



Symposium Article

# The Collective Behavior of Ant Groups Depends on Group Genotypic Composition

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## Abstract

Recently, researchers have documented variation between groups in collective behavior. However, how genetic variation within and between groups contributes to population-level variation for collective behavior remains unclear. Understanding how genetic variation of group members relates to group-level phenotypes is evolutionarily important because there is increasing evidence that group-level behavioral variation influences fitness and that the genetic architecture of group-level traits can affect the evolutionary dynamics of traits. Social insects are ideal for studying the complex relationship between individual and group-level variation because they exhibit behavioral variation at multiple scales of organization. To explore how the genetic composition of groups affects collective behavior, we constructed groups of pharaoh ants (*Monomorium pharaonis*) from 33 genetically distinct colonies of known pedigree. The groups consisted of either all workers from the same single colony or workers from two genetically different colonies, and we assayed the exploration and aggression of the groups. We found that collective exploration, but not aggression, depended on the specific genotypic combination of group members, i.e., we found evidence for genotype-by-genotype epistasis for exploration. Group collective behavior did not depend on the pedigree relatedness between genotypes within groups. Overall, this study highlights that specific combinations of genotypes influence group-level phenotypes, emphasizing the importance of considering nonadditive effects of genotypic interactions between group members.

**Subject Area:** Quantitative genetics

**Key words:** aggression, exploration, genotype  $\times$  genotype epistasis, group-level phenotypes, social insects

Collective behavior, defined as the behaviors of groups of individuals that operate without central control (Gordon 2014, 2016), is widespread in nature. Recent research on collective behavior has focused on documenting behavioral variation between groups that is consistent across time or context (i.e., collective or group personality) (Gordon 1991; Bengston and Jandt 2014; Jandt et al. 2014). For example, harvester ant (*Pogonomyrmex barbatus*) colonies differ consistently across years in their regulation of foraging rate (Gordon 2002; Greene and Gordon 2007; Gordon et al. 2007; Gordon et al.

2011). Although there has been a large focus on understanding mechanistically how local behavioral interactions between individuals produce emergent group-level behavior (Sumpter 2010), the genetic architecture of collective behavior remains largely unknown (Walsh et al. 2020b), including the degree to which it is heritable and how genetic variation within and between groups contributes to population-level variation for collective behavior.

Additionally, it is unclear exactly how variation in individual-level traits leads to variation in group-level traits (Pinter-Wollman 2012;

LeBoeuf and Grozinger 2014; Ulrich et al. 2021). For example, we do not know whether each group member contributes equally to determining group-level behavior or if instead some individuals (i.e., “keystone individuals”) have a disproportionately large effect. Furthermore, it is unclear how the genotypic make-up of groups affects group-level traits. For example, we do not know if individuals of a particular genotype have a consistent impact on the collective behavior of the group regardless of the genotype of other group members, or if the effect of the genotype of group members depends on the specific genotype of other group members (i.e., whether there are genotype-by-genotype interactions for collective behavior). Understanding how the genotypes of group members map to group-level phenotypes is evolutionarily important because there is increasing evidence that group-level behavioral variation influences fitness and that the genetic architecture of group-level traits can affect the evolutionary dynamics of traits (Wray et al. 2011; Modlmeier et al. 2012; Gordon 2013; Blight et al. 2016a; Blight et al. 2016b; Walsh et al. 2020b).

Social insects are ideal for elucidating the complex relationship between individual and group-level variation because they exhibit behavioral variation at multiple scales of organization (i.e., between workers, castes, colonies, species) (Jandt et al. 2014). Variation between individuals within a colony results in a division of labor, where queens reproduce while workers perform all other tasks, including foraging for food and caring for larvae (Wilson 1971; Oster and Wilson 1978; Beshers and Fewell 2001). Workers can further specialize on a wide range of tasks, resulting in division of labor within the worker caste (Oster and Wilson 1978; Robinson 1992; Beshers and Fewell 2001; Mikheyev and Linksvayer 2015; Walsh et al. 2018). Social insects exhibit consistent colony-level variation for a wide range of collective behaviors, including aggression, foraging, and exploration (Bengston and Jandt 2014; Jandt et al. 2014; Jeanson and Weidenmüller 2014).

There is increasing evidence that colony-level behavioral variation is influenced by genotype as numerous studies have estimated the heritability of social insect collective behavior (Snyder 1993; Linksvayer 2006; Hunt et al. 2007; Gordon 2013; Greenwood et al. 2015; Friedman and Gordon 2016; Walsh et al. 2020b) and candidate gene studies have linked allelic variation to variation in collective behavior (Krieger 2005; Wang et al. 2008; Wang et al. 2013; Tang et al. 2018). The amount of genetic variation within social insect colonies depends on whether the colony includes one (monogyny) or multiple queens (polygyny), whether queen(s) mates with one (monandry) or multiple males (polyandry), and the amount of inbreeding (Keller 1993; Bourke and Franks 1995; Boomsma and Ratnieks 1996; Oldroyd and Fewell 2007; Haag-Liautard et al. 2009). Both polyandry and polygyny are widespread and are thought to be favored because genetic variation within a colony may increase disease resistance (Baer and Schmid-Hempel 1999; Schmid-Hempel and Crozier 1999; Tarpay 2003; Hughes and Boomsma 2004; Reber et al. 2008) or the efficiency of division of the labor (Crozier and Page 1985; Oldroyd and Fewell 2007).

A small number of studies have attempted to better understand how behavioral variation in workers influences colony-level behavioral variation by creating mixed groups that included workers with different behavioral tendencies. In these cases, one behavioral type exhibited “behavioral dominance” over the other and caused individuals within the group to behave more similarly to the dominant type. For example, in mixed colonies of docile European honey bees and aggressive Africanized honey bees, honey bees increased their aggression with the number of Africanized bees in the colony

(Guzmán-Novoa and Page 1994). Similarly, fire ant workers will accept either one or multiple queens based on their genotype in the *Gp-9* nonrecombining region, and workers from monogyny (one queen) colonies will accept multiple queens if just 5–10% of the colony consists of workers from polygyny colonies (Ross and Keller 2002; Gotzek and Ross 2008).

To better understand the evolution of collective behavior, we must understand how the genotypic composition of the group affects variation in collective behavior. In general, few studies have utilized an experimental design creating mixed groups and assaying continuous, rather than discrete, behavioral variation, including in social insects (but see Linksvayer 2006, 2007; Wang et al. 2008; Linksvayer et al. 2009; van Zweden et al. 2010; Linksvayer et al. 2011; Teseo et al. 2014; Vojvodic et al. 2015 for studies on non-behavioral traits), which would allow us to understand how the genotypic composition of the group affects social insect collective behavior (Moore et al. 1997; Agrawal et al. 2001; Bijma et al. 2007a; Bijma et al. 2007b; Bijma and Wade 2008; McGlothlin et al. 2010; Bijma 2011). When individuals interact within a group, they can affect each other's traits and group-level traits in an additive (i.e.,  $G + G$ ) or nonadditive manner (i.e.,  $G \times G$ ) (Wolf et al. 1998; Wade 2000). Nonadditive effects between interacting individuals have been called  $G \times G$  epistasis because epistatic interactions can exist between loci in the genomes of two interacting individuals (Wolf et al. 1998; Culumber et al. 2018; Jaffe et al. 2020; Wade 2000). Such additive and nonadditive interaction effects are predicted to play an especially large role in the evolution of behavior because behavior, more so than other phenotypes, is flexible, depending on biotic and abiotic environmental conditions (Bailey et al. 2017). Furthermore, these effects are predicted to play a larger role in the evolution of behavior in social insects because of their highly complex societies that rely on social interactions (Linksvayer and Wade 2005; Linksvayer 2006; Linksvayer 2015).

In this study, we used pharaoh ant (*Monomorium pharaonis*) colonies from a pedigreed laboratory population. Previous work on colonies from this same population found that colonies consistently varied in collective behavior and that this variation was heritable and associated with colony productivity (Walsh et al. 2020b). Understanding how genetic variation within a group affects group-level phenotypes is especially important in unicolonial species (i.e., “super colonies”; species that span beyond a simple colony structure but instead spread across many nest sites) like *M. pharaonis*. Such colonies contain many queens, and workers may move between neighboring colonies in nature, so that genetic variation within a colony is expected to be relatively high and various genotypes may interact and influence colony function and productivity. To explore the effect of the genotypic composition of group members on the resulting collective behavior of the group, we set up groups of workers that contained either workers all from the same colony (control colonies) or from two different colonies, and we assayed the aggression and exploration of these groups. We used 33 colony genotypes in total and each colony genotype was paired with at least 5 other colony genotypes. We considered aggression (our aggression assay measured the aggregate aggression of *M. pharaonis* workers toward *Tetramorium immigrans* workers, see Methods) and exploration to be collective behaviors, rather than individual-level behaviors, because they both consist of emergent patterns of individual workers operating through local interactions, either through the influence of pheromones or through direct antennal contact (Adler and Gordon 1992; Gordon and Mehdiabadi 1999; Gordon 2002; Greene and Gordon 2007; Gordon 2010; Pinter-Wollman et al. 2013; Kleineidam et al. 2017).

## Methods

### Experimental Design

We used 33 *M. pharaonis* colonies, which we subsequently refer to as “colony genotypes,” from our heterogeneous stock mapping population. This pedigreed population was started by intercrossing 8 distinct lineages collected across North America, Asia, Africa, and Europe for 9 generations (Schmidt 2010; Pontieri et al. 2017; Walsh et al. 2020a; Walsh et al. 2020b). To control for the age of the workers used in our study, we collected newly eclosed workers and used these age-matched workers to construct experimental groups for our study: we anesthetize source colonies with carbon dioxide and carefully removed 450 worker pupae from each colony genotype using a paint brush. We separated these worker pupae into 3 separate petri dishes (150 pupae per dish) and monitored the dishes daily for the eclosion of callow workers for each colony genotype. We collected the callows and placed 9 callows into separate petri dishes for each colony genotype. Five days after the callows eclosed, we combined 2 groups of 9 callows each, either both from the same colony (“same colony groups”) or from different colonies (“mixed groups”), to form a larger group of 18 workers that we subsequently assayed for collective behavior. We refer to the two groups of workers that made up the larger group of 18 workers as “genotype one” and “genotype two.” Experimental designs including just two, rather than multiple, genotypes/families within a group have been shown theoretically to be optimal for estimating the genetic effects of group members (Bijma 2010). We were able to mix workers from different colonies because *M. pharaonis* workers show little to no aggression toward conspecifics from other colonies (Schmidt et al. 2010; Pontieri 2014). We included a total of 33 colony genotypes in our study, and the behavioral assays (see details below) were divided into 6 blocks that each ran for about two weeks from May to August of 2018. Within each block, containing 3 to 6 total colony genotypes, each colony was paired with itself (i.e., same colony group) and with each of the other colonies 3 times (i.e., 3 replicates for each combination). We fed all groups of workers with an agar-based synthetic diet (Dussutour and Simpson 2008) and provided water ad libitum via a glass tube plugged with cotton. We kept all groups of workers on a 12:12 hour light:dark cycle and at  $27 \pm 1$  °C and 50% relative humidity.

### Behavioral Assays

Two or three days after combining the 2 groups of 9 workers into one larger group, we assayed the exploratory and aggressive behavior of the larger groups following the protocols from Walsh et al. (2020a). Because some of the ants died during the aggression assay, we always conducted the exploratory assay before the aggression assay. We conducted the exploratory assay inside of a filming box with white LED lights along the walls and a camera mounted to the top (Walsh et al. 2020a). To prevent trail pheromones from previous assays influencing future assays, we covered the floor of the filming box with a poster board that we replaced between each assay. We placed the 18 workers inside a Petri dish and placed the Petri dish upside down in the middle of a circular arena in the center of the filming box and waited 5 min for the ants to settle down after being handled. Next, we removed the petri dish, allowing the ants to explore the entirety of the circular arena, and used the camera to record the ants exploring the arena for 10 min. Finally, we collected all 18 workers and returned them to their Petri dish. We analyzed the videos using the R package “trackR” (<https://github.com/swarm-lab/trackR>), which tracked the location of all the ants in each frame of

the video. We calculated the percent of the arena explored by the groups of ants by determining how many pixels were visited at least once across all frames of the video divided by the total number of pixels inside the circular arena (Walsh et al. 2020a).

We began the aggression assay at least 2 h after the completion of the exploratory assay. Because *M. pharaonis* workers only show transient to no aggression toward conspecifics (Schmidt et al. 2010; Pontieri 2014), we quantified aggression of the *M. pharaonis* workers toward a second species, *Tetramorium immigrans* (Wagner et al. 2017). We collected the *T. immigrans* colony on the campus of the University of Pennsylvania during May of 2018 and maintained and fed the colony using the same methods we used for the *M. pharaonis* groups. We moved the 18 *M. pharaonis* workers to a small Petri dish and 18 *T. immigrans* workers to a second small Petri dish and placed the Petri dishes upside down inside a larger petri dish. We waited five minutes to give the ants time to acclimate after being handled and then lifted the small Petri dishes, allowing the ants of the 2 species to interact with each other. Every five minutes for one hour, we recorded the number of *M. pharaonis* workers biting *T. immigrans* workers. We defined the aggression of the groups as the average number of *M. pharaonis* workers biting *T. immigrans* workers across all observations within an hour. We froze all *T. immigrans* workers used in the assay so that we did not reuse the same workers in subsequent assays. We only managed to record aggression data for 5 of the 6 experimental blocks because our *T. immigrans* colony started to run out of workers to use in the assays.

### Statistical Analyses

We performed all analyses in R version 3.6.0 (R Core Team 2014). The colonies included in this study were all from the same pedigreed lab population (Pontieri et al. 2017; Walsh et al. 2020a; Walsh et al. 2020b) and therefore were all related to each other to some degree. First, we tested whether observed collective behavior across all genotypic combinations depended on the pedigree relatedness of the 2 interacting genotypes. We conducted Spearman rank correlation tests between the behavior (either exploration or aggression) and the pairwise relatedness estimates between genotypes. The pairwise relatedness estimates were calculated using the R package “MCMCglmm” (Hadfield 2010). Furthermore, to better understand how the interaction between the 2 genotypes making up the mixed groups affected group behavior, we built animal models to estimate the best linear unbiased predictors (BLUPs) for each genotype given the observed trait values (i.e., for the 2 collective behaviors) and known pedigree relatedness among colony genotypes. These BLUPs correspond to the additive expectation for trait values for each genotype. Next, we asked if the observed behavior of the group was correlated with the combined BLUP for each genotype combination using Spearman rank correlation tests (see [Supplementary Materials](#) for more information).

Given that the relatedness between interacting genotypes did not affect collective behavior (see Results), we next determined how observed collective behavior depended on the specific additive and nonadditive combinations of each pair of genotypes, not considering pedigree relatedness between genotypes. To estimate the effects of the 2 genotypes individually and the additive and interaction effects between them, we used generalized linear models. We included the experimental block and either the additive or interaction effects between the 2 genotypes as fixed effects. Our exploration data was normally distributed (Shapiro Wilk test;  $W = 0.991$ ,  $P = 0.080$ ) and we used generalized linear models with Gaussian distributions. We

conducted likelihood ratio tests to compare the models and evaluate the significance of the effects. Because our aggression data showed overdispersion with the initial Poisson distribution (dispersion test;  $z = 8.68$ ,  $P < 0.001$ ), we used generalized linear models with negative binomial distributions. To determine effect size of each term included in each model, we used the “r.squaredGLMM” function of the R package “MuMIn” (Barton 2009).

## Results

Neither group exploration nor group aggression depended on the pairwise pedigree relatedness of the 2 genotypes comprising each group (exploration, 2-tailed Spearman rank correlation,  $\rho = -0.031$ ,  $P = 0.657$ ; aggression  $\rho = -0.132$ ,  $P = 0.072$ ), indicating that the pedigree relatedness between group members, per se, did not affect collective behavior. Observed group exploration and aggression were significantly correlated with the expected level based on the additive combination of genotypes (exploration,  $\rho = 0.363$ ,  $P = 0.001$ ; aggression,  $\rho = 0.507$ ,  $P < 0.001$ ), as estimated as the sum of BLUPs for the 2 genotypes, indicating that group behavior can be predicted by the combined additive expected phenotype of each genotype making up groups (Supplementary Figure 1). However, the observed phenotypes for many genotypic combinations, in particular for exploration, deviated from BLUP additive expectations, suggesting that nonadditive interactions may also be important (Supplementary Figure 1); see Supplementary Material for more information).

To explore the potential contribution of additive and non-additive effects of genotype in more detail, we treated genotypes as being independent (i.e., ignoring assumed pedigree relationships). For aggression, neither the additive nor nonadditive effects were significant (Table 1). For exploration, both additive ( $G + G$ ) and nonadditive ( $G \times G$ ) effects were significant (Table 1), indicating that the specific genotypic makeup of each group influenced collective exploration (Figure 1). The estimated proportion of variance explained by the nonadditive ( $G \times G$  epistasis) effect was 0.383, which was greater than the estimated proportion of variance explained by the additive effect which was 0.283 (Table 2). Block was also significant for both aggression and exploration (Table 1).

## Discussion

In this study, we used a phenotypically and genetically variable laboratory population of the pharaoh ant *M. pharaonis* to study the importance of intra-group genetic composition in the production of emergent group-level behavior. In groups of ants composed of workers from 2 different colony genotypes, the effect of the genotype of group members was conditional on the other group members

(i.e., the interaction term was significant) for exploration, suggesting  $G \times G$  epistasis is important. Overall, our results highlight the importance of specific genotypic combinations of group members on collective phenotypes in general.

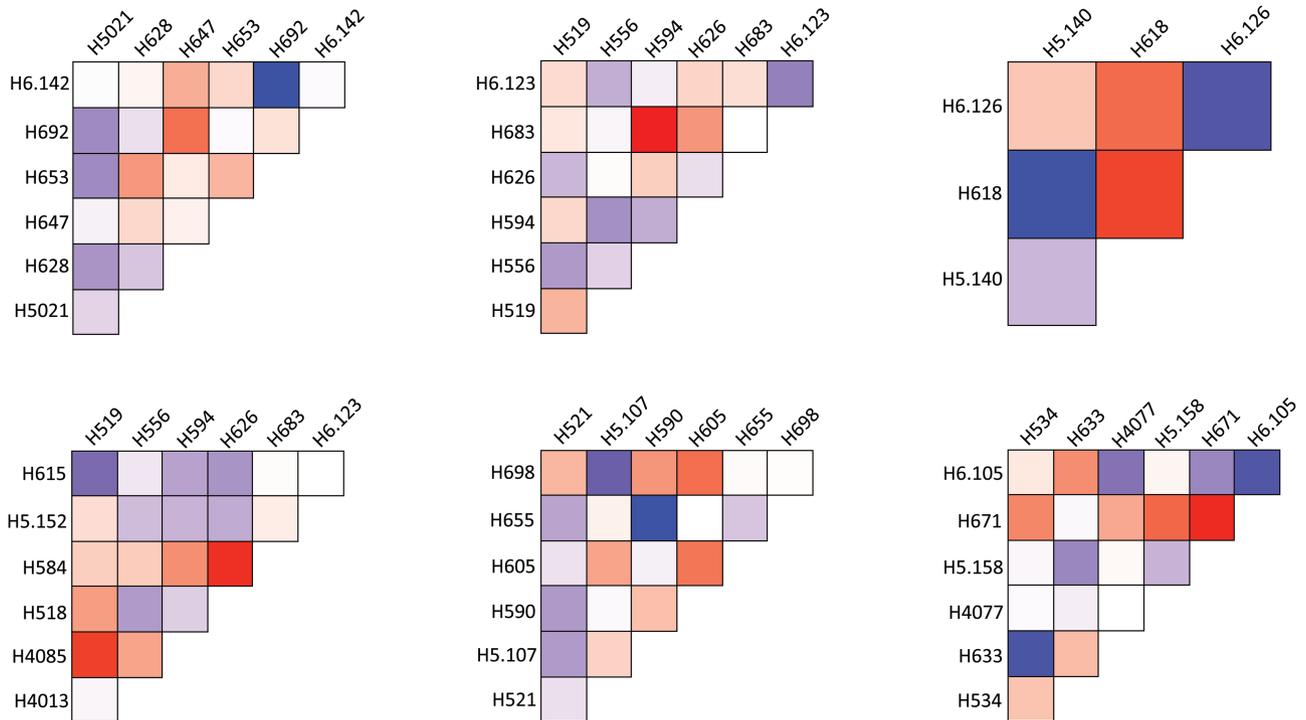
For exploration, nonadditive effects of genotypic combinations (i.e.,  $G \times G$  epistasis) explained a larger proportion of variance than additive effects, suggesting that the specific combinations of genotypes within a group affects variation in collective behavior (Figure 1). In general in nature, group-level phenotypes depend on potentially complex genetic interaction effects between group members, so that models only considering additive genetic variance among individuals may often not be adequate (Moore et al. 1997; Wolf et al. 1998; Agrawal et al. 2001; McGlothlin et al. 2010). Our results suggest that models explicitly incorporating  $G \times G$  epistasis will be useful for understanding the evolution of group-level traits (Bijma 2014; Bailey et al. 2017). Notably, the indirect genetic effects framework considers how the phenotypes of individuals are affected directly by their own genotype and indirectly by the genotype(s) of social partners (Moore et al. 1997; Wolf et al. 1998; Agrawal et al. 2001; McGlothlin et al. 2010). While these indirect genetic effect models are focused on individual-level traits and do not explicitly consider how the genetic makeup of group members affects group-level traits, they are conceptually closely related and likely readily extended to include group-level traits (McGlothlin et al. 2010).

Although social interactions, and therefore the potential for additive effects and  $G \times G$  epistasis, occur between conspecifics of almost every species, these effects are predicted to be especially important for social insects (Linksvayer 2015). Social insect colonies are characterized by a division of labor between individuals within the colony, which requires frequent communication between individuals, and by cooperative brood care by workers (Oster and Wilson 1978; Beshers and Fewell 2001; Linksvayer and Wade 2005; Linksvayer 2006; Linksvayer 2015). The occurrence and biological importance of  $G \times G$  epistasis for colony traits and colony performance likely depends on the amount of genetic variation within the colony. The amount of genetic diversity within a social insect colony depends on a number of factors including whether the colony is monogynous or polygynous, the level of polyandry, and the amount of inbreeding (Keller 1993; Bourke and Franks 1995; Boomsma and Ratnieks 1996; Oldroyd and Fewell 2007; Haag-Liautard et al. 2009). We would expect the effect of  $G \times G$  epistasis on colony-level phenotypes to be larger in colonies with higher levels of within-colony genotypic variation, due to more queens, higher levels of polyandry, and low levels of inbreeding. Additionally, in unicolonial species, including *M. pharaonis*, individual workers can freely move between neighboring colonies, leading to more genetic diversity (Giraud et al. 2002) and a greater potential for  $G \times G$  epistasis between genotypes. Finally, genetic diversity within social insect colonies allows for “social heterosis,” the maintenance of genetic diversity through a mutualistic benefit of the interindividual expression of multiple alleles at a single locus (Nonacs and Kapheim 2007).

The response-threshold model postulates that workers within a social insect colony differ intrinsically, possibly due to genotypic differences (Page and Robinson 1991), in the stimulus level at which they begin to behaviorally respond (Wilson 1976; Robinson 1987; Beshers and Fewell 2001). Increased genetic diversity within a social insect group would increase the likelihood that at least some individuals would respond to a stimulus at a given level. Therefore, we might expect collective behavior to be correlated with the pairwise relatedness within a group. For example, *M. pharaonis* group-level aggression could potentially increase as genetic diversity increases

**Table 1.** A summary of GLM results on exploration and aggression

Exploration	$\chi^2$	df	P
Block	18.71	5	0.002
Genotype 1 + Genotype 2	98.57	54	<0.001
Genotype 1 $\times$ Genotype 2	87.72	55	0.003
<b>Aggression</b>			
Block	57.38	4	<0.001
Genotype 1 + Genotype 2	57.60	44	0.082
Genotype 1 $\times$ Genotype 2	49.34	41	0.174



**Figure 1.** Heatmaps showing the average level of exploration between genotypic combinations across 6 different blocks. Blue signifies high levels and red signifies low levels of exploration. Darker shades of either color signify more extreme values.

**Table 2.** Estimates of additive and nonadditive combinations of interacting genotypes for exploration

Exploration	R <sup>2</sup>
G + G	0.283
G × G epistasis	0.383
Block	0.061

(as relatedness within the group decreased) because there would be a greater chance that some group members would respond aggressively to the threat and recruit others, through the use of alarm pheromones, to also respond aggressively. Similarly, collective exploration could increase with genetic variation because there may be a greater chance that some individuals may venture out into open areas, rather than staying close to the starting point, and begin laying trail pheromone, which may encourage others to follow. However, we found that group-level aggression and exploration were not associated with within-group relatedness. This result is perhaps not surprising, given that unicolonial species like *M. pharaonis* show little to no aggression toward conspecifics (Schmidt 2010; Schmidt et al. 2010; Pontieri 2014) and workers from neighboring colonies often intermix.

Overall, this study highlights the importance of the specific combinations of genotypes in shaping collective behavior. We detected an effect of G × G epistasis when only studying small groups of 18 workers, which are much less complex than typical *M. pharaonis* colonies that can include thousands of workers in addition to multiple queens and brood at different developmental stages, suggesting that these effects are widespread, as predicted. Furthermore, our small groups included only 2 distinct genotypes, while real colonies may have a wider range of genotypes. Additionally, our study was conducted in the laboratory, under carefully controlled environmental conditions. In a natural setting, genotype-by-environment

interaction effects are likely very common, further complicating how genetic composition affects group-level traits. Future studies should tease apart how the genotypic composition of group members influences social interactions through different types of social communication (e.g., pheromones, physical interactions, trophallaxis) and how social interactions influence phenotypic variation across all colony members (e.g., queens, workers, brood).

## Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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## Data Availability

The data accompanying this manuscript are available as online Supplementary Material.

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