d\hat{W}}{d\bar{g}}\right|^2 = SE^2(\bar{g})\frac{4}{(2\bar{g} + 2)^4} \tag{S2}$$

The predicted queen optimum (see text) is (Trivers and Hare 1976, Pamilo 1991):

$$\hat{Q} = 0.50 \tag{S3}$$

And, when all colonies are assumed to have a single queen, the exact variance of $\hat{Q}$ is:

$$SD^2(\hat{Q}) = 0 \tag{S4}$$

Cost ratio

The cost ratio (i.e. the ratio of the productivity and maintenance costs to the colony of raising a single gyne versus a single male to adulthood) can be estimated as (Boomsma 1989, Boomsma et al. 1995):

$$c_k = \left(\frac{\bar{f}}{\bar{m}}\right)^k \tag{S5}$$

The value of 0.7 for the metabolic parameter k is commonly used for estimating the cost ratios of ants (Bourke and Franks 1995, Helms 1999, Fournier et al. 2003, Bourke 2005). Assuming that the masses are normally distributed, the variance of the cost ratio can be approximated with the standard formula for the propagation of errors in any composite function of two variables (Colquhoun 1971, pp. 39–41):

$$SD^2(c_k) \approx SE^2(\bar{f})\left(\frac{\partial c_k}{\partial \bar{f}}\right)^2 + SE^2(\bar{m})\left(\frac{\partial c_k}{\partial \bar{m}}\right)^2 + 2COV(\bar{f}, \bar{m})\left(\frac{\partial c_k}{\partial \bar{f}} \frac{\partial c_k}{\partial \bar{m}}\right) \tag{S6}$$

Which is equivalent to:

$$SD^2(c_k) \approx SE^2(\bar{f})\left(\frac{k\bar{f}^{k-1}}{\bar{m}^k}\right)^2 + SE^2(\bar{m})\left(\frac{-k\bar{f}^k}{\bar{m}^{k+1}}\right)^2 + 2COV(\bar{f}, \bar{m})(-k^2\bar{f}^{2k-1}\bar{m}^{-2k-1}) \tag{S7}$$

Assuming that COV $(\bar{f}, \bar{m})$ is zero, Eq. S7 simplifies to:

$$SD^2(c_k) \approx SE^2(\bar{f})\frac{k^2\bar{f}^{2(k-1)}}{\bar{m}^{2k}} + SE^2(\bar{m})k^2\frac{\bar{f}^{2k}}{\bar{m}^{2(k+1)}} \tag{S8}$$

Equation S6, which is commonly used as an approximation for error propagation (Weisstein 1999), yields a negligible ($\leq 0.05\%$ in the present case for $k = 0.7$) overestimation of the variance, relative to the exact expression for the variance of the product of two functions (Colquhoun (1971; formula 2.7.15 on p. 40). We therefore refrained from deriving all variances with the exact, more complicated, expression.

Operational population sex allocation ratio

In the present study, the operational population numerical sex ratio and its variance were estimated from the minimum adequate logistic model, when all covariates were entered as deviations from their mean value. The corresponding operational population sex allocation ratio can then be calculated as:

$$I_k = \frac{c_k N}{(c_k - 1)N + 1} \tag{S9}$$

To estimate the variance of I_k, we first need to introduce a variable v:

$$v = \frac{1}{I_k} = 1 - \frac{1}{c_k} + \frac{1}{c_k N} \tag{S10}$$

The variance of v can be approximated – analogously to above – as follows:

$$SD^2(v) \approx SD^2(c_k)\left(\frac{\partial v}{\partial c_k}\right)^2 + SD^2(N)\left(\frac{\partial v}{\partial N}\right)^2$$
$$+ 2COV(c_k, N)\left(\frac{\partial v}{\partial c_k} \frac{\partial v}{\partial N}\right) \tag{S11}$$

Because there is no reason to expect colinearity between our estimates for the cost ratio and the operational population numerical sex ratio, this can be simplified to:

$$SD^2(v) \approx SD^2(c_k)\left(\frac{\partial v}{\partial c_k}\right)^2 + SD^2(N)\left(\frac{\partial v}{\partial N}\right)^2$$
$$= SD^2(c_k)\left(\frac{1}{c_k^4}\right)\left(1 - \frac{1}{N}\right)^2$$
$$+ SD^2(N)\left(\frac{1}{c_k^2 N^4}\right) \tag{S12}$$

The variance of I_k can now be approximated as:

$$SD^2(I_k) = SD^2(v)\left|\frac{dI_k}{dv}\right|^2 = SD^2(v)\left|\frac{-1}{v^2}\right|^2$$
$$= SD^2(v)I_k^4 \tag{S13}$$

Which yields:

$$SD^2(I_k) = \left(SD^2(c_k)\left(\frac{1}{c_k^4}\right)\left(1 - \frac{1}{N}\right)^2\right.$$
$$\left. + SD^2(N)\left(\frac{1}{c_k^2 N^4}\right)\right)I_k^4 \tag{S14}$$

Sensitivity of the population sex allocation ratio to the metabolic parameter

We can use a z-test to determine whether the operational population sex allocation ratio is significantly different from $\hat{W}$ and $\hat{Q}$. For worker control, the z- statistic is calculated as:

$$z_1 = \frac{\hat{W} - I_k}{SD(\hat{W} - I_k)} = \frac{\hat{W} - I_k}{\sqrt{SD^2(\hat{W}) + SD^2(I_k)}} \tag{S15}$$

And for queen control as:

$$z_2 = \frac{\hat{Q} - I_k}{SD(\hat{Q} - I_k)} = \frac{\hat{Q} - I_k}{SD(I_k)} \tag{S16}$$

We can use the command $= NORMSDIST(z)$ in Microsoft Excel to calculate the one-tailed significances $p(Z_1)$ for worker control and $p(Z_2)$ for queen control, which need to be multiplied by two if a two-tailed test is desired. The parameter k, which has its own uncertainty, will affect the estimated value of I and thus the significance of the differences with $\hat{W}$ and $\hat{Q}$. It is therefore recommended (Boomsma and Nachman 2002) to formally test whether the conclusions about the relative likelihoods of queen or worker control are robust to the choice of k. In the present study, we did this for the first time ever by plotting four one-tailed probabilities for every value of k (Fig. 3):

$$p\{I \geq \hat{Q}\} = p_1 = p(z_1) \tag{S17}$$
$$p\{I < \hat{Q}\} = p_2 = 1 - p(z_1) \tag{S18}$$
$$p\{I \geq \hat{W}\} = p_3 = p(z_2) \tag{S19}$$
$$p\{I < \hat{W}\} = p_4 = 1 - p(z_2) \tag{S20}$$

As these probabilities are based on one-tailed z-tests, while we are interested in the two-tailed probabilities, the critical value (dotted horizontal lines in Fig. 3) has been set at 0.025 rather than 0.05.

Maximum possible fitness pay-off to workers from split sex ratios

Boomsma and Grafen (1991) showed that workers can optimize their inclusive fitness by raising exclusively gynes (sisters) when the effective mating frequency of their mother queen is below average, and exclusively males (brothers) when her mating frequency is above average. The ratio A_i of the inclusive fitness pay-offs of raising 1 mg gynes versus 1 mg males to workers in colony is:

$$A_i = \frac{x_F r_{i,F}}{c_k x_M r_{i,M}} \tag{S21}$$

The mating successes of gynes and males are inversely proportional to their respective frequencies in the population (Boomsma and Grafen 1991, Pamilo 1991), so that:

$$A_i = \frac{(1 - N)r_{i,F}}{c_k N r_{i,M}} \tag{S22}$$

The ratio between the average relatedness of workers in colony i towards sisters versus brothers depends on g_i, the mating frequency of their mother queen:

$$\frac{r_{i,F}}{r_{i,M}} = \frac{g_i + 2}{g_i} \tag{S23}$$

Which yields:

$$A_i = \frac{(1 - N)(g_i + 2)}{c_k N g_i} \tag{S24}$$

Let Y_i be the sex allocation ratio of colony i. The inclusive fitness ω_i of workers in colony i per mg produced sexuals is then given by:

$$\omega_i = A_i Y_i + 1 - Y_i = Y_i(A_i - 1) + 1 \tag{S25}$$

If colony i has the same sex ratio as the population, the following will apply:

$$Y_i = I_k \Rightarrow \omega_i = I_k(A_i - 1) + 1 \tag{S26}$$

In contrast, if the workers in colony i perfectly adapt the sex ratio to the mating frequency of their mother queen, their inclusive fitness per mg sexuals becomes:

$$\lambda_i = \begin{cases} 1(A_i - 1) + 1 = A_i & \text{for } g_i \leq \bar{g} \\ 0(A_i - 1) + 1 = 1 & \text{for } g_i > \bar{g} \end{cases} \tag{S27}$$

The average (over all n colonies in the sample) inclusive fitness gain (in %) to workers if they produce perfectly split sex ratios, compared to their inclusive fitness if the sex ratio in their colony is equal to the population sex ratio, then becomes:

$$\bar{R} = -100 + \frac{100}{n} \sum_i^n \frac{\lambda_i}{I_k(A_i - 1) + 1} \tag{S28}$$

where is A_i calculated with Eq. S24 and λ_i with Eq. S27.

Additional references

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