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RESEARCH ARTICLE

Colony fitness and garden growth in the asexual fungus-growing ant *Mycocepurus smithii* (Attini, Formicidae)

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Abstract

Nest-founding in the fungus-growing ant *Mycocepurus smithii* is typically by single queens (monogyny) and colonies transition to multiple queens (polygyny) as they grow larger. Here, we study the transition from monogyny to polygyny of *M. smithii* under lab conditions. We hypothesize that the worker-to-queen ratio affects colony growth, fungus-garden growth, and colony survival. Monogyne colonies with small gardens (0.1 g) had greater garden growth than polygyne colonies, suggesting that monogyny may be the superior strategy for small colonies after initial nest establishment. In monogyne colonies with small gardens and either 30, 60, or 90 workers, colonies with 60 workers produced the largest gardens, suggesting that an intermediate worker-to-queen ratio is optimal for monogyne colonies with small gardens. For monogyne colonies with larger gardens (0.45 g) and with either 6, 18, 60, or 90 workers, colonies with 60 or 90 workers had significantly greater garden growth than those with 6 or 18 workers. New daughter queens were produced only by colonies with a worker-to-queen ratio of 60 or 90, suggesting that only colonies with sufficient worker numbers and garden growth are stimulated to produce new queens. A single queen lays only 1.12 ± 0.06 (SE) eggs within 24 h, therefore, limiting growth of monogyne colonies. The transition from monogyny to polygyny through the addition of supernumary queens, as well as a worker-to-queen ratio of around 60, are, therefore, critical for colony growth and reproduction in *M. smithii*.

Keywords Caste determination · Fungus-growing ants · Monogyny · Nest-founding · Polygyny · Survival strategy

Introduction

After the nuptial flight of ants, a mated foundress queen faces many threats (e.g., predation, competition and opportunistic infection) that endanger the success of colony foundation (Weber 1966; Oster and Wilson 1978; Porter and Tschinkel 1986; Hölldobler and Wilson 1977; Keller 1991; Marti et al. 2015). These threats are exacerbated during nest-founding in fungus-growing ants (Attini), because queens need to sustain a symbiotic fungal garden in addition to nourishing the first brood of workers (Wheeler 1907; Weber 1958; Mueller et al. 1998; Fernández-Marín et al. 2005; Marti et al. 2015). During the first 2 days of nest-founding, an attine foundress expels from her infrabuccal pocket a pelleted inoculum of fungal hyphae, brought by the foundress from her natal nest, as starter inoculum of her incipient garden (Weber 1982; Mueller et al. 2001; Meirelles et al. 2016). Using nutrients stored in her body (in fat body and degenerating flight muscles) and, in some attine species, some plant material brought into the nest as substrate for fungiculture, the queen then carefully nurtures her incipient garden and raises her first cohort of workers (Weber 1958; Weber 1966; Mueller et al. 1998; Marti et al. 2015). Only after eclosion of the first cohort of workers, does the queen cease to tend the garden and the workers then assume responsibility for cultivation of the fungus that is essential for the success of the incipient colony. In fungus-growing ants, the biomass of fungus garden, queen fecundity (number of eggs laid), and the resulting number of workers are, therefore, key fitness components (Mehdiabadi et al. 2006; Seal and Tschinkel 2008; Himler et al. 2009; Shik et al. 2014; Kellner et al. 2018).

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In the fungus-growing ant *Mycocepurus smithii*, low fecundity and possibly asexual reproduction by *M. smithii* foundresses are two disadvantages that impact the success of the typical monogynous nest-founding (Fernández-Marín et al. 2005; Himler et al. 2009; Rabeling et al. 2009; Rabeling et al. 2011; Kellner et al. 2013). A colony founded by single-queen transitions to a polygynous phase only after surviving this haplometrotic nest-founding stage. For example, of 74 new *M. smithii* colonies surveyed by Fernández-Marín et al. (2005), 85% were monogyne colonies and 15% were polygyne colonies with 2–4 foundresses. In a second sample of additional 109 incipient colonies, each colony contained between 0 and 5 eggs, 0–4 larvae, 0–2 pupae, and 0–4 workers during this phase of early colony establishment (Fernández-Marín et al. 2005). Colonies of *M. smithii*, therefore, exhibit relatively low growth rates (Fernández-Marín et al. 2003, 2004), compared to other attine ants such as *Atta* where a single foundress rears around 100 workers in the first 3 months (Marti et al. 2015).

In addition to low fecundity, a second potential disadvantage during colony founding is the obligate asexuality (thelytoky) in most populations of *M. smithii*, where queens produce both daughter workers and new reproductive females (gynes) asexually (Fernández-Marín et al. 2005; Himler et al. 2009; Rabeling et al. 2009; Rabeling et al. 2011; Kellner et al. 2013). Unlike ants with sexual reproduction, there exists, therefore, no within-nest genetic variation between workers within an asexually reproducing colony of *M. smithii* that is founded by a single queen, potentially reducing disease resistance of the colony (Hughes and Boomsma 2004) or precluding genetically based behavioral specializations (Julian and Fewell 2004). Moreover, for those populations lacking sexual recombination, deleterious mutations may accumulate that entail long-term evolutionary disadvantages (Butlin 2002; Rice 2002; Rabeling et al. 2009). Rabeling et al. (2011) found both sexual and asexual *M. smithii* populations in Amazonian Brazil, whereas only asexual populations have been found so far elsewhere across the range of *M. smithii* from northern Argentina to northern Mexico and across the Caribbean islands (Kempf 1963; Mackay et al. 2004; Fernández-Marín et al. 2005; Rabeling et al. 2011). Despite the potential disadvantages of asexual reproduction, asexually reproducing *M. smithii* have a wider geographic distribution than the sexual populations (Rabeling et al. 2011). The ecological and genetic factors that contribute to the success of asexual colonies throughout most of the range of *M. smithii* are unknown (Rabeling et al. 2011).

Mature *M. smithii* colonies are highly polygynous (Rabeling 2004; Fernández-Marín et al. 2005; Rabeling et al. 2009, 2011). In field colonies, for example, between 1 and 23 queens can occur in the same reproductive nest (Fernández-Marín et al. 2005), and laboratory colonies can accumulate dozens to hundreds of reproducing queens coexisting in the same colony (Fang and Mueller, unpublished observations). However, Fernández-Marín et al. (2004, 2005) observed that 85–88% of incipient colonies in Panamá are founded by a single queen, indicating that most multi-queen nests in *M. smithii* become secondarily polygynous after a single-queen nest-founding phase. This suggests the hypothesis that *M. smithii* optimize colony fitness by transitioning at a specific queen-to-worker ratio from a monogynous phase to a subsequent polygynous phase.

To further elucidate factors that optimize colony fitness and garden growth in *M. smithii*, we varied the queen-to-worker ratio in small experimental colonies set-up with a standardized fungus-garden biomass. Our experiments aimed to test (1) whether a very small colony (i.e., an established incipient colony) with multiple queens is able to grow and sustain a larger biomass of fungus garden than a comparable colony with a single queen; (2) whether there exists an optimal number of workers that a very small colony can support with a given garden biomass; and (3) whether there exists a specific queen-to-worker ratio when colonies transition from monogyny to polygyny.

Methods

Ant colonies

All experimental colonies were derived through successive splitting from a single asexual ant colony, the so-called A5 clone of *M. smithii* (Kellner et al. 2013, 2018), collected originally in 2001 in Gamboa, Panamá, using methods described previously (Rabeling et al. 2009; Kellner et al. 2013, 2015, 2018). Colonies were maintained in a lab at the University of Texas at Austin in a temperature-controlled room (25 ± 1 °C). Two years before the start of the experiments, five source colonies were split from one nest of the A5 clone of *M. smithii*, and then, each of these five source colonies was allowed to grow to yield sufficient garden and colony sizes to set up replicates for the experiments. Each colony was kept with its fungus gardens in a $7.8 \times 7.8 \times 3.0$ cm^3 plastic container with a bottom of hydrated plaster, and this nest chamber was connected to a dry foraging chamber by a transparent vinyl tube, as described by Sosa-Calvo et al. (2015). As gardening substrate, each colony was given in the foraging chamber a mixture of polenta and finely minced ground oats (3:1 ratio polenta-to-oats by volume). The plaster in the garden chamber was hydrated weekly to generate a humid environment for the fungus in the garden chamber.

Preparation of brood-free fungus-garden fragments

To generate brood-free garden for the below experiments, we removed garden fragments of about 4 cm^3 along with about 300 workers from a source colony, removed all female reproductives (winged gynes and dealate queens) to return them to their source colony, and then waited 60 days so brood in these fragments could develop to eclosion. This waiting time is a superior method to generate brood-free garden fragments compared to searching several rounds through a garden to remove all brood, because the garden is sensitive to a low humidity environment, and carefully searching for embedded eggs and larvae requires teasing apart the garden into small fragments, thus harming the garden. Sixty days is a sufficient time to rear all brood, and then remove any remaining un-eclosed pupae, because the total development from an egg to an adult is 60 days under 25 ± 1 °C (Fang and Mueller, unpublished observation).

Basic experimental protocol

To set up replicates for experiments, we randomly selected queens and workers from five source colonies (see above) of the so-called A5 ant clone used in the previous experiments by Kellner et al. (2013, 2018). Ants and garden from the five different source colonies were not mixed to set up a particular experimental replicate; this minimized aggressive interactions between ants within an experimental replicate. Because colony fitness of lower attine ants such as *M. smithii* is dependent on fungal garden weight (Mehdiabadi et al. 2006; Kellner et al. 2018), we standardized the weights of initial gardens by giving each colony a standardized amount of fungus garden at the beginning of an experiment (either 0.1 g or 0.45 g, depending on the experiment). The total mass of each garden was weighed weekly for the duration of the experiment (13–31 weeks, depending on the experiment), weighing gardens blindly with respect to experimental treatment to minimize observer bias (Kardish et al. 2015). Chitin or carbohydrate content of gardens can be superior proxies of colony fitness than garden weight under some conditions (Seal and Tschinkel 2007), but we chose garden weight because of the simplicity of quantifying this fitness proxy. For weighing of gardens, we anesthetized the ants by filling the chamber briefly with carbon dioxide, then gently removed all ants from each garden with soft forceps or moistened paintbrushes, then weighed the garden on a weighing dish, and then returned all ants to their garden chamber.

We generated experimental nests with different combinations of number of queens and number of workers to test for the effect of queen-to-worker ratio on garden growth.

Experiment 1: effect of the number of mature queens per colony

According to the field survey by Fernández-Marín et al. (2005), founding colonies of *M. smithii* contain 1–4 foundresses (average of 1.16 foundresses). To explore the effect of foundress queen number on colony growth and garden growth, we set up 26 experimental colonies with different number of queens, either no queen (6 replicates), 1 queen (7 replicates), 5 queens (5 replicates), or 10 queens (8 replicates). Each colony was given 30 workers and 0.1 g of garden (prepared as described above). Because workers of *M. smithii* continue to maintain gardens even in the absence of a queen, it was possible to conduct this experiment with queenless colonies as a control. Starting in January 2017, all colonies were observed and weighted weekly (see above) for a total of 31 weeks. During these 31 weeks, no replicate had a garden that filled up more than ≈ 30% of the nest chamber (garden growth is inherently slow in *M. smithii*, see also below), so experimental colonies were not space-constrained in their nest chamber.

Experiment 2: effect of the number of workers per colony

In September 2017, we set up experimental colonies each with 0.1 g of garden and 1 queen, but varied worker numbers, either 30 workers ($N = 9$ colonies), 60 workers ($N = 3$ colonies), or 90 workers ($N = 2$ colonies). Each colony was observed for 13 weeks. The low sample sizes in some treatments were because we feared larger sample sizes would deplete our source colonies too much. By January 2018, however, our source colonies from which garden and workers had been taken in September 2017 had re-grown, and we were, therefore, able to add seven more replicate colonies to each treatment, and then observed these for 13 weeks. Combining the samples from 2017 and 2018, the total experimental colonies with 30, 60, and 90 workers were 16, 10, and 9, respectively.

Experiment 3: effect of the number of workers per colony with different initial fungus weight

To further determine the effects of worker number and a garden larger than the one used in Experiment 2, we set up colonies with single queens and with either 6, 18, 60, or 90 workers, and provided each colony with an initial garden size of 0.45 g ($N = 3$ for each treatment). Starting in April 2018, each colony was monitored for 13 weeks, as described above.

Experiment 4: testing cues for production of new queens

In Experiment 2, new daughter queens (gynes) were produced in some colonies by the fourth month (January 2018) from 3 colonies each with 60 workers and 1 queen, and from 1 colony with 90 workers and 1 queen. These colonies had reached by then fungus-garden weights averaging about 0.45 g. To test the roles of worker number and garden size, we set up new colonies, each with 1 queen and either 60 or 90 workers, and gave each of those 0.45 g of initial fungus garden ($N = 3$ for each of these 2 treatments). Starting in June 2018, each colony was monitored for 13 weeks as described above. We combined this data set with the data set from Experiment 2 and compared garden weights from colonies with 60 workers ($N = 10$ colonies, 3 colonies from 2017 and 7 from 2018) and 90 workers ($N = 9$ colonies, 2 colonies from 2017 and 7 from 2018).

Experiment 5: quantification of egg-laying capacity of single queens

To estimate the number of eggs laid by a single queen of *M. smithii*, we randomly selected 313 queens of *M. smithii* from the five source colonies of the most productive ant clone (the so-called A5-clone; Kellner et al. 2013) and set up three replicate experimental runs (run A with $N = 98$ individual queens; run B with $N = 104$ queens; run C with $N = 111$ queens) (Fig. 6). Each queen was set up individually in a Petri dish (6 cm diameter) with a bottom of 1% agarose to maintain humidity and allow easy visual identification of any eggs laid by the queen on the agarose. We counted the number of eggs laid by each queen after 24 h.

Table 1 Pairwise comparisons of garden weights over 31 weeks between all treatments in Experiment 1, analyzed using emmeans::emmeans() in R

Contrast	Estimate	SE	*df*	*t* ratio	*p* value
0Q30W–1Q30W	−0.00612	0.0113	772	−0.541	0.9489
0Q30W–5Q30W	0.04731	0.0123	772	3.844	0.0008
0Q30W–10Q30W	0.09262	0.011	772	8.438	< 0.0001
1Q30W–5Q30W	0.05343	0.0119	772	4.49	< 0.0001
1Q30W–10Q30W	−0.09874	0.0105	772	−9.387	< 0.0001
5Q30W–10Q30W	−0.04531	0.0116	772	−3.911	0.0006

The *p* values are adjusted using a Tukey method for comparing differences between four treatments (*M. smithii* treatments: 0Q-30W, 1Q-30W, 5Q-30W, and 10Q-30W). Figure 1 presents the corresponding data graphically

Statistical analysis

For Experiments 1 and 3, we used a Poisson generalized linear mixed model (GLMM) with worker number as the fixed effect and time (week) as the random effect (Bolker et al. 2009). For Experiment 2, we combined the small data set from 2017 with a data set (seven additional replicates) from 2018, and analyzed this combined data set with the same GLMM also used in Experiments 1 and 3, but with two random effects, week, and year. For Experiment 4, as explained above, we also combined the data set from Experiment 2 (10 and 9 replicates for 60 and 90 workers provided with 0.1 g of garden) with the data set from June 2018 (3 and 3 replicates for 60 and 90 workers provided with 0.45 g of garden), and analyzed this combined dataset with the same linear mixed model and again with two random effects, week, and year. We performed statistical analyses in RStudio, version 1.1.463 (R Core Team 2017), and generated plots using ggplot2. We include R scripts used for analyses (Online Resource 1), statistics for pairwise comparisons (Tables 1, 2, 3, 4, 5, 6), and the raw data as.csv files in Online Resource 2 (Tables S1–S6). For Experiment 5, we analyzed the data with analysis of variance (ANOVA) and a Tukey's post hoc test for identifying differences among the three replicate runs.

Table 2 Pairwise comparisons of garden weights over 13 weeks between all treatments in Experiment 1, analyzed using emmeans::emmeans() in R

Contrast	Estimate	SE	*df*	*t* ratio	*p* value
1Q30W–5Q30W	0.0616	0.0215	245	2.862	0.0126
5Q30W–10Q30W	−0.0532	0.0209	245	−2.542	0.0312
1Q30W–10Q30W	−0.1148	0.019	245	−6.038	< 0.0001

The *p* values are adjusted using a Tukey method for comparing differences between three treatments (*M. smithii* treatments: 1Q-30W, 5Q-30W, and 10Q-30W). Figure 2 presents the corresponding data graphically

Table 3 Pairwise comparisons of garden weights over 13 weeks between all treatments in Experiment 2, analyzed using emmeans::emmeans() in R

Contrast	Estimate	SE	*df*	*t* ratio	*p* value
1Q30W–1Q60W	−0.066	0.0176	440	−3.744	0.0006
1Q30W–1Q90W	−0.0518	0.0181	440	−2.852	0.0126
1Q60W–1Q90W	0.0142	0.0181	438	0.785	0.7124

The *p* values are adjusted using a Tukey method for comparing differences between three treatments (*M. smithii* treatments: 1Q-30W, 1Q-60W and 1Q-90W). Week and year (2017 and 2018) were treated as random effects. Figure 3 presents the corresponding data graphically

Table 4 Pairwise comparisons of garden weights over 13 weeks between all treatments in Experiment 3, analyzed using emmeans::emmeans() in R

Contrast	Estimate	SE	*df*	*t* ratio	*p* value
1Q6W–1Q18W	0.0276	0.0334	140	0.828	0.841
1Q6W–1Q60W	0.2157	0.0334	140	6.464	< 0.0001
1Q6W–1Q90W	− 0.3024	0.0334	140	− 9.061	< 0.0001
1Q18W–1Q60W	− 0.1881	0.0334	140	− 5.635	< 0.0001
1Q18W–1Q90W	− 0.2747	0.0334	140	− 8.232	< 0.0001
1Q60W–1Q90W	− 0.0867	0.0334	140	− 2.597	0.0504

The *p* values are adjusted using a Tukey method for comparing differences between four treatments (*M. smithii* treatments: 1Q-6W, 1Q-18W, 1Q-60W, and 1Q-90W). Figure 4 presents the corresponding data graphically

Table 5 Pairwise comparisons of garden weights over 13 weeks between all treatments in Experiment 4, analyzed using emmeans::emmeans() in R

Contrast	Estimate	SE	*df*	*t* ratio	*p* value
1Q60W_0.1–1Q60W_0.45	− 0.2627	0.0307	308	− 8.56	< 0.0001
1Q60W_0.1–1Q90W_0.1	0.0116	0.0209	308	0.555	0.945
1Q60W_0.1–1Q90W_0.45	− 0.3494	0.0307	308	− 11.384	< 0.0001
1Q60W_0.45–1Q90W_0.1	0.2743	0.0307	308	8.941	< 0.0001
1Q60W_0.45–1Q90W_0.45	− 0.0867	0.037	308	− 2.342	0.0909
1Q90W_0.1–1Q90W_0.45	− 0.361	0.0307	308	− 11.766	< 0.0001

The *p* values are adjusted using a Tukey method for comparing differences between four treatments (*M. smithii* treatments 1Q-60W + 0.1 g fungus, 1Q-90W + 0.1 g fungus, 1Q-60W + 0.45 g fungus, and 1Q-90W + 0.45 g fungus). Week and year (2017 and 2018) were treated as random effects. Figure 5 presents the corresponding data graphically

Table 6 Pairwise comparisons of garden weights over 13 weeks between all treatments in Experiment 2, analyzed using emmeans::emmeans() in R

Contrast	Estimate	SE	*df*	*t* ratio	*p* value
1Q30W–1Q60W	− 0.07537	0.0142	258	− 5.29	< 0.0001
1Q30W–1Q90W	0.00966	0.0142	258	0.678	0.7766
1Q60W–1Q90W	0.08503	0.0142	258	5.968	< 0.0001

The *p* values are adjusted using a Tukey method for comparing differences between three treatments (*M. smithii* treatments 1Q-30W, 1Q-60W, and 1Q-90W). Week was treated as random effect. Figure 7 presents the corresponding data graphically

Results

We use the following notation to abbreviate experimental treatments that vary the number of workers and the number of queens: 0Q-30W denotes an experimental set-up of no queen and 30 workers; 5Q-30W denotes 5 queens and 30 workers, and likewise for other queen-to-worker ratios. Raw data used to generate Figs. 1, 2, 3, 4, 5, 6, and 7 are, respectively, in Tables S1–S6 (Online Resource 2).

Experiment 1: effect of the number of mature queens per colony

We found no significant difference in fungus-garden weight between colonies in the 0Q-30W (control) and 1Q-30W treatments ($P = 0.5885$, Fig. 1). The significant differences were found between 0Q-30W colonies with both 5Q-30W colonies ($P < 0.01$; Fig. 1; Table 1) and 10Q-30W colonies ($P < 0.01$; Fig. 1; Table 1). The presence of multiple queens (5Q-30W and 10Q-30W) significantly reduced colonies fitness compared to the 1Q-30W colonies ($P < 0.01$; Fig. 1; Table 1). The estimated intercept was 0.1668, and the estimated coefficients for 1Q-30W colonies, 5Q-30W colonies, and 10Q-30W colonies were 0.0061, − 0.0473, and − 0.0926, respectively. By week 31, only one replicate each survived for 0Q-30W colonies and for 1Q-30W colonies; all the replicates of 5Q-30W colonies and 10Q-30W colonies had died by weeks 19 and 27, respectively. To analyze how colony fitness was influenced by the different numbers of queen(s) at the founding colony, we used a GLMM to analyze the first 13 weeks of the experiment. We only compared treatment 1Q-30W, 5Q-30W, and 10Q-30W treatments, which most closely resemble the queen-to-worker ratios observed in field colonies (Fernández-Marín et al. 2003). The GLMM analyses show that 1Q-30W colonies had significantly greater garden weights than 10Q-30W colonies ($P < 0.01$; Fig. 2), and 5Q-30W colonies had significantly greater garden weights than 10Q-30W colonies ($P = 0.01$; Fig. 2). All pairwise comparisons between treatments are listed in Table 2.

Experiment 2: effect of the number of workers per colony

Because Experiment 1 indicated that founding colonies with single queens had the highest fitness (Figs. 1, 2), we used in Experiment 2 single-queen colonies but varied worker number from 30, 60, and 90 workers, giving each colony 0.1 g fungus garden when starting the experiment in September 2017. We generated 9, 3, and 2 replicate colonies, respectively, for the 1Q-30W, 1Q-60W, and

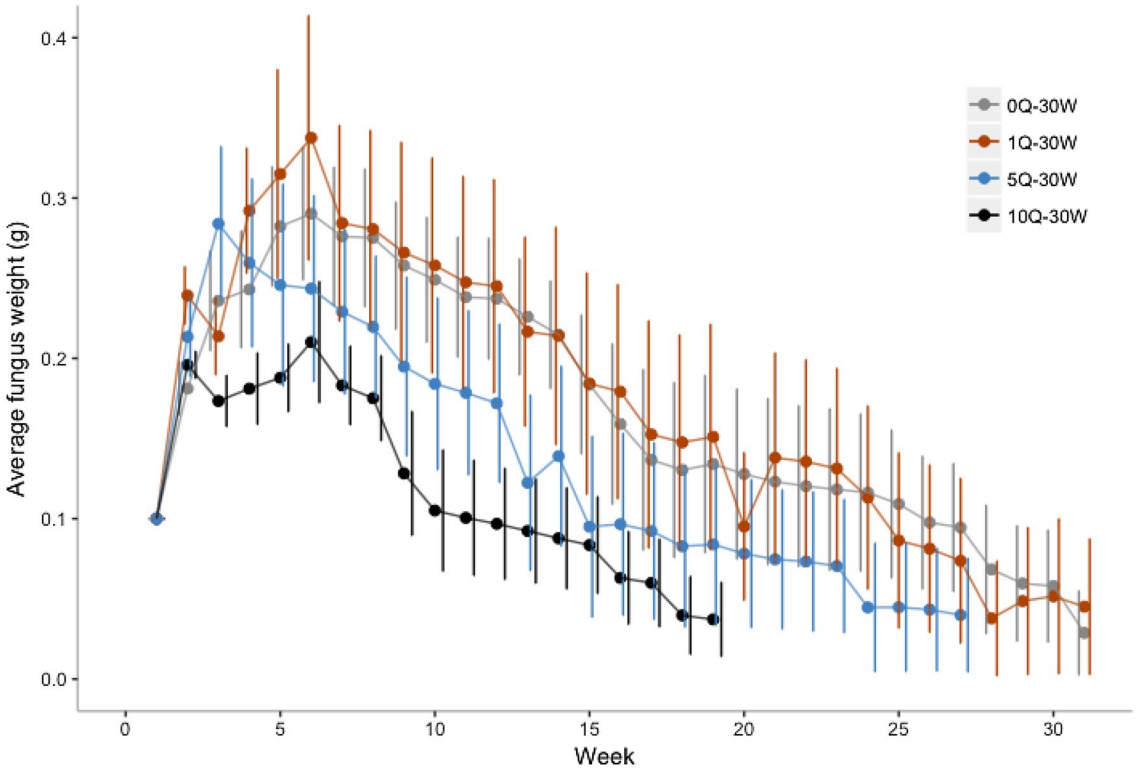

Fig. 1 Weekly fungus-garden weight (in g; mean ± SEM) over 31 weeks for *Mycocepurus smithii* nests starting with different number of queens (0, 1, 5, or 10 queens) and 30 workers. Overall, there was no significantly difference between 0Q-30W colonies (control, gray) and 1Q-30W colonies (burnt orange) ($P = 0.5885$). The estimated intercept was 0.1668, and the estimated coefficients for 1Q-30W colonies (gray), 5Q-30W colonies (blue), and 10Q-30W colonies (black) were 0.0061, −0.0473, and −0.0926, respectively. Negative estimated coefficients indicate a decrease of fungus-garden weight over time. The colonies with either five queens or ten queens had significantly lower garden weights than the garden weights of 0Q-30W control colonies (both $P < 0.01$). Garden weights of 1Q-30W colonies were significantly greater than garden weights of 5Q-30W colonies ($P < 0.01$, Table 1). Treatment differences were analyzed using a linear mixed model which treated queen number as the fixed effect, and week as the random effect. For 0Q-30W and 1Q-30W colonies, there was only one replicate alive in week 31. All the replicate colonies in the 5Q-30W and 10Q-30W treatments had died by week 19 and 27, respectively. Sample sizes were $N = 6$, 7, 5, and 8 for treatments 0Q-30W, 1Q-30W, 5Q-30W, and 10Q-30W, respectively. *Q* number of queens, *W* number of workers

1Q-90W treatments. Because the small-sample size of colony 1Q-90W ($N = 2$) seemed insufficient, we increased samples sizes by starting in January 2018 seven additional replicate colonies for each treatment. Combining the replicates from 2017 and 2018, the total sample sizes were, therefore, $N = 16$, 10, and 9 for 1Q-30W, 1Q-60W, and 1Q-90W treatments, respectively. In an analysis using a linear mixed model that treated both week and year as the random effects in the combined data set, 1Q-60W colonies had significantly greater garden weights than 1Q-30W colonies ($P < 0.01$; Fig. 3), and 1Q-30W colonies had significantly greater garden weights than 1Q-90W colonies ($P < 0.01$; Fig. 3). When analyzing only the 2018 data with a linear mixed model with only one random effect (i.e., week), 1Q-60W colonies had significantly greater garden weights than 1Q-30W colonies ($P < 0.01$, Fig. 7). There was no significant difference in garden weights between 1Q-30W colonies and 1Q-90W colonies ($P = 0.4984$, Fig. 7; Table 6). All pairwise comparisons between Experiment 2 treatments are listed in Table 6.

Among the 14 experimental colonies ($N = 9$, 3, 2 for 1Q-30W, 1Q-60W, and 1Q-90W, respectively) started in September 2017, new daughter queens (winged females, i.e., gynes) were first observed in the fourth month (January 2018) in three 1Q-60W colonies and in one of the 1Q-90W colonies. The first new daughter queen appeared in the eighth month (May 2018) in two colonies of the 1Q-30W treatment. In the replicate colonies added in 2018 ($N = 7$ for 1Q-30W, 1Q-60W, and 1Q-90W), no new daughter queens appeared from the 1Q-60W or 1Q-90W colonies by the fourth month (May 2018) of the experiment.

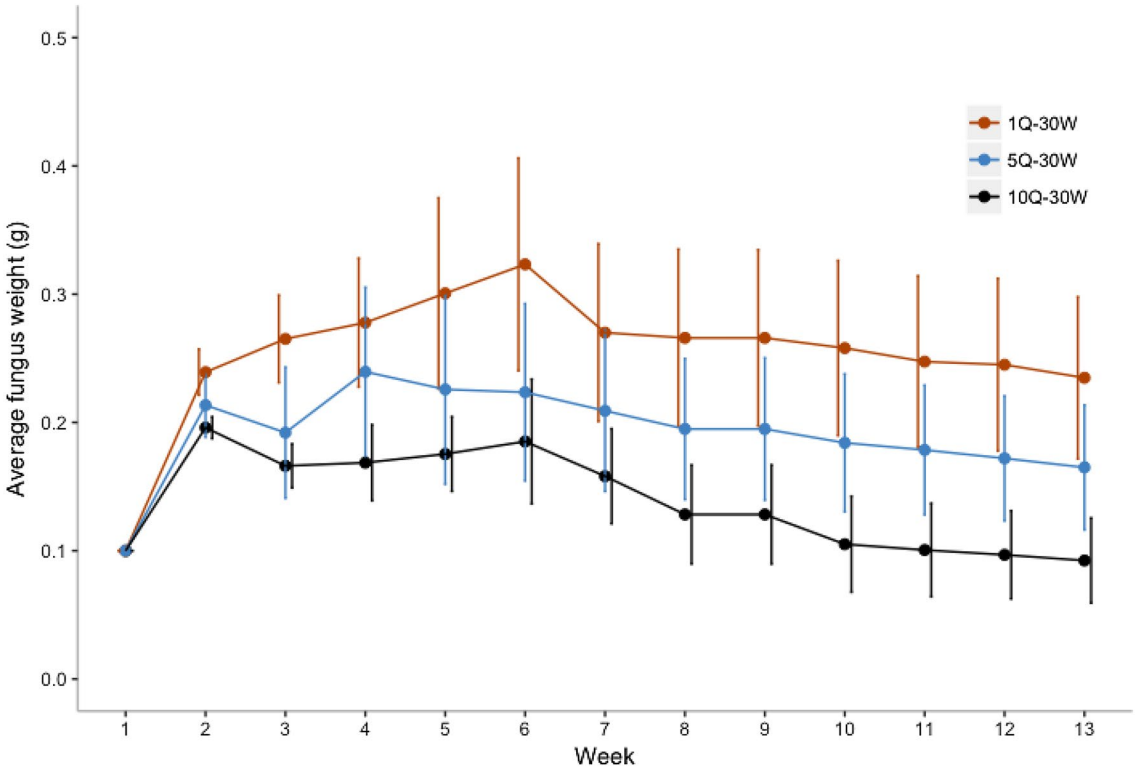

Fig. 2 Weekly fungus-garden weight (in g; mean ± SEM) over 13 weeks for *Mycocepurus smithii* nests starting with different number of queens (1, 5, or 10 queens), 30 workers, and an initial garden of 0.1 g. The 1Q-30W colonies had significantly greater garden weights than 10Q-30W colonies ($P < 0.01$). The 5Q-30W colonies had significantly greater garden weights than 10Q-30W colonies ($P = 0.01$). Treatment differences were analyzed using a linear mixed model where queen number was treated as the fixed effect and week was treated as the random effect. Sample sizes were $N = 7$, 5, and 8 for treatments 1Q-30W, 5Q-30W, and 10Q-30W, respectively. *Q* number of queens, *W* number of workers

Experiment 3: effect of the number of workers per colony with different initial fungus weight

There was no significant difference in garden weights between 1Q-6W and 1Q-18W colonies ($P = 0.409$; Fig. 4). 1Q-60W and 1Q-90W colonies had significantly greater garden weights than 1Q-18W colonies ($P < 0.05$; Fig. 4). All pairwise comparisons between treatments are listed in Table 4.

Experiment 4: testing cues for production of new queens

In Experiment 2, new daughter queen appeared during the fourth month (January 2018) in three 1Q-60W colonies, and in one of the 1Q-90W colonies. The average garden weight in these four colonies was around 0.45 g. We, therefore, used in Experiment 4 colonies with either 60 or 90 workers along with 1 queen, but increasing the initial the garden weight to 0.45 g garden ($N = 3$ for each of these 2 treatments). The experiment was started in June 2018, and each colony was observed for 13 weeks. There was no significant difference in garden weight between 1Q-60W colonies with 0.1 g fungus garden and 1Q-90W colonies with 0.1 g fungus garden ($P = 0.579$; Fig. 5). Both 1Q-60W and 1Q-90W colonies with initially 0.45 g fungus had greater garden weights than 1Q-60W colonies with initially 0.1 g fungus ($P < 0.01$; Fig. 5). All pairwise comparisons between treatments are listed in Table 5. We observed no new daughter queens during the 4 months of this experiment.

Experiment 5: quantification of egg-laying capacity of single queens

On average, each queen laid 1.12 ± 0.06 eggs within 24 h. There were no significant differences in eggs laid among the three replicate runs ($P = 0.2168$). A Tukey's post hoc test also showed no significant difference when comparing each pair of replicate run ($df = 2$; A–B: $P = 0.998$; A–C: $P = 0.307$; B–C: $P = 0.273$). When combining three replicate runs, the percentage for 0, 1, 2, 3, 4, and 5 eggs laid in 24 h were 33.86%, 35.14%, 19.16%, 8.62%, 2.55%, and 0.63%, respectively (Fig. 6). About 90% of the queens, therefore, laid two or fewer eggs within 24 h.

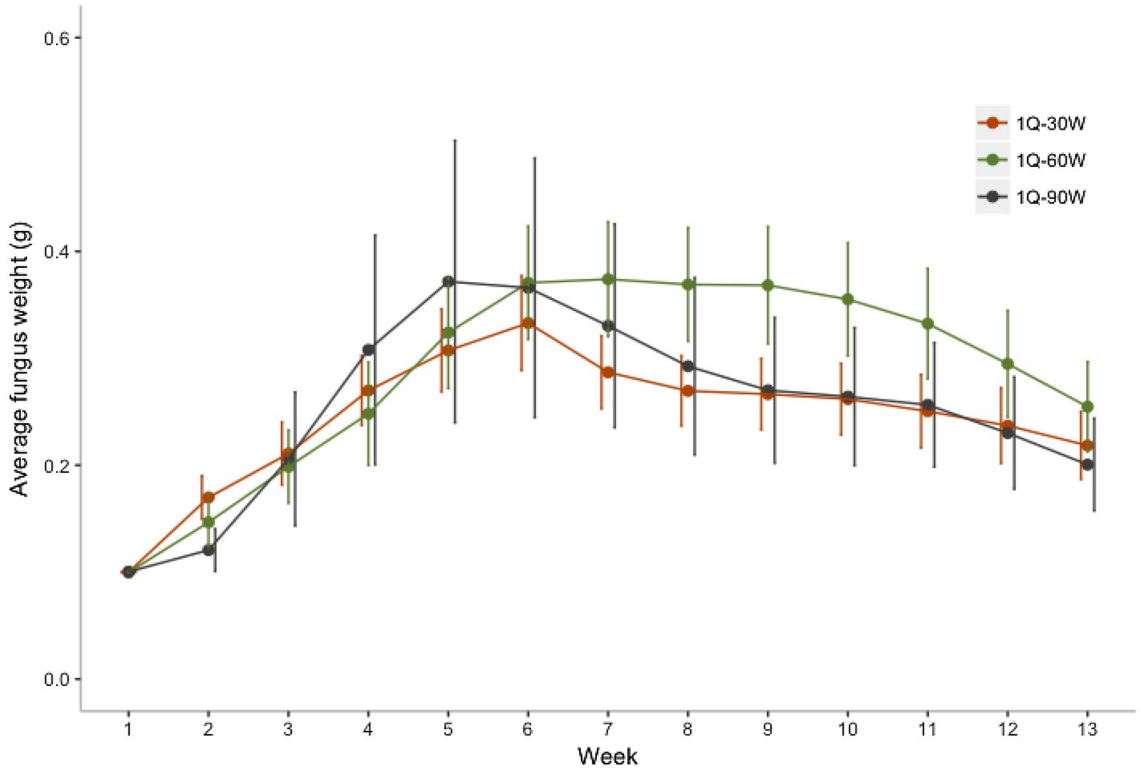

Fig. 3 Weekly fungus-garden weight (in g; mean ± SEM) over 13 weeks for *Mycocepurus smithii* nests starting with different number of workers (30, 60, or 90 workers), 1 queen per colony, and an initial garden of 0.1 g. The data combine experimental replicates from 2017 (14 replicates) and 2018 (21 replicates). The 1Q-60W colonies had significantly greater garden weights than 1Q-30W colonies ($P < 0.01$). In addition, there is a significant difference in garden weights between 1Q-30W colonies and 1Q-90W colonies ($P < 0.01$). Treatment differences were analyzed using a linear mixed model where queen number was treated as the fixed effect; week and year (2017 and 2018) were treated as the random effects. Sample sizes were $N = 16$, 10, and 9 for treatments 1Q-30W, 1Q-60W, and 1Q-90W, respectively. *Q* number of queens, *W* number of workers

Discussion

Our experiments aimed to compare colony fitness in established small nests with either multiple queens or single queens of the asexual, polygynous fungus-growing ant *M. smithii*, to understand why most colonies of *M. smithii* are founded by single queens under natural conditions (Fernández-Marín et al. 2005) and to elucidate the transition from single queen to multiple queens in *M. smithii*. Colony fitness was greater for single-queen colonies than multi-queen colonies over the course of a 31-week experiment (Fig. 1), and the presence of too many queens appears to have stressed a limited resource, the small fungal garden of a very small colony after nest establishment. Specifically, increased brood in colonies with supernumary queens, in addition to the queens themselves, may have led to overconsumption of garden, diminished garden growth, and thus resource shortage. Consequently, during the first 13 weeks of colony growth in Experiment 1 (Fig. 2), single-queen colonies produced larger gardens, a proxy of fitness. Following nest establishment, faster garden growth of single-queen colonies compared to multi-queen colonies may also be true for natural field conditions (but see also discussion below), where most nests are founded by single queens (85% haplometrosis and 15% pleometrosis; Fernández-Marín et al. 2005). In the leafcutter ant *Acromyrmex versicolor*, incipient colonies with single foundresses are less likely to establish a successful fungus garden than colonies with cooperating co-foundresses (Cahan and Julian 1999), in contrast to our experiments that did not test garden establishment but focused on somewhat older, very small nests that transition from single to multiple queens. In social insects, cooperating queens typically conserve energy reserves, which translate into increased brood production, faster colony growth, more efficient foraging behavior, and higher colony survival (Tschinkel and Howard 1983; Adams and Tschinkel 1995; Johnson 2004; Overson et al. 2014; Chiu et al. 2018).

Transition from monogyny to polygyny

Although haplometrosis is typical for *M. smithii* (Fernández-Marín et al. 2005), mature *M. smithii* colonies are invariably

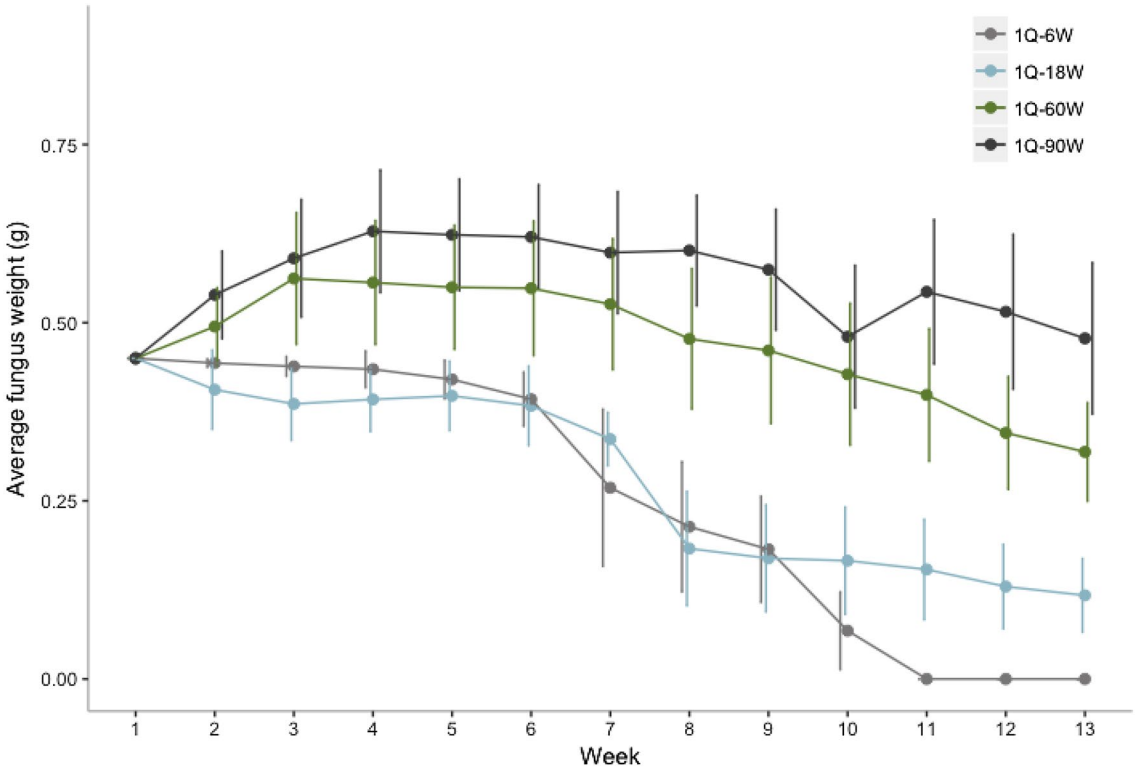

Fig. 4 Weekly fungus-garden weight (in g; mean ± SEM) over 13 weeks for *Mycocepurus smithii* nests starting with different number of workers (6, 18, 60, or 90 workers), 1 queen per colony, and an initial garden of 0.45 g. There is no significant difference in garden weights between 1Q-6W colonies and 1Q-18W colonies ($P = 0.409$). Garden weights of both 1Q-60W and 1Q-90W colonies were significantly greater than garden weights of 1Q-18W colonies ($P < 0.05$). There is no significant difference in garden weights between colonies with 1Q-60W and colonies with 1Q-90W ($P = 0.0504$, Table 4). Treatment differences were analyzed using a linear mixed model where worker number was treated as the fixed effect and week was treated as the random effect. Sample sizes were $N = 3$ for each of the four treatments 1Q-6W, 1Q-18W, 1Q-60W, and 1Q-90W. *Q* number of queens, *W* number of workers

highly polygynous (Rabeling 2004; Fernández-Marín et al. 2005; Rabeling et al. 2009, 2011). Fernández-Marín et al. (2005), therefore, hypothesized that *M. smithii* nests become secondarily polygynous after a monogyne nest-founding stage. This colony ontogeny must involve a crucial transition point where the addition of supernumary queens increases growth of the colony and the fungus garden, unlike the early nest-founding stage were supernumery queens may reduce garden growth (Figs. 1, 2). That is, to support supernumerary queens, the colony needs to possess sufficient fungus garden, which cultivated and maintained by a sufficient number of workers. In our second experiment, the optimal switch point from a single-queen to a multiple-queen phase is when the colony reaches a ratio of about 1 queen to 60 workers (1Q-60W), a ratio at which colonies can sustain the largest biomass of fungus garden when initially given 0.1 g of fungus garden (Fig. 3, 7). Our third experiment confirmed that queen-to-worker ratios of 1Q-60W and 1Q-90W maximize garden growth when colonies were started with, respectively, 0.45 g of fungus garden (Fig. 4). An optimal queen-to-worker ratio appears, therefore, critical for the incipient colonies observed by Fernández-Marín et al. (2005), the very small colonies tested in our experiments to understand the transition from single to multiple queens, and the mature, highly polygynous colonies of *M. smithii* typically collected by researchers. An inadequate number of workers for a given size of garden result in deficient garden maintenance, whereas an excess number of supernumerary queens reduces garden growth, because the food demands by the queens and their brood exceeds the carrying capacity of a garden. Our findings are consistent with the previous studies, showing that the initial worker number contributes critically to colony survival and competitive ability in both monogynous and polygynous ant species (Bartz and Hölldobler 1982; Rissing and Pollock 1991; Tschinkel and Howard 1983; Hölldobler and Wilson 1990).

Production of new queens

In our experiments, new daughter queens (gynes) were found only in colonies with specific queen-to-worker ratios. For example, in the fourth month of Experiment 2, new daughter

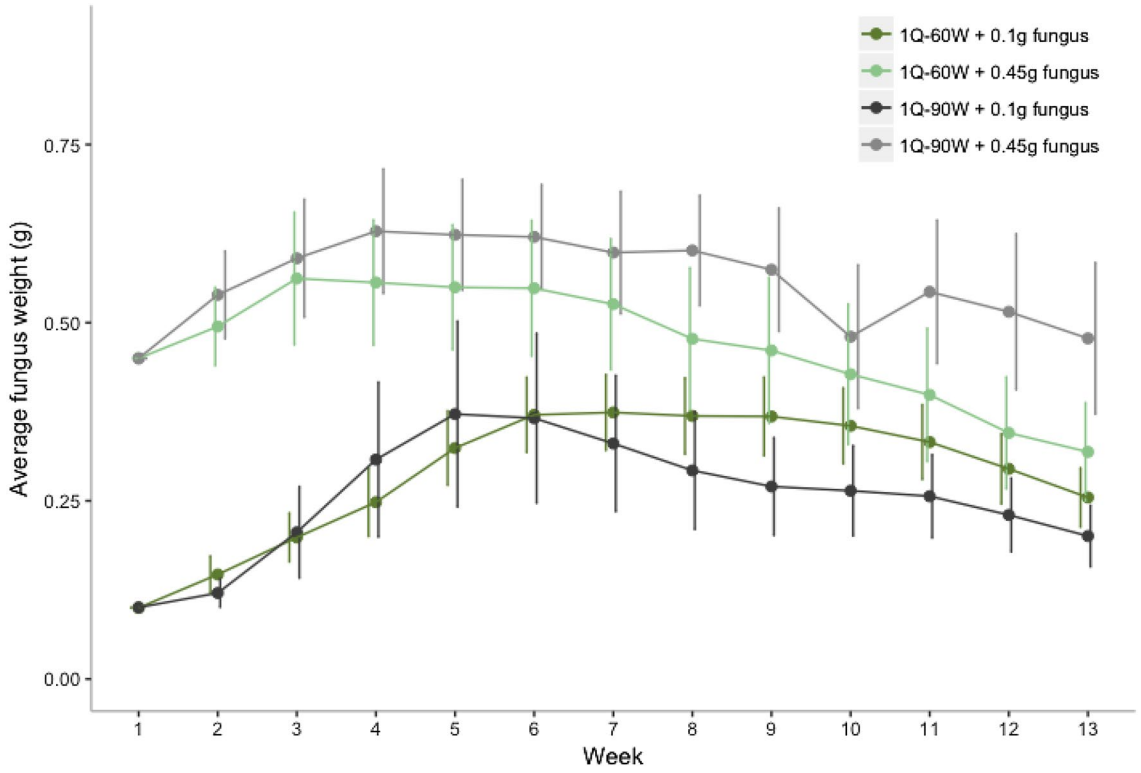

Fig. 5 Weekly fungus-garden weight (in g; mean ± SEM) over 13 weeks for *Mycocepurus smithii* nests starting with different number of workers (60 or 90 workers), 1 queen per colony, and initial garden weights of either 0.1 or 0.45 g. There is no significant difference in garden weights between colonies with 1Q-60W + 0.1 g fungus and colonies with 1Q-90W + 0.1 g fungus ($P = 0.579$). In addition, there is no significant difference in garden weights between colonies with 1Q-60W + 0.45 g fungus and colonies with 1Q-90W + 0.45 g fungus ($P = 0.0909$, Table 5). Not surprising, both colonies starting with 1Q-60W + 0.45 g fungus and colonies with 1Q-90W + 0.45 g fungus had significantly greater garden weights than colonies starting with 1Q-60W + 0.1 g fungus ($P < 0.01$). Treatment differences were analyzed using a linear mixed model that worker number was treated as the fixed effect, and both week and year were treated as the random effects. Sample sizes were $N = 10$, 9, 3, and 3 for treatments 1Q-60W + 0.1 g fungus, 1Q-90W + 0.1 g fungus, 1Q-60W + 0.45 g fungus, and 1Q-90W + 0.45 g fungus, respectively. *Q* number of queens, *W* number of workers

queens (gynes) were produced by colonies with 1Q-60W and 1Q-90W that started with 0.1 g of fungus garden. In contrast, colonies with 1Q-30W and 0.1 g garden needed twice the time (8 months) to produce the first daughter queens, and also by the fourth month, no gynes appeared in colonies with the same queen–worker combinations that started initially with 0.45 g of fungus in Experiment 4 (Fig. 5). We, therefore, hypothesize that new daughter queens are added in *M. smithii* under specific queen-to-worker ratios of ants tending a specific size of fungus garden. Our finding is the first such detailed study for *M. smithii* documenting the complete stepwise transitions from monogyny to polygyny through the production of supernumary queens.

Limits on egg-laying capacity and colony growth rate in *Mycocepurus smithii*

For eusocial insects, fitness is maximized by optimizing the colony output of female and male reproductives and the quality of these reproductives (Ratnieks and Reeve 1992). In the asexual *M. smithii*, overall colony output of female reproductives is limited in part by the low fecundity of queens, which, under optimal laboratory conditions (25 ± 1 °C; well-fed mature colonies), can produce only 1.12 ± 0.06 (SE) eggs per day (Fig. 6). Each queen of *M. smithii* has one pair of ovaries, each ovary is composed of three ovarioles, and each of the total of six ovarioles can generate only a single egg per day. This limits the number of eggs that a single queen can lay per day to maximally six eggs, but most queens exhibit a much lower daily fecundity (Fig. 6). In our experiment quantifying egg-laying potential, 69% of the queens (216 of a total of 313 queens tested) laid one or no egg within 24 h (Fig. 6). Because the queens used in this experiment were selected from the most productive ant clone A5 in our laboratory, this low fecundity suggests that queens in typical field colonies may exhibit even lower productivity than in our experiment.

A second reason for the low growth rate of *M. smithii* colonies is the relatively long developmental time from egg to adult. *M. smithii* eggs take around 20 days from oviposition

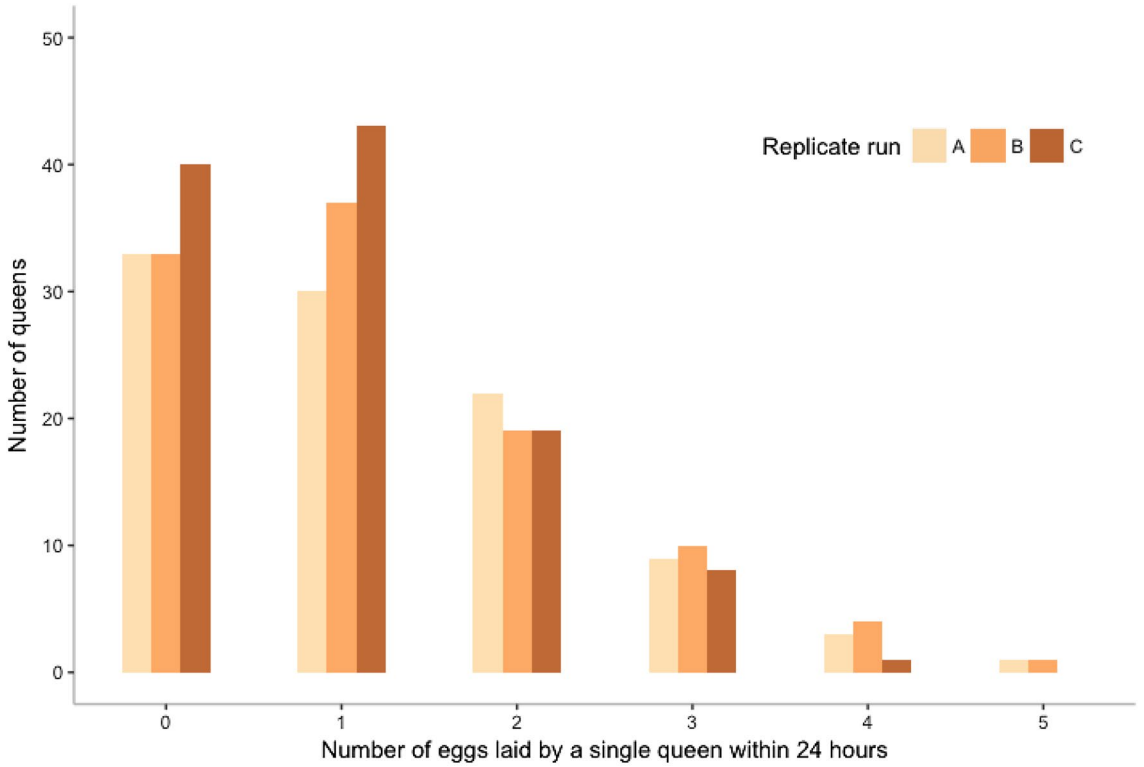

Fig. 6 Queen egg-laying productivity within 24 h in *Mycocepurus smithii*. Each queen was randomly selected from the fungus garden of a large source colony. We placed each queen in a separate container and counted the number of eggs laid within 24 h. The experiment was replicated in three separate runs, and egg-laying rates did not differ in any comparison between any two replicate runs (ANOVA, $df=2$, $P=0.217$). There was also no significant difference between any two replicate runs in the number of eggs laid using a Tukey's post hoc test (A–B: $P=0.9987$; A–C: $P=0.3076$; B–C: $P=0.2731$). The number of the queens tested in replicate run A, B, and C were, respectively, 98, 104, and 111. On average, each queen laid 1.12 ± 0.06 (SE) eggs within 24 h

to hatching, and development from first instar larva to a worker adult lasts around 40 days at 25 °C. Because gynes are slightly larger than workers, total development requires 60–70 days for a gyne at 25 °C in the laboratory (Fang and Mueller, unpublished observations). This slow development time is similar to the one observed for *M. smithii* in the field in Panamá (Fernández-Marín et al. 2005) where the first workers emerge 2–5 months after colony founding. This suggests the hypothesis that a mother queen produces new gynes to overcome the slow colony-growth rate by adding supernumary queens that are clonally identical to the mother queen in *M. smithii* (Fernández-Marín et al. 2005; Himler et al. 2009; Rabeling and Kronauer 2013), and these additional queens, therefore, contribute to brood production and, consequently, accelerate colony growth. This model of life-history strategy and optimal colony growth likely applies to other clonal ant species, as well (Rabeling and Kronauer 2013).

Implications for caste determination

When and how queen and worker castes are determined in ants—at an early developmental stage or late larval instar stage—is still unclear (Wheeler 1986; Grbic et al. 1997; Khila and Abouheif 2008, 2010; Fang 2019). Some evidence suggests that caste could be determined during the earliest embryological stages involving gene regulation that channels development into either a worker or a queen path already at the egg stage (Khila and Abouheif 2008, 2010). Other evidence suggests that caste can be influenced late in larval development, for example in those ant species where environmental factors and a nest's social environment affect caste determination in brood (Hölldobler and Wilson 1990; Wheeler 1994). Likewise, nutrients can influence caste determination (Richards and Packer 1994; Wheeler 1994), but also the queen-to-worker ratio and other such factors that depend on colony size (Boulay et al. 2009; Schmidt et al. 2011; Ruel et al. 2012). In addition, cues from eggs, larvae, and workers can influence caste determination in brood (Boulay et al. 2007; Endler et al. 2004; Warner et al. 2016). Our experiments on *M. smithii* add more evidence that caste determination is influenced by factors in the nest environment. Specifically, *M. smithii* gynes were produced in our experiments only after a colony cultivated a garden

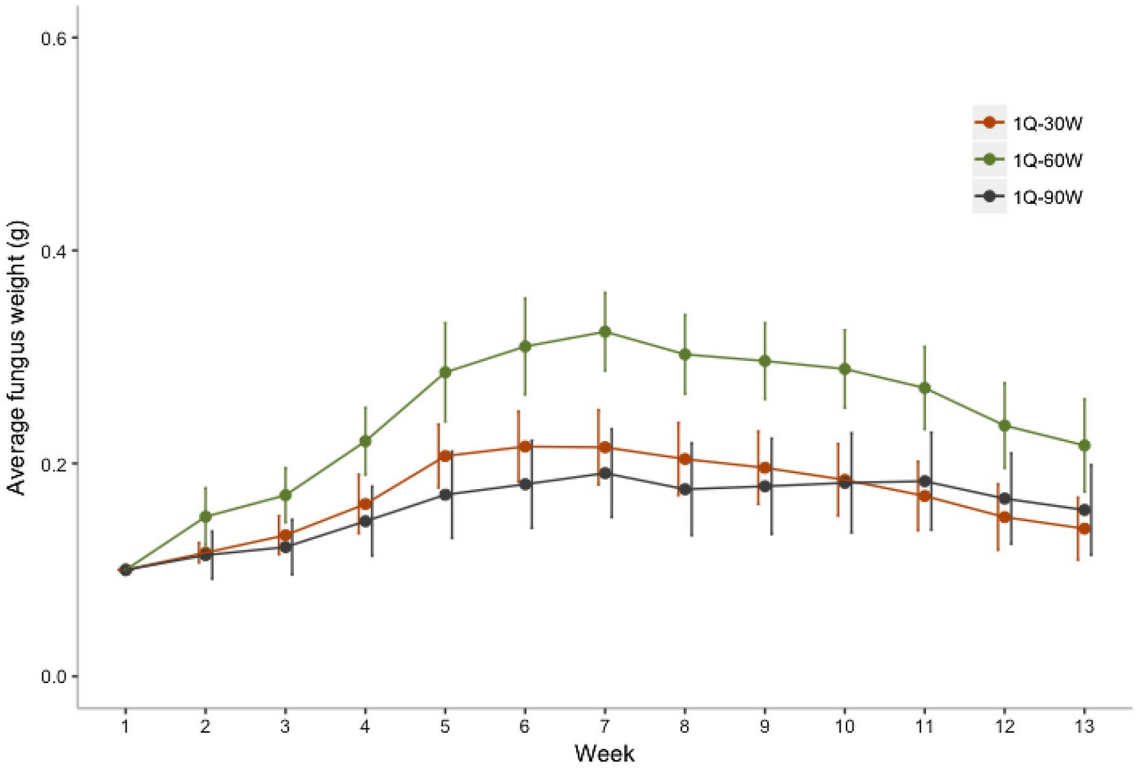

Fig. 7 Weekly fungus-garden weight (in g; mean ± SEM) over 13 weeks for *Mycocepurus smithii* nests starting with different number of workers (30, 60, or 90 workers), 1 queen per colony, and an initial garden of 0.1 g. The experimental series here includes only the replicate colonies from 2018 (7 replicates for each of the 3 treatments). The 1Q-60W colonies had significantly greater garden weights than 1Q-30W colonies ($P < 0.01$). There was no significant difference in garden weights between 1Q-30W colonies and 1Q-90W colonies ($P = 0.4984$). There is no significant difference in garden weights between colonies with 1Q-30W and colonies with 1Q-90W ($P = 0.7766$, Table 6). Treatment differences were analyzed using a linear mixed model where queen number was treated as the fixed effect and week was treated as the random effect. Sample sizes were $N = 7$, 7, and 7 for treatments 1Q-30W, 1Q-60W, and 1Q-90W, respectively. *Q* number of queens, *W* number of workers

of sufficient size, which was dependent on an optimal queen-to-worker ratio for maximal garden growth. While these findings document the importance of environmental factors in the caste determination of *M. smithii*, this does not bear on the controversy regarding the timing of early versus late-caste determination in brood development (Wheeler 1986; Grbic et al. 1997; Khila and Abouheif 2010), because it is possible that either (a) queens of *M. smithii* prime caste-development early by laying queen-destined eggs when a fungus garden reaches a sufficient size, or alternatively and independent of any queen factors, (b) the nest environment directly influences brood development at either egg, early larval, or late larval stages. Because of the ease of manipulating queen-to-worker ratios and garden size in *M. smithii*, and because of easy propagation in the laboratory due to its clonal reproduction, *M. smithii* could be a model system to elucidate the above processes of queen-mediated versus environmental caste determination.

Shortcomings of our study and recommendations for future research

Our study has several shortcomings, which do not invalidate the above findings, but which can be addressed in future investigations of *M. smithii*. First, we used CO_2 to anesthetize ants for weekly weighing of colonies; this procedure may have reduced the productivity of *M. smithii* queens, as exposure of honey bee queens to CO_2 reduces ovary activity (Berger et al. 2015; Koywiwattrakul et al. 2005). However, although we used CO_2 treatment, because it simplifies the weighing procedure, all experimental replicates were treated the same, so the observed differences between experimental treatments cannot be attributed to CO_2 exposure per se, but must be attributed to other parameters varied in the experiments (e.g., queen-to-worker ratios). Second, because we selected wingless *M. smithii* queens randomly from our laboratory colonies, and not winged foundress queens that may disperse from their natal nest under natural conditions, it is

possible that the behavior and fecundity of queens in our experiments differed from those of true foundress queens (e.g., garden consumption rate and fecundity of foundress queens under natural conditions may be somewhat different than the those of the average queen used in our experiments, but fecundity is still very low compared to other attine ants; Fig. 6). Founding queens of *M. smithii* observed in nature (semi-claustral queens) exhibit behaviors that distinguish them from queens under laboratory conditions (Fernández-Marín et al. 2004). In the field, semi-claustral foundress queens of *M. smithii* forage near their incipient colony for garden substrate rather than staying always in the safety of their nest (Fernández-Marín et al. 2004), whereas mature queens presumably do not forage in established nests with workers. This difference in queen behavior between our laboratory colonies and natural colonies may limit the extent to which our findings can be extrapolated to wild *M. smithii* foundresses. Third, we supplied our lab colonies with artificial gardening substrate (mix of polenta and oats) and lab colonies reared on artificial diet may show different ant-fungus allometries compared to field colonies, similar to what has been observed in *Trachymyrmex septentrionalis* (Seal and Tschinkel 2007, 2008). Altered allometries could mean that the absolute differences documented in Figs. 1, 2, 3, 4, and 5 may be greater or less pronounced under natural conditions, but the relative order of treatment effects will presumably be the same. Fourth, both queens and workers used in this study were randomly chosen from mature, healthy lab colonies, and such individuals may be physiologically in a better condition compared to queens and workers from resource-stressed young colonies in the field. If so, garden growth documented in Figs. 1, 2, 3, 4, and 5 for our lab colonies may be better than under field conditions, although again, the relative order of treatment effects will likely be the same. Fifth, fungus-garden growth exhibited an inverted *U* curve in all our experiments (Figs. 1, 3, 4, 5, 7), where garden weights increased initially for a period of the first 4–6 weeks, and then, garden weights declined until the end of each experiment. We do not know the exact reason for the eventual decline in garden weights in our experiments, but we suspect that a gradually increasing pathogen load may cause the reduction of fungus garden. Although the plastic containers used to house the experimental colonies were sterilized at the start of each experiment, pathogens (e.g., pathogenic fungi or bacteria) may accumulate during the multi-month experiments and eventually impede garden growth. Pathogens infect incipient attine colonies also in the field, as queens that found nests of social insects generally experience high mortality rates (Marti et al. 2015), but laboratory colonies may be plagued by higher pathogen loads than those found under natural conditions in the safety below ground. An improved experimental design, therefore, could regularly switch experimental colonies to new, sterilized containers (e.g., every 2–3 months). In fact, such a colony maintenance regime switching colonies regularly to new containers has allowed us to keep the same colony clones of *M. smithii* for nearly 20 years in the laboratory.

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