

Old and wise but not size: factors affecting threat response behaviour and nestmate recognition in *Acromyrmex echinator* leaf-cutting ants

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Abstract Detecting and responding to threats are of prime importance for social species which need to be able to distinguish nestmates from intruders to protect the resources of their colony. However, individuals may differ in their propensity to recognise threats due to factors, often inter-correlated, such as caste, age and experience, and the ability to separate these is important for understanding why behaviours are expressed. Here, we use leaf-cutting ants in a controlled behavioural assay to tease apart the factors which likely affect threat response behaviours in social insect workers. We show that foraging workers respond to threats more readily than do within-nest workers. The response of all workers was greater towards more foreign stimuli—nestmates rarely stimulated a response, whereas ants of a different genus stimulated a response in most cases. We show that age and experience act separately to increase an individual's ability to perceive the threat. This suggests that where multiple, compounding factors affect the expression of certain behaviours it is important to realise that these factors can also have independent effects, particularly those which correlate with age. Separating the influence of correlating factors experimentally, as shown here, is particularly useful for understanding why individuals may differ in their behavioural profile.

Keywords Caste · Nestmate recognition · Social insects · Mandible opening response

Introduction

In group-living organisms, the defence of shared resources is of vital importance to group security. Such resources may include shelter, nesting habitat, food or the individuals themselves, particularly those that contribute more towards the reproduction of the group (Hölldobler and Wilson, 1990; Seeley, 2009; Wenseleers et al., 2013). The first step in any defence is for individuals to be able to recognise a threat and communicate this to fellow group members. Without this stage, the group as a whole will not be able to mount a suitable response, be it fight or flight (Verheggen et al., 2010). While the ability of organisms to recognise nestmates from non-nestmates has been much addressed (e.g., Sturgis and Gordon, 2012a), few studies have disentangled the various interacting processes influencing response behaviours. Here, we investigate these processes under controlled laboratory settings.

Social insects in particular have evolved efficient recognition systems whereby workers can detect and subsequently defend their colony from external threats such as robbery or parasitism (Gamboa, 1978; Walker and Hughes, 2009). Division of labour within social insect colonies is well known (Oster and Wilson, 1979; Robinson, 1992; Wilson, 1990), and this also extends to workers differing in their propensity to perform specific nest defence behaviours. Examples vary from species with a morphologically specialised soldier caste (Grüter et al., 2012), to behaviourally specialised guard workers (Butler, 1952; Moore et al., 1987). However, the ability of workers outside these task groups to perceive threats is also crucial to the

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colony being able to respond to a threat quickly and appropriately. The recognition of nestmates from non-nestmates in most social insects is thought to be based on cuticular hydrocarbons (Bonavita-Cougourdan et al., 1987; Liang and Silverman, 2000; Wagner et al., 2000), although colony differences in composition and behavioural bioassays suggest that alarm pheromones may also be informative for nestmate recognition in some taxa such as leaf-cutting ants (Brandstaetter et al., 2008; Francelino et al., 2006, 2008; Hernández et al., 2006; Hughes et al., 2001a; Whithouse and Jaffe, 1996).

There are many factors which may affect an individual's propensity to exhibit threat response behaviour, but being able to independently test each factor can be challenging. Important factors in social insects include size (Hölldobler and Wilson, 2010; Huang, 2010; Nowbahari et al., 1999) and age (Jaisson, 1991; Morel et al., 1988; Seeley, 1982; Waddington and Hughes, 2010), with, for example, larger ants being expected to act more aggressively, or older ants to have a more developed ability to distinguish between nestmate and non-nestmate. Experience has also been shown to be a contributory factor in some aspects of nestmate recognition (Beshers and Fewell, 2001; van Wilgenburg et al., 2010). The difficulty, however, is that age and experience are often correlated and few studies have looked at whether these factors act separately. Laboratory colonies enable these factors to be separated. In this setting, older ants are no more experienced in interacting with foreign individuals than younger ants, something which is not necessarily the case in field colonies.

Studies regarding nestmate recognition often use aggression to score how successful an individual is at discriminating friend from foe (e.g., D'Ettorre et al., 2006; Downs, 2000; Guerrieri et al., 2009; Kikuchi et al., 2007; Van Wilgenburg et al., 2010). Whilst aggression is indicative of the detection and rejection of a non-nestmate, it is just one part of the nestmate recognition process; individuals may perceive the threat but exhibit a different behaviour, such as an alarm response or a panic response (Verheggen et al., 2010). However, in all of these responses, individuals open their mandibles to release alarm pheromone from the mandibular glands and to prepare to bite (Stoeffler et al., 2007; Wilson and Regnier, 1971). This makes the mandible opening response (MOR) an excellent indicator of threat detection and responses in general because it is expressed during aggression, alarm and panic, rather than solely aggression (Guerrieri and D'Ettorre, 2008). This specific mandible opening behaviour indicates that a threat has been perceived by the focal ant (Hölldobler and Wilson, 1990; Hughes et al., 2001b), making it highly biologically relevant for studies concerning how and why individuals differ in their tendency to recognise threats. The MOR is, in some ways, similar to the proboscis extension

response and sting extension response used in honey bees, in being an assay allowing an objective evaluation using a categorical response (yes/no) (Balderrama et al., 2002; Kuwabara, 1957; Page et al., 1998; Takeda, 1961). It allows ants to be tested individually, thus avoiding trials involving multiple individuals at once which may confound and complicate the response of individuals (Kikuchi et al., 2007; Pamminger et al., 2011; Sturgis and Gordon, 2012b).

Here, we use the MOR assay to tease apart the importance of task, caste, age and experience in threat response behaviour in a controlled and standardised way. We study the propensity of workers from the leaf-cutting ant *Acromyrmex echinator* to exhibit a threat response behaviour to non-nestmates in a number of contexts (assessing the roles of cuticular hydrocarbons versus alarm pheromone in recognising a non-nestmate from nestmates), and test the importance of task, caste, age and experience on how this behaviour is expressed.

Methods

Eight *A. echinator* colonies were used for Experiment 1 (Ae1103, Ae1102, Ae088, Ae399, Ae1003, Ae396, Ae603, Ae084) and six for Experiments 2, 3 and 4 (Ae1102, Ae1105, Ae399, Ae088, Ae396, Ae603), all collected from Gamboa, Panama between 2008 and 2011. Colonies had been kept in the laboratory for at least two years before the experiments were conducted. Colonies were kept at 80 ± 5 % relative humidity, 26 ± 2 °C and 12:12 h light/dark cycle. All fungus chambers were housed in sealed plastic nest boxes. They were fed twice weekly on privet leaves (*Ligustrum* spp.) placed in a foraging pot (c. 100 mm × 80 mm × 60 mm) and provided with water ad libitum.

Mandible opening assay

The MOR assay was conducted following Guerrieri and d'Ettorre (2008). Ants were cooled on ice until immobilised and were then harnessed, leaving only the head, antennae, and mouthparts free to move. The harness was made using a 0.2-ml pipette tip (Starlab, Bucks, UK), cut at the apex through which the ant's head was passed and secured with a thin strip of masking tape. Ants were left for 2 h to acclimatise to the harness and recover from the anaesthesia before experiments began. There was no mortality over the 2-h period. All experimental treatments were presented in a random order to each subject with at least a 5-min interval between stimuli. Each stimulus was presented to the ant for a 20-s period. Ants occasionally opened their mandibles very briefly (<1 s) when any object contacted their antennae, so we only recorded a MOR if the ant opened its mandibles widely for a period of >1 s.

Experiment 1: does threat response behaviour differ between within-nest workers and foragers?

Foragers were removed from the foraging pot and nest workers were taken from the fungus garden after being observed tending to the fungus. Between four and six ants of each caste were taken from each of the eight colonies, giving a total of 38 foragers and 42 within-nest workers. The cuticle of leaf-cutting ant workers darkens with age (Armitage and Boomsma, 2010), and individuals at the extreme ends of the range of cuticular colouration were avoided to minimise differences in ages between ants. Each ant was presented with the following five stimulus types in a random order: (1) a nestmate worker, (2) a non-nestmate worker of the same species (conspecific), (3) a worker of the same genus but a different species (congeneric; *Acromyrmex octospinosus*), (4) a worker of a different genus (*Atta cephalotes*), and (5) a control. All five stimulus types (1–5) were presented in three ways to each ant as either: (a) a dead ant that contacted the antennae, (b) a live ant that did not contact the antennae (presented ~10 mm away from the focal ant; held in forceps but not otherwise immobilised), or (c) a live ant that contacted the antennae. The different presentation methods, therefore, respectively, exposed the focal ants to cuticular hydrocarbons only (dead ant contact), alarm pheromones (live ant but no contact; the ants generally gaped their mandibles, indicating the release of alarm pheromone), or both (live ant contact), from the stimulus ant. Dead ants were killed by freezing and then defrosted immediately before use in the assays. Both live and dead stimulus ants were medium-sized and medium-aged workers collected from near the entrance to the fungus chamber. The control was a clean metal ball bearing, washed in hexane, then rinsed in water and allowed to dry between each trial. A blind trial was also carried out with 20 ants from two *A. echinator* colonies (40 in total) with three of the treatment ants: a nestmate, a non-nestmate of the same species and a non-nestmate of a different species as well as the same control stimulus used in previous experiments, to confirm that the results were not subject to confirmation bias of the observer (van Wilgenburg and Elgar, 2013). Forceps used to present the stimuli were rinsed in hexane and allowed to dry between each presentation.

Experiment 2: the effect of age on threat response behaviour

To look at the effects of age on the threat response, ants of the same size class (1.2- to 1.8-mm head width) but of three different age classes (young, medium and old) were chosen based on their cuticular colour. Six ants of each age class were chosen from each of the six colonies, giving 108 ants in total. Each ant was photographed dorsally using a digital SLR with

constant camera settings and lighting conditions. Images were imported into ImageJ software (Schneider et al., 2012) and converted to grayscale, giving a reading of 0 (pure black)–256 (pure white). Cuticular colour was quantified using the mean value of the middle third of the femur of one of the rear legs, as in Armitage and Boomsma (2010). Mean \pm standard deviation colour for the three age categories was: young (0–20 days) 155 ± 12.5 , medium (20–40 days) 133 ± 8.2 and old (40+ days) 108 ± 8.6 . The MOR was tested as described for Experiment 1. The treatments were: a control metal ball, a freshly killed nestmate and a freshly killed *A. cephalotes* worker, which contacted the focal ant's antennae.

Experiment 3: the effect of size on threat response behaviour

To determine how size affects threat response behaviour we used ants of different sizes but the same age. We investigated small (<1.2 mm), medium (1.2–1.8 mm) and large (>1.8 mm) size classes (Waddington and Hughes, 2010). All ants used for this experiment were of a medium cuticular colour (and therefore of similar age). Six ants of each size class were collected from each of the six colonies, with each ant being dorsally photographed so that size could be quantified by measuring width between the eyes using ImageJ software, a commonly used index of size in ants (e.g. Huang, 2010; Wilson, 1983). Mean \pm standard deviation head widths were: small 1.09 ± 0.085 mm, medium 1.53 ± 0.158 mm and large 1.92 ± 0.099 mm. The MOR of the ants responding to antennal contact with either the control, a freshly killed nestmate and a freshly killed *A. cephalotes* worker, were tested as before.

Experiment 4: the effect of experience on threat response behaviour

To determine whether the threat response of ants increased with their experience of stimuli, workers were repeatedly tested once every hour for 6 h on 2 consecutive days, giving 12 repeated trials in total. All ants used in this experiment were of a medium cuticular colour and in the small size range (<1.2 mm head width). Eighteen ants were taken from each of the six colonies and split into three equal groups. This gave 36 ants for each treatment group. All colonies had no previous experience with non-nestmates. Each group received one of three treatments: a metal ball control, a freshly killed nestmate and a freshly killed *A. octospinosus* worker. This species was chosen rather than *A. cephalotes* because it stimulated lower threat responses in preliminary trials and were thus a better stimulus for detecting the hypothesised increase in the MOR with experience. Harnessed ants were hand-fed a 10 % sucrose solution between days one and two to ensure they were not starved during the trial period.

Statistical analyses

The responses of individual ants in Experiments 1 and 2 were analysed using generalised linear mixed models (GLMM) with binomial distributions and log link functions. The analysis for Experiment 1 examined the effects of treatment (foreignness of the presented ant), method of presentation of the ant (method one, two or three described above), and caste (forager, nest-worker). The analyses for Experiments 2 and 3 examined the effects of treatment (relatedness of the presented ant), and either age (young, medium and old: Experiment 2) or size (small, medium and large: Experiment 3). Colony identity was included as a random factor in all GLMMs. For Experiment 4, we instead analysed the effects of treatment and experience on the total numbers of ants from each colony that showed a MOR over the twelve time periods (i.e., with colonies as replicates rather than a random factor), using a repeated measures general linear model. A Greenhouse-Geisser correction was used to control for deviations from the assumption of sphericity. In all models, non-significant interaction terms were removed in a step-wise manner, using AIC values, to give minimum adequate models. Post hoc pairwise comparisons of treatments used the sequential Bonferroni method to control for multiple comparisons. All statistics were performed in SPSS (v.20 SPSS Inc., Chicago, IL, USA).

Results

Experiment 1: does threat response behaviour differ between within-nest workers and foragers?

We found a significant difference in MOR between foragers and within-nest leaf-cutting ant workers ($F_{1, 1,184} = 45.9$, $P < 0.001$). Foragers showed the MOR more often than within-nest workers in all cases, regardless of the stimulus or presentation method (Fig. 1). There was a significant interaction between the effects on MOR frequency of stimulus type (nestmate, conspecific, congeneric ant, different genus, or control), and the method of presentation (dead ant contact, live ant contact, or live ant no contact; $F_{8, 1,184} = 4.97$, $P < 0.001$). When the stimuli contacted their antennae, the proportions of ants showed a MOR was, in general, greatest to ants of a different genus or congenics, and least to nestmates, with the conspecifics and control treatments stimulating intermediate MOR frequencies (Fig. 1a, c). This was not the case when the stimuli did not contact their antennae, with responses in this case being low regardless of treatment (Fig. 1b). There was no significant difference between blind and non-blind trials ($F_{1, 279} = 0.148$, $P = 0.701$; Fig. S1), confirming that the effect seen was not an artefact of observer bias.

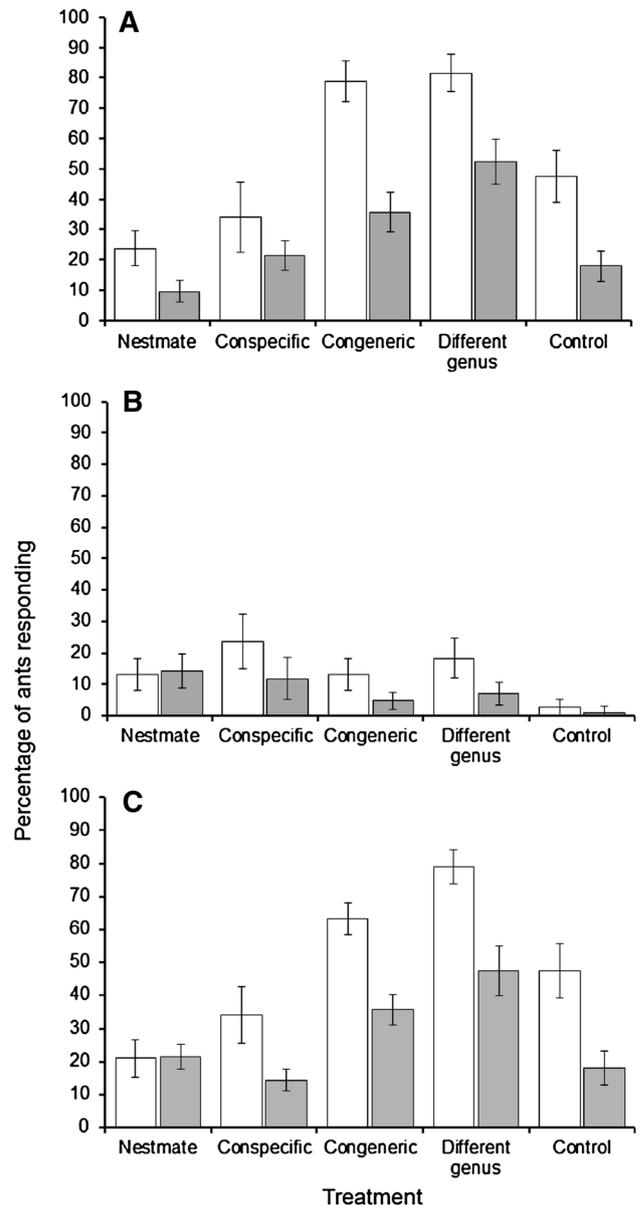


Fig. 1 The mean \pm SE percentage of *Acromyrmex echinatior* leaf-cutting ant foragers (white columns) and nest workers (grey columns) from eight colonies that showed a mandible opening response to all five stimuli types (a nestmate, a non-nestmate of the same species, a non-nestmate of the same genus but different species, a non-nestmate of a different genus and a control). Stimuli ants were presented as either: **a** a dead ant (or control) that contacted the antennae, **b** a live ant (or control) that did not contact the antennae, or **c** a live ant (or control) that contacted the antennae. The different presentation methods thus exposed the focal ants, respectively, to the cuticular hydrocarbons, alarm pheromones, or both, from the stimulus ant

Experiment 2: the effects of age on threat response behaviour

Both age and treatment significantly affected the MOR of ants ($F_{2, 319} = 7.03$, $P = 0.001$, and $F_{2, 319} = 39.10$, $P < 0.001$, respectively). Medium and old workers more

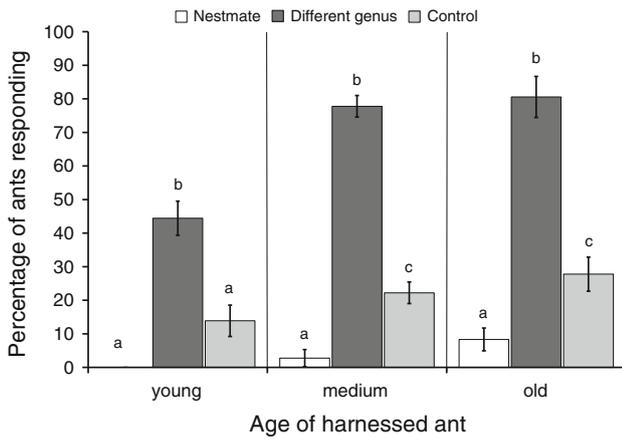


Fig. 2 The mean ± SE percentage of young, medium-aged and old *Acromyrmex echinator* leaf-cutting ant workers from six colonies that showed a mandible opening response to a nestmate (white columns), an ant of a different genus (dark grey) or a control (light grey columns). Stars represent significant differences within each age category ($P < 0.05$). Stimuli ants were presented as dead ants that contacted the antennae

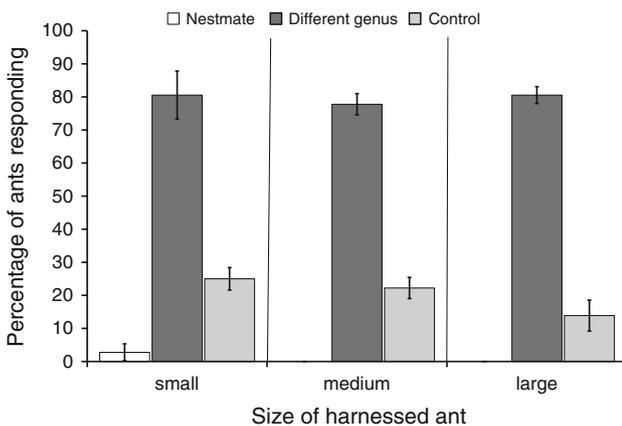


Fig. 3 The mean ± SE percentage of small, medium and large *Acromyrmex echinator* leaf-cutting ant workers from six colonies that showed a mandible opening response to a nestmate (white columns), an ant of a different genus (dark grey) or a control (light grey columns). Stimuli ants were presented as dead ants that contacted the antennae

frequently showed the threat response behaviour than did young workers (Fig. 2). For all ages of ants, the MOR was stimulated significantly more frequently by ants of a different genus than by the other stimuli, with nestmates only rarely stimulating a response (Fig. 2).

Experiment 3: the effect of size on threat response behaviour

Size did not significantly affect the response of the ants to the stimuli ($F_{2, 319} = 0.48, P = 0.358$; Fig. 3). The response to ants of a different genus was extremely similar for small, medium and large workers, and the response to

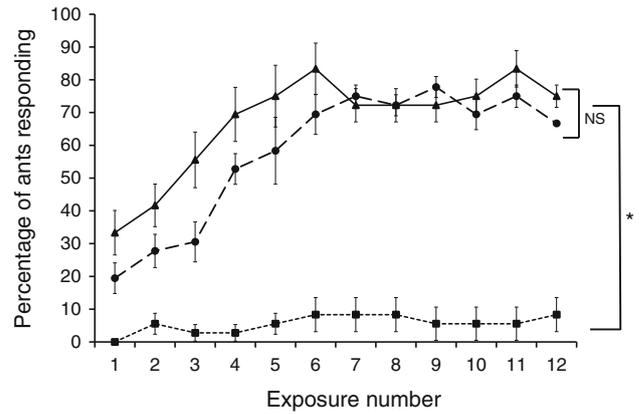


Fig. 4 The mean ± SE percentage of leaf-cutting ant workers from six colonies that showed a mandible opening response to either a nestmate (squares), an ant of a different species (triangles) or a control (circles) in 12 encounters repeated over two days. Stimuli ants were presented as dead ants that contacted the antennae

the control stimulus was only moderately higher for small than large workers. Including an ‘extra-large’ category (ants with head widths >2 mm) for the analysis also did not reveal a significant effect on the response ($F_{3, 320} = 1.061, P = 0.304$).

Experiment 4: the effect of experience on threat response behaviour

Experience increased the response of the ants with there being a significant interaction between treatment and time ($F_{8, 62} = 6.82, P < 0.001$). Both the control stimulus and ants of a different species stimulated significantly more frequent responses than nestmate ants (Fig. 4), with the responses to the controls and ants of a different species increasing substantially over time. The response of ants to nestmates, in contrast, stayed relatively constant over the repeated exposures, with no more than 10 % of ants responding at any point. Responses to ants of a different species and to the control treatment did not differ significantly from one another and reached a plateau after seven exposures (Fig. 4).

Discussion

Not much is known about the factors influencing threat response behaviour, which is surprising given the large literature on nestmate recognition. We show that individual workers differ strongly in their propensity to display the MOR to threats. Foraging workers responded more readily compared to within-nest workers. Age and experience both positively, and independently, correlate with threat response

behaviour. Surprisingly, we show that size did not predict an individual's propensity to show this behaviour. All ants gave increased responses to increasingly foreign stimuli ants, which was also the case in a blind trial and therefore not due to observer bias (van Wilgenburg and Elgar, 2013). Although the foreign control stimuli used stimulated relatively high responses themselves on occasion, the responses to the most foreign ants were consistently greater. Interestingly, experience had a strong effect on the strength of the response to foreign ants compared to nestmates. These results suggest that the controlled setting of the MOR assay is ideal to test hypotheses on intercorrelating factors affecting an individual's propensity to exhibit threat response behaviour. This assay would also be a useful tool for the study on insect behaviour in general as it could be transferred to any insect which can show behaviours using their mouthparts.

In *Atta* leaf-cutting ants, the composition of the alarm pheromone can differ between colonies (Francelino et al., 2006; Hughes et al., 2001a), and ants have been shown to distinguish between nestmate and non-nestmate alarm pheromone in some assays (Francelino et al., 2008; Hernández et al., 2002; Whitehouse and Jaffe, 1995). However, we find here that *Acromyrmex* leaf-cutting ants display significantly greater MOR to treatment ants when they contact their antennae than to volatile cues alone. This could suggest that ants use cues from cuticular hydrocarbons more readily than alarm pheromone in the context in this assay, or that any levels of alarm pheromone produced, even by nestmates, were not sufficient to elicit a response here (Guerrieri et al., 2009; Ozaki et al., 2005; Wagner et al., 2000).

Threat response behaviour increased with the foreignness of the focal ant to the treatment ant meaning ants can apportion their threat response according to how similar the intruder is to the receiver. This is most likely because the cuticular hydrocarbon profile of more foreign ants differs more from the responding ant's nestmate template, as found by Guerrieri and D'Etorre (2008). Ants were always least responsive to nestmates, suggesting that being in a harness did not impair their ability to distinguish nestmate from non-nestmate. What is surprising, however, is the lack of significant discrimination between nestmates and individuals of the same species, given the importance of nestmate recognition in leaf-cutting ants and social insects more generally (Hamilton, 1964; Hernández et al., 2006, 2002; Hölldobler and Wilson, 1990). Laboratory colonies of ants are known to be less aggressive to conspecifics than field colonies (Crosland, 1989; Obin and Vander Meer, 1988). Different food sources are also known to modify cuticular hydrocarbons, and subsequent nestmate recognition, in *Acromyrmex subterraneus subterraneus* and *A. octospinosus* leaf-cutting ants, as well as in the Argentine ant

Linepithema humile and *Camponotus herculeanus* (Guerrieri et al., 2009; Jutsum et al., 1979; Liang and Silverman, 2000; Richard et al., 2004). All laboratory colonies were reared on the same diet, perhaps leading to smaller differences in cuticular hydrocarbons between colonies than found in natural settings.

Surprisingly, given the role of larger individuals in nest defence (Grüter et al., 2012; Huang, 2010; Nowbahari et al., 1999; Wilson, 1983), size was not found to affect the response of ants to threats. However, the MOR assay only tests threat response behaviour in general, not specifically aggression. All sizes of individuals recognise conspecifics in equal amounts but it is still possible that larger individuals may react more aggressively following threat recognition. In *Atta* species, the larger, soldier caste responds more readily in response to vertebrate predation, although they are unable to distinguish conspecifics from separate nests (Salzemann and Jaffe, 1991; Wilson, 1980), whereas smaller ants may be recruited more readily in response to a conspecific threat (Hölldobler and Wilson, 2010; Hughes and Goulson, 2001; Whitehouse and Jaffe, 1996).

In contrast, age did affect threat response behaviour, with young ants less frequently showing a MOR to all treatments compared to medium- and old-aged ants. This fits in with other nestmate recognition studies which found that younger individuals, especially callows, require a learning period to develop the nestmate template that they need to compare all other chemical templates to (Gamboa et al., 1986; Jaisson, 1991; Sturgis and Gordon, 2012a). Many social insects, leaf-cutting ants included, also exhibit age-based polyethism in which older workers carry out more dangerous, external tasks (Camargo et al., 2007; Julian and Fewell, 2004; Waddington and Hughes, 2010; Wakano, 1998), that may make recognising threats from non-nestmates potentially more important. The effect of age may therefore be a physiological constraint and also adaptive.

What is most interesting, however, is that the effect of experience (independent of age) also showed an increase in threat response behaviour. Experience is known to affect behaviour expression in social insects, for example, its effects on foraging are particularly studied (Beshers and Fewell, 2001; Hagbery and Nieh, 2012; Robinson et al., 2012). Artificially created experience over the experimental time period in medium-aged ants caused an increase in threat response behaviour towards both the congeneric ant and control treatment but not the nestmate treatment. However, the causal mechanism behind this is speculative. Some studies have found that experience of a non-nestmate lowers the response threshold for aggression (van Wilgenburg et al., 2010), while others have reported a raising of the response threshold to non-nestmates due to habituation (Stroeymeyt et al., 2010). Our results agree with the former, with an increase in threat response behaviour during the

experiment and no evidence of habituation. It is worth noting that in the context of other aspects of this experiment experience is not the only factor affecting an individual in their ability to show an appropriate threat response.

The fact that age and experience act separately indicates a more complex expression of these types of behaviours than previously thought, with both factors seeming to have separate underlying mechanisms. It is likely that lowering of the threshold is coupled with other mechanisms, such as potential physiological effects of age which may explain why certain individuals tend to react more strongly to non-nestmates. The results suggest that experience may be a crucial factor in nestmate recognition and highlight the complexity of factors contributing to the expression of behaviours; where those that appear to be similar can act as separate entities. The importance of assessing the individual contribution of effects that act synergistically will be particularly useful in assessing the reasons why individuals differ in their behaviours.

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