

# Late-instar ant worker larvae play a prominent role in colony-level caste regulation

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**Abstract** The success of social insect societies is often attributed to an efficient reproductive division of labor between queen and worker castes. At the group level, social insect colonies must decide both the timing and amount of resources to allocate to each caste. Queen production is typically restricted by factors such as season or the presence of fertile queens in colonies. In the absence of such inhibition, the relative production of new queens versus workers varies between and is regulated by colonies. Here, we investigate social regulation of caste in the ant *Monomorium pharaonis* through a series of experiments manipulating the environment in which developing larvae are reared. The number of new queens produced depended strongly on diet as well as the number of late-instar worker larvae in the colony at the time of caste regulation. Given that these late-instar larvae are known to process solid protein for the rest of the colony, their stimulatory effect on queen production is likely a result of their contribution to a colony's nutrient assimilation. Our results emphasize the need to consider late-instar larvae as critical social players and effectors of colony regulatory dynamics.

**Keywords** Reproductive division of labor · Caste regulation · Caste ratio · Late-instar worker larvae

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## Introduction

The phenomenon of caste determination and caste development in social insects has long been the focus of active research (for reviews, see Anderson et al. 2008; Schwander et al. 2010). Alternative reproductive castes (i.e., queens versus workers in hymenoptera) form the basis of the reproductive division of labor that defines eusocial insect societies. In many species, caste is mainly dependent on environmental, usually nutritional factors (Haydak 1970; Wheeler 1986; Hölldobler and Wilson 1990; Richards and Packer 1994). The genotype of individual developing larvae can also directly affect larval caste fate and, in some cases, is the sole determinant of caste (Anderson et al. 2008; Schwander et al. 2010). Finally, maternal age or condition can influence caste fate (Libbrecht et al. 2011), as can the genotype of care-giving worker nurses (Linksvayer 2006; Linksvayer et al. 2011; Teseo et al. 2014; Villalta et al. 2016b).

As each individual larva develops into either a queen or worker, caste fate can be considered an individual-level trait. However, caste fate is also socially regulated (e.g., via control of nutritional inputs; Linksvayer et al. 2011; Vojvodic et al. 2015), with the overall number of queens and workers produced by colonies determined by colony-level regulatory mechanisms, which we label “caste regulation”. In species that reproduce by colony fission, queen production is often restricted when colonies contain fertile queens, likely via pheromone (Vargo and Fletcher 1986; Edwards 1987; Vargo and Passera 1991; Boulay et al. 2009). When colonies do produce queens, they differ in their allocation of resources to different castes, defined here as “caste ratio”: the ratio of gynes (virgin queens) produced to the total number of gynes and workers produced (Schmidt et al. 2011). Caste ratio is a colony-level phenotype that varies

genetically between colonies of the same species (Linksvayer 2006; Libbrecht et al. 2011; Villalta et al. 2016b) and is affected by social factors such as colony size (Schmidt et al. 2011; Ruel et al. 2012).

The pharaoh ant *Monomorium pharaonis* is a globally widespread polygynous (multi-queen) tramp ant of unknown tropical origins (Berndt and Kremer 1986). It is considered to be one of the oldest and most successful invasive ants (Wetterer 2010). Like other species that reproduce by colony fission, the production of gynes and males in *M. pharaonis* is inhibited in colonies containing fertile queens. When queens are removed, egg production is halted completely, as workers lack ovaries (Bolton 1995). In this case, colonies begin to rear gynes and males from existing young brood but continue to produce workers concurrently (Edwards 1987; Schmidt et al. 2011). In this paper, we carry out a series of experiments to investigate the social mechanisms behind caste regulation in *M. pharaonis*.

As colonies with abundant eggs restrict male and gyne production but retain the ability to rear them from existing larvae, caste must be regulated at a relatively early larval stage. To identify the developmental timing of caste regulation, we first determined the developmental stages of larvae that may mature as adult gynes and males in newly queenless colonies. In doing so, we distinguish between the terms caste regulation and caste determination: our experiments do not investigate the precise timing of caste determination (i.e., at what developmental point an individual embarks on a caste-specific developmental trajectory and begins the process of caste differentiation). Rather, our experiments seek to identify the timing of caste regulation, which is the result of collective behavior that determines the number of each caste that are produced by the colony. Next, we attempted to identify social factors affecting differential allocation of resources to new reproductives (males and gynes) and workers. Late-instar worker larvae have been termed the “social stomach” of ant colonies due to their ability to process protein (Petralia et al. 1980; Tschinkel 1988; Cassill et al. 2005) and are known to have positive effects on queen fecundity (Tschinkel 1988; Børgesen 1989; Børgesen and Jensen 1995; Tschinkel 1995). Because differences in caste determination and differentiation in social insects are often associated with differences in nutrition (Haydak 1970; Wheeler 1986; Richards and Packer 1994; Aron et al. 2001; Smith and Suarez 2010; Schmidt et al. 2012), we hypothesized that late-instar larvae may affect caste regulation in *M. pharaonis*. We investigated whether the presence of such larvae affects male and gyne production before queen removal (via a possible maternal effect) or after queen removal. Lastly, to comprehensively evaluate the relative contribution of each demographic class to caste regulation after queen removal, we varied the

number of eggs, late-instar larvae, and adult workers and measured the relative production of each caste.

## Methods

### Basic setup

All colonies of *M. pharaonis* used in the current study were constructed by mixing several, genetically similar stock colonies originally obtained through the sequential crossing of eight separate lineages. Colonies were maintained at  $27 \pm 1$  °C, with 50 % RH and a 12:12 LD cycle. Colonies were fed ad libitum with an agar-based synthetic diet containing sugars and protein in a 3:1 ratio by mass (Dussutour and Simpson 2008) and supplemented with dried mealworms (*Tenebrio molitor*). Food was provided twice weekly. All surveys and manipulations were performed using dissecting microscopes. When individual brood stages were manipulated, ants were anesthetized using carbon dioxide and brood was gently moved using paintbrushes.

Larval instars were determined by hair presence and morphology as well as body size and shape (Berndt and Kremer 1986). Third instar larvae were defined as “small” until they reached  $\frac{3}{4}$  the size of an adult worker pupa, “medium” until the size of a worker pupa, and “large” thereafter. Demographic classes were defined as follows: young brood (eggs and 1st instar larvae), middle-aged larvae (2nd instar and small 3rd instar; see size 3rd instar size classification below), late-instar larvae (combining medium 3rd instar and large 3rd instar larvae), pupae and pre-pupae, and workers. The egg stage in *M. pharaonis* lasts about 8 days (Peacock and Baxter 1950), with each larval stage noted lasting about 3 days (personal observation, MRW).

In experiments 1, 2, and 4, colonies were created using ~3 mL of mixed brood, workers, and queens (typically resulting in 300–400 workers). This colony size was chosen because it is typically sufficient for the colony to produce gynes and males but small enough to make demographic manipulation feasible (personal observation, MRW). To induce colonies to shift from investment in only workers to new queens and males as well as workers, all queens were removed from the colonies. At the start of each experiment following queen removal, colonies were surveyed for all demographic classes. In all experiments, surveys following queen removal lasted about 4 weeks, until all individuals had matured to at least the pupal stage.

### Experiment 1: timing of caste regulation

Twenty-one colonies were created in September 2014. Seven replicates were assigned to each of three treatments, named by the type of young brood present following queen

removal: (1) “No young brood”, created by removing all eggs and 1st instar larvae, (2) “1st instar only”, created by removing eggs, and (3) “Control.” No individuals were removed from the latter, but eggs and 1st instar larvae were picked up with a paintbrush and placed at the nest entrance to simulate disturbance experienced by the other treatments. We focused on eggs and 1st instar larvae because worker- and queen-destined larvae can be distinguished visually by the 2nd instar (Berndt and Kremer 1986), so workers likely regulate caste before the 2nd instar. All colonies contained similar numbers of older colony members (i.e., older larvae, pupae, workers). To quantify the timing of caste regulation, the number of worker, gyne, and male pupae produced by each replicate colony was recorded.

### Experiment 2: effect of nutrition on caste regulation

Previous work has shown that social insect reproductive individuals (i.e., gynes and males) are fed diets composed of higher quantities of protein while they develop as larvae (Smith and Suarez 2010; Schmidt et al. 2012). To determine if diet affects caste regulation in *M. pharaonis*, 24 queenless colonies were reared with (“Supplemented”) and without (“Unsupplemented”) an insect protein supplement in September 2014. All colonies received were fed the standard 3:1 carb:protein diet from the same basic recipe used in all experiments (Dussutour and Simpson 2008) but also a 1:3 carb:protein diet. This diet contains whey protein, egg powder, and calcium caseinate as protein sources. 12 “Supplemented” colonies were fed dried mealworms. To quantify the effect of nutrition on caste regulation, the number of worker, gyne, and male pupae produced by each colony was counted.

### Experiment 3: effect of the presence of late-instar larvae on caste regulation

To assay the effect of late-instar larvae on caste regulation, their presence in colonies was manipulated before and after queen removal and reproduction was measured. In July 2014, 32 colonies were constructed and assigned to two types of treatment each: larval presence/absence before removal and larval presence/absence after removal, resulting in eight colonies of each treatment combination. Larval presence was manipulated before queen removal to determine if their presence results in a maternal effect, in which eggs laid by queens in the presence of larvae are caste biased in comparison to those laid by queens in the absence of larvae. Larval presence was manipulated afterwards to study their effect during male and gyne development (i.e., during the period of caste regulation).

16 colonies (“Larvae present, pre-queen removal”) were constructed with 400 adult workers, 15 queens, 100 pupae,

and 100 large and 50 medium 3rd instar larvae. 16 colonies (“Larvae absent, pre-queen removal”) were constructed with the same composition but lacking larvae. After 8 days, all queens, larvae, and pre-pupae were removed, eggs were counted, and worker pupae number was standardized to 100. Pupae number was standardized to control for the number of young workers that would eclose over the course of the experiment, since young workers are known to act as nurses (Mikheyev and Linksvayer 2015). 100 large and 50 medium 3rd instar larvae were immediately added to “Larvae present, post-queen removal” colonies to determine the effect of larvae on caste regulation while potential males and gynes develop. No larvae were added to “Larvae absent, post-queen removal” colonies. To quantify the effect of late-instar larvae on caste regulation, numbers of male and gyne pupae produced by each colony were recorded.

### Experiment 4: demographic effects on caste regulation

To assay the relative contribution of each demographic class to caste regulation, the number of eggs, late-instar larvae, and workers was manipulated and reproduction was measured. In June 2015, 36 colonies were constructed and queens were removed. After 14 days, all brood other than late-instar worker larvae were removed and 10 queens were added to each colony. Queens were allowed to lay eggs for 3–4 days and then removed. By this method, queenless colonies lacking brood stages other than eggs and late-instar worker larvae were created. Subsequently, these experimental colonies were supplemented with either eggs, larvae, or workers. No individuals were added to the control colonies, but disturbance was simulated by the same method as experiment 1. To supplement a colony, all individuals of the given demographic class (late-instar larvae, workers, or eggs) were added from two donor colonies, approximately tripling the number of individuals in that demographic class. Six replicate colonies of each of the four experimental conditions were created: “larvae added,” “workers added,” “eggs added,” and “control.” To quantify the effect of each demographic class on caste regulation, the number of worker, gyne, and male pupae produced by each colony was measured.

### Statistical analysis

All statistical analyses were performed using R, version 3.1.3 (R Core Team 2014). For all experiments, data were fitted using generalized linear models (GLM), using measured demographic classes and/or treatments as predictors of gyne, male, or worker production. In experiment 3, a full model was constructed with both larval presence/absence before and after and their interaction as well as the number

of eggs at queen removal as predictors. Because each colony corresponded to a single observation for a given measured quantity (i.e., number of gynes produced), no random factor was used. In experiment 4, replicates within treatments varied widely with regard to the starting number of each demographic class, so data were analyzed using initial worker, late-instar larvae, and egg numbers as continuous predictors, rather than treatment as a categorical predictor. Data for experiment 4 were also analyzed using Pearson's product-moment correlation.

Count data were analyzed using a Poisson error distribution (log-link) or quasipoisson if the residuals were overdispersed based on the function "dispersiontest" from the R package AER (Zeileis and Kleiber 2008). Caste ratio was analyzed with a binomial model (logit-link) or quasibinomial when overdispersed. The significance of models was compared using a likelihood ratio test (LRT). A Tukey's post hoc Test was implemented using the R package multcomp (Hothorn et al. 2008). All plots were generated using ggplot2 (Wickham 2009). All data and R scripts are included as supplemental material.

## Results

### Experiment 1: timing of caste regulation

Colonies without eggs or 1st instar larvae produced no gynes (Fig. 1b), indicating that caste is determined at latest by the end of the 1st larval instar. Such colonies also produced no males (Fig. 1a). "Control" colonies containing eggs and 1st instar larvae did not differ in male or gyne production from those containing only 1st instar larvae (Fig. 1; Tukey's post hoc test;  $df = 2$ ; males produced:  $P = 0.161$ , gynes:  $P = 0.413$ , caste ratio:  $P = 0.963$ ). The number of new workers produced was not affected by the treatment or other colony demographic quantities (LRT;  $P > 0.05$ ), indicating that colony-level caste regulation is at least initially focused on the recognition and removal of reproductive-destined brood (see Villalta et al. 2016a for a similar conclusion).

### Experiment 2: effect of nutrition on caste regulation

The availability of dried mealworms affected the number of gynes, workers, and males produced, as well as the caste ratio (LRT; males  $\chi^2 = 171.85$ ,  $P < 0.001$ ; gynes  $\chi^2 = 156.60$ ,  $P < 0.001$ ; workers  $\chi^2 = 9.14$ ,  $P = 0.003$ ; caste ratio  $\chi^2 = 56.78$ ,  $P < 0.001$ ); colonies supplemented with mealworms produced more of each individual (Fig. 2; GLM; males  $t_{22} = 7.51$ ,  $P < 0.001$ ; gynes  $t_{22} = 6.88$ ,  $P < 0.001$ ; workers  $t_{22} = 3.37$ ,  $P < 0.001$ ) and a higher caste ratio (GLM; caste ratio  $t_{22} = 4.09$ ,  $P < 0.001$ ). While

it is unsurprising that supplemented colonies performed better overall, it is notable that colonies continued to produce workers in unsupplemented colonies but nearly completely ceased gyne and male production (Fig. 2).

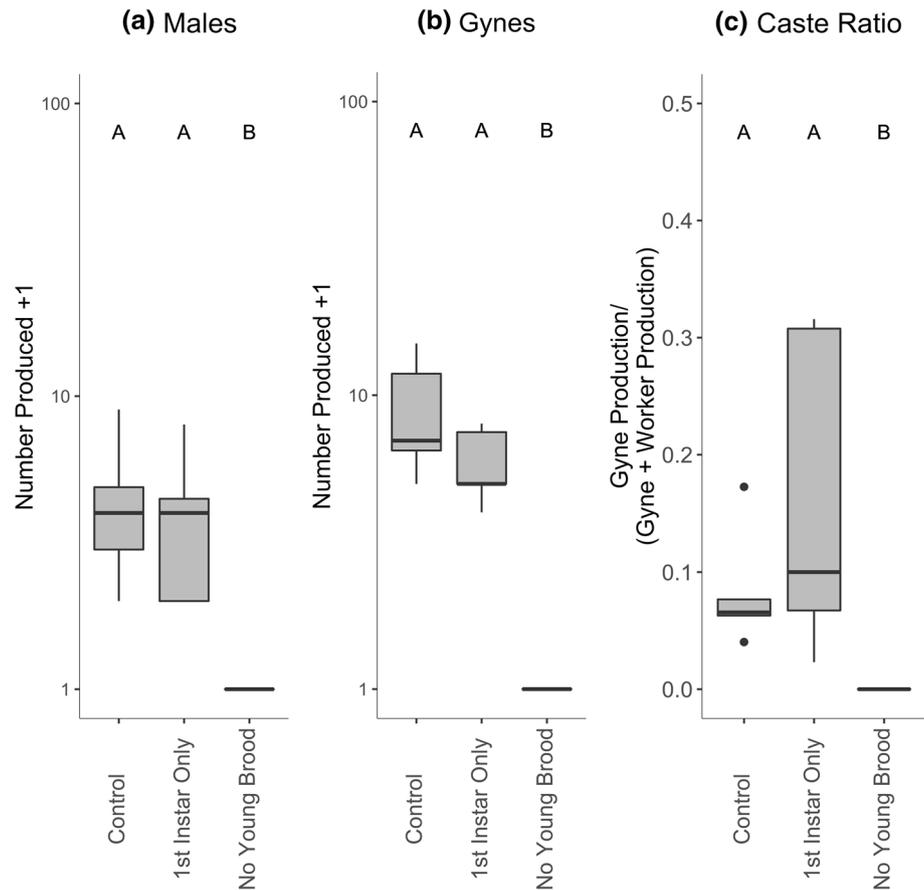
### Experiment 3: effect of the presence of late-instar larvae on caste regulation

The presence of late-instar worker larvae before queen removal affected the number of eggs at removal (LRT;  $\chi^2 = 9.47$ ,  $df = 1$ ,  $P = 0.002$ ); colonies supplemented with late-instar larvae produced  $683.15 \pm 59.45$  (mean  $\pm$  SE) eggs compared to  $443.79 \pm 49.92$  for control colonies. The number of eggs and larval presence after queen removal affected male and gyne production (LRT; effect of number of eggs on male production  $\chi^2 = 7.40$ ,  $P = 0.007$ , on gynes  $\chi^2 = 11.93$ ,  $P < 0.001$ ; effect of larval presence after removal on males  $\chi^2 = 7.16$ ,  $df = 1$ ,  $P = 0.008$  on gynes  $\chi^2 = 17.28$ ,  $df = 1$ ,  $P < 0.001$ ). Both larval presence after queen removal (Fig. 3) and number of eggs stimulated male and gyne production (GLM, egg effect on males  $z_{23} = 2.776$ ,  $P = 0.006$ , on gynes  $t_{23} = 3.53$ ,  $P = 0.002$ ; larval presence after effect on males  $z_{23} = 2.50$ ,  $P = 0.012$ , on gynes  $t_{23} = 3.76$ ,  $P = 0.001$ ). The presence of larvae before removal had no effect on male or queen production ( $P > 0.05$ ). Therefore, while late-instar larvae boost queen fecundity, there is no evidence of a maternal effect on caste fate driven by such larvae. However, it is possible that such an effect would only be present with a longer treatment than 8 days. There was no significant interaction between larval presence before queen removal and larval presence after queen removal on the number of males or gynes produced (LRT;  $df = 1$ ; males  $P = 0.487$ , gynes  $P = 0.227$ ). Five colonies were removed from the experiment due to errors in colony creation.

### Experiment 4: demographic effects on caste regulation

Gyne production and caste ratio were positively correlated with initial larvae number (Fig. 4; Pearson correlation; gynes produced  $r = 0.632$ ,  $P < 0.001$ ; caste ratio  $r = 0.540$ ,  $P = 0.007$ ) but not correlated with initial egg or worker number ( $P > 0.05$ ). Male production was not correlated with any initial demographic variable ( $P > 0.05$ ), and worker production was correlated positively with initial egg number ( $r = 0.746$ ,  $P < 0.001$ ), negatively with initial worker number ( $r = -0.459$ ,  $P = 0.024$ ), and not correlated with initial number of larvae ( $P > 0.05$ ). When data were analyzed using the full generalized linear model, the number of larvae affected gyne production and caste ratio (LRT; gynes produced  $\chi^2 = 11.37$ ,  $P < 0.001$ ; caste ratio  $\chi^2 = 6.97$ ,  $P = 0.008$ ), but not male or worker production

**Fig. 1** Reproduction as a function of the young brood present in colony after manipulation. Letters correspond to differences between treatments based on Tukey's post hoc test. **a** Colonies lacking eggs and 1st instar (*No Young Brood*) larvae produced zero males, while control colonies (containing eggs and 1st instar larvae) and those containing only first instar larvae did not differ. **b** Colonies lacking eggs and 1st instar larvae produced zero gynes, while control colonies (containing eggs and 1st instar larvae) and those containing only first instar larvae did not differ. **c** Colonies lacking eggs and 1st instar larvae produced a lower caste ratio, due to a lack of gynes produced, while control colonies (containing eggs and 1st instar larvae) and those containing only first instar larvae did not differ. Counts were rescaled by one so they could be plotted on a log scale.  $N = 7$  for all treatments



( $P > 0.05$ ). Larvae increased both gyne production and caste ratio (GLM; gynes  $t_{20} = 3.58$ ,  $P = 0.002$ , caste ratio  $t_{20} = 2.75$ ,  $P = 0.012$ ). Worker number did not have an effect on the production of any pupal type ( $P > 0.05$ ). The number of eggs had a positive affect on worker production (LRT;  $\chi^2 = 3.434$ ,  $P = 0.0026$ . GLM;  $t_{20} = 3.43$ ,  $P = 0.003$ ) but not on gyne or male production or caste ratio ( $P > 0.05$ ).

## Discussion

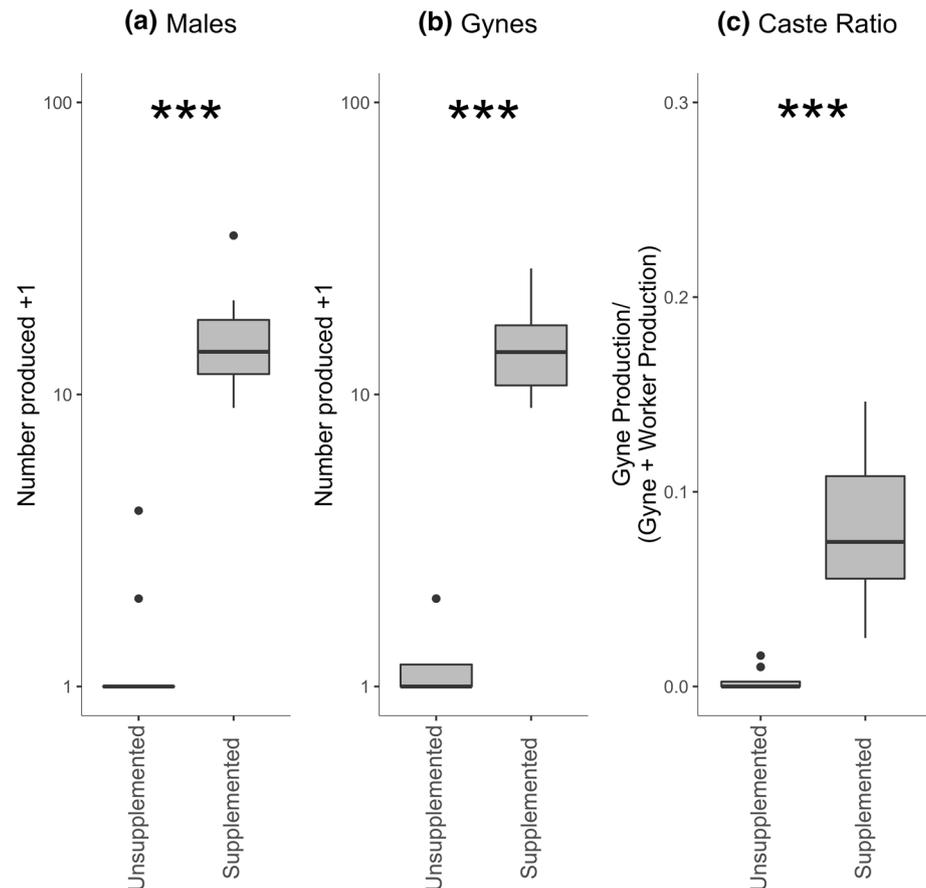
Social insect colonies must efficiently allocate resources to workers and gynes to maximize growth and reproduction. Caste regulation is thus a colony-level collective behavior that may have a major impact on colony fitness. Here, we show that late-instar worker larvae are critical players in caste regulation in *Monomorium pharaonis*. Eggs and 1st instar larvae that matured to pupae were more likely to develop into gynes than workers with increasing numbers of late-instar larvae. The numbers of eggs and workers had no effect on gyne production in experiment 4, while worker production was stimulated by the number of eggs. These results suggest that worker production is limited by eggs,

but gyne production is limited primarily by the number of late-instar larvae a colony contains.

Because late-instar worker larvae are known to be an essential protein processing part of the colony (Petralia et al. 1980; Tschinkel 1988; Cassill et al. 2005), it is likely that their stimulatory effect is mediated by nutrition. As their capacity to process proteins increases, colonies can feed developing larvae more proteinaceous food. Both harvester ant (*Pogonomyrmex badius*) and yellowjacket wasp (*Vespa maculifrons*) gynes and males are fed a diet from a higher trophic level as larvae than their worker counterparts (Smith and Suarez 2010; Schmidt et al. 2012). In experiment 2, supplementing colonies with insect protein, which increased diet quality and quantity, allowed the production of males and gynes. Unsupplemented colonies produce a lesser number of workers. In experiment 3, colonies with late-instar larvae present during male and gyne development produced more individuals of each sex, so it seems likely that these two results are linked: the presence of late-instar larvae allows colonies to more efficiently process insect protein and thereby rear more males and gynes.

The effect of late-instar larvae on caste regulation could exist purely because of nutritional limitation: colonies lacking or with few late-instar larvae simply

**Fig. 2** Reproduction as a function of availability of mealworms. Colonies supplemented with mealworms in addition to an agar-based diet produced more **a** males, **b** gynes, and a higher **c** caste ratio. \*\*\*Indicates  $P < 0.001$  by LRT (see “Results”). Counts were rescaled by one so they could be plotted on a log scale.  $N = 24$



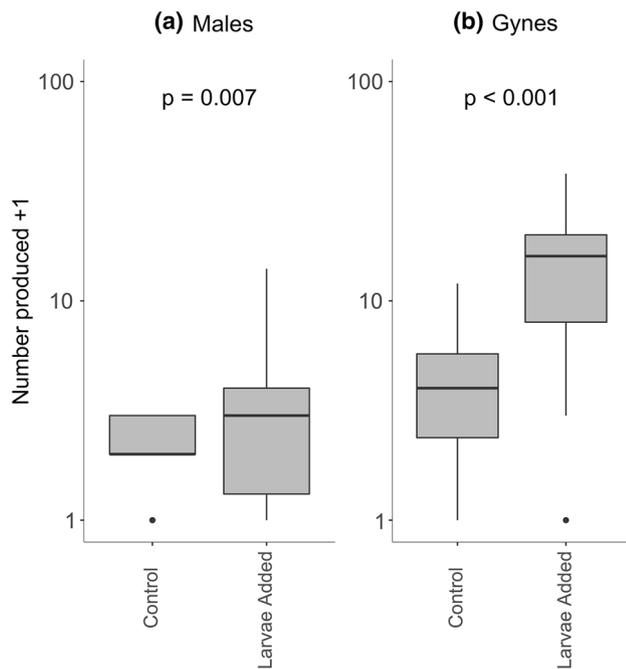
cannot produce as many reproductives. However, this regulation may also be adaptive. The number of adult workers a colony will contain in the near future is likely highly correlated with the number of late-instar it contains at the present. Therefore, colonies with few perspective adult workers (late-instar larvae) may concentrate on worker production, while those with many may allocate more resources to reproductives.

While the number of reproductives produced depended on aspects of their rearing environment, the identity of specific individuals may still be entirely dependent on their genotype. For males, this must be the case as sex is determined by ploidy (Peacock et al. 1954; i.e., ants are haplodiploid). Therefore, haploid, male-destined, brood must be present in a colony before queen removal in order for the colony to produce males. Because colonies were only able to produce males if they contained eggs or 1st instar larvae, male-destined haploid individuals are likely constantly produced but culled prior to maturing to the 2nd larval instar in queen-present colonies. The selective removal of male larvae by workers has been noted in other ant species (e.g., Aron et al. 1994, 1995; Keller et al. 1996; Passera and Aron 1996). Interestingly, male larvae do not appear to be culled as 1st instar larvae, so workers may be

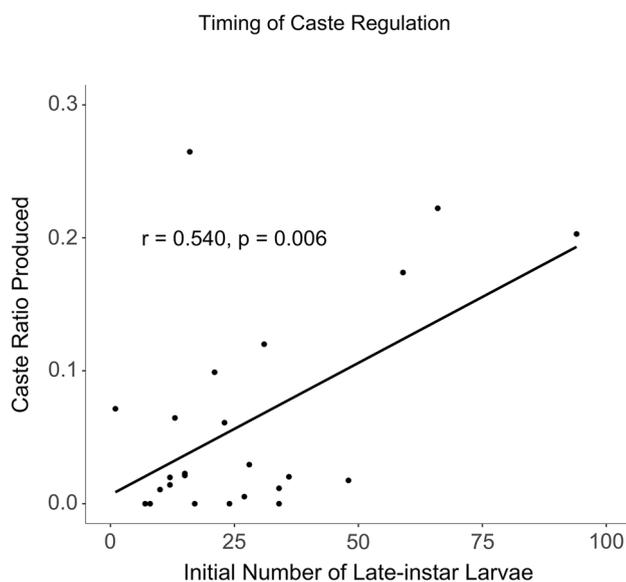
unable to recognize males (possibly via pheromonal differences) until the 2nd larval instar.

It is possible that workers similarly cull excess gyne-destined larvae. Indeed, in the fission-performing *Aphaenogaster senilis*, workers selectively remove gyne-destined larvae in the presence of a fertile queen (Villalta et al. 2016a). *M. pharaonis* colonies normally contain many queens, so egg production may be cheap enough that colonies can simply hedge their bets by producing all types of eggs they may need and cannibalizing excess reproductive-destined eggs. Whatever the mechanism of caste determination, colonies retain remarkable allocation flexibility; colonies containing only 1st instar larvae did not differ in caste ratio or caste production from those containing eggs as well as 1st instar larvae, despite having fewer total individuals that may become reproductives.

In the future, it will be valuable to work to identify the precise mechanism of caste determination in *M. pharaonis*. *Monomorium emersoni*, whose workers also lack ovaries, shows possible evidence of caste determination occurring during the egg stage. *M. emersoni* eggs from colonies producing only workers lack germ-line development characteristic of worker eggs in species whose adult workers have ovaries, suggesting these eggs are pre-destined to be



**Fig. 3** Reproduction as a function of the presence of larvae after queen removal. Colonies marked “Larvae Added” were supplemented with late-instar larvae after queen removal, while those marked *Control* contained 0 late-instar larvae after queen removal. Colonies containing late-instar larvae after queen removal produced more **a** gynes and **b** males than those without late-instar larvae. *P* values were calculated by LRT based on GLM model (see “Results”). Counts were rescaled by one so they could be plotted on a log scale.  $N = 27$  (14 *Larvae Added* and 13 *Control*)



**Fig. 4** Caste ratio [gyne production/(gyne + worker production)] produced by colonies as a function of the number of late-instar larvae they contained after queens removed. Colonies produced more gynes relative to total number of females when they contained more late-instar larvae. Trendline shown in *black* is calculated from linear model. Pearson correlation,  $df = 22$

workers (Khila and Abouheif 2010). Our study suggests that caste is determined prior to the second larval instar, so it is possible that a similar mechanism exists in *M. pharaonis*, and that larvae are simply not noticeably morphologically different until the second larval instar.

Two main discrepancies exist between the results of experiments 3 and 4. First, while the number of eggs did not affect gyne or male production in experiment 4, it did affect the production of both in experiment 3. In general, colonies had many more eggs in experiment 3— $559.04 \pm 44.52$  (mean  $\pm$  SE) versus  $125.83 \pm 17.39$  in experiment 4. It is unlikely that the differential effect of eggs is due to their limitation, as the opposite effect would be expected: a positive relationship between egg number and male/gyne production in colonies with fewer eggs. Alternatively, colonies with more eggs in experiment 3 may have used excess eggs as nutrition, increasing their ability to rear reproductives in experiment 3. It is possible that there were not sufficient numbers of eggs in experiment 4 for this effect to be present.

A second discrepancy between the experiments is that the number of late-instar larvae positively affected male production in experiment 3 but had no effect on male production in experiment 4. Late-instar larvae may have more of a qualitative than quantitative effect on male production. All colonies contained at least some late-instar larvae in experiment 4, while colonies lacking larvae in experiment 3 had none at all. Therefore, a small number of late-instar larvae may have a stimulatory effect on male production, while increasing larval number beyond some threshold has no effect. This would be the case if male production is limited, for example, by the processing of some vital nutrient that is only required in small quantities. In this case, it may be that a small number of late-instar larvae are sufficient to make such a nutrient non-limiting. *M. pharaonis* males mate in the nest and can mate with multiple females (Fowler et al. 1993), so it may be adaptive for colonies to produce only a small number of males while continuing to increase gyne production with the number of late-instar larvae. If male production is dependent on late-instar larvae by such a threshold mechanism, it is likely that the results of experiment 4 more closely resemble the effect of late-instar larvae on male production in natural colonies.

Caste regulation as presented here is the combination of inhibitory and stimulatory factors. When colonies contain fertile queens, gyne and male production is inhibited (Edwards 1987). When this inhibition is released, colonies retain the ability to produce reproductives, and late-instar larvae have a stimulatory effect on their production. The combination of inhibitory and stimulatory factors on caste regulation may be a general phenomenon in fission-performing species, even if the precise mechanisms differ among species. For example, while eggs inhibit gyne and

male production in *M. pharaonis* (Edwards 1987), they have no effect on caste regulation in *Solenopsis invicta* (Vargo and Fletcher 1986), *Linepithema humile* (Vargo and Passera 1991), and *Aphaenogaster senilis* (Ruel et al. 2013). In these three species, all of which also reproduce by fission (note that the *S. invicta* colonies studied were polygynous), pheromone on queens seems to regulate caste development. Inhibition in *M. pharaonis* could be still driven by surface hydrocarbons present on eggs, as queens in the species *Camponotus floridanus* regulate worker reproduction via such secretions (Endler et al. 2004).

In the future, it will be valuable to see if the effect of late-instar worker larvae on caste regulation applies to other species, both those that reproduce by fission and found colonies independently. Studying species with known genetic caste determination would be particularly illuminating. Larvae also may play more general roles. Recent research discovered an effect of larvae on reproduction and foraging in a clonal ant, a species that completely lacks castes (Ulrich et al. 2015), and larvae are known to inhibit worker reproduction in four ant species (Heinze et al. 1996; Teso et al. 2013; Ebie et al. 2015; Villalta et al. 2015). Here, our results suggest that regardless of the system of caste determination in *M. pharaonis*, eggs that may become gynes are over-abundant and late-instar larvae play the most prominent role in caste regulation in queenless colonies. It is possible that this is a general phenomenon, and that even in species with genetic caste determination gynecastid eggs are abundant so social partners actually play the strongest role in regulation. In general, this study shows that while larval behavior is more subtle than worker behavior, they nonetheless are important social partners that contribute to colony-level processes.

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