Egg production and caste allocation in the clonally reproductive ant *Vollenhovia emeryi*

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Sex ratio theories based on relatedness asymmetries among colony members have been tested in eusocial Hymenoptera. The haplodiploid sex determination system leads to differences in optimal sex allocation between queens and workers. Queens should ideally produce sexual eggs in accordance with relatedness asymmetries. One of the methods to test this hypothesis is to examine sex allocation in species where relatedness asymmetries are extremely different between female and males produced by the queens. We investigated sex allocation patterns in the polygynous ant *Vollenhovia emeryi* where both sexes are produced clonally so that the relatedness of the queens to their female and male offspring is 1.0 and 0, respectively. In this situation, queens invest most of their resources in female production. Furthermore, caste allocation patterns were observed in egg production. This study involved genotype analysis of colony members using genetic polymorphic markers and revealed clonal reproduction in the *V. emeryi* population. In this population, castes were identified in 1,060 eggs produced by 70 queens in 8 colonies. Queens invested more resources in female sexual eggs, though male eggs were also produced depending on resource availability. In addition, the proportion of female eggs negatively correlated with that of worker eggs, suggesting that this correlation is a trade-off. However, male egg production did not affect the egg production of other castes. This observation indicates that male egg production is independent of that for other castes. This study is the first to document the real primary sex ratio and caste allocation in eusocial Hymenoptera. **Key words:** ant, clonal reproduction, egg, queen, sex ratio.  

Social Hymenoptera have been used as model organisms historically to test hypotheses of sex ratio theories (Fisher 1930). Many studies have emphasized that sex allocation at the colony and population levels is strongly dependent on relatedness asymmetries and resource availability (Bourke and Franks 1995; Crozier and Pamilo 1996). The male haploid and female diploid sex-determining systems of ants cause relatedness asymmetries among colony members, leading to differences in sex allocation between queens and workers. In general, queens are equally related to their daughters and sons; hence, queens favor equal allocation of resources to both females and males. In contrast, workers are more related to their female siblings than males in a colony headed by a single mated queen; therefore, workers allocate more resources to females than to males. As a result, ant societies harbor internal conflicts over sex allocation between queens and workers (Trivers and Hare 1976). Some studies have demonstrated the existence of such conflicts (Aron et al. 1995).  

One method to test the theory of sex allocation is to examine sexual allocation in species where queens and workers differ in their relatedness to newly produced queens and males (Meunier et al. 2008). Parthenogenesis has been recently discovered in some ant species (Pearcy et al. 2004; Fournier et al. 2005; Himler et al. 2009). In addition, queens use sexual reproduction for workers and clonal reproduction for female sexuals in *Wasmannia auropunctata* (Fournier et al. 2005) and *Cataglyphis cursor* (Pearcy et al. 2004). Males in *C. cursor* arise from unfertilized eggs as seen in Hymenoptera in general (Peary and Aron 2006), whereas males in *W. auropunctata* arise from fertilized eggs that reflect only the paternal genome with no essential genetic exchange between males and females during reproduction (Fournier et al. 2005; Queller 2005). In such reproduction systems, all castes (including workers as well as female and male sexuals) are genetically determined.

The clonal reproduction system in *W. auropunctata* and *C. cursor* gives rise to specific patterns of relatedness asymmetries between colony members. Hence, it is possible to test the hypotheses of sexual allocation in their colonies. In clonal reproduction, female sexuals inherit their whole genome from mother queens. In *W. auropunctata*, in particular, males never inherit the queen’s genome. The queen’s relatedness to her female sexuals, worker, and male offsprings is 1.0, 0.5, and 0, respectively. Considering the uncommon relatedness asymmetries among colony members, the most adaptive sexual allocation for queens is to utilize most of their resources in producing female sexuals. However, males are essential for the queen to produce workers that develop from inseminated eggs. Therefore, queens must produce male offsprings to ensure mating partners for their female offsprings. In addition, queens acquire a powerful mode of controlling sex allocation because queen and worker castes arise from unfertilized and fertilized eggs, respectively. Queens can bring about preferred sex allocation by controlling the caste allocation of eggs. This observation indicates that caste determination is controlled by the reproducing queen and is not influenced by environmental factors or worker manipulation at the egg stage (Passera et al. 2001).

*Vollenhovia emeryi* exhibits a clonal reproduction system like that of *W. auropunctata* (Ohkawara et al. 2006). Although clonal reproduction remains to be established as a general phenomenon in this ant, the castes were genetically determined. Assuming that related asymmetries between females and males are based on clonal reproduction and that control of the egg caste rests with the queen, we hypothesize that the sex ratio in eggs is female biased and that male-destined eggs

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are produced with low frequency. To test this hypothesis, we identified all castes of eggs produced by each queen in *V. emeryi* using a genetic polymorphic marker. Furthermore, this hypothesis also facilitates investigating the proportion of workers, new queens, and males present in the eggs, although this investigation is generally difficult to perform in ordinary ants.

**MATERIALS AND METHODS**

**Field collection and sampling**

*Vollenhovia emeryi* is found in forest areas throughout Japan. This ant is polygynous, and the number of queens per colony is 7.6 ± 6.3 (mean ± standard deviation [SD]) (Ohkawara et al. 2002). The queens exhibit polymorphism in wing length with long- and short-winged queens (Kinomura and Yamauchi 1994). New individuals emerge by reproduction from late summer to autumn. The long-winged queens sexually reproduce with new mates during a nuptial flight in spring, whereas short-winged queens almost exclusively mate with males in their nests. Previous studies have demonstrated clonal reproduction in these ants, especially in the short-winged queen population (Ohkawara et al. 2006; Kobayashi et al. 2008); therefore, short-winged queens and their colonies were investigated in this study.

Colonies were collected from a secondary forest of Kanazawa city (lat 36°35′ N, long 136°35′ E) from June to October 2008. The collection site was about 5 km away from the site where clonal reproduction was confirmed by Ohkawara et al. (2006). The population was composed of short-winged queen colonies, and a census of all members of collected colonies was taken.

**Reproduction system of V. emeryi in the study population**

To assess the reproduction system of the *V. emeryi* population used in this study, we randomly chose 11 of the 27 collected colonies. We collected all colony members and examined the genotype of all dealate queens, all sexes, and 2–10 workers chosen randomly by microsatellite analysis from each of the 11 colonies. In addition, paternal genotypes were determined by examining the sperms in the spermathecae of dealate queens. All individuals were killed by crushing their heads. DNA was then extracted from the heads and thoraces by homogenization using a digestive solution in 1.5-ml tubes (50 μl cell lysis solution and 2.0 μl proteinase K; Qiagen, Valencia, CA) and incubated for 3 h at 55 °C. Sperm DNA was extracted in a similar manner using a digestive solution in 1.5-ml tubes after dissecting the gasters of all dealate queens and extracting sperms from the spermathecae. After incubation, genomic DNA was purified by phenol/chloroform protocols and precipitated with ethanol following standard protocols. It was then suspended in 30 μl TE (10 mM Tris with HCl, 1 mM EDTA, pH 8.0) buffer. We amplified a DNA microsatellite locus using primers originally developed for *Tennatohorax nylanderi* (L-5, L-18; Foitzik et al. 1997) and *Myrmica tahomensis* (Myr-3; Evans 1993). These 3 loci differed remarkably among castes in *V. emeryi* in the previous study by Ohkawara et al. (2006). Polymerase chain reaction (PCR) was performed in 12.25 μl extract using the following: 0.25 μl 6.25 pmol L⁻¹ dye-labeled forward and 1.0 μl unlabeled reverse primers, 2.25 μl deoxyribonucleotide triphosphate mixture (2.5 mmol L⁻¹ each), 1.25 μl 10X Ex Taq buffer, 1.1 μl 25 mM MgCl₂, 5.5 μl distilled water, 0.4 μl 500 U Ex Taq DNA polymerase (Fermentas, Germany), and 0.5 μl template DNA. PCR was performed for 5 min at 94 °C followed by 35 denaturation cycles for 75 s at 94 °C, 75 s of annealing at 53 °C, and 75 s of extension at 72 °C. The last extension at 72 °C was prolonged for 30 min to complete the extension. Fluorescent PCR fragments were visualized by capillary electrophoresis on an ABI 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA).

**Observation of caste composition in eggs produced by queens**

Caste composition of eggs produced by queens was examined by oviposition experiments and genotype analysis. In 8 colonies, all dealate queens were isolated with 5 workers and 5 larvae in artificial nests made of plaster (6.0 × 4.0 × 1.5 cm). They were maintained at a constant temperature of 23.5 °C and fed a dead grasshopper for 2–10 days. During the experiment, the number of eggs produced was counted daily. All eggs produced per queen were then preserved in 99% ethanol, following which all queens were dissected to observe ovarian development and insemination conditions. The eggs’ DNA, the queens’ bodies, and the sperm in spermathecae were extracted, and the genotypes were determined by the same method used for investigating the reproduction system. The number of eggs destined for each caste was estimated for each queen from the genotype data.

**RESULTS**

**Reproduction system of the *V. emeryi* population**

In 27 colonies, 7 (26%) produced only males, 2 (7.4%) produced only females sexuals, and 9 (33.3%) produced both sexes. The average numbers of female sexuals and males per colony were 5.4 ± 9.9 (mean ± SD) and 5.0 ± 8.2, respectively, in 27 colonies. Although investigating the reproduction system in 11 colonies, L-5 microsatellites (4 alleles: a111 bp, b113 bp, c109 bp, and e105 bp), Myr-3 (3 alleles: a126 bp, b234 bp, and c238 bp), and L-18 (4 alleles: a176 bp, b188 bp, c194 bp, and d226 bp) were amplified as described by Ohkawara et al. (2006). Table 1 lists the genotype composition in colony members at the locus L-5. This locus showed high allelic diversity among castes. Genotype and allele frequencies differed strikingly among queens, workers, and sexuals. Almost all queens and female sexuals were homozygous at the L-5 locus and had the cc genotype (queens: 100% and female sexuals: 91.5%). In contrast, workers tended to be heterozygous and had ac (97.1%) and bc (2.9%) genotypes. Furthermore, almost all sperms from the spermathecae and all males had a genotype (sperms: 81.3% and males: 100%). Genotype composition at the locus Myr-3 also showed a similar tendency. The results suggest that *V. emeryi* in this population exhibited clonal reproduction system to *W. auropunctata*.

**Genotypes of eggs produced by queens**

Genotype analysis of the social system indicated obvious differences between genotypes among castes at the L-5 locus (Figure 1, Table 1). Therefore, the castes of eggs were determined by their genotypes at the L-5 locus. Eight colonies consisting of 2–19 queens were used for sex ratio and caste composition observations in eggs. Seventy dealate queens were collected and isolated from the colonies. Although 2 queens did not produce eggs, 68 others produced a total of 1060 eggs (mean: 15.1 ± 6.9 eggs, range: 3–31 eggs) during the experimental period. Four alleles (a, b, c, and e) at the L-5 locus were amplified in a genotype analysis similar to that performed for the reproduction system. All 68 queens had the cc genotype, whereas the sperm from spermathecae had a (98.5%) and b (1.5%) genotypes. A total of 991 eggs had 4 genotypes:
cc (8.8%), ac (79.2%), bc (0.75%), and a (4.7%). The genotype of the remaining 69 eggs (6.5%) is unknown. Based on the results of the analyses for the reproduction system, cc and ac eggs were designated as female- and male-destined eggs, respectively. Furthermore, almost all ac and bc eggs were deemed as worker eggs. In particular, bc eggs were produced by cc queens with b sperm. However, genotype analysis of the reproduction system suggested that some female sexuals had heterozygous ac genotype (Table 1). Therefore, we estimated the number of queen-destined eggs with ac genotype by estimating the frequency of sexuals produced by the collecting queens in field (40.7%) and the frequency of ac queens in all samples (8.5%). The frequency of queen-destined eggs in ac egg was thus estimated to be 0.035, and we calculated the number of queen-destined eggs using this value. We deemed the queen-destined eggs to be entirely absent when the estimated number of queen-destined eggs was less than 1.0.

Sex ratio and caste composition in eggs

The proportion of each caste in eggs was investigated for each queen. All queens produced worker-destined eggs, and the proportion of workers was 21.4–100% of all eggs. In 68 queens, 30 (44.1%) produced worker-destined and sexual eggs, whereas the other 38 (55.9%) produced only worker-destined eggs. Figure 2 shows the frequency distribution of the sex investment ratio in queens producing sexual eggs. In 68 queens, 12 (17.6%) produced only male-destined eggs. 3 (4.4%) produced only queen-destined eggs, and 15 (22.1%) produced both sexes. The investment in sex production tended to be male biased.

The sex allocation patterns were analyzed in 30 queens producing sexuals. The number of queen-destined eggs was positively correlated with the total number of sexual eggs (female: \( P < 0.0001, r^2 = 0.88; \) Figure 3a). In addition, the number of male-destined eggs positively correlated with the total number of sexual eggs (\( P = 0.006, r^2 = 0.27; \) Figure 3b). However, the correlation was more significant for the production of queen-destined eggs than for male-destined eggs; a significant difference was observed between the slopes of correlation lines (\( P < 0.01, t\)-test). This suggests that the greater the number of sexual eggs produced, the greater is the number of queen-destined eggs among them.

Resource allocation patterns in caste production

In the eggs produced by each queen, the proportion of queen-destined eggs was negatively correlated with that of worker-destined eggs (\( P < 0.0001, r = 0.69; \) Figure 4), whereas the proportion of male-destined eggs showed no correlation (\( r = 0.23 \)). Furthermore, no correlation was observed between the proportion of queen- and male-destined eggs (\( r = 0.55 \)). Figure 5 indicates a comparison of worker-destined egg numbers among queen groups. To investigate whether the number of worker-destined eggs laid by queens was influenced by the various castes they produced, we classified queens into 3 groups: 1) queens producing only worker-destined eggs; 2) worker- and male-destined eggs; and 3) worker and both sexual eggs. The average number of worker eggs per queen was significantly lower in the queen group both sexual eggs than in the other 2 groups (\( P = 0.031, F_{2,62} = 3.68, \) one-way analysis of variance). However, no significant difference was observed between the group producing both worker- and male-destined eggs and those producing only worker-destined eggs (both worker and sexual eggs to only worker-destined eggs: \( P = 0.01 \); both sexual eggs to worker- and male-destined eggs: \( P = 0.052 \); and worker- and male-destined eggs to only worker-destined eggs: \( P = 0.9 \), Student’s t-test). These results demonstrate that queen-destined egg production reduced worker egg production, but male egg production did not affect the latter.

DISCUSSION

Investigating clonal reproduction in ants that demonstrate this reproduction systems provide new approaches to test sex ratio theories in social insects (Himler et al. 2009). In C. cursor, males and females are produced by arrhenotokous and thelytokous parthenogenesis, respectively (Pearcy et al. 2004). Thus, relatedness asymmetries among colony members are not affected by the mating frequency of mother queens, and little variation is observed in asymmetries between
colonies in a population. By excluding factors that affect sex allocation, Pearcy and Aron (2006) demonstrated local resource competition to cause highly male-biased sex allocation in *C. cursor* at both colony and population levels. *V. emeryi* also has an uncommon reproduction system that can be used to investigate caste allocation. In ants, it is hard to investigate caste ratio at the egg stage because female castes are determined by brood-rearing conditions. This study is the first to document sex allocation and caste ratio in eggs influenced by the queen.

In *V. emeryi*, queens invested more resources in producing queen-destined eggs than those of other castes according to relatedness asymmetries based on the clonal reproduction system. Male-destined eggs were also produced. However, the correlation was more significant for queen-destined eggs than for male-destined eggs; the slope of the correlation line was higher in queen-destined eggs. As we hypothesized, our results suggest that queens invested more resources in more related castes when they could control the caste ratio in the process of egg production. However, it is unclear whether more investment in queen-destined eggs is caused by only relatedness asymmetry. The production pattern of sexual eggs may be affected by other factors, such as the resources invested in producing female and male eggs, the physiological

\[ r^2 = 0.88 \quad \text{and} \quad r^2 = 0.27 \]

However, a significant difference is observed between the slopes in the correlation ($P < 0.01$), suggesting that the investment in female- and male-destined eggs produced per queen is different.
status of queens including age and mating number, social structure, and environmental conditions. Such factors also should be considered when explaining the factors influencing the production of queen-destined eggs.

The proportion of queen-destined eggs is negatively correlated with the proportion of worker-destined eggs produced, suggesting that this correlation is a trade-off. It also indicates that the frequency of fertilized eggs produced is different among fertile queens. A trade-off in female caste production has been reported in a few ant species (Ross 1993; Pamilo and Seppa 1994; Fournier et al. 2004; Kümmerli and Keller 2007). Fournier et al. (2004) successfully investigated that greater female production contributes significantly to lesser worker production in queens of the polygynous ant *Pheidole pallidula*. This suggests a trade-off between developmental frequencies in female sexuals and workers that originated from diploid (fertilized) eggs in ants with a nonclonal reproduction system, such as *P. pallidula*. Observations of *V. emeryi* indicate a trade-off between the production of fertilized and unfertilized eggs. Furthermore, Fournier et al. (2004) hypothesized that a trade-off was caused by the properties of sexual reproduction in queens at the individual level. Our data demonstrates that the trade-off occurs at the individual level. Although the physiological mechanism and factors causing the trade-off remain to be elucidated, it is possible that the production processes of female sexual and worker-destined eggs interact.

Few queens produced only queen-destined eggs in sexual reproduction, but many queens (17.6%) produced only male-destined eggs. In *V. emeryi*, queens cannot gain fitness through male-destined egg production, so it is puzzling why the frequency of queens producing only male-destined eggs is so high. One possible reason is that queens have to produce a given number of male-destined eggs to provide mating partners for their daughters in order to produce workers. In *V. emeryi*, multiple fertile queens exist in nests, and relatedness among queens is extremely high because of clonal reproduction. In addition, short-winged queens mate almost exclusively with their brothers in nests. It is possible that queens produce male-destined eggs according to the total number of female sexuals in nests, suggested by the weak correlation between the number of male-destined eggs and the total sexual investment.

However, all queens produced only male-destined eggs in 2 colonies. This observation indicates that the production of male-destined eggs cannot be explained by the queens' mating behavior. Another possible reason is that male production is controlled by the male parent. Some hypotheses have been proposed with regard to the mechanism of male clonality in *W. auropunctata*. Fournier et al. (2005) hypothesized that males arise from the male genetic contribution in the sperm by removing the maternal genetic material from the egg during fertilization (maternal genome elimination). In other words, male clonality may result from manipulation by the male parent. In addition, some *V. emeryi* queens may also be manipulated to produce a given number of male-destined eggs by their mating partners' sperm. However, this study demonstrated no correlation between male-destined and worker-destined egg production. This observation indicates that the production of male-destined egg was independent of female-destined egg production and is controlled by the male parent. In addition, this also means that a queen cannot completely control the caste of egg. However, this hypothesis should be tested carefully. Foucaud et al. (2007) suggested that male clonality to result from anucleate ovules that are fertilized later and that male-destined egg production has evolved as one of the reproductive strategies of queens. We have no evidence leading us to support these hypotheses from our data. In the future, it will be necessary to directly observe the development process from the egg stage for each caste by means of physiological experiments.

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