Testing the adjustable threshold model for intruder recognition on Myrmica ants in the context of a social parasite

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Social insect colonies are like fortresses, well protected and rich in shared stored resources. This makes them ideal targets for exploitation by predators, parasites and competitors. Colonies of Myrmica rubra ants are sometimes exploited by the parasitic butterfly Maculinea alcon. Maculinea alcon gains access to the ants’ nests by mimicking their cuticular hydrocarbon recognition cues, which allows the parasites to blend in with their host ants. Myrmica rubra may be particularly susceptible to exploitation in this fashion as it has large, polydomous colonies with many queens and a very viscous population structure. We studied the mutual aggressive behaviour of My. rubra colonies based on predictions for recognition effectiveness. Three hypotheses were tested: first, that aggression increases with distance (geographical, genetic and chemical); second, that the more queens present in a colony and therefore the less-related workers within a colony, the less aggressively they will behave; and that colonies facing parasitism will be more aggressive than colonies experiencing less parasite pressure. Our results confirm all these predictions, supporting flexible aggression behaviour in Myrmica ants depending on context.

Keywords: aggression; defence; social parasite; Maculinea

1. INTRODUCTION

The ability to discriminate between relatives and non-relatives should give an advantage to the inclusive fitness of an individual, and is therefore expected to be favoured by kin selection [1,2]. Inclusive fitness can only increase if the benefits of shared labour can be directed towards colony members or close relatives rather than towards foreigners [2]. Social insects, such as ants, have achieved sophisticated nest-mate recognition skills, and high levels of aggression are generally expressed towards non-nest-mates, which potentially pose a cost to the society. Even though social insects have evolved an array of discrimination cues, odours are thought to be the predominant cues for the discrimination of nest-mates and non-nest-mates. Considerable direct and indirect evidence has been collected in favour of cuticular hydrocarbons serving as the main cues for nest-mate recognition in ants [3–5]. Several models of the proximate recognition mechanisms have been put forward to explain how ants in huge societies are able to tell friends from foes. Crozier & Dix [6] proposed the ‘Gestalt’ model, which assumes that colony members mix their odours and recognize a composite odour for their own colony, which allows for discrimination of workers from other colonies that have a different distribution of odour locus alleles. Reeve [7] proposed an additional action component to the model, triggering aggression according to cues perceived by the actor exceeding or falling below a certain threshold. The model aims to adjust the threshold to minimize acceptance errors (acceptance of a non-nest-mate) and rejection errors (rejection of a nest-mate) according to the needs of a colony. The more costly an acceptance or rejection error will be, the more likely it is that the thresholds will shift in order to avoid any costly mistake. To test the implications of this model, we examined colonies from a population of the ant Myrmica rubra, at a site where it co-occurs with its social parasite Maculinea alcon. Myrmica rubra is a common Palaearctic ant species, with a highly variable colony structure, ranging from monogynous and monodomous to highly polygynous and polydomous populations [8], with queen number within populations being a very variable trait [9,10]. Relatedness among My. rubra nest-mates has been shown to be directly correlated with the number of reproductively active queens in the colony [11]. Myrmica rubra sexuals tend to mate close to the home nest, and new nests are usually started when the mother colony fissions (i.e. the colony splits and workers leave the mother colony together with at least one inseminated queen) [12].

Maculinea alcon is a socially parasitic butterfly whose larvae take advantage of the shelter and resources of ant nests. The butterfly caterpillars feed on their host plant Gentiana pneumonanthe for their first three instars before they leave the plant in their fourth and final instar, and await carriage back to the nest by a Myrmica ant. This is facilitated by mimicking the ant brood’s cuticular hydrocarbon cues [13]. Once inside the nest, the ants will treat the caterpillars like their own larvae, feeding them by trophallaxis. Additionally, the caterpillars feed directly on ant brood. Since G. pneumonanthe is patchily distributed relative to the common Myrmica host ants, areas where ants face strong parasitism by My. alcon are

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typically small ‘islands’ defined by gentian patches within a local Myrmica population. Nevertheless, it has been shown [14] that the viscous population structure of My. rubra has allowed M. alcon to enter into a coevolutionary arms race with its host ants, altering their cuticular hydrocarbon profiles.

In the present study, we compared how aggressively workers from colonies of My. rubra behave towards conspecific intruders from areas of the same population where the social parasite M. alcon is present with areas where it is absent. We collected data on the genetic structure and the chemical profiles of colonies to be able to test several predictions arising from Reeve’s [7] optimal acceptance threshold model:

— If neighbouring colonies are more closely related to each other than to colonies further away, recognition cue similarity should decline with geographical distance. Without a change in acceptance threshold, aggression is therefore expected to rise with distance (production component; figure 1).

— With more queens in a colony, genetic diversity will rise. As a consequence, unless there is perfect mixing of the gestalt odour within colonies, the recognition cue distribution will broaden. We thus expect the acceptance threshold to right-shift (ants get less aggressive) in order to avoid too many costly rejection errors (action component; figure 1).

— In populations that suffer from social parasitism, the acceptance threshold is expected to shift to the left, since acceptance errors are more costly. Colonies from areas where Maculinea is present are therefore expected to act more aggressively compared with colonies that live in areas where the Maculinea parasite is absent (action component; figure 1).

Figure 1. Frequency distribution of cue dissimilarities between the actor template (left of acceptance threshold) and recipients (non-nest-mates) phenotype (right of the acceptance threshold). Queen effect (dashed lines) causes the cue dissimilarity distribution (CDD) to broaden and as a result to shift the acceptance threshold to the right in order to avoid rejection errors (rejection of nest-mates); at the same time, with the right shift the risk of acceptance errors (acceptance of non-nest-mates) increases. Social parasites can take advantage of this ‘open door’. In order to avoid social parasites, the threshold needs to be set to more restrictive values (action component; dashed arrow). With increasing distance (physically, chemically or genetically; dotted lines) overlaps between template CDD and recipient phenotype CDD are lower, since the CDDs diverge (production component) and it becomes safer to unleash higher aggression towards an encountered recipient.

2. MATERIAL AND METHODS
(a) Ant collection and housing
We collected a total of eight My. rubra colonies (hereafter ‘test colonies’) in June 2006 from the Nordmarken area of the Danish island of Læsø. Myrmica rubra colonies were collected along two parallel transects, 50 m apart. One of the transects was laid out within a 1.1 hectare patch of G. pneumonanthe plants, where the social parasite Maculinea alcon is common and My. rubra faces severe parasitism [15]. The second transect ran through an area separated from the flying areas of M. alcon by low shrubs, and too dry for G. pneumonanthe to grow. Along the second transect, Maculinea parasitism has not been observed since we started monitoring in 1998 and is highly unlikely since My. rubra do not forage more than a few metres from their nests [16]. Our collections took place at two sampling points 100 m apart along each transect. At each sampling point, we completely excavated two neighbouring Myrmica rubra nests (resulting in four colonies per transect).

Colony social structure was examined based on the number of excavated queens and using three highly variable microsatellite loci (for more detailed information see the electronic supplementary material).

In natural colonies, the number of queens observed in a nest and the genetic diversity among the workers in the nest (which is expected to influence cue diversity), while correlated, often diverge from each other owing to reproductive skew, variation in mating frequency among queens and queen turnover. To try to tease apart the effects of genetic relatedness and queen number per se, we collected an
additional nine *My. rubra* colonies from the area without parasitism just after hibernation in March 2008, and controlled queen number (matrilines) within the nests (hereafter ‘matrine colonies’) by removing queens and allowing a new cohort of workers to develop. This allowed us to decouple the possible effect of innate aggressiveness on genetic diversity that could arise if less aggressive colonies are more likely to adopt new queens (for further information see the electronic supplementary material).

(b) Aggression assays

Directed aggression tests in a controlled, neutral arena were performed with the eight test colonies using a full factorial design, with each colony acting as either defenders (five workers per encounter) or intruders (one worker per encounter) in interactions with every other colony. Three replicates were conducted for each of the 64 possible pairs of colonies. For the matrine colonies, we were not able to test the colonies in a full factorial design since too few workers were produced in the small laboratory colonies, but instead each colony was tested against every other as either defender or intruder. For each test, an aggression index was calculated for how aggressively the defenders responded to the intruder (for further information see the electronic supplementary material).

(c) Surface chemistry

The cuticular hydrocarbons of ten of the genetically analysed workers were extracted and analysed on a gas chromatograph with mass spectrometer (GC-MS) detector, using standard methods (for further information see the electronic supplementary material).

(d) Statistical analysis

Correlations between geographical, genetic and chemical distances between colonies were assessed using Mantel tests, as implemented in Genailex v. 6.2 [17].

To test the various hypotheses generated by the adjustable threshold model, we assessed the relationship between the measured index of aggression and transect (i.e. presence or absence of *M. alcon*), three different measures of distance between colonies (geographical, chemical and genetic) and three different measures of colony social structure (the number of queens excavated with the colony, and the number of queens and the within-colony relatedness estimated from microsatellite markers). The aggression index was log-transformed for the analysis, to normalize its distribution, and nine separate models were run for each of the combinations of different distance and social structure measurements.

For the matrine colonies, the effect of number of matrilines and geographical distance on loge aggression index was of different distance and social structure measurements. Nine separate models were run for each of the combinations transformed for the analysis, to normalize its distribution, and from microsatellite markers (*M. rubra*). The aggression index was loge-number of queens and the within-colony relatedness estimated number of queens excavated with the colony, and the three different measures of colony social structure (the PCA), which yielded two principal components (PCs) explaining significantly more of the variance than expected under a broken stick model [18]. Discriminant analysis based on those two PCs was performed to distinguish between our predefined groupings, the colonies, the sampling points and the transects. Chemical distance between colonies was calculated as the Euclidean distance between the samples from pairs of colonies in PC space.

3. RESULTS

Two of the four colonies collected within the parasitized transect had one and four *M. alcon* caterpillars present, respectively. The number of queens collected with the test colonies ranged from 1 to 17 (mean 8.6). However, based on three microsatellite markers, mean number of queens per colony was estimated as 11.25, ranging from 7 to 15. The correlation between excavated number of queens and estimated number of mother queens was +0.558 (*n* = 8, *p* = 0.125). Within-colony relatedness estimates ranged from 0.020 to 0.314 (mean 0.139), and were significantly negatively correlated with both the excavated number of queens (*r* = −0.731, *p* = 0.040) and the number of queens estimated from microsatellite markers (*r* = −0.806, *p* = 0.016).

For the analysis of cuticular hydrocarbon profiles, the first PC explained 59.0 per cent of the total variance and the second PC explained an additional 13.9 per cent. A discriminant analysis based on the two PCs and grouping by colony, sampling point or transect found significant diversification, with colonies, sampling points and transects being significantly different from each other (colonies: Wilks’ *λ* = 0.247, *F* = 10.54, *p* < 0.0001; sampling points: Wilks’ *λ* = 0.412, *F* = 14.32, *p* < 0.0001; transects: Wilks’ *λ* = 0.845, *F* = 7.24, *p* = 0.0013).

As predicted, based on the previously reported genetic viscosity of *M. rubra* populations, genetic, chemical and geographical distances between the test colonies were significantly correlated (Mantel tests: geographical versus genetic distances: *r* = +0.373, *p* = 0.001; geographical versus chemical distances: *r* = +0.388, *p* = 0.036; genetic versus chemical distances: *r* = +0.417, *p* = 0.032).

Overall, test colonies showed significant differences in aggression levels (figure 2; ANOVA, *F* = 6.91, *p* < 0.0001). Colonies collected from the transect where *M. alcon* was present were significantly more aggressive than those from the transect where *M. alcon* was absent, and the difference between transects explained 65 per cent of the variance in aggression that could be attributed to differences between colonies (nested ANOVA: between transects, *F* = 11.3, *p* = 0.015; colonies nested within transects, *F* = 2.79, *p* = 0.013). When we constructed ANCOVA models using measures of distance between defender-colony and intruder-colony, defender-colony social structure, and transect from which the defender-colony was collected, some general patterns emerged for all estimates of distance and social structure (table 1). Once again, transect was highly significant in all analyses, with aggression being higher on the *M. alcon* transect. In six of the nine analyses, there was also a significant interaction between transect and social structure of the defender colony, and this effect was close to significance in the other three models. This arose because there was no relationship between social structure and aggression on the transect with *M. alcon* present (where aggression was uniformly
high), but on the transect with M. alcon absent, aggression decreased as the number of queens increased and within-colony relatedness decreased (figure 2). Geographical distance between home and intruder colonies also explained a significant portion of the variance in aggression index in the analysis in which it was included, but there was no effect of the other distance measures (i.e. genetic and chemical distances; table 1). The effect of distance was primarily because aggression was significantly reduced, and rejection and acceptance errors are less likely to occur. The aggression level rises gradually and is not an ‘all or nothing’ response (figure 1), as has been shown previously in other studies (e.g. [21,22]). Selection should generally favour narrow CDDS with high frequencies of desirable recipients, since this allows for restrictive thresholds and at the same time minimizes rejection errors [7]. Genetically diverse ant societies achieve this by mixing their recognition cues throughout the colony, to form a ‘gestalt’ odour [6,23], narrowing down the CDD. This mechanism is less efficient in more diverse and larger colonies. Monogamous monogyny is thought to be the ancestral state in social insects [24], and also the condition under which CDDs can be expected to be narrowest. Polygyny has evolved later [24,25] to improve colony efficiency [26], and perhaps reduce parasite pressure [27]. Our source Myrmica rubra population was highly polygynous, as is typical for this species [28,29]. We collected only one apparently monogynous colony, which contained multiple matrilines after estimating queen number using genetic markers. Overall, our queen numbers estimated using genetic markers were higher than the numbers of queens found during excavation, particularly when one considers that estimates based on genetic markers are minimum estimates. This pattern could emerge if workers in polydomous colonies were to move and mix freely between the nests [30] or if queen turnover was so high that we collected workers from queens that were no longer alive [30,31]. We can also not rule out that some queens escaped our collection routine.

**4. DISCUSSION**

We demonstrated that aggressive behaviour of Myrmica rubra workers is a variable trait that depends on both external and internal factors. The three hypotheses that we tested were all largely verified. In accordance with Reeve’s [7] optimal acceptance threshold model, we found that acceptance thresholds became more restrictive as fitness costs for the colony rise.

In our aggression assays, five ‘defender’ ants encountered one intruder in a neutral area. The five ants should have a higher eagerness to fight since the presence of nest-mates is very likely to indicate the proximity of the home colony, and aggressive defence behaviour should therefore be more beneficial to the ‘defender’ ants. The intruder should benefit from retreat, and is less likely to act aggressively [19]. Buczkowski & Silverman [20] showed that acceptance thresholds shift according to the presence of nest-mates or nest material indicating colony proximity in Linepithema humile. We performed our assays under standardized conditions in a neutral arena to keep the influence of the surrounding conditions to a minimum. Differences in aggression are therefore expected to be caused by something other than the presence of nest-mates or nest material.

We confirmed our first hypothesis: that aggression will increase with increasing distance. The rise in geographical distance is correlated with a rise in genetic distance as well as chemical distance. These differences in cue distribution represent the production component in Reeve’s [7] optimal acceptance threshold model. Colonies further apart have more dissimilar recognition cue distributions, the overlap in cue dissimilarity distribution (CDD) is reduced, and rejection and acceptance errors are less likely to occur. The aggression level rises gradually and is not an ‘all or nothing’ response (figure 1), as has been shown previously in other studies (e.g. [21,22]). Selection should generally favour narrow CDDS with high frequencies of desirable recipients, since this allows for restrictive thresholds and at the same time minimizes rejection errors [7]. Genetically diverse ant societies achieve this by mixing their recognition cues throughout the colony, to form a ‘gestalt’ odour [6,23], narrowing down the CDD. This mechanism is less efficient in more diverse and larger colonies. Monogamous monogyny is thought to be the ancestral state in social insects [24], and also the condition under which CDDs can be expected to be narrowest. Polygyny has evolved later [24,25] to improve colony efficiency [26], and perhaps reduce parasite pressure [27]. Our source Myrmica rubra population was highly polygynous, as is typical for this species [28,29]. We collected only one apparently monogynous colony, which contained multiple matrilines after estimating queen number using genetic markers. Overall, our queen numbers estimated using genetic markers were higher than the numbers of queens found during excavation, particularly when one considers that estimates based on genetic markers are minimum estimates. This pattern could emerge if workers in polydomous colonies were to move and mix freely between the nests [30] or if queen turnover was so high that we collected workers from queens that were no longer alive [30,31]. We can also not rule out that some queens escaped our collection routine.

In accordance with our second hypothesis, aggression level decreased with increasing genetic diversity within colonies, but only in those areas where the social parasite Maculinea alcon was absent (see below). We see the same
increased genetic diversity (in terms of queen number) was in itself not an effect of low aggressiveness, we reared colonies from different combinations of laboratory queens, performed the same aggression assays and obtained the same results: aggression level dropped with increasing numbers of queens. We are therefore confident that genetic diversity determines aggressiveness and not vice versa.

Ants in areas where the social parasite *M. alcon* is present were far more aggressive than ants collected in habitat where this parasite is absent. However, the pattern of decreasing aggression with increasing colony genetic diversity was not apparent in these areas (figure 2). This is initially surprising, since Thomas Als (results reported in [5]) was able to show that within-nest relatedness of parasitized *My. rubra* colonies was lower than that of unparasitized colonies collected on the island of Læsø, implying that increased genetic diversity facilitates exploitation. From these results, we would thus expect to find lower aggression levels in colonies with high queen numbers facilitating the acceptance of the parasite. The lack of this pattern in the parasite area might be caused by colonies having reached the upper limit of their aggression level, rendering any more aggression too costly for a colony. Another explanation would be our somewhat low sample size not picking up the pattern.

As hypothesized, we found strong context-dependent aggression in *Myrmica* ants, with large differences in aggression index for the test colonies. Significant effects ($p < 0.05$) are highlighted in bold.

### Table 1. Results of ANCOVA examining the effect of distance between home and intruder colonies, social structure of home colonies and area of origin (transect) of home colonies on loge of the aggression index for the test colonies.

<table>
<thead>
<tr>
<th>distance measure</th>
<th>geographical</th>
<th>genetic</th>
<th>chemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>social structure as number of queens counted during excavation</td>
<td>$F_{1,160}$</td>
<td>$p$</td>
<td>$F_{1,160}$</td>
</tr>
<tr>
<td>distance (D)</td>
<td>19.1</td>
<td>$&lt;0.001$</td>
<td>1.08</td>
</tr>
<tr>
<td>no. queens (Q)</td>
<td>2.72</td>
<td>0.101</td>
<td>0.849</td>
</tr>
<tr>
<td>transect (T)</td>
<td>38.9</td>
<td>$&lt;0.001$</td>
<td>24.3</td>
</tr>
<tr>
<td>D × Q</td>
<td>0.429</td>
<td>0.514</td>
<td>0.465</td>
</tr>
<tr>
<td>D × T</td>
<td>1.41</td>
<td>0.238</td>
<td>0.450</td>
</tr>
<tr>
<td>Q × T</td>
<td>4.62</td>
<td>0.033</td>
<td>3.79</td>
</tr>
<tr>
<td>D × Q × T</td>
<td>0.224</td>
<td>0.637</td>
<td>0.068</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.332</td>
<td>0.252</td>
<td>0.263</td>
</tr>
</tbody>
</table>

| social structure as number of queens estimated from microsatellites | $F_{1,160}$ | $p$ | $F_{1,160}$ | $p$ |
|------------------|--------------|---------|----------|
| distance (D) | 11.3 | $<0.001$ | 0.129 | 0.720 | 3.07 | 0.081 |
| no. queens (Q) | 0.001 | 0.986 | 0.005 | 0.942 | 0.013 | 0.909 |
| transect (T) | 36.9 | $<0.001$ | 23.5 | $<0.001$ | 22.9 | $<0.001$ |
| D × Q | 0.808 | 0.370 | 0.460 | 0.355 | 0.299 | 0.585 |
| D × T | 0.676 | 0.412 | 1.25 | 0.265 | 0.732 | 0.393 |
| Q × T | 7.47 | 0.007 | 5.29 | 0.023 | 5.37 | 0.022 |
| D × Q × T | 2.00 | 0.160 | 1.22 | 0.272 | 0.013 | 0.908 |
| $r^2$ | 0.347 | 0.266 | 0.277 |

| social structure as within-colony relatedness | $F_{1,160}$ | $p$ | $F_{1,160}$ | $p$ |
|------------------|--------------|---------|----------|
| distance (D) | 16.3 | $<0.001$ | 1.02 | 0.314 | 2.26 | 0.134 |
| relatedness (R) | 0.996 | 0.320 | 1.33 | 0.251 | 0.780 | 0.378 |
| transect (T) | 44.4 | $<0.001$ | 29.0 | $<0.001$ | 28.7 | $<0.001$ |
| D × R | 0.003 | 0.955 | 0.349 | 0.556 | 0.133 | 0.716 |
| D × T | 1.71 | 0.192 | 0.486 | 0.487 | 1.64 | 0.203 |
| R × T | 5.30 | 0.023 | 1.94 | 0.165 | 3.65 | 0.058 |
| D × R × T | 1.26 | 0.263 | 0.831 | 0.364 | 0.006 | 0.939 |
| $r^2$ | 0.327 | 0.247 | 0.252 |
aggression within only a few metres between the two transects. Chemical analysis showed a significant diversification between ants from parasite areas and ants outside parasite areas, which is in accordance with the findings of Nash et al. [14], who could confirm a coevolutionary arms race between the low-dispersal *My. rubra* ants and *M. alcon*. While we believe this to be a likely consequence of parasite pressure by *Maculinea* butterflies, we cannot rule out that habitat differences also play a role. While the transect with the parasite present was rather boggy, the transect without the parasite was relatively dry; whether this has any effect on the aggression is unknown, although it may have an influence on the chemical profile [20]. Our whole-model approach showed that geographical distance and transect explain aggression best, while genetic and chemical distances do not explain aggression. Our study makes an interesting comparison with that of Lorenzi [32] on the wasp *Polistes biglumis* and its social parasite *Polistes atritumodulies*. Wasps learn new compounds added by the parasite to the colony as being from their own colony, and therefore widen their template. Lorenzi found colonies with parasites to be more permisive towards non-nest-mates, but also less permisive to nest-mates, compared with unparasitized colonies. While this apparently illogical result was explained in terms of impaired template learning abilities of the parasitized wasp colonies, it can also be readily interpreted in terms of Reeve’s [7] adjustable threshold model. As the template gets wider, the host workers need to shift towards a more permisive overall template, and more acceptance errors will occur when compared with unparasitized colonies. At the same time, this threshold should shift, relative to the unparasitized colonies, towards a less permisive acceptance level caused by the parasite pressure, explaining the relative higher number of nestmate rejections.

In summary, the variable aggression shown by *My. rubra* colonies can be largely explained by Reeve’s [7] adjustable threshold model. The additional costs of failure to recognize intruders imposed by social parasites appear from our results to have a major influence on acceptance or rejection thresholds, which are adjusted in an adaptive manner. We thank T. A. Hansen and E. Stafflinger for assistance in the field, J. J. Boomsma for helpful comments on the manuscript, and the Centre for Social Evolution for providing such a stimulating work environment. The project was founded via a Danish National Research Foundation grant to the Centre for Social Evolution.

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