IMMUNE DEFENSE IN LEAF-CUTTING ANTS: A CROSS-FOSTERING APPROACH

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To ameliorate the impact of disease, social insects combine individual innate immune defenses with collective social defenses. This implies that there are different levels of selection acting on investment in immunity, each with their own trade-offs. We present the results of a cross-fostering experiment designed to address the influences of genotype and social rearing environment upon individual and social immune defenses. We used a multiply mating leaf-cutting ant, enabling us to test for patriline effects within a colony, as well as cross-colony matriline effects. The worker’s father influenced both individual innate immunity (constitutive antibacterial activity) and the size of the metapleural gland, which secretes antimicrobial compounds and functions in individual and social defense, indicating multiple mating could have important consequences for both defense types. However, the primarily social defense, a Pseudonocardia bacteria that helps to control pathogens in the ants’ fungus garden, showed a significant colony of origin by rearing environment interaction, whereby ants that acquired the bacteria of a foster colony obtained a less abundant cover of bacteria: one explanation for this pattern would be co-adaptation between host colonies and their vertically transmitted mutualist. These results illustrate the complexity of the selection pressures that affect the expression of multilevel immune defenses.

KEY WORDS: Acromyrmex echinatior, antibacterial activity, cross-fostering, ecological immunology, metapleural gland, Pseudonocardia.

If we are to understand phenotypic plasticity and condition-dependent expression of traits, it is necessary to examine how the interplay between nature and nurture influences these traits (Pigliucci 2001). Immune defenses are well known for their conditional expression, because they have both maintenance (e.g., McKean et al. 2008) and usage costs (e.g., Poulsen et al. 2002a; Armitage et al. 2003). Net selection on immune traits can be complex when various selection regimes affect the same trait, as for example when gender differentially shapes immune investment (Rolff 2002; McKean and Nunney 2005). Other complexities arise when different levels of selection are involved, for example when defenses at the group level partly compensate for individual immunity (Anderson and May 1985; Cremer et al. 2007). Although sterile workers are in some ways comparable to somatic cells in multicellular organisms (e.g., Wheeler 1911; Cremer and Sié 2009), and as such are disposable to a degree, they have highly effective immune defenses to protect the propagation of the germline (Cremer et al. 2007). Eusocial insects provide instructive examples of immunity at different levels, as they have both individual immune defenses and social defenses that are used at the colony level (Cremer et al. 2007). This may impose trade-offs for resource allocation to alternative immune functions, and also potential evolutionary conflict because the phenotypes of workers are influenced not only by their own genes (direct genetic effects), but also by the social environment and the genes of the individuals that have reared them and with whom they continue to interact as adults (indirect genetic effects).
A fruitful approach to understand the effects of individual and social environment upon phenotypic immunity traits is cross-fostering (e.g., Pankiw et al. 2002; Linksvayer 2006), an experimental technique that we apply here for the first time to analyze variation in individual and social immune defenses. We use the leaf-cutting ant *Acromyrmex echinatior*, to investigate the interplay of genotype and the social environment during pupal and callow nursing on the expression of three components of immune defense. The strength of using this study organism is that the single queen in each colony mates with multiple males (Villesen et al. 2002; Sumner et al. 2004), so that a colony is a dynamic chimera of full sister patrilines that are half sisters to one another. This provides a unique opportunity to study the genetic components of disease resistance by comparing variation in immune traits between patrilines within colonies, as well as across-colony matriline effects, in home-reared as well as cross-fostered social environments. The social environment of leaf-cutting ants further involves an obligate mutualistic symbiosis with a crop-fungus that provides all nourishment for the ant larvae (Bass and Cherrett 1995). Callow workers do not leave the fungus garden to forage and so must obtain their food directly from the fungus garden, or potentially indirectly via trophyllaxis from older workers (Moreira et al. 2006). As a result, in this experiment, the social environment consists of the genetic and environmental components of sister or non-sister nest mates plus the fungus garden (Wcislo 2000).

The first component of immune defense that we considered was individual innate immunity. In simple terms, this is haemolymph-mediated defense consisting of haemocytes that can phagocytose and encapsulate parasites and humoral molecules with antimicrobial activity, plus the phenoloxidase cascade (for reviews see Siva-Jothy et al. 2005; Lemaitre and Hoffmann 2007). These defenses combine constitutively expressed (always ready to act) and induced (expressed only after parasite infection) mechanisms (Hamilton et al. 2008). Constitutive immunity can be a rapid response to infection (e.g., Haine et al. 2008) that is present in the background prior to any immune challenge, and tends to be unsppecific in that it is elicited or effective against a variety of immune challenges (Schmid-Hempel 2005). Here, we assayed the constitutive antibacterial activity of haemolymph in ant workers.

The second defense component that we assayed was the paired exocrine metapleural glands, a phylogenetically ancient trait of the Formicidae that is present in all but a few ant species (Brown 1968). The metapleural glands exude a secretion with broad-spectrum antimicrobial activity (Beattie et al. 1986; Do Nascimento et al. 1996; Bot et al. 2002). The secretion from *Acromyrmex* contains many compounds (Do Nascimento et al. 1996; Ortius-Lechner et al. 2000), which via focused grooming behavior primarily protect the ants (Poulsen et al. 2002a; Fernández-Marín et al. 2009), but also the fungus garden and the leaf substrate brought in by foragers (Fernández-Marín et al. 2006). We therefore consider the metapleural glands as a defense that acts at both the individual and social level and measured the diameter of the externally visible gland (e.g., Hughes et al. 2008) as this is highly correlated with the number of secretory cells (Bot et al. 2001), and thus presumably also with the quantity of the secreted product.

Third, *Pseudonocardia* filamentous bacteria (Actinomycetales) are reared in cuticular crypts, supported by unicellular exocrine glands (Currie et al. 2006). They are vertically transmitted via the founding queen (Currie et al. 1999), but recent evidence from ant and *Pseudonocardia* phylogenies suggests that these bacteria may also sometimes be acquired from the environment (Mueller et al. 2008). The bacteria are absent from the workers’ cuticle when they eclose (Poulsen et al. 2003a), and reach their greatest abundance on the cuticle of approximately two- to three-week old callows of the large worker caste (Poulsen et al. 2003a,b), which at that age do not yet forage but move around in the older and lower parts of the fungus garden, where the Escovopsis infections that the bacteria inhibit (Poulsen et al. 2003b; Poulsen and Currie 2010) are most prevalent (Poulsen et al. 2002b). In addition to targeting *Escovopsis*, some *Pseudonocardia* may also inhibit the in vitro growth of other fungi, including some strains of entomopathogens (e.g., *Beauveria bassiana*; Sen et al. 2009), although it is as yet unclear whether this also happens in vivo. Similar uncertainty exists about the possible inhibition of fungus garden growth by *Pseudonocardia*, which has been shown in vitro (Sen et al. 2009), whereas in vivo it does not reduce fungus garden biomass (Poulsen and Currie 2010). However, for the purpose of the present study, all that matters is that *Pseudonocardia* is an immune defense that largely functions at the collective, social level in contrast to the individual antibacterial immune defense and to the “intermediate” metapleural gland defense that has both individual and collective functions. Importantly, both of the social defenses have been shown to have measurable costs: ants with open metapleural glands (as opposed to experimentally closed glands) and those carrying the full cover of *Pseudonocardia* bacteria had significantly increased respiration rates relative to controls (Poulsen et al. 2002a, 2003a, respectively), implying that individual workers should be selected to reduce the costs of using these defenses when they are not needed, and that there may be trade-offs between these social defenses and individual immune defense.

**Methods**

We performed a short-term cross-fostering experiment in which large workers were removed from their mother colony as newly moulted pupae and kept with fungus garden and nestmates from their own or a foster colony until three weeks after imaginal eclosion. After this time, we assayed the three defense components. By
using microsatellite markers to genotype the workers, we could examine how the social environment, maternal colony, and paternal genotype influence the investment in individual and social defenses.

Two colonies (Ae227 and Ae266) of *A. echinatior* collected in Gamboa, Panama, in 2003 and 2004, respectively, were chosen out of a larger sample of colonies because these colonies contained the most distantly related fungus gardens (AFLP data from preliminary work for Ivens et al. 2009). All colonies and experimental ants were maintained at 25 °C and 75% humidity in a 12 h light:dark cycle, with a diet of fresh bramble leaves (*Rubus fruticosus*), apple and dry rice. Over 158 days in spring and summer 2007, 994 large worker pupae were removed from the two colonies, randomly assigned to control or cross-foster groups, and kept in large Petri dishes until adult eclosion. Each large Petri dish contained 10 minor, 10 media, and 2 major workers, plus 0.61 g of fungus garden from one of the two colonies. Four Petri dishes were set up per colony, two control dishes received pupae from the same colony as the fungus garden and workers, and two cross-foster dishes received pupae from the other colony. After eclosion, control and cross-foster workers were paint-marked with a different color combination for each day of emergence, and transferred to larger subcolonies. We established two large subcolonies per mother colony, each randomly assigned as either control or cross-foster. Each of these contained 2.71 g of fungus garden and 155 minor, 50 media, and 20 major workers from one mother colony. Nonpaint-marked workers that died were replaced, but the fungus garden size was not modified after set-up, to disturb the subcolonies as little as possible. Over the course of the experiment we noted small fluctuations in fungal growth across subcolonies, but there was no consistent pattern. We use the term social environment to cover the sum of dietary effects from the fungus and care received from the ant workers. Because the two colonies that we used were of the same size and had been kept in the laboratory for several years prior to the experiments, we believe we have largely eliminated environmental differences in colony performance. We therefore assume that differences that we detect at the colony level largely reflect genetic differences.

Painted ants were removed from the subcolonies 21 ± 1 days after adult eclosion. They were anaesthetized on ice, weighed to the nearest 0.1 mg, the distance between the eyes was measured with a stereomicroscope (Leica, Ballerup, Denmark), and the body-coverage of the *Pseudonocardia* bacteria acquired from either the cross-fostering colony or mother colony was quantified using a scale from 1 to 12 (see Poulsen et al. 2003a for details). When the ants emerge as adults they shed their pupal exoskeleton, thus we assume that *Pseudonocardia* on the adult ants are derived from the nest in which they emerge as adults. Immediately after examination, 0.2 μL of haemolymph was collected for the antibacterial assay in glass capillaries drawn to a fine point. Capillaries were calibrated with a Hamilton syringe and flushed with sterile anticoagulant buffer (0.098 M NaOH, 0.186 M NaCl, 0.0051 M EDTA, 0.041 Citric Acid, pH 4.5) before use. Haemolymph from each individual was added to 3 μl anticoagulant buffer, immediately frozen in liquid nitrogen, and stored at −80 °C for the antibacterial zone of inhibition assay. All ants were sampled between 1230 and 1930; time was included as a covariate in statistical analyses as the number of hours after midday at which the haemolymph was removed.

The zone of inhibition assay that we used was modified from Moret and Schmid-Hempel (2000). In short, we added 6 mL sterile broth medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl per 1000 mL dH2O, pH 7.5) at 42 °C, containing 1% bacto-agar and 10⁵ *Arthrobacter globiformis* (strain CIP 105365, Institut Pasteur, Paris, France) bacterial cells per milliliter, to 85 mm diameter Petri dishes. Holes with a 2-mm diameter were punched into the agar, evenly spaced, in a single circle 30 mm from the edge of each plate, and hence were in agar of uniform thickness (based on tests prior to the assay). One microlitre of ethanol saturated with phenylthiourea (PTU, Sigma-Aldrich, Brøndby, Denmark), was added to each hole to prevent melanization of the haemolymph, and the ethanol was allowed to evaporate. Randomly selected haemolymph samples were defrosted on ice, and 2 μL of briefly centrifuged haemolymph-buffer mix was allocated at random to each hole. The plates were incubated for 19 h at 28 °C and during this time the antibacterial activity of the haemolymph inhibited the growth of the *A. globiformis* culture and resulted in clear circular zones around each of the holes. The diameters of the clear zones are proportional to the strength of the inhibition of the bacteria (Moret and Schmid-Hempel, 2000). Digital images of the plates were obtained with a scanner and we measured two perpendicular diameters of the zone of inhibition using Paintshop Pro 7.0.0 with three times magnification, and used the mean value of these two diameters for statistical analyses. These antibacterial methods were repeatable (Lessells and Boag, 1987), using haemolymph from one ant and measuring the clear zone in two different holes (intraclass correlation coefficient: r = 0.887, F₁₀₀,₂₀ = 160.227, P < 0.0001).

For genotyping, DNA was extracted from one leg per ant by boiling it for 15 min in a 20% Chelex® (Fluka 22477, Sigma-Aldrich, Brøndby, Denmark) ddH₂O solution. The extracted DNA was amplified at four polymorphic microsatellite loci: Ech1390, Ech3385, Ech4126, and Ech4225 (Ortius-Lechner et al. 2000). All PCR reactions contained 4 μL GATC mix, 1 μL PCR buffer (Applied Biosystems, Naerum, Denmark), 0.1 μL AmpliTaq Red polymerase (Applied Biosystems), and 1 μL DNA extract. In addition, reactions for Ech1390 and Ech4126 contained 1.9 μL ddH₂O and 0.5 μL primer; reactions for Ech3385 contained 3.1 μL ddH₂O and 0.4 μL primer, and reactions for Ech4225 contained 1.5 μL ddH₂O and
1 μL primer as well as 0.4 μL MgCl₂ (Applied Biosystems). DNA was amplified by multiplexing Ech1390 and Ech4126, and running Ech3385 and Ech4225 separately, on a Hybaid PCR Express Thermal Cycler. For the first three primers the program was as follows: an initial denaturing step of 94°C for 4 min followed by a sequence of six cycles (92°C for 30 sec, 65°C for 30 sec, 72°C for 45 sec), a sequence of seven cycles (92°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec), and a sequence of 20 cycles (92°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec) followed by a final elongation step of 72°C for 15 min, except for Ech3385 where this final step lasted 60 min. The program for Ech4225 consisted of an initial denaturing step of 94°C for 4 min, then 35 cycles (92°C for 30 sec, 52°C for 30 sec, and 72°C for 45 sec) followed by a final elongation step of 72°C for 60 min. PCR products were analyzed on a Hitachi Applied Biosystems 3130XL Genetic Analyzer. The microsatellite data were analyzed with Genemapper® software (version 4.0, Applied Biosystems) and individuals were assigned to patrilines using Matesoft 1.0 software (Moilanen et al. 2004).

Metapleural gland diameter was measured blindly on a subset of 192 ants following the methods of Hughes et al. (2008). Measurements included an approximately equal number of ants from each of the most common patrilines and rearing environments, and similar numbers of individuals from across the time course of the experiment.

In all groupings, Box–Cox transformed \((y^{1.587} - 1)/1.587\) antibacterial activity (mean zone of inhibition diameter) was normally distributed or approaching normality (Kolmogorov–Smirnov tests), and had equal variances (Levene’s test). Loge *Pseudonocardia* cover, and loge, metapleural gland width were not normally distributed in all groupings but did have equal variances. These deviations from normality were not considered substantial enough to preclude parametric statistical analyses. Using JMP 7.0.2 (SAS Institute, Cary, NC), we ran separate models for the three variables, Box–Cox antibacterial activity, loge *Pseudonocardia* cover, and loge, metapleural gland size, and we also tested for trade-offs between the immune traits. Because 186 (24%) of the individuals for which we obtained zone of inhibition and *Pseudonocardia* data either did not belong to one of the more common patrilines or could not be unambiguously assigned to a single patriline, one set of models without patriline was applied to the whole dataset, and another set of models was applied to a smaller dataset including patrilines that were common enough to give acceptable sample sizes. Where patriline was included it was assigned as a random factor and nested within colony of origin. The term environment indicates the identity of the colony in which the individuals were raised (i.e., Ae227 or Ae266). Covariates included in the models with antibacterial zone of inhibition and *Pseudonocardia* cover were body size (The first principal component from a PCA of loge body mass and loge head width), day of sampling, and day of sampling squared, to allow the dependent variables to show curvilinear responses over time. For the antibacterial activity model, hour of haemolymph sampling was also included. Although the size of the metapleural gland is fixed at adult eclosion (Bot and Boomsma 1996) we cannot discount the possibility that some changes may occur during the pupal stage, so we included pupal rearing environment in the model with metapleural gland. To test for correlations or trade-offs between the various disease defense traits, we ran models for all three combinations of defenses, and included colony of origin, patriline, and interactions. For these analyses, we used residuals for metapleural gland width from the correlation with body size. Figures are plotted using untransformed data and all data and figures show means ± one standard error.

**Results**

Colonies Ae227 and Ae266 contained workers from eight and 14 patrilines respectively, a range that is comparable to previous estimates for this species (Sumner et al. 2004). Only the most common patrilines (denoted as I, II, and III for Ae227 and i, ii, iii, and iv for Ae266) were used for the analyses that included patrilines.

**ANTIBACTERIAL ACTIVITY**

The individual immune defense, haemolymph antibacterial activity measured as the diameter of the zone of inhibition, was significantly different between ants originating from the two colonies and the rearing environment had a weaker but also significant effect (Table 1, Model A). However, when we included patriline in the reduced dataset, colony of origin was no longer a significant predictor, and there was instead a significant effect of patriline upon the zone of inhibition (Table 1, Model B; Fig. 1A) whereby patrilines within, as well as between, colonies differed significantly from one another. There were also significant positive relationships in both models between the zone of inhibition and worker size, day of sampling, and day of sampling squared (Table 1, Models A and B), but most of the latter were due to an unplanned increase in worker size as the experiment progressed.

**METAPLEURAL GLAND SIZE**

Metapleural gland width did not differ significantly between the two colonies, and there was no effect of rearing environment or an interaction between colony of origin and rearing environment (Table 2). However, similar to the antibacterial defense, patrilines had significantly different gland sizes to one another (Table 2; Fig. 1B), both within and between colonies. Once again, there were significant positive relationships between metapleural gland size and worker size, day of sampling, and day of sampling squared.
Table 1. The effects of genotype and rearing environment upon antibacterial activity measured as the diameter of the zone of inhibition. Patriline was included in model B, but not in model A. Where appropriate the sign of the relationships is indicated with (+) or (−). P-values less than 0.05 are in bold.

<table>
<thead>
<tr>
<th>Tested effect</th>
<th>Model A</th>
<th></th>
<th>Model B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony of origin</td>
<td>1,632</td>
<td>8.30</td>
<td>0.004</td>
<td>1,546</td>
</tr>
<tr>
<td>Treatment (Own vs. Foster)</td>
<td>1,632</td>
<td>4.30</td>
<td>0.039</td>
<td>1,446</td>
</tr>
<tr>
<td>Colony of origin × Treatment</td>
<td>1,632</td>
<td>0.343</td>
<td>0.559</td>
<td>1,446</td>
</tr>
<tr>
<td>Day</td>
<td>1,632</td>
<td>28.9</td>
<td>&lt;0.001 (+)</td>
<td>1,446</td>
</tr>
<tr>
<td>Day²</td>
<td>1,632</td>
<td>15.2</td>
<td>0.001 (−)</td>
<td>1,446</td>
</tr>
<tr>
<td>Worker size</td>
<td>1,632</td>
<td>16.0</td>
<td>&lt;0.001 (+)</td>
<td>1,446</td>
</tr>
<tr>
<td>Hours after midday</td>
<td>1,632</td>
<td>0.661</td>
<td>0.417</td>
<td>1,446</td>
</tr>
<tr>
<td>Patriline nested within colony of origin</td>
<td>5,446</td>
<td>8.08</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. (A) Antibacterial activity measured as the diameter of the zone of inhibition across patrilines. The filled and open squares to the right of the dashed line are the mean values for the ants that were from an uncommon patriline or which could not be assigned to a patriline; they were not included in the statistical model with patriline (Table 1, Model B) but are included here for visual comparison. (B) Mean metapleural gland size across patrilines. For both (A) and (B), filled symbols are patrilines from Ae227 and open symbols are patrilines from Ae266; when letters above the means are not the same the means are significantly different from one another; numbers in parentheses below the datapoints indicate the number of ants sampled.

Table 2. Effects of genotype and rearing environment upon metapleural gland size. Where appropriate the sign of the relationships is indicated with (+) or (−). P-values less than 0.05 are in bold.

<table>
<thead>
<tr>
<th>Tested effect</th>
<th>df</th>
<th>F-ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony of origin</td>
<td>1,507</td>
<td>0.017</td>
<td>0.900</td>
</tr>
<tr>
<td>Treatment (Own vs. Foster)</td>
<td>1,180</td>
<td>2.53</td>
<td>0.114</td>
</tr>
<tr>
<td>Colony of origin × Treatment</td>
<td>1,180</td>
<td>0.120</td>
<td>0.278</td>
</tr>
<tr>
<td>Day</td>
<td>1,180</td>
<td>5.88</td>
<td>&lt;0.001 (−)</td>
</tr>
<tr>
<td>Day²</td>
<td>1,180</td>
<td>6.58</td>
<td>&lt;0.001 (+)</td>
</tr>
<tr>
<td>Worker size</td>
<td>1,180</td>
<td>54.90</td>
<td>&lt;0.001 (+)</td>
</tr>
<tr>
<td>Patriline nested within colony of origin</td>
<td>5,180</td>
<td>8.08</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

PSEUDONOCARDIA COVER

Because the callow workers eclose with no Pseudonocardia cover, the bacteria we measured were assumed to have been acquired from the subcolony in which the ants had eclosed. Pseudonocardia cover was the only response variable to show a significant colony of origin by social environment interaction (Table 3, Fig. 2). Ants reared by their own colony, and hence with their

Table 3. Effects of genotype and rearing environment upon Pseudonocardia cover. There was no significant effect of patriline so this level was removed from the model. Where appropriate the sign of the relationships is indicated with (+) or (−). P-values less than 0.05 are in bold.

<table>
<thead>
<tr>
<th>Tested effect</th>
<th>df</th>
<th>F-ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony of origin</td>
<td>1,753</td>
<td>4.21</td>
<td>0.041</td>
</tr>
<tr>
<td>Treatment (Own vs. Foster)</td>
<td>1,753</td>
<td>4.50</td>
<td>0.034</td>
</tr>
<tr>
<td>Colony of origin × Treatment</td>
<td>1,753</td>
<td>12.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Day</td>
<td>1,753</td>
<td>22.5</td>
<td>&lt;0.001 (−)</td>
</tr>
<tr>
<td>Day²</td>
<td>1,753</td>
<td>14.7</td>
<td>&lt;0.001 (+)</td>
</tr>
<tr>
<td>Worker size</td>
<td>1,753</td>
<td>10.0</td>
<td>0.002 (+)</td>
</tr>
</tbody>
</table>

E V O L U T I O N J U N E 2 0 1 1  1 7 9 5
own Pseudonocardia strain, had significantly higher cover than when they were cross-fostered and had “foreign” Pseudonocardia growing on their cuticle. In addition there was a significant effect of environment and colony of origin, as well as a significant positive influence of worker size, and a negative correlation with day of sampling and day of sampling squared (Table 3). However, in contrast to the individual antibacterial defense, patriline did not have a significant effect upon the abundance of Pseudonocardia cover (all P > 0.25), and so the model including patriline is not presented.

**TRADE-OFFS BETWEEN THE TRAITS**

Assaying multiple traits from the same individual allowed us to test for possible within-individual trade-offs between defense traits (Table 4a–c). However, rather than signs of a trade-off, we found a weak positive relationship between metapleural gland size and Pseudonocardia cover (Table 4a; Fig. 3). There was no statistically significant correlation between antibacterial activity and metapleural gland size (Table 4b), although there was a significant effect of patriline upon antibacterial activity (Table 4b) as also seen in Table 1. There was no statistically significant correlation between Pseudonocardia cover and antibacterial activity (Table 4c).

**Discussion**

This is the first study, to our knowledge, to use a cross-fostering approach to examine how colony of origin and social rearing environment influence investment in individual and social immune defenses. Social environment did not affect antibacterial activity or metapleural gland size, but there were direct genotype effects

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**Figure 2.** Pseudonocardia cover on the cuticle of ants from each of four subcolonies after being reared in their own social environment or after being cross-fostered by another colony. The x-axis shows the social environment in which the ants were raised, and the black (Ae227) and open (Ae266) symbols indicate the genetic colony of origin of the pupae. Numbers in parentheses are the number of ants sampled. Pupae eclosing in their own colony acquired a higher Pseudonocardia coverage as callow workers compared to cross-fostered workers.
shown by significant differences between patrilines, both within and between the two colonies. In contrast, patriline did not influence Pseudonocardia cover, and cover was significantly affected by the social environment in which the ants were raised.

When we tested antibacterial activity against the bacterium A. globiformis in the absence of patriline information, we found that there was variation at the colony level, a trait deriving from both maternal and paternal genes. Furthermore, there was potential for plasticity as immune defense varied according to rearing environment. However, when we included patriline in the model, we found no environmental rearing effect upon antibacterial activity. This apparent discrepancy is possibly caused by the difference in sample size, whereby the excluded individuals (28%) were driving this relationship in the simpler model. More importantly, the colony of origin no longer significantly affected antibacterial activity and this variation could be better explained by variation between patrilines. This suggests that paternal genes affect individual immune defense, independent of the colony of origin (maternal genes). Indeed an hypothesized advantage to multiple mating is differential resistance to disease in the offspring from different patrilines (Hamilton 1987; Sherman et al. 1988; for other advantages of multiple mating, for example, ensuring a sufficient and good quality sperm supply, reducing the risk of producing diploid males, and increased ergonomic efficiency/homeostasis through a more complex task system: see Oldroyd and Fewell 2007; Boomsma et al. 2009). Our results are supported by previous work that found significant variation between patrilines in survival after infection with a low dose of entomopathogenic fungi (Hughes and Boomsma 2004), indicating genetic variation for disease resistance. Infection by a fungus is a multistage process, and ant behavioral responses can interfere with the successful penetration of the cuticle before the fungus is faced with the defenses of the haemolymph. Our present experiment is novel in that we show that also haemolymph-derived defense factors show patriline variation in immunity. An extension of this would be to test haemolymph defense against different microorganisms, to uncover whether there are patrilines that are consistently good at defending against a multitude of microorganisms or whether there are trade-offs within patrilines. Although we do not know which antimicrobial substances were responsible for the antibacterial activity, we can discount phenoloxidase and direct mediation by haemocytes (see methods).

Similar to the antibacterial defense, direct genetic effects in terms of patriline had a significant effect upon metapleural gland size. Despite this similarity, it is noteworthy that there was no within-individual trade-off or correlation between these traits. Our patriline result corroborates a recent study on metapleural gland size in A. echinatior and A. octospinosus (Hughes et al. 2010), and once more suggests that increased variation in defense strategies could be a benefit of multiple mating. Previous work has shown that when metapleural glands were experimentally closed, the basic metabolic rate was significantly reduced compared to control workers with open glands, indicating that production of the secretion incurs a considerable cost (Poulsen et al. 2002a). In addition when workers are deprived of food they quickly stop producing the secretion, which also suggests that the secretions are costly in some way (Bot and Boomsma 1996). If patrilines vary in gland size and there are costs to the production of the secretion, it is conceivable that there are patriline-specific trade-offs between production of the secretion and other life-history traits or immune functions. However, the size of the metapleural gland is only one factor that will influence its efficacy; also important are the chemical composition of the secretion and the behavioral manner in which the gland is used. Interestingly, in a closely related species, A. octospinosus, although patrilines showed differences in the quantitative chemical composition of the secretion from the metapleural gland, these were not consistent or statistically significant (Ortius-Lechner et al. 2003), and it is not yet clear if this variation relates to differences in the quality of the secretion, or whether there is a patriline influence upon behavioral use of the gland. Perhaps unsurprisingly, cross-fostering at the pupal stage did not influence metapleural gland size, although it would be interesting to test for plasticity in this trait, for example when there is parasite pressure during an earlier developmental stage.

One of our assumptions was that the mutualistic Pseudonocardia bacteria come from the colony in which the adult ecloses, therefore our experimental set-up allowed us to test how well Pseudonocardia strains grow on a novel host colony, which is pertinent given a recent finding that the ants can acquire novel Pseudonocardia from their environment (Mueller et al. 2008; Sen et al. 2009). Pseudonocardia cover showed a significant colony of origin by rearing environment interaction, that is, ants raised in their home environment had higher Pseudonocardia cover compared to those raised in foster environments. This
indicates that *Pseudonocardia* strains may indeed be swapped between colonies, but that newly acquired strains may grow less well (Boomsma and Aanen 2009). The generalizability of reduced *Pseudonocardia* growth on a novel ant colony should be extended by testing more colonies, but if the pattern is upheld it could have important ramifications regarding the evolution of this host–mutualist interaction. Interestingly there was no effect of patriline on the baseline *Pseudonocardia* levels. The abundance of *Pseudonocardia* on the cuticle is increased after infecting the colony with *Escovopsis* (Currie et al. 2003), so it would be interesting to test whether differences between patrilines become evident after infection. Given that social defense by *Pseudonocardia* and metapleural gland secretions can be costly, and that innate defenses have also been shown to be costly, one may predict individual-level trade-off between the defenses. However, we found no evidence for trade-offs, at least in the absence of infection, when the costs of the defenses will be low. Our data instead suggest a weak positive relationship between metapleural gland size and *Pseudonocardia* cover.

To conclude, we present a novel application of the classical cross-fostering technique to investigate the basis of variation in individual and social immune defenses. Nature had a significant effect on individual innate immunity: the worker’s father significantly influenced constitutive antibacterial activity, and it influenced the size of the metapleural gland. However, the primarily social defense, *Pseudonocardia* bacteria, showed a significant colony of origin by rearing environment interaction, where ants that acquired the bacteria of a foster colony had a less abundant cover of bacteria. Future work to cross-foster as larvae or after parasite infection, along with using more colonies, could prove a powerful technique to further our understanding of the underlying selective forces of nature and nurture upon immune defense.

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