



Regulation of worker egg laying by larvae in a fission-performing ant



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Ants provide one of the best examples of the division of labour in animal societies. In many species, although workers still have ovaries, they refrain from laying (haploid) eggs when a queen is present in the colony and, instead, dedicate themselves exclusively to domestic tasks. In monogynous species, workers generally begin laying eggs once the queen dies, which allows the colony's remaining resources to be invested in the workers' offspring. However, we found that in the fission-performing ant *Aphaenogaster senilis*, worker reproduction was also inhibited by brood pheromones; the presence of larvae, irrespective of their ploidy or origin, inhibited egg laying by orphaned workers in a dose-dependent manner. The end result was that workers allocated resources to the larvae that were present in the colony before starting to lay their own eggs. We also found that, while the number of workers reared was proportional to the number of larvae provided, the number of queens reared tended to plateau at six, regardless of the number of totipotent larvae present. This finding concurs with what has been observed in colonies in the field and fits with what the theory of local resource competition predicts. One explanation is that one of the queens produced is likely to mate and replace her dead mother as the head of the colony. Therefore, by rearing the queen's brood after her death, workers may have an opportunity to prolong the life of the colony.

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The evolution of reproductive division of labour marks the transition from solitary to social life (Buss, 1987; Chapuisat, 2010; Field, Paxton, Soro, & Bridge, 2010; Maynard Smith & Szathmari, 1997; Simpson, 2011). Particularly intriguing is the way in which individuals transmit information about their physiological condition and reproductive potential; by so doing, they influence collective decisions. Colonies of social hymenopterans (ants as well as some bees and wasps) are excellent model systems for studying this phenomenon. Their queens and workers are adapted to carry out highly specialized tasks. While the former have efficient reproductive systems and spend much of their time laying eggs, the latter perform nonreproductive tasks and thus largely control resource allocation. By manipulating food availability and environmental conditions, workers can control whether diploid larvae develop into workers or queens (Oster & Wilson, 1978).

In many species, although workers cannot mate, they do have small ovarioles and can thus potentially lay haploid eggs that will develop into males. Yet, they generally refrain from doing so when a fertile queen is present in the colony (Hammond & Keller, 2004;

Wenseleers & Ratnieks, 2006). In addition, policing mechanisms prevent worker reproduction in queenright colonies (Beekman & Ratnieks, 2003; Ratnieks, 1988). These mechanisms include the selective removal of worker-laid eggs (D'Ettorre, Heinze, & Ratnieks, 2004; Foster & Ratnieks, 2001; Kikuta & Tsuji, 1999; Monnin & Peeters, 1997) and aggressive behaviour directed at egg-laying workers (Monnin & Ratnieks, 2001). These mechanisms are selected for because they enhance colony efficiency either because egg-laying workers spend less time caring for the brood or because they engage in conflicts (Foster & Ratnieks, 2001; Hammond & Keller, 2004; Hartmann, Wantia, Torres, & Heinze, 2003; Iwanishi, Hasegawa, & Ohkawara, 2003; Ohtsuki & Tsuji, 2009; Pirk, Neumann, & Ratnieks, 2003; Teseo, Kronauer, Jaisson, & Châline, 2013; Wenseleers & Ratnieks, 2006). For example, workers that reproduce have significantly shorter life spans than workers that do not reproduce (Tsuji, Kikuta, & Kikuchi, 2012). Moreover, in polyandrous species, workers are less related, genetically to other workers' sons than to the queen's sons (Ratnieks, 1988; Ratnieks & Wenseleers, 2008; Wenseleers & Ratnieks, 2006). Interestingly, it has been suggested that worker policing depends on colony demography and ontogeny (Ohtsuki & Tsuji, 2009). Hence, because worker egg laying in small colonies may hamper colony growth and future reproduction, there may be

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selection for both self-restraint and policing (Bonckaert, van Zweden, D'Etorre, Billen, & Wenseleers, 2011; Moore & Liebig, 2010).

In many monogynous species, the queen's death puts an end to policing behaviour and triggers ovariole maturation and egg laying in workers (Bourke, 1988; Choe, 1990; Holman, Jørgensen, Nielsen, & d'Etorre, 2010; Van Oystaeyen et al., 2014). This shift allows the colony to invest its remaining resources in producing workers' sons before it vanishes entirely (Hölldobler & Wilson, 1990). However, in some species that disperse through colony fission (or, more generally, through dependent colony founding), the dead queen may be replaced by one of her daughters (Boulay et al., 2007; Cronin, Molet, Doums, Monnin, & Peeters, 2013). In such species, queens do not participate in large nuptial flights; instead, they mate near the entrances of their nests. If the mother queen is still in the colony, the newly mated queen leaves with a group of workers to create a new colony (walking distance away). However, if the mother queen is dead, the newly mated queen can replace her as the head of the colony. Thus, in fission-performing ants, the life span of the colony may be much longer than that of the queen, provided a replacement queen is produced rapidly enough after the death of the current queen (Bourke & Franks, 1995). In such species, one may expect orphaned workers to allocate resources to the remaining diploid brood so as to produce a few replacement queens (an excessive number would be useless and only increase local resource competition) and as many workers as possible to guarantee colony survival (Bourke & Franks, 1995; Cronin et al., 2013; Pamilo, 1991; Percy & Aron, 2006), even though this investment comes at the expense of laying their own male eggs.

Using a fission-performing ant species, *Aphaenogaster senilis*, we examined the collective reproductive decisions of orphaned workers in relation to the presence of totipotent larvae. In this species, the queen signals her presence to workers by means of contact pheromones (Ruel, Hefetz, Cerdá, & Boulay, 2013). When the workers perceive this signal, they refrain from laying eggs and rear almost all young larvae as workers (Boulay, Cerda, Fertin, Ichinose, & Lenoir, 2009; Boulay et al., 2007). However, if the queen disappears, workers rear a few larvae as new queens, which allows requeening (Chéron, Doums, Federici, & Monnin, 2009). The mechanism of larval caste determination is unknown in this species but probably involves nutritional and hormonal factors (Barroso, Amor, Cerdá, & Boulay, 2013; Ledoux, 1977). To investigate a trade-off between egg laying by orphaned workers and raising already existing larvae to adulthood, we sought to answer the following questions. First, we asked whether larvae number affected larval fate and/or egg laying by workers in queenless colonies. To date, the presence of larvae has been shown to inhibit egg laying by workers in two ant species, *Cerapachys biroi* (Teseo et al., 2013) and *Pachycondyla villosa* (Heinze, Trunzer, Oliveira, & Hölldobler, 1996), and in the honeybee *Apis mellifera* (Arnold et al., 1994; Mohammedi, Paris, Crauser, & Le Conte, 1998; Oldroyd, Wossler, & Ratnieks, 2001; Traynor, Le Conte, & Page, 2014). In *P. villosa*, young larvae were also observed eating worker-laid eggs (Heinze et al., 1996). Second, we asked whether worker-laid eggs suffered higher mortality in the presence of larvae, either directly as a result of larval predation or indirectly through worker policing. Third, we asked whether egg laying by workers was affected by the sex of the larvae. Male larvae might be predicted to have no effect on worker egg-laying decisions since males do not contribute to colony survival. Fourth, we asked whether young diploid larvae inhibited egg laying by workers via chemical signals. Thus far, most studies on reproductive inhibition in workers have focused on queen pheromones; larval pheromones have largely been ignored, particularly in ants. Finally, although orphaned workers may collectively decide to rear diploid larvae

before producing sons, some individuals might none the less selfishly lay eggs before all the larvae reach the pupal stage. Therefore, the fifth question we asked was whether orphaned workers punished potential cheaters when larvae were present. By answering this series of questions, we aimed to determine whether and how orphaned ant workers trade individual reproduction for raising existing larvae to adulthood and, potentially, to queens.

METHODS

Model Species and Rearing Conditions

Aphaenogaster senilis is a common species distributed along the western Mediterranean Basin. All the stock colonies used in this study were collected in Doñana National Park (southwestern Spain). In this area, colonies are active from March to December. At least three larval stages have been identified in this species (Boulay et al., 2009). All three stages are present in the colonies year round, but the first instars are more abundant from September to April and the second and third instars from March to July. New workers are produced from May to November (Boulay et al., 2009). Males are produced in large numbers in colonies with more than 500 workers; this process occurs mostly in the summer, although males can be found until November. Virgin queens are produced in much smaller numbers. They can be found in queenless colonies throughout the year and in large queenright colonies (of greater than 2000 workers) in July and August (Boulay et al., 2007).

Each colony used in this study had a queen and 1000–2500 workers. They were housed in plastic boxes (dimensions: 13 × 32 cm and 15 cm high) that contained three or four test tubes (dimensions: 2 × 20 cm). The groups used in the following experiments were placed in test tubes half filled with water blocked by a cotton plug. The test tubes were connected to a 9 × 9 cm circular arena, whose inner walls had been coated with Fluon to prevent ants from escaping. Both the stock colonies and the experimental groups were kept at 25 ± 1 °C and fed mealworms, *Tenebrio molitor*, twice a week. Under these laboratory conditions, colonies continually produced worker pupae but only rarely generated males. Experiments 1, 2, 3 and 5 were conducted using colonies that had been kept in the laboratory for 2–5 months. Experiment 4 was conducted using colonies that had been kept in the laboratory for 3 months to 2 years.

Experiments

The first experiment aimed to determine whether orphaned workers delayed producing their own sons because of the presence of queen-derived larvae. Five queenless groups composed of 200 workers were created from each of 10 large stock colonies. The groups received 160, 120, 60, 20 or no first-instar larvae from the same stock colonies. They were all checked 24 h after set-up to confirm that no eggs had been introduced unintentionally. The experimental groups were then kept in the above-mentioned rearing conditions. The groups were checked three times a week for 11 weeks; all the pupae produced were collected, and their sex and caste were determined. Worker-laid eggs and surviving workers were counted at weeks 7 and 11. This experiment was conducted in November 2011 with colonies collected 2 months earlier.

The second experiment aimed to determine whether the larvae directly or indirectly increased the mortality of worker-laid eggs. Three experimental groups of 50 workers were created from each of 15 stock colonies. The first group received 30 freshly laid workers' eggs and 60 first-instar larvae from the same stock colony, the second group received 30 freshly laid workers' eggs but no

larvae and the third group received neither eggs nor larvae. The worker-laid eggs were obtained from groups of 150 workers that had been separated from their queens 2 weeks before the beginning of the experiment. If the presence of larvae among workers increases egg mortality, either directly (through the consumption of eggs by the larvae) or indirectly (by stimulating egg destruction by the workers), then we predicted that the number of eggs would decrease more rapidly in the first than in the second group. The third group served as a control, to confirm that no eggs were laid by workers over the course of the experiment. A previous study has shown that it takes about 10 days for orphaned workers to start laying eggs (Ruel, Cerdá, & Boulay, 2012). To avoid the confounding effects of worker egg laying, eggs and surviving workers were counted only after 7 days had passed.

The third experiment aimed to determine whether egg laying by workers was differentially affected by the presence of queen-derived larvae versus worker-derived larvae. Three groups of 150 workers were created from each of 10 stock colonies. The first group received no brood. The second group received 60 first-instar larvae; these larvae were mostly diploid since males are rare in queenright colonies kept under laboratory conditions. The third group received 60 first-instar larvae produced by a group of 200 workers that had been queenless for 2 months; these larvae were the workers' sons. The larvae used in groups 2 and 3 were from the same stock colonies; however, the workers came from different stock colonies than the larvae. Thus, workers were in contact with their respective queens until the beginning of the experiment (so they did not start laying eggs), and they were given diploid and haploid larvae with which they were equally unfamiliar. Worker-laid eggs and surviving workers were counted after 3 weeks as this was sufficiently long to allow egg laying.

The fourth experiment tested whether larvae emitted chemical signals that reduced egg laying by workers. Four experimental groups of 50 workers were created from each of 18 stock colonies. They were kept in small cylindrical containers (8 × 4 cm) and experienced one of four treatments. (1) LV-right: ants received 60 first-instar larvae from their stock colony of origin with which they were allowed to interact fully. (2) LV-up: ants were allowed to have antennal contact with 60 first-instar larvae located in a compartment above them, although ants and larvae were separated by a fine wire mesh. The ants could reach the larvae by climbing up the container's wall (it was not coated with Fluon); however, no exchange of food could take place. The larvae were housed with 50 other workers that took care of them. (3) LV-down: ants could perceive any volatiles emitted by 60 first-instar larvae located in a compartment below them. As in the LV-up treatment, a fine wire mesh separated the two compartments. However, because the larvae were below the ants, the ants could not touch them. The larvae were housed with 50 workers that took care of them. (4) LV-less: ants had no contact with larvae. All the ants in the experiment had access to a water-soaked piece of cotton and were given sliced mealworms three times a week. Worker-laid eggs and surviving workers were counted after 3 weeks had passed. Fourteen groups of 10 ants were randomly sampled from each experimental condition and rapidly frozen at -20°C ; their ovaries were then dissected to measure the length and the width of the longest and widest ovarioles, respectively. Ovarian development in experimental ants was compared to that in 40 ants sampled from eight queenright colonies. Ovariole length and width were measured using a stereomicroscope equipped with an ocular micrometre.

The aim of the last experiment was to determine whether the presence of small larvae among queenless workers triggered mutual policing. In groups of orphaned workers, only a small proportion of individuals tend to lay eggs. Because this proportion probably decreases with group size, we decided to introduce small

groups of orphaned 'foreign' workers in which at least one worker had started laying eggs into larger groups of resident ants that were (1) in contact with larvae, (2) in contact with larvae and the queen or (3) had no contact with larvae or the queen. To do so, we initially created 12 groups of 10 foreign workers from each of 10 stock colonies (this was to ensure that at least three groups would start laying eggs at the same time). These groups of foreign workers were kept in 2×20 cm test tubes and given access to water. On the same day, three groups of 100 resident workers were created from the same stock colonies. Groups of residents were placed in 8×4 cm containers. The first resident group received 60 first-instar larvae from the same stock colony and the queen, the second resident group received 60 first-instar larvae but no queen and the third resident group received neither larvae nor queen. The groups of foreign workers were checked daily for eggs. When eggs were observed in three groups of foreign workers from the same stock colony, the ants in these groups were marked individually with a dot of paint and introduced into the three respective resident groups. The remaining groups of foreign workers that had not yet started laying eggs were discarded. For each stock colony, foreign workers were reunited with resident workers of their colony of origin on the same day in order to control for the potential confounding effects of separation duration on aggressiveness. Interactions between the resident workers and the foreign workers were observed every 20 min for 4 h (total 12 observation sessions); acts of aggression (bites and threats, i.e. opening the mandibles with the gaster bent below the thorax) were noted. The foreign workers were then frozen at -20°C ; they were subsequently dissected, and the length and the width of their longest and widest ovarioles were measured.

Ethical Note

The authors adhered to ASAB/ABS's Guidelines for the Treatment of Animals in Behavioural Research and Teaching. All experiments comply with Spanish laws on animal experimentation. We minimized the number of colonies and replicates as much as possible while maintaining statistical power. All the ants were treated carefully. During behavioural observations, agonistic interactions were not harmful. Ants were euthanized by freezing before dissection of the ovaries.

Statistics

The results of experiments 1, 2, 3 and 4 were analysed by fitting linear mixed-effects (LME) models using the lme function in the R nlme package (R Core Team, 2012). In all models, stock colony identity was included as a random factor. In the experiment 1 models, the number of larvae provided was the linear predictor variable; the number of worker-laid eggs at 7 and 11 weeks (Eg07 and Eg11, respectively), the total numbers of worker, queen and male pupae at 11 weeks (Wk11, Qu11 and Ma11, respectively), and the number of surviving workers at 7 and 11 weeks (Su7 and Su11, respectively) were the response variables. In addition, a nonlinear Michaelis-Menten model ($y = ax/(1 + bx)$) was fitted to test whether the number of queens produced (y) approached an asymptote (a) as the number of larvae provided (x) increased; b is an unknown parameter. This model was fitted using the SSmicmen function in the R stats package. An LME model was also fitted to test the effect of the number of larvae provided (Lv), larva caste (Ca) and the interaction between these two factors on time to pupa production (measured in weeks from the beginning of the experiment). In the LME models for experiments 2, 3 and 4, treatment was the predictor variable and the numbers of eggs produced (Eg) and surviving workers (Su) were the response variables. For experiment

4, LME models were used in which treatment (levels: LV-right, LV-up, LV-down, LV-less and queenright) was the independent variable and ovariole length and width were the dependent variables. For each LME, we first tested the main effects of the predictors using *F* tests. For significant predictors we then conducted pairwise comparison using *t* statistics.

In experiment 5, the number of aggressive acts directed at foreign workers was low and highly unequally distributed. The response variable was therefore recoded as a binary variable as follows: a score of 1 was assigned to ants that were attacked at least once during the 12 observation sessions and a score of 0 was assigned to ants that were not attacked. The results were analysed using a generalized linear mixed-effects model (GLME) with a binomial distribution and a logit link function (lme4 package in R). We used LME models to compare ovariole maximum length (Ol) and maximum width (Ow) for foreign ants that did and did not receive aggression. All estimates are means \pm SE.

RESULTS

Experiment 1: Caste Production as a Function of Larval Density

Over the first 7 weeks of the experiment, the groups of orphaned workers that were not provided with larvae (0-larvae groups) laid, on average, 48.48 ± 3.57 eggs. The number of worker-laid eggs in the other experimental groups decreased significantly and linearly as a function of the number of larvae provided (Fig. 1a, Table 1; LME_{Eg07} : $F_{1, 39} = 82.95$, $P < 0.0001$). A similar pattern was observed for the results at 11 weeks (LME_{Eg11} : $F_{1, 39} = 4.81$, $P = 0.034$); however, the value of the intercept was smaller and the slope of the linear regression was less pronounced (Table 1, Fig. 1a). This is probably because fewer workers were alive at 11 weeks than at 7 weeks.

Most of the pupae produced during experiment 1 were workers (81%; 2217 out of 2737). As expected, no worker pupae were produced in the 0-larvae groups and worker pupa production increased linearly with the number of larvae provided (Table 1, Fig. 1b; LME_{Wk11} : $F_{1, 39} = 607.53$, $P < 0.0001$). Overall, only 5% of the pupae became queens. No queens were produced in the 0-larvae groups, and the number of queens produced also increased as the number of larvae provided increased (Table 1; LME_{Qu11} : $F_{1, 39} = 116.58$, $P < 0.0001$). However, this relationship was nonlinear: queen production rapidly plateaued even when many totipotent larvae remained available (Fig. 1b). The shape of the curve was well described by a Michaelis-Menten model: $y = ax/(1 + bx)$, where $a = 6.16 \pm 0.70$ ($t = 10.27$, $P < 0.0001$) and $b = 34.02 \pm 11.46$ ($t = 3.06$, $P = 0.004$). In contrast, worker production was not asymptotic ($a = 32.33 \pm 22.35$, $t = 1.45$, $P = 0.149$ and $b = 409.31 \pm 372.81$, $t = 1.098$, $P = 0.273$).

The production of male pupae was highest in the 0-larvae groups and decreased as a function of the number of larvae provided (Fig. 1c; LME_{Ma11} : $F_{1, 39} = 24.27$, $P < 0.0001$). The numbers of surviving workers at weeks 7 and 11 were not significantly affected by the number of larvae provided (Table 1; LME_{Su07} : $F_{1, 39} = 0.36$, $P = 0.55$; LME_{Su11} : $F_{1, 39} = 0.92$, $P = 0.34$).

The time to pupa production depended on caste (LME_{Ca} : $F_{1, 2722} = 218.38$, $P < 0.0001$), the number of larvae provided (LME_{Lv} : $F_{2, 2722} = 325.37$, $P < 0.0001$) and the interaction between these two factors (LME_{Ca*Lv} : $F_{2, 2722} = 218.57$, $P < 0.0001$). Hence, the time it took to produce worker and queen pupae (4.92 ± 0.12 and 5.13 ± 0.32 weeks, respectively) increased as the number of larvae provided increased (Fig. 1d). This was probably because of the lower per capita amount of care received by the larvae as they increased in number, which may have reduced their developmental rate. In the 0-larvae group, male pupae were produced in

9.65 ± 0.12 weeks, but production time decreased as the number of larvae provided increased. Males were produced in just 5.36 ± 0.25 weeks in the 160-larvae group. This pattern was probably because the few males produced in the 120- and 160-larvae groups (4 and 3% of the total pupae, respectively) probably came from the larvae that were initially provided; they thus reached the pupal stage before those produced in the 0- and 20-larvae groups, which mostly came from worker-laid eggs.

Experiment 2: Egg Survival

After 7 days, no eggs were found in the worker-only groups, which fits with previous findings that no eggs were laid by workers during the first week after they had been orphaned (Ruel et al., 2012). An average of 15.53 ± 1.78 and 15.13 ± 1.62 eggs (LME_{Eg} : $F_{1, 14} = 0.06$, $P = 0.81$) were observed in the groups provided with 30 eggs and 30 eggs plus 60 larvae, respectively. Therefore, approximately 50% of the eggs survived in both groups and egg survival rate was not affected by the presence of larvae. Worker survival was not significantly different between the three groups (LME_{Su} : $F_{2, 28} = 1.23$, $P = 0.31$).

Experiment 3: Worker Versus Queen-derived Larvae

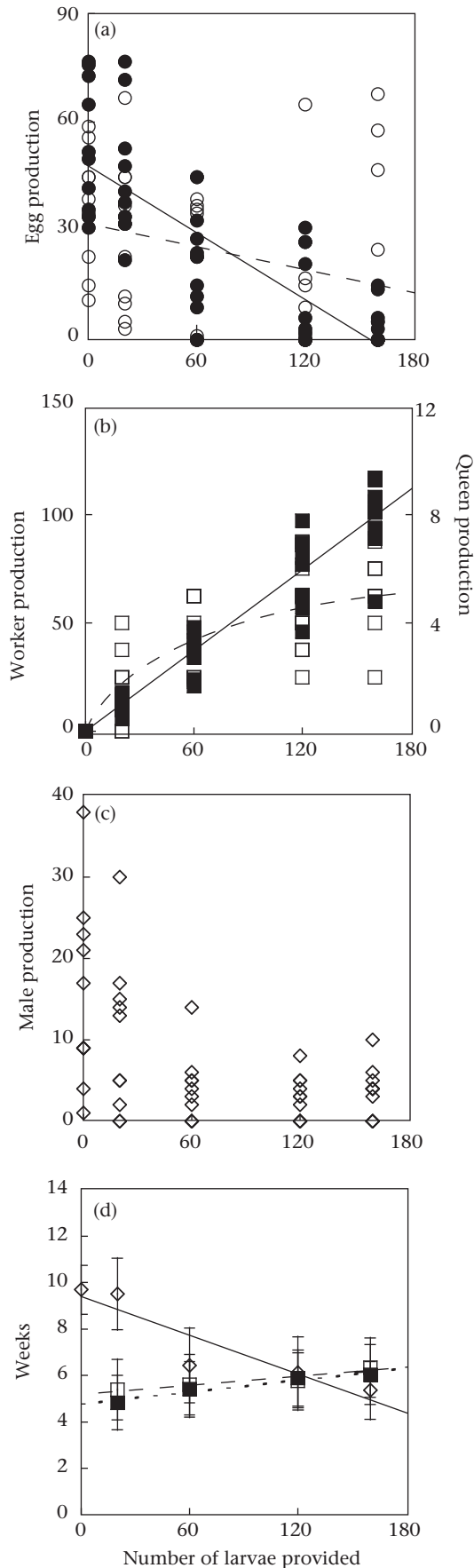
The treatment of the third experiment had a significant effect on egg production (LME_{Eg} : $F_{2, 28} = 16.87$, $P < 0.0001$). However, larva origin (worker versus queen) did not affect egg laying by workers. The average number of eggs produced over the 3 weeks of the experiment was not significantly different between the groups that received 60 queen-derived larvae and the groups that received 60 worker-derived larvae (21.00 ± 6.64 versus 24.63 ± 6.55 , respectively; $t_{14} = 0.43$; $P = 0.67$). However, egg production was about half that observed in the 0-larvae groups (49.37 ± 9.17 ; $t_{14} = 3.37$, $P = 0.005$ and $t_{14} = 2.94$, $P = 0.011$, respectively). Worker survival was not significantly different between the three groups (LME_{Su} : $F_{1, 28} = 1.15$, $P = 0.33$).

Experiment 4: Larval Chemical Signals

The treatments experienced by the ants significantly affected egg laying by workers ($F_{3, 51} = 11.23$, $P < 0.0001$). The number of eggs laid was significantly higher when no larvae were present or when the larvae were located below the workers than when the larvae were above or with the workers (Fig. 2a, Table 2). Worker survival was not significantly affected by treatment ($F_{3, 51} = 0.42$, $P = 0.74$). Ant ovariole width and length varied dramatically between ants within the treatment (queenless) groups and did not differ significantly between the treatment groups and the queen-right groups (Fig. 2b; LME_{Ol} : $F_{4, 567} = 0.99$, $P = 0.41$; LME_{Ow} : $F_{4, 567} = 0.92$, $P = 0.45$). The large variation in ovarian development observed in the treatment groups may reflect that only a small number of workers developed ovaries, thus increasing variance among workers.

Experiment 5: Mutual Policing

The probability that a foreign ant was attacked at least once by the residents during the 12 observations (biting and threats) was not significantly different between the queenless resident groups, irrespective of the presence of larvae (Fig. 3; QL versus QL-LV: GLME: $z = 0.326$, $P = 0.74$). However, when the queen was present, aggressive acts were significantly less common (Fig. 3; GLME: $z = 2.323$, $P = 0.020$ and $z = 2.088$, $P = 0.037$). Foreign ants did not experience equal levels of aggression; one or two individuals, whose ovarioles were significantly longer (LME_{Ol} : $F_{1, 227} = 10.05$,



$P = 0.002$; difference in ovariole length: 0.49 ± 0.15 mm; $t_{227} = 3.17$, $P < 0.002$), were more frequently targeted. However, ovariole width did not differ between targeted and nontargeted foreign ants (LME_{OW}: $F_{1, 227} = 0.20$, $P = 0.66$; difference in ovariole width: 0.007 ± 0.016 mm; $t_{227} = 0.44$, $P = 0.65$).

DISCUSSION

In many ant species, workers do have ovaries but refrain from laying eggs when a queen is present in the colony (Hammond & Keller, 2004; Hölldobler & Wilson, 1990). It is generally thought that they initiate egg laying, and thus invest in producing sons, only after the queen dies (Hölldobler & Wilson, 1990). Previous studies have focused on identifying the chemical nature and glandular origin of the pheromones that allow queens to signal their presence and inhibit worker reproduction (Van Oystaeyen et al., 2014 and literature therein). Our results show that, in *A. senilis*, worker reproduction is also modulated by larval pheromones. In this species, the function of larval pheromones differed from that of queen pheromones in at least two respects: larval pheromones did not prevent workers from rearing new queens (experiment 1) and workers given only larvae did not behave like queenright workers (experiment 5). The fact that orphaned workers reared larvae before starting to lay their own male eggs could maximize worker production and could increase the probability that a new queen will be produced before the whole colony vanishes.

In the many monogynous species that display independent colony founding, the death of the queen is catastrophic. However, the situation is not hopeless for fission-performing species such as *A. senilis*, provided that young diploid larvae are present in the colony. Orphaned workers are able to rear larvae as queens which, after mating, can replace their dead mother. However, workers seem to be unable to produce female offspring themselves (via thelytoky), as no workers or queens were produced in the 0-larvae groups; this result concurs with those of previous studies (Boulay et al., 2007). Interestingly, in orphaned colonies, colony-level investment in queens versus workers differed. While there was a positive linear relationship between the number of workers produced and the number of larvae present, the number of queens plateaued at around 6.16 ± 0.70 individuals. This finding matches what has been observed in the field: colonies never contain more than three virgin queens (Boulay et al., 2007). Our results also support the hypothesis that producing one queen might be enough for requeening in strictly monogynous species. The production of a few extra queens may serve as 'life insurance', in the event that the first queen fails to mate or dies (Chéron et al., 2009). However, if the production of queens was proportional to the number of larvae, an overabundance of queens would result and lead to increased local resource competition (i.e. competition between sister queens to monopolize resources; Bourke & Franks, 1995; Pamilo, 1991; Percy & Aron, 2006). In contrast, there is an incentive to produce as many workers as possible (Pamilo, 1991), particularly given the highly competitive conditions that characterize ant communities, in which larger colonies dominate smaller ones (Boulay et al., 2010). Although the mechanism limiting queen production is unknown, one hypothesis may be that the first larva to develop into a queen

Figure 1. Number of eggs and pupae produced and time to pupa production in experiment 1 as a function of the number of larvae provided. (a) Number of eggs at 7 (closed symbols and plain line) and 11 weeks (open symbols and broken line). (b) Number of worker (closed symbols and plain line) and queen pupae (open symbols and broken line) produced over the 11 weeks of the experiment. (c) Number of male pupae produced over the 11 weeks of the experiment. (d) Time (mean \pm SE) to production for worker (closed squares and dotted line), queen (open squares and broken line) and male pupae (open diamonds and plain line).

Table 1
Results of the linear mixed-effect models conducted using the data from experiment 1

Response variable	Intercept±SE (t value)	Significance	Slope±SE (t value)	Significance
Eg07	48.48±3.57 (13.56)	***	-0.31±0.03 (-9.10)	***
Eg11	32.04±4.55 (7.04)	***	-0.11±0.05 (-2.19)	*
Wk11	0.10±2.52 (0.04)	NS	0.61±0.03 (24.64)	***
Qu11	0.81±0.34 (0.02)	*	0.03±0.00 (10.79)	***
Ma11	12.16±1.96 (6.19)	***	-0.07±0.01 (-4.93)	***
Su07	148.31±8.18 (18.27)	***	0.05±0.09 (0.69)	NS
Su11	86.93±8.42 (10.33)	***	0.08±0.09 (0.96)	NS

Eg07 and Eg11: number of eggs at 7 and 11 weeks; Wk11, Qu11 and Ma11: number of worker, queen and male pupae produced over 11 weeks; Su07 and Su11: number of surviving workers at 7 and 11 weeks. There were 39 degrees of freedom for each analysis.

* $P < 0.05$; *** $P < 0.0001$.

somehow prevents other larvae from developing into queens, thus exerting a sort of colony level feedback on queen development.

The results of experiment 1 show that the presence of larvae delayed egg laying by workers in a dose-dependent manner. This was probably because the larval developmental rate is negatively affected by the ratio between the number of larvae and the number of workers (Ruel et al., 2012). Hence, groups of workers provided with a small number of larvae were able to rear them more rapidly than those provided with a large number of larvae. Thus, the inhibition on worker egg laying disappeared more rapidly in the groups with fewer larvae. In contrast to what has been seen in other species (Boulay et al., 2009; Masuko, 2003), larval presence did not affect egg survival in experiment 3. If it had, we would have observed fewer worker-laid eggs in groups with larvae than in groups without larvae. The fact that there was no significant difference between the groups ($P = 0.81$) indicates that the larvae did not directly consume the eggs nor did they induce egg destruction by the workers (policing). In this experiment, the larvae and the workers were collected in different colonies. Although workers may treat non-nestmate larvae differently from nestmate larvae, this is unlikely to have affected the result of the experiment since the same stock colonies were used in the two treatments containing larvae.

In experiment 4, there was no difference in worker ovarian development as a result of larval presence, even though there was a clear difference in the number of worker-laid eggs. This finding concurs with what previous studies have found in *A. senilis* (Boulay et al., 2007; Ruel et al., 2012) and other species (Cini, Meconcelli, & Cervo, 2013; Dietemann & Peeters, 2000): ovarian development may be a better indicator of overall reproductive potential than current reproductive activity (Cini et al., 2013). One explanation might be that, once the queen has been removed, ovarian development occurs in only a few individuals which lay the vast majority of eggs. If so, the average ovariole size of a large group of workers may not reflect egg production levels.

Full contact between adult workers and larvae was not required for egg laying by workers to be suppressed. In experiment 4, contact cues appeared to suffice to significantly reduce the number of worker-laid eggs. In contrast, volatiles seemed to have no effect on egg laying by workers. Such cues might be contained in the hydrocarbon layer that covers the larval integument (Allan, Capon, Brown, & Elgar, 2002; Bonavita-Cougourdan, Clément, & Lange,

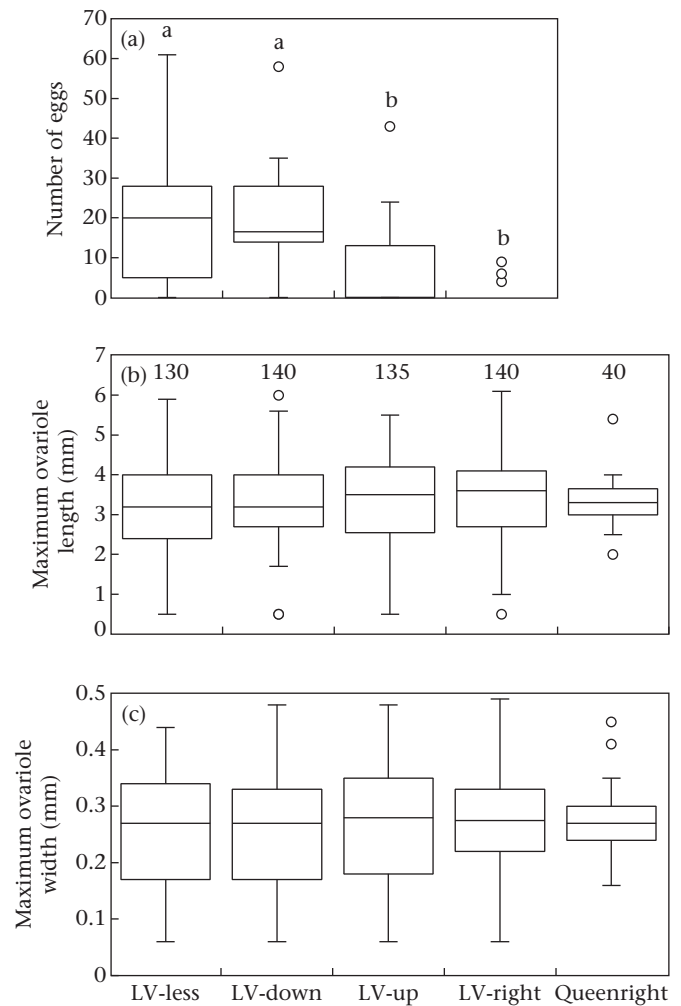


Figure 2. (a) Egg production and (b) ovariole length and (c) width in different treatment groups in experiment 4. LV-less: no larvae were provided to the ants; LV-down: larvae were located in a compartment below the ants, which could not touch the larvae but could perceive any volatiles produced; LV-up: larvae were located in a compartment above the ants, and antennal contacts could take place via a fine wire mesh; LV-right: ants had full contact with the larvae. The values provided are the median, quartiles and 5th and 95th percentiles. Open circles are values below or above these percentiles. The different letters denote significant differences between experimental treatments. Note that there were no data for the number of eggs in the queenright condition. Numbers are sample sizes for both ovariole length and width.

1989). Indeed, cuticular hydrocarbons have been shown to constitute one of the main means of communicating information in ants (Lahav, Soroker, Hefetz, & Vander Meer, 1999). For example, the queen's hydrocarbons inhibit worker ovarian development and egg-laying behaviour in several species (Van Oystaeyen et al., 2014; Vargo, 1998). The hydrocarbons that cover queen-laid eggs have

Table 2

Pairwise comparisons of the number of eggs produced in the four treatment categories in experiment 4

	LV-less	LV-down	LV-up	LV-right
LV-less		0.471	0.001	0.0001
LV-down	0.72		0.005	0.0001
LV-up	3.70	2.97		0.210
LV right	4.97	4.24	1.27	

The numbers to the right and the left of the diagonal are the P and t values, respectively, given by the linear mixed-effects model.

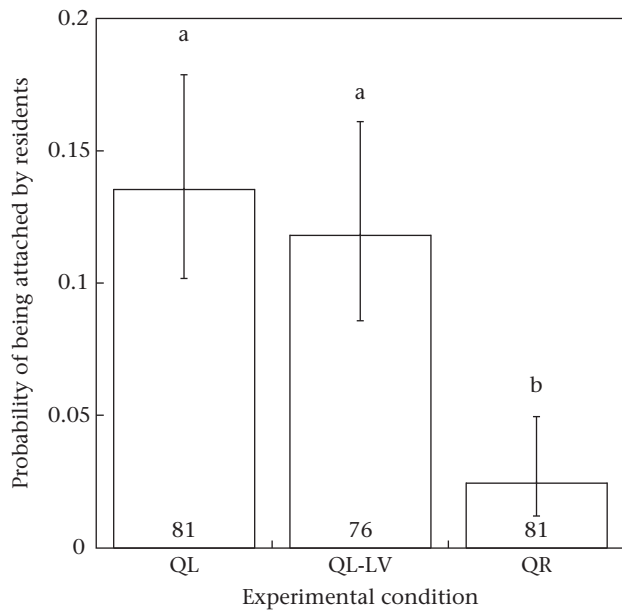


Figure 3. Probability (mean \pm SE) of a foreign ant being attacked by residents that were queenless (QL), queenless with larvae (QL-LV) or queenright (QR). The different letters denote significant differences between experimental treatments. Numbers are sample sizes.

similar effects (Endler, Liebig, & Hölldobler, 2006; Endler et al., 2004) in *Camponotus floridanus* but not in *A. senilis* (Ruel, Lenoir, Cerdá, & Boulay, 2013). In *A. mellifera*, brood pheromones largely composed of fatty acid esters have been shown to inhibit worker reproduction (Mohammedi et al., 1998). However, this chemical signal may not honestly reveal larva sex in *A. senilis*; deceptive signalling has already been observed in other ant species (Nonacs & Carlin, 1990). Hence, the results of experiment 3 show that diploid queen-derived larvae and haploid worker-derived larvae equally inhibited egg laying by workers. Orphaned workers do not gain any apparent benefits from raising their sisters' sons instead of their own. However, situations in which orphaned workers are surrounded by abundant worker-laid eggs might be rare in nature. Once some workers have overcome their sisters' potential policing and egg predation, the surviving haploid larvae may constrain reproduction by their mothers and aunts.

Several studies have shown that mutual policing is a major means of controlling selfish reproduction by workers in ants and other social insects (Iwanishi et al., 2003; Monnin & Ratnieks, 2001; Ratnieks & Wenseleers, 2008; Schmid, Kaltenpoth, Strohm, & Heinze, 2013; Tsuchida et al., 2003; Wenseleers & Ratnieks, 2006). Queenless workers with developed ovaries are often attacked when they are reintroduced into queenright nests (Monnin & Ratnieks, 2001; Ratnieks & Wenseleers, 2008; Ruel et al., 2012). In contrast to what we might have expected, we found that queenless workers with developed ovaries were systematically the targets of aggression when they were introduced into groups of other queenless ants, regardless of the number of larvae; however, they almost never faced aggression when they were introduced into queenright groups. The lack of aggression under queenright conditions is difficult to interpret. Thus far, no mechanisms for controlling worker reproduction, such as egg policing, have been observed in this species (Boulay et al., 2007; this study). There may be no need for aggressive behaviours if workers naturally refrain from laying eggs in the presence of the queen. However, aggression among reunified queenless ants could be explained by the fact that queenless ants often form hierarchies

in which individuals with the greatest reproductive potential are also behaviourally dominant (Monnin & Peeters, 1997). By directing their aggression towards low-ranking ants, high-ranking ants may enhance their direct reproduction (selfish policing, sensu Stroeymeyt, Brunner, & Heinze, 2007). Introducing egg-laying workers may thus have disturbed the hierarchy that had already been established among residents, which led to high levels of aggression towards foreign workers with the largest ovarioles. Workers with more developed ovaries have slightly different hydrocarbon profiles than those with less developed ovaries (Ichinose & Lenoir, 2009; Ruel, Hefetz, et al., 2013). These chemical differences may be the basis for recognition and aggression among high-ranking workers.

In orphaned colonies of *A. senilis*, the allocation of resources to diploid brood allows the production of numerous workers and a few queens, and one of the latter may replace her dead mother. It might therefore be advantageous for workers to delay egg laying until most of the diploid larvae have reached an advanced developmental stage. If workers do so, the colony may survive the death of the queen. However, this possibility is likely to be limited to species that perform colony fission. In contrast, in species with independent colony foundation, young queens mate during nuptial flights and are unlikely to return to their original nests after mating. Therefore, the colony has the same life span as its queen. In this type of situation, where colony persistence is impossible, the collective decision made by workers to start laying haploid eggs should be less dependent upon the presence of larvae. Further studies should be conducted on a wider range of ant species in order to better understand how ant larvae, via either behavioural or pheromonal mechanisms, affect collective reproductive decisions at the colony level. Such studies would very likely contribute to our understanding of the transition from solitary to social life.

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