

# GENETIC COMPATIBILITY AFFECTS DIVISION OF LABOR IN THE ARGENTINE ANT *LINEPITHEMA HUMILE*

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Division of labor is central to the organization of insect societies. Within-colony comparisons between subfamilies of workers (patrilines or matriline) revealed genetic effects on division of labor in many social insect species. Although this has been taken as evidence for additive genetic effects on division of labor, it has never been experimentally tested. To determine the relative roles of additive and nonadditive genetic effects (e.g., genetic compatibility, epistasis, and parent-of-origin imprinting effects) on worker behavior, we performed controlled crosses using the Argentine ant *Linepithema humile*. Three of the measured behaviors (the efficiency to collect pupae, the foraging propensity, and the distance between non-brood-tenders and brood) were affected by the maternal genetic background and the two others (the efficiency to feed larvae and the distance between brood-tenders and brood) by the paternal genetic background. Moreover, there were significant interactions between the maternal and paternal genetic backgrounds for three of the five behaviors. These results are most consistent with parent-of-origin and genetic compatibility effects on division of labor. The finding of nonadditive genetic effects is in strong contrast with the current view and has important consequences for our understanding of division of labor in insect societies.

**KEY WORDS:** Behavior, division of labor, *Linepithema humile*, social insects.

Division of labor is characterized by the performance of different tasks or roles by different groups of individuals. The most striking examples of division of labor in nonhumans occur in insect societies. By enhancing colony performance and homeostasis, division of labor is thought to be one of the main factors responsible for the tremendous ecological success of ants, termites, bees, and wasps (Wilson 1971; Oster and Wilson 1979; Holldobler and Wilson 1990).

Task specialization within a colony results from workers differing in their responses to environmental signals indicating colony needs for specific tasks (Wilson 1971; Oster and Wilson 1979; Robinson and Page 1989a). Four main factors are known to influence worker thresholds for particular tasks. The first is worker size and morphology, both of which correlate with worker

behavior in almost all species in which this has been studied (e.g., Wilson 1980; Detrain and Pasteels 1991; Robinson et al. 2009). The second is age, as evidenced by workers frequently moving from one task to another as they become older (Wilson 1971; Seeley 1982). The third is individual experience, which has been shown to influence task preference in a few species (Theraulaz et al. 1998; Ravary et al. 2007). Finally, in almost all species studied, the genetic background of workers seems to affect their likelihood to undertake different tasks (Oldroyd and Fewell 2007). Besides some reports of artificial selection for honeybee worker behaviors (Rothenbuhler 1964; Pérez-Sato et al. 2009), evidence for genetic effects on task specialization comes primarily from the finding of differences between subfamilies (matriline or patriline) in the worker likelihood and efficiency to perform different



tasks in species where colonies contain either several queens or one multiply-mated queen. Such genetic effects on task performance have been shown to influence a wide range of behaviors in ants (Stuart and Page 1991; Snyder 1992, 1993; Blatrix et al. 2000; Julian and Fewell 2004; Schwander et al. 2005; Waddington et al. 2010), bees (Calderone and Page 1988; Frumhoff and Baker 1988; Robinson and Page 1988, 1989b; Estoup et al. 1994; Kryger et al. 2000; Jones et al. 2004) and termites (Kaib et al. 1996; Goodisman and Crozier 2003).

The finding of between-matriline and between-patriline differences in task performance has led to the realization that task specialization and division of labor are influenced by genetic effects. Authors usually discussed these effects as if they were simple additive genetic effects. However, the mere demonstration of maternal or paternal effects on division of labor in colonies with several matriline or patriline does not allow one to discriminate between additive and nonadditive genetic effects such as parent-of-origin specific effects associated with imprinting, epistasis, and genetic compatibility effects between parental genomes (Schwander and Keller 2008; Libbrecht et al. 2011). In both cases, one would expect an association between matriline and patriline affiliation and the performance of specific tasks. The only way to discriminate between additive and nonadditive effects is to conduct controlled crosses to quantify paternal and maternal effects, as well as the interaction between parental effects.

So far such controlled crosses have only been used twice in social insects, the aim being to investigate the effects of parental genetic backgrounds on various aspects of caste allocation. In the seed-harvester ant *Pogonomyrmex rugosus*, the process of caste determination was shown to be strongly influenced by interactions between the parental genomes, but neither by the paternal nor the maternal colony of origin (Schwander and Keller 2008). Similarly, in the Argentine ant *Linepithema humile*, controlled crosses revealed both significant parent-of-origin influences and interactions between parental genomes on various components of caste allocation (Libbrecht et al. 2011). Although there have been numerous studies investigating the roles of paternal and maternal effects on division of labor, surprisingly no controlled crosses have been conducted so far and it remains completely unknown whether genetic effects on division of labor stem from additive effects or more complex, nonadditive effects such as parent-of-origin influences or interactions between the parental genomes.

The aim of this study was to conduct controlled crosses in the Argentine ant *L. humile* to investigate the genetic components affecting worker behavior and patterns of division of labor. In contrast to most other ants, it is possible to obtain both males and queens in the laboratory and to mate them under controlled conditions (Keller and Passera 1992; Libbrecht et al. 2011). By conducting controlled crosses, we investigated the effects of both

the maternal and paternal genetic backgrounds, as well as their interaction, on five measures of worker behavior that were indicators of the likelihood of workers to perform several tasks and their efficiency to perform them.

## Methods

### PRODUCTION OF PARENTAL LINEAGES

The first step of our experiment consisted in producing sufficient numbers of unmated queens and males to conduct the controlled crosses in the laboratory. To this end, we collected *L. humile* colonies on 11 February 2008 in Port-Leucate (3°2'20"E, 42°51'22"N), southern France and established 13 single queen colonies with each 2.5 cm<sup>3</sup> (ca. 1000) workers in the laboratory. Colonies were kept under a 12h:12h artificial light:dark cycle at 25°C, 60% humidity, and were fed a mixture of mealworms, eggs, honey, and vitamins three times a week. To ensure that colonies contained only brood from the mother queen, we removed all the brood present during the first 2 weeks. The queens were then allowed to lay eggs during 8 weeks before being removed so as to stimulate the production of new queens and males (Keller and Passera 1992, 1993). Colonies were then regularly checked to transfer all the male and queen pupae produced to queenless and broodless recipient colonies, set up to each receive the pupae of only a single sex and colony. This design allowed us to obtain large numbers of unmated queens and males of the same lineage (i.e., produced by the same mother queen). These individuals were used to conduct the controlled crosses.

### CONTROLLED CROSSES

Of the 26 (13 female and 13 male) recipient colonies, six produced enough new queens and four produced enough males to conduct replicate crosses between these maternal and paternal lineages. Mating was obtained by placing one unmated queen with four to six males overnight in a 6.5-cm diameter vial (Keller and Passera 1992). Several lines of evidence show that queens were singly inseminated, despite the presence of several males. First, genetic analyses of the sperm from spermathecae of 87 *L. humile* queens collected from the field were consistent with all queens being singly inseminated (Krieger and Keller 2000). Second, laboratory experiments showed that, when several males were given access to one virgin queen in *L. humile*, only one was found to have emptied its seminal vesicles at the end of the experiment in 44 cases out of 47. Moreover, the number of sperm found in queen spermathecae was consistent with the number of sperm transferred by one male only (Keller et al. 1992; Keller and Passera 1992). Finally, in experimental conditions similar to that of the present study in which virgin *L. humile* queens were given access to several males, genetic analyses of queens and the sperm stored in

their spermathecae revealed no evidence of multiple mating (more than 450 samples genotyped at eight microsatellites) (Fournier and Keller 2001; Keller and Fournier 2002). Importantly, all these studies were done with ants collected from the same population (Port-Leucate) as in the present study. The crosses allowed us to obtain between two and eight singly inseminated queens for 22 of the 24 possible maternal-by-paternal lineage combinations. The 110 newly mated queens were then overwintered with ca. 1000 workers for 3 months in the dark at  $10 \pm 2^\circ\text{C}$ , 60% humidity to trigger the production of sexual offspring (Vargo and Passera 1992), which were used in another study (Libbrecht et al. 2011).

### SINGLE-COHORT COLONIES

Worker behavior is known to be affected by age (Wilson 1971; Seeley 1982) and experience (Theraulaz et al. 1998). To control for these factors, all experiments were performed on single-cohort colonies (Giray and Robinson 1994) consisting of groups of workers of the same age with similar nursing and foraging experience. To produce such workers, each mated queen was placed with a new set of ca. 600 workers (collected randomly in a stock colony composed of a mix of several field colonies collected on February 16, 2009) and no brood (hence ensuring that all the brood later collected in each colony was produced by the mated queen), in  $20 \times 14 \times 5$  cm transparent plastic boxes. To set up the single-cohort colonies, 50 worker pupae were collected from each colony and isolated with five to seven marked workers (collected randomly in the above-mentioned stock colony) that took care of them, ensuring the emergence of adult workers. The marked workers were removed from the single-cohort colonies as soon as the first workers emerged from the pupae. The behavioral experiments were all performed in the same environmental conditions with workers collected in the single-cohort colonies 4 weeks after the isolation of pupae. We quantified the efficiency of workers to feed larvae, their speed to collect pupae, their foraging propensity, and their average distance to the brood.

### EFFICIENCY TO FEED LARVAE

The efficiency to feed larvae was assessed by measuring the mass gained by larvae when tended by workers for 3 days. For each single-cohort colony, 20 workers were collected and introduced in a 5-cm diameter plastic box containing 10 third-instar larvae randomly collected from a stock colony. The workers and larvae were kept for 3 days in complete darkness with water and food very close to the larvae, so that the workers did not have to forage. The larvae were weighed before and after the 3 days using a microbalance (Mettler Toledo MT5, Mettler Toledo, Columbus, OH) to a precision of  $1 \mu\text{g}$ . The difference between the initial and final larval mass was used as a proxy for the efficiency of worker feeding behavior.

### EFFICIENCY TO COLLECT PUPAE

The efficiency to collect pupae was quantified as the time needed by 20 workers to collect 12 pupae. For each single-cohort colony, 20 workers were anesthetized with  $\text{CO}_2$  and introduced in an arena ( $5 \times 10.5$  cm) containing 12 pupae evenly distributed around the center of the arena. All these pupae were randomly collected from a stock colony. We videotaped the arena in the dark using an infrared camcorder (Sony HDR-XR 200, Sony, Japan) and recorded the time needed to collect the 12th pupae once the first worker woke up.

### FORAGING PROPENSITY

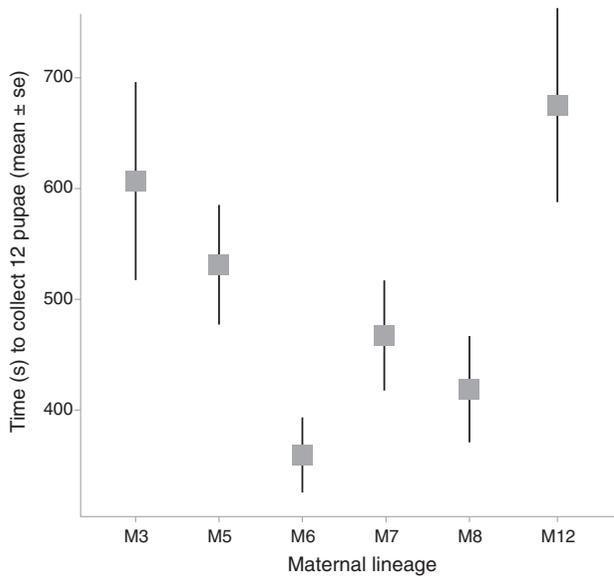
Once the workers had gathered the pupae in one or several piles, some workers tended to stay close to the brood whereas others tended to move away from the brood and walk around the arena. Once the workers had gathered all the pupae we extracted, for each colony, screenshots for 20 time points in the dark (every 30 s for 10 min) and 12 time points in the light (every 10 s for 1 min after the light was switched on and every 30 s for the next 3 min). For each screenshot, we recorded the numbers of brood-tenders, defined as any worker with less than one ant-length to the nearest pupa, and non-brood-tenders, defined as all other workers with more than one ant-length to the nearest pupa. The proportion of non-brood-tenders was used as a proxy for the foraging propensity.

### DISTANCE TO THE BROOD

The distance between workers and brood was measured for both brood-tending and non-brood-tending workers. We used the same screenshots (32 time points) as for the foraging propensity to record the distance between workers and brood in the dark and in the light. The software ImageJ (<http://rsbweb.nih.gov/ij>) was used to collect the spatial coordinates of each worker, as well as the center of each pile of pupae. These coordinates were then used to infer the distance between each worker and the center of the closest pile of pupae. Brood-tenders and non-brood-tenders were analyzed separately.

### STATISTICAL ANALYSIS

Among the 110 colonies that overwintered successfully, four colonies did not produce any offspring, 11 colonies did not produce any females (perhaps because the queens were not inseminated), 25 colonies did not produce enough brood to set up a single-cohort colony with 50 pupae and five colonies lost their queen. Therefore, a total of 65 single-cohort colonies could be used for all behavioral experiments, except the nursing of larvae and collection of pupae experiments for which 2 and 7 colonies, respectively, were discarded for technical reasons. To test for the effect of paternal and maternal lineages on worker behavior (as well as time when required), we conducted two-way analyses of variance (ANOVAs) on linear models optimized to fit our data.



**Figure 1.** The time to collect pupae (mean ± SE) is significantly affected by the maternal lineage ( $F_{5,52} = 2.71, P = 0.03$ ).

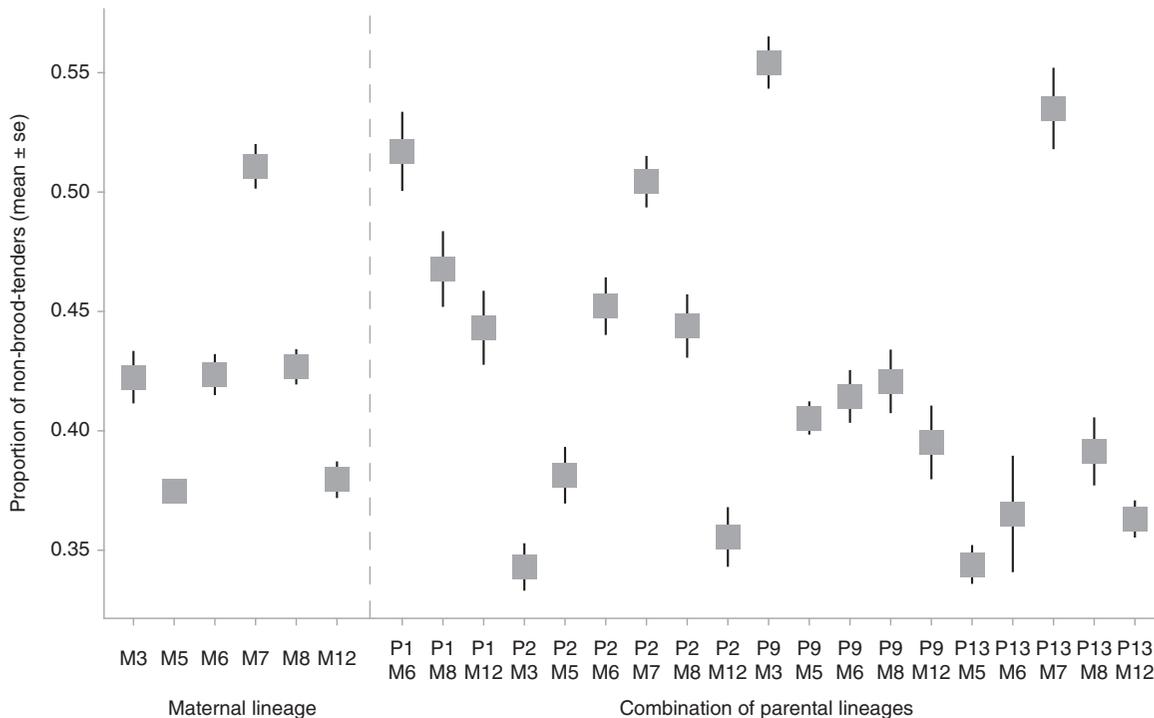
When needed, the data were transformed so that the residuals of the models followed a normal distribution. The proportion of non-brood-tenders and the distance between workers and brood were analyzed using a linear mixed-effect model with a Satterthwaite compilation of degrees of freedom. For each response variable, we used a single value (proportion or mean) per time point per

colony and we specified the colony as a random factor to avoid pseudoreplication and take into account the nonindependence of repeated measures. The time to collect pupae and the difference in larval mass were analyzed using a linear model. Correlation tests were carried out using Spearman rank correlation tests.

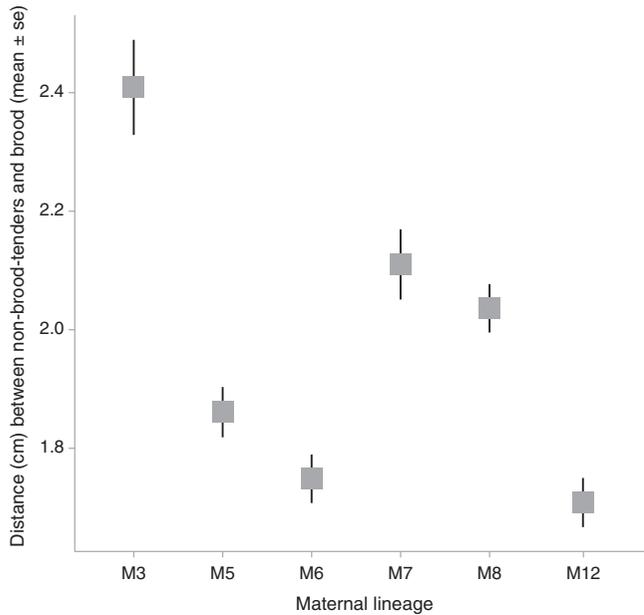
### Results

Our measures of worker behavior revealed that worker likelihood and efficiency to perform tasks varied greatly among the single-cohort colonies. The change in larval mass over 3 days varied from  $-0.44$  to  $0.69$  mg ( $0.17 \pm 0.23$ , mean ± SD), the time to collect pupae from 227 to 1063 s ( $515 \pm 221$ ), the proportion of non-brood-tenders from 0.1 to 0.8 ( $0.41 \pm 0.12$ ), the distance between workers and the center of the pile of pupae from 0.01 to 1.07 cm ( $0.28 \pm 0.14$ ) for brood-tending and 0.21–8.6 cm ( $2.19 \pm 1.74$ ) for nonbrood-tending individuals. There was a significant positive correlation between normal conditions (darkness) and stressful conditions (light) for the foraging propensity ( $\rho = 0.64, P < 0.0001$ ), and the distance between brood and either brood-tenders ( $\rho = 0.83, P < 0.0001$ ) or non-brood-tenders ( $\rho = 0.59, P < 0.0001$ ).

The maternal lineage had a significant effect on three of the five behavioral measures. These were the time needed to collect the 12 pupae (Fig. 1,  $F_{5,52} = 2.71, P = 0.03$ ), the proportion of non-brood-tenders (Fig. 2,  $F_{5,341} = 3.62, P = 0.003$ ), and the



**Figure 2.** The proportion of non-brood-tenders (mean ± SE) is significantly affected by the maternal lineage ( $F_{5,341} = 3.62, P = 0.003$ ) and by the interaction between parental lineages ( $F_{10,341} = 2.3, P = 0.01$ ).



**Figure 3.** The distance between non-brood-tenders and the brood (mean  $\pm$  SE) is significantly affected by the maternal lineage ( $F_{5,872} = 6.75$ ,  $P < 0.0001$ ).

distance between non-brood-tenders and the brood (Fig. 3,  $F_{5,872} = 6.75$ ,  $P < 0.0001$ ). The paternal lineage had no significant effect on any of these measures but significantly affected the two others, namely the change over time in the larval mass (Fig. 4,  $F_{3,44} = 3.30$ ,  $P = 0.026$ ) and the distance between brood-tenders and the center of the closest pile of pupae (Fig. 5,  $F_{3,141} = 8.98$ ,  $P < 0.0001$ ).

The interaction between parental lineages significantly affected three of the five measures of behavior. These were the change in larval mass over 3 days (Fig. 4,  $F_{10,44} = 2.22$ ,  $P = 0.033$ ), the proportion of non-brood-tenders (Fig. 2,  $F_{10,341} = 2.3$ ,  $P = 0.01$ ), and the distance between brood-tenders and the brood pile center (Fig. 5,  $F_{10,141} = 2.79$ ,  $P = 0.0035$ ).

## Discussion

This study demonstrates strong effects of the maternal and paternal lineages on worker behavior in the Argentine ant *L. humile*. Although the maternal lineage affected the efficiency to collect pupae, the foraging propensity and the distance between non-brood-tenders and the brood, the paternal lineage influenced the efficiency to feed larvae and the distance between brood-tenders and the brood pile center. Furthermore, there was a significant interaction between maternal and paternal lineages in the efficiency to feed larvae, the foraging propensity, and the distance between brood-tenders and the brood pile center.

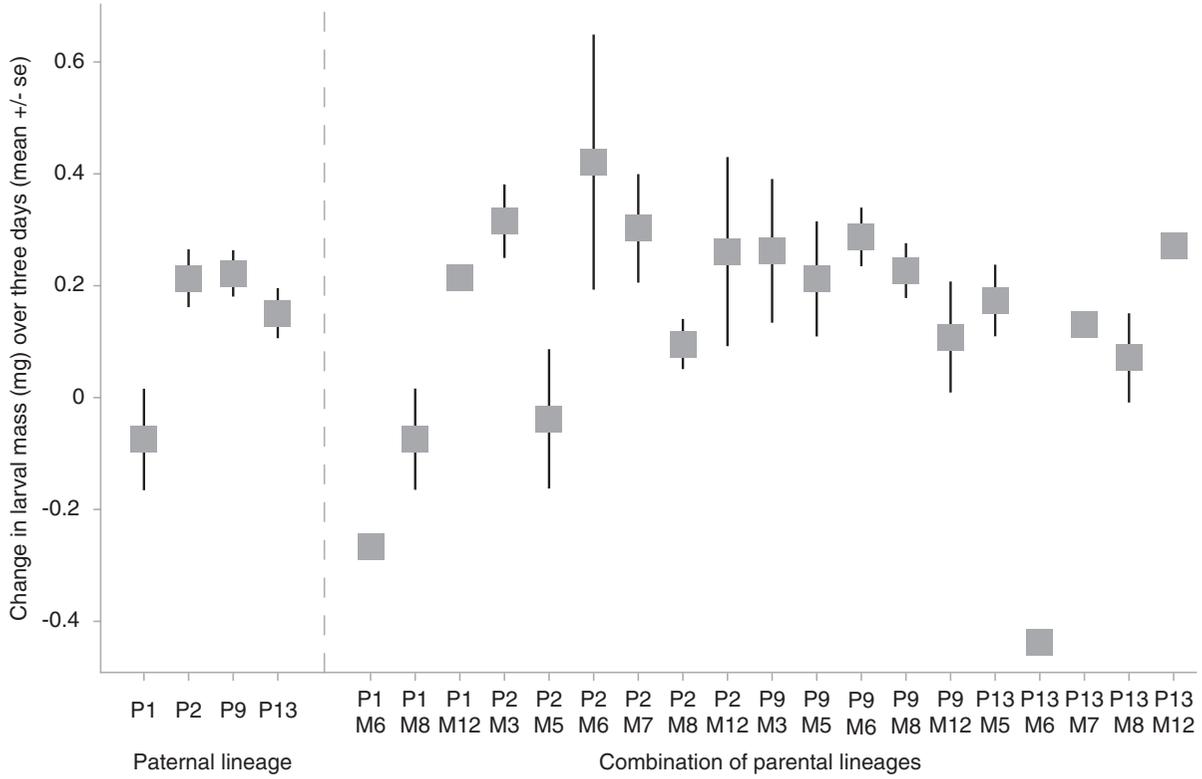
Three lines of evidence indicate that the effects of the parental lineages are mostly or only due to genetic influences. First, the

experiments were performed on workers that developed in colonies that experienced the same environment (colony size, food, temperature, humidity, light cycle). Second, the use of single-cohort colonies allowed us to ensure that the workers were of the same age and had similar nursing and foraging experience at the time of the experiments. Finally, although we cannot completely rule out the existence of maternal effects during oviposition or larval development, the paternal effects must have been of genetic nature only, as males died after mating and never encountered their progenies.

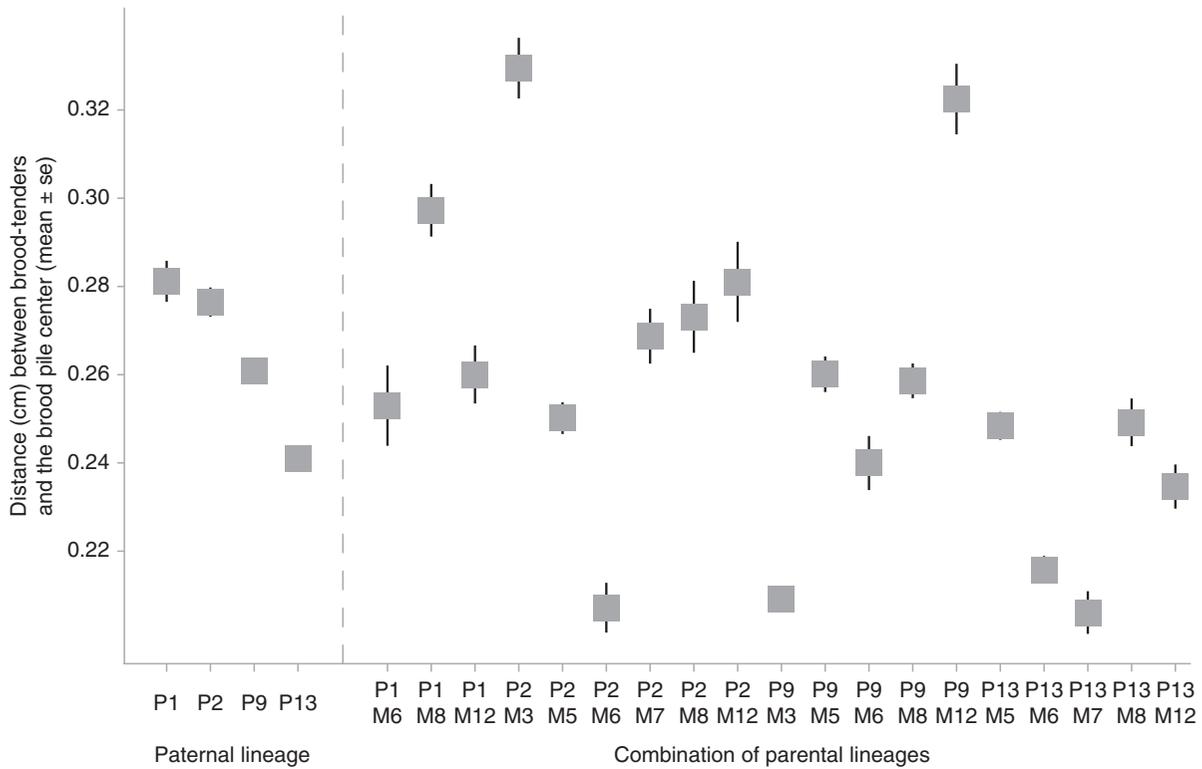
The finding that three of the five behavioral measures were affected by the maternal lineage and the two others by the paternal lineage is surprising. If there were additive genetic effects on worker behavior, one would expect a given behavior to be similarly affected by the maternal and paternal lineages (Schwander and Keller 2008; Libbrecht et al. 2011). The finding of all tested behaviors being affected by either the maternal or the paternal lineage is best explained by parent-of-origin specific effects through epigenetic changes. This interpretation is consistent with recent findings indicating that social insects are capable of epigenetic modifications. First DNA methylation was found to occur widely among ants, bees, and wasps (Kronforst et al. 2008). Second, the genome sequences of several bee and ant species revealed the conservation of a DNA methylation toolkit across social Hymenoptera (Wang et al. 2006; Bonasio et al. 2010; Nygaard et al. 2011; Smith et al. 2011a, 2011b; Suen et al. 2011; Wurm et al. 2011; Gadau et al. 2012). Parent-of-origin specific effects could occur if genes that affect behavior were imprinted and differed in expression depending on whether they were maternally or paternally inherited, as found in mammals (Isles et al. 2006; Garfield et al. 2011).

Importantly, this study also revealed that three of the five measured behaviors (the efficiency to feed larvae, the foraging propensity and the distance between brood-tenders and the brood pile center) were significantly affected by the interaction between parental lineages. This indicates widespread genetic compatibility effects on worker behavior. These findings are important, in particular because the worker intrinsic propensity and efficiency to perform a given task are the basis of division of labor in social insects (Wilson 1971; Oster and Wilson 1979).

The finding of nonadditive genetic effects, such as parent-of-origin and genetic compatibility effects on behavior have important implications for our understanding of the role of genetic diversity in social insects. Genetic effects on division of labor have been documented in many species of social insects, primarily through the finding of within-colony behavioral differences between matriline or patriline (Oldroyd and Fewell 2007). As these genetic effects were typically assumed to be additive, it was argued that an increase in genetic diversity among the worker force would increase behavioral diversity and colony performance



**Figure 4.** The change in larval mass over 3 days (mean  $\pm$  SE) is significantly affected by the paternal lineage ( $F_{3,44} = 3.30, P = 0.026$ ) and by the interaction between parental lineages ( $F_{10,44} = 2.22, P = 0.033$ ).



**Figure 5.** The distance between brood-tenders and the brood pile center (mean  $\pm$  SE) is significantly affected by the paternal lineage ( $F_{3,141} = 8.98, P < 0.0001$ ) and by the interaction between parental lineages ( $F_{10,141} = 2.79, P = 0.0035$ ).

(Crozier and Page 1985). In line with this view, a greater genetic diversity has been shown to facilitate division of labor (Page and Robinson 1991; Mattila and Seeley 2007; Oldroyd and Fewell 2007) and enhance colony performance (Oldroyd et al. 1992; Fuchs and Schade 1994; Page et al. 1995; Costa and Ross 2003; Jones et al. 2004; Wiernasz et al. 2004; Mattila and Seeley 2007; Oldroyd and Fewell 2007) in some social insects. However, in some other species, studies failed to find a link between genetic diversity and colony performance (Sundstrom and Ratnieks 1998; Fjerdingstad et al. 2003; Rosset et al. 2005; Fournier et al. 2008). The discrepancy between these studies could stem from between-species differences in the genetic architecture underlying division of labor. Of interest would be to investigate whether additive and nonadditive genetic effects on division of labor produce different links between genetic diversity and colony performance, and to what extent such differences impact colony performance.

In conclusion, the use of controlled crosses allowed us to investigate for the first time the genetic architecture of division of labor in a social insect species. Maternal and paternal effects were found to each affect several behaviors but surprisingly, no behavior was simultaneously affected by both the maternal and paternal lineages. Such a pattern is not expected under simple additive genetic effects and rather suggests parent-of-origin specific effects on behavior. Our study also revealed genetic compatibility effects between parental genomes on three of the five behaviors recorded. These findings, together with the recent empirical (Schwander and Keller 2008; Libbrecht et al. 2011) and theoretical (Dobata and Tsuji 2012) documentation of similar effects on caste and sex allocation, indicate that nonadditive genetic effects play an important and unrecognized role in the organization of social insect colonies. Such effects are likely to have important implications in our understanding of social organization, the resolution of kin conflicts, the maintenance of multiple mating, and other important issues in social insect biology.

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