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Ant Pupae Employ Acoustics to communicate Social Status in their Colony's Hierarchy

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Running head: Acoustic signaling of ant brood status

Summary

The possession of an efficient communication system is an essential attribute that enables ants and other eusocial insects to live in complex integrated societies. Although ants communicate primarily via chemicals, it is increasingly clear that acoustical signals are an important means of conveying information, including social status, between adult nestmates in many species. However all immature stages were believed to be mute. Here we show that the larvae and recently formed (soft) pupae of *Myrmica* ants are indeed mute, yet once they are sclerotised, the pupae possess a fully-developed functioning stridulatory organ on the nascent adult beneath the pupal integument. The sounds generated by sclerotised worker-pupae were similar in frequency and intensity to those of adult workers, but were emitted as single pulses rather than in long sequences characteristic of adults.When recordings of pupal acoustics were played to unstressed workers, they induced the same range and intensity of benevolent behaviours – including attraction, antennation and guarding - as when workers experienced their own adult sounds. Both white and sclerotised pupae have a higher social status than larvae within *Myrmica* colonies, but the latter fell to the bottom of the brood's hierarchy after they were made mute. We suggest that acoustical signals supplant semio-chemicals as a means of identification in sclerotised pupae, perhaps because their hardened integuments block the secretion of brood pheromones or have a reduced ability to absorb colony odours.

Highlights

- We present first evidence that maturing pupae of *Myrmica* ants can stridulate
- Stridulations are similar in frequency to worker sounds but differ in structure
- Experiments show that stridulations convey a high social status among the brood

Introduction

The possession of a sophisticated communication system is a key trait that enables eusocial insects such as ants to live in the complex societies that dominate most terrestrial ecosystems [1,2]. An essential component of this is the ability of workers to recognize and discriminate between their young stages: indeed, the core definition of eusociality is of an organism with distinct reproductive castes that engages in co-operative brood care by relatives from earlier generations [3]. The adaptive advantages of brood recognition, and hence care, are many, and include the selective grooming, feeding, transport, segregation, rescue or defence of particular immature stages by nurse workers in response to distinctive brood signals received in the context of the available resources, changing state or threats to their colony [1,4-7].

The main means of recognition and communication between ants is by chemical cues, often modulated by tactile stimuli [1]. Members of the same society typically share a cocktail of hydrocarbons which provides a distinctive 'gesthalt' odour across the colony, allowing workers to discriminate between kin and strangers [8,9]. Additional variation between individuals' profiles permits recognition of - and appropriate responses to – nestmates of different sex, caste and developmental stage [3,10-14]. For example, when a colony is perturbed, the workers quickly rescue and retrieve the brood, including dummies treated with larval extracts [15,16]. In the well-studied Myrmicine genus *Myrmica*, brood recognition by pheromones is supplemented by tactile cues, including larval turgidity, hairiness, size, shape and surface properties [17]; and a social hierarchy exists between the different young stages: small larvae are killed and fed to larger larvae in times of food shortage, and a distinct order of rescue occurs – starting with pupae, followed by large larvae and finally small larvae and eggs – whenever a colony is disturbed [18].

Notwithstanding the predominant use of semiochemicals in ant communications, many species generate acoustical signals through a stridulatory organ or by drumming their gaster. Once considered a weak form of communication, restricted to spreading alarm or modulating responses to other signals [1,19,20], it is increasingly clear that acoustics is used to convey a greater variety of information between nestmates as well as signalling an individual's social status [21-23]. For example, in the genus *Myrmica*, the stridulations made by queens differ from those of workers in both their dominant and pulse repetition frequencies, and queen calls elicit a suite of enhanced benevolent responses from workers, including guarding these prized members of society [22-24]. However, to our knowledge, there was no evidence that the young stages of any ant could communicate using acoustics. On the contrary, previous studies indicated that the immature stages of *Myrmica* were mute [25], although older, sclerotised pupae may not have been investigated.

Here we test the possibility that the hardening cuticles of sclerotised (brown) pupae of *Myrmica scabrinodis* can generate sound. We first searched for the presence of a nascent stridulatory organ on or beneath the outer cuticle, and then recorded the putative acoustical signals of unstressed individuals before playing them back to adult workers to observe any responses. Finally, we tested the hypothesis that the observed seniority shown to pupae (which are static) over larvae (which can attract attention by begging) in the hierarchy of a *Myrmica* society [18] might be explained by an ability of pupae to interact acoustically with worker ants. Surprisingly we find that maturing pupae that can not make sound drop in the social hierarchy suggesting that the acoustic signals compensate for the loss of another, most likely chemical signal to maintain a high social status.

Results

Pupal Sound Production

Scanning electron microscopy revealed the presence of a fully formed stridulatory organ on the developing imago within sclerotised ant pupae, similar to that on adult workers and queens (Figure 1). The organ consists of a minutely ridged file (Figures 1C, D; *pars stridens*) located on the mid-dorsal edge of the fourth abdominal segment, and by a spike (*plectrum*) projecting from the rear edge of the post-petiole. However, compared with adults, the scope for the pupa to play one surface rapidly against the other was constrained due to the thin pupal cuticle that encompassed it (Figures

1A, B). Emerging stridulatory organs were also recognizeable on the soft abdomens of newly formed white pupae, but were absent from larvae.

We recorded larvae and white pupae for a total of 40 hours, but no sounds or substrate-borne vibrations were detected. In contrast, sclerotised (nascent worker) pupae readily produced acoustic signals which resembled those of adult workers and, to a lesser extent, queens in their frequency and intensity, but which consisted of single pulses rather than the streams of 'song' emanating from both adult castes (Figure 2A). Using a multivariate approach over three sound parameters, the normalised Euclidean distances (mean ± s.d.) within samples of *M. scabrinodis* pupae, queens and workers were respectively 0.88 ± 0.32, 0.52 ± 0.30 and 1.00 ± 0.59 (Fig 2A). Principal component analysis (PCA) was also conducted on the three sound parameters recorded from groups of 6 *M. scabrinodis* sclerotised pupae, 1 individual worker and 1 queen from each of 10 *M. scabrinodis* nests: the first and the second principal components accounted for 79.1% and 20.9% respectively, i.e. explaining all the variance (Figure 2B). Nested ANOSIM analysis of the Eucledian distance matrix showed a clear separation between the signals of sclerotized pupae, workers and queens (Overall: $R = 0.778$, $p < 0.001$; and for component distances: sclerotised pupae: $distance_{workers} = 2.52 \pm 1.00$, ANOSIM $R = 0.941$, $p = 0.001$; $distance_{queens} = 3.16 \pm 0.96$, ANOSIM $R = 1$, $p = 0.001$). As expected, the signals emitted by sclerotised (nascent worker) pupae were significantly closer to the stridulations of workers than to those of queens (2 sample t-test: $t = 10.198$, $df = 198$, $p < 0.001$).

Worker Ant Responses to Pupal Sounds

The responses of otherwise undisturbed *M. scabrinodis* workers to recordings of the sounds emitted by their sclerotised pupae were compared with play-backs of their own (worker) recordings and of white noise in three randomly assigned containers, simultaneously replicated twenty times.

No antagonistic or alarmed ant behaviour occurred during play-back experiments, but five benevolent responses were observed, the first two involving attraction and the rest involving reactions: (i) *walking* – the worker was attracted to the speaker but walked over it without stopping on it; (ii) *alerting* – the worker abruptly changed direction to pass onto the speaker; (iii) *antennating* – the worker antennated the speaker for at least 3 seconds; (iv) *guarding* – the workers rested in an alert on-guard poise (*sensu* [22]) on the speaker for at least 5 sec; (v) *digging* – the worker dug into the soil surrounding the speaker.

Linear mixed effect models showed that worker reactions to the three sound stimuli were significantly different for all observed behaviours except digging, which, however, was never elicited by white noise (Figure 3). Thus compared with white noise, both pupal and worker sounds always induced significantly more instances of walking, alerting, antennating and guarding by *Myrmica* worker ants, with values of p ranging from 0.019 to <0.0001. Yet despite the fact that pupal calls consisted of single pulses whereas worker stridulations were broadcast in streams, no significant difference was found in worker responses to these two sound stimuli (Figure 3).

Social Status of Normal and Mute *Myrmica* Pupae

As has been reported for other *Myrmica* species [18], we found that *M. scabrinodis* workers rescued living pupae, as a class (i.e. brown + white), significantly more quickly than their larvae (Wilcoxon Mann-Whitney, $Z = 6.822$, $p = 0.009$) after their nest was disturbed (Figure 4A). However, within these assays using normal (i.e. non-muted) brood items, the white pupae were rescued ahead of both sclerotised pupae ($Z = 2.118$, $p = 0.026$) and larvae ($Z = -3.177$, $p < 0.001$), with no significant difference being found between sclerotised pupae and larvae ($Z = -1.399$, $p = 0.168$) although the latter were, on average, rescued last (Figure 4A).

The pattern of rescue changed with recently killed brood: i.e. brood still coated with its full cocktail of recognition pheromones [26,27] but which was mute and immobilised (Figure 4B). Now, the mute sclerotised pupae were the last item to be rescued, significantly behind both white pupae ($Z = 3.326$, $p < 0.001$) and larvae ($Z = 2.306$, $p = 0.021$). White pupae were again on average rescued first, but not significantly ahead of larvae ($Z = 1.5875$, $p = 0.107$). Wilcoxon signed rank tests were also used to directly compare the shift in order in each brood type between the normal and mute trials: sclerotised pupae shifted to being rescued significantly after the other brood in the mute trials (Z = -24.500, df =10, p = 0.0098) but there was no significant shift in the order of recovery of white pupae or larvae between the two experiments (Z = -4.500, df =10, p = 0.6719 and Z = -15.500, df =10, p = 0.1309 respectively).

Discussion

Our results reveal the presence of a functional organ that generates strong acoustical signals from the sclerotized pupae of *Myrmica scabrinodis*, and they support the hypothesis that stridulations signal the social status of different members of an ant colony as well as inducing a suite of benevolent and alarm behaviours. Whilst the adults of many ant species stridulate to nestmates [28,29], acoustical communication by their immature stages has not, to our knowledge, been previously described. Since the active organ formed part of a nascent adult developing inside the sclerotised *M. scabrinodis* pupa, we might expect similar acoustical communications, after the first few days of pupal lives, among the four sub-families of ant (Ponerinae, Nothomyrmecinae, Pseudomyrmecinae, Myrmicinae) that possess a stridulatory organ. In other eusocial insects, the larvae of certain wasps use noise to demand food from adults, but produce it by rubbing their mandibles against their cell walls rather than from a specialised organ [30-32].

In the ant genus *Myrmica*, the stridulatory organ is caste-specific rather than species-specific [23], and unsurprisingly, the structure we found in *M. scabrinodis* pupae was identical to that of an eclosed adult worker (Figures 1, 2). Similarly, we predict that the stridulatory organ of a gyne pupa will produce similar sounds to an adult queen, and will induce similar royal treatment from nurse workers [22]. The constraint of an enveloping integument may explain why the pupal sounds occurred in single pulses rather than the complex repetitions that characterize an adult ant's diagnostic patterns (Figure 2A). We recorded the sounds of unstressed but isolated pupae and workers, and played them back to small groups of unstressed workers: the brief pulses of pupae triggered the same frequent occurrence and wide range of benevolent responses by workers as occurred when they received their own adult sounds (Figure 3). It is worth noting that our test environment was simple and constant, and that in nature adult ants are capable of both producing different sounds [33] or of reacting in different ways to the same acoustics [34], depending on the context in which the signal is transmitted or received. Furthermore, our acoustics were tested in isolation, whereas in nature they may be modulated by chemical or tactile cues, and *vice versa* [1,2]. Thus we suspect that tended pupae in natural colonies may possess a wider acoustical repertoire than observed here, and that worker responses to them may be more complex.

It was impractical to record the acoustics of distressed *Myrmica* pupae during the rescue experiment (Figure 4), but the likelihood that sound production helps workers to locate alarmed sclerotised pupae was indicated by the observation that – when killed by freezing – the mute brown pupae became the last brood items to be rescued, significantly behind dead larvae (Figure 4B), whereas they were chosen marginally ahead of larvae when alive (Figure 4A). Of course, dead brood cannot move either, e.g. larvae cannot beg, but the lack of any significant difference in the relative order of rescue of white pupae and larvae during the mute assays compared with the living trials supports previous conclusions [26,27] that the chemical and tactile signals involved in brood recognition are not compromised by this treatment.

The preference afforded to living white pupae after colony perturbation was, however, unexpected (Figure 4A). We had predicted that the calls of sclerotised pupae would attract preferential worker attention, perhaps explaining why pupae as a group were selected ahead of larvae or eggs in previous ant rescue experiments [18]. A possible explanation is that, rather than elevating the social level of sclerotized pupae through the possession of an additional cue, their acoustics may replace brood-recognition pheromones, perhaps because the hardened integument blocks the secretions from their own glands or reduces their ability to absorb colony odours.

More generally, our results support a growing body of work – facilitated by the increased sophistication of affordable sound equipment – that suggests that acoustical communication plays a greater and more varied rôle in influencing ant social behaviour than was previously thought (e.g. [22,23,29,33,35,36]). While not disputing the prime importance of semio-chemicals, mediated by tactile signals, we suspect that acoustics may be a more flexible means of signalling and conveying information between ants than is generally recognised [33].

Experimental Procedures

Field Collection and Culture

Myrmica scabrinodis nests (*n* = 10) were collected in July 2011 at Wallingford (UK) and set as standardised laboratory ant colonies with >100 workers in 12.5cm x 8cm x 2cm Perspex containers, maintained on a diet of sugar and *Drosophila* larvae [37]. All colonies contained at a minimum of 10 larvae, 10 white pupae and 10 sclerotised pupae.

Scanning Electron Microscopy

We used dissection and Scanning Electron Microscopy to investigate the presence of stridulatory organs on ant brood. Two *M. scabrinodis* larvae, 2 white and 2 sclerotised pupae from two ant colonies were kept in 70% ethanol and one item per category was dissected between the post-petiolum and the abdomen to expose the *pars stridens* and the *plectrum*. The whole individuals and the two ant parts were mounted on the same steel stub, coated with gold, and the samples were scanned using a Cambridge Stereoscan S360 SEM. *M. scabrinodis* white pupae and larvae were dried in hexamethyldisilazane to avoid cell structure disruption before coating. The SEM operated at 20-25 kV.

Sound Recordings

We recorded sounds of clusters of 6 *M. scabrinodis* larvae, 6 white and 6 sclerotised pupae from 10 *M. scabrinodis* nests. Separate recordings were made of individual queens and workers taken from the same test colonies. The recording equipment consisted of a 12.5cm x 8cm x 2cm recording chamber with a moving-coil miniature microphone attached through the centre. A second microphone of the same type was used to record ambient noise but in anti-phase. An amplifier was attached to each microphone and calibrated to maximize the noise cancellation of ambient noise from the two microphones, leaving the signal from the recording chamber. The resulting signal was processed through two-stage low-noise amplification before being digitally recorded on a laptop computer, using Audacity v. 1.3 Beta (http://audacity.sourceforge.net/). To further reduce ambient noise and interference, the equipment was powered by a 12V gel cell battery, and the recording chamber and microphones were placed inside an anechoic chamber. Sounds were recorded for 20 min periods starting 10 min after items were introduced into the recording chamber.

Recordings were sampled at 44.10 kHz and 32-bit resolution. Frequency information was obtained through Fast Fourier Transformation (FFT; width 1024 points). Spectrograms were obtained at Hanning window function with 512 bands resolution. We selected 20 good quality pulses from each track and measured dominant frequency (Hz), pulse length(s) and sound amplitude (dB), using Audacity 1.3Beta. Based on the 3 sound parameters, single pulses were ordinated by principal components analysis (PCA). To test whether sound differed between groups, we calculated the pairwise normalized Euclidean distance over all three parameters and used a nested ('Colony' within 'Group') ANalysis Of SIMilarity implemented in Primer v. 6. (Primer-E Ltd.). The sound parameters were log(x+1) transformed. We calculated the average pairwise distances and used a two-sample t-test to compare differences in between group distances.

Worker ant responses to sound recordings

Behavioural assays were carried out in three 7cm x 7cm x 5cm perspex arenas with the speaker attached at the bottom of the box and sealed on the outside with Blu-tac™. The speaker was covered with a thin layer of slightly wet soil. Ten workers from the same *M. scabrinodis* colony were placed in each arena and allowed to settle for 10 minutes before being played one of the three test sounds (*M. scabrinodis* worker, sclerotised pupae sound and white noise). The sounds were produced by MP3 players playing loops of the original recordings, with each volume adjusted to the natural level by attaching the speaker to the microphone of the recording equipment and by calibrating to the same levels reached during recording. Each trial lasted 30 minutes: counts were made of all instances of antagonistic or attractive behaviours, during periods of one minute for each box and in sequence between the three treatments, i.e. Σ10 minutes for each sound per trial. Each play-back experiment was repeated twenty times, using fresh ants from 10 different *M. scabrinodis* colonies (i.e. twice for each colony). The source of sound for each arena was randomly assigned before each trial was replicated to control for possible positional effects. Between each trial, new soil was introduced and all the equipment, including speakers and arenas, was cleaned with absolute alcohol and rinsed with distilled water. The effect of sound stimulus on the 5 worker ant behaviours was analysed in a linear mixed effects model with “colonies” as a random factor using the software R-2.15.0. [38].

Experiment to measure the order in which workers rescued different brood items

The arena used for the brood-rescue experiment consisted of two adjacent chambers of 7 x 2 cm communicating at one end. We placed 8 *Myrmica* larvae, 8 white pupae, 8 sclerotised pupae and 10 workers on a 0.4 cm^3 moist sponge (to maintain humidity) at the end of one chamber which was then covered with a transparent glass. The other chamber was covered with a dark glass. After 10 minutes of resting in the dark, we shone a 60 W light placed 10 cm away onto the chamber containing the worker ants and brood, to create a high level of stress which induced workers to rescue the exposed brood and carry it into the dark chamber. The order in which each item of brood was rescued was recorded. The experiment was then repeated after placing all brood items from a colony in a freezer (-20°C) for twenty minutes, thus killing the brood to make them mute (and immobile). Brood items were then left at room temperature for five minutes to return to normal temperature. Immediately after this period, the same procedures as before were used to make rescue-experimnets. Previous studies [26,27] have established that in assays conducted only a short time after immature ants are killed, the chemicals responsible for brood recognition remain present in approximately the same quantities as in the live brood.

Statistical analyses were performed using the package ‘coin’ provided with the software R-2.15.0 [38,39]. Kruskal-Wallis tests were used to compare the rescue orders of different brood categories between non-mute and mute treatments. Subsequent pairwise comparisons of median rescue order between brood categories within the same treatment were made using Wilcoxon Mann-Whitney tests; *p*-values were calculated against a null distribution generated from data using a Monte Carlo resampling. Direct comparisons of the same brood categories between normal and mute treatments were made using paired Wilcoxon signed rank tests.

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Figure legends

Figure 1. The stridulatory organ of sclerotised pupae of *Myrmica scabrinodis*. (A, B) Location of the acoustical organ (arrow) beneath the integument of an intact pupa. (C) Pupa with integument removed. (D) *Pars stridens* on pupa with integument removed.

Figure 2. Comparison of the acoustics of queen, worker and sclerotised pupae of *Myrmica scabrinodis*. (A) Oscillogram, spectrogram and single pulse parameters. (B) Combined effect of the three sound parameters (pulse length, frequency and intensity) shown as the first and second component plot of a principal components analysis over all individual pulse measurements.

Figure 3. Responses of *Myrmica scabrinodis* workers to broadcasts of worker and pupal acoustics and white noise. Five benevolent but no antagonistic behaviours were observed: the same letter indicates no significant difference within each type of behaviour, different letters indicate a significantly different response. Compared with white noise, linear mixed effect model likelihood ratios are: (i) walking $LR_{pupa} = 11.082$, $df = 4$, $p = 0.001$; $LR_{worker} = 8.097$, $df = 4$, $p = 0.004$; (ii) alerting $LR_{pupa} = 23.232$, $df = 4$, $p < 0.0001$; $LR_{worker} = 20.518$, $df = 4$, $p < 0.0001$; (iii) antennating $LR_{pupa} = 8.425$, $df = 4$, $p = 0.004$; $LR_{worker} = 17.154$, $df = 4$, $p < 0.0001$; (iv) guarding $LR_{pupa} = 5.476$, $df = 4$, $p = 0.019$; $LR_{worker} = 11.419$, $df = 4$, $p = 0.001$. Likelihood ratios comparing pupal and worker acoustics are: (v) walking $LR = 0.296$, $df = 4$, $p = 0.587$; (vi) alerting $LR = 0.145$, $df = 4$, $p = 0.704$; (vii) antennating $LR = 2.278$, $df = 4$, $p = 0.131$; (viii) guarding $LR = 1.441$, $df = 4$, $p = 0.230$.

Figure 4. The hierarchical status of *Myrmica* brood items. Box plots illustrate the order in which worker ants rescued sclerotised (brown) pupae, young white pupae and larvae after their nest was perturbed by exposure to light: vertical line = median order of rescue, box = 25th-75th percentiles, whiskers = one standard deviation below and above the mean of the data. (A) ‘Normal’ live ant

brood (Kruskal-Wallis $H_n = 11.182$, $df = 2$, $p = 0.003$. (B) ‘Mute’ recently dead brood ($H = 26.347$, $df = 2$, $p < 0.001$).

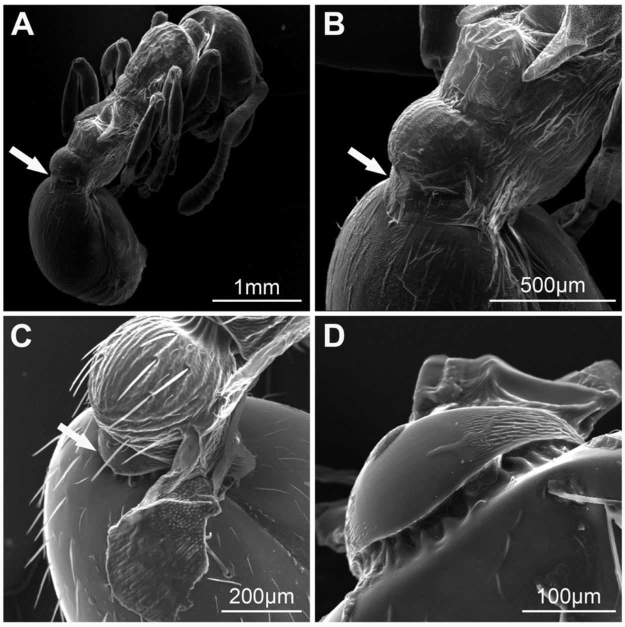

Figure 1

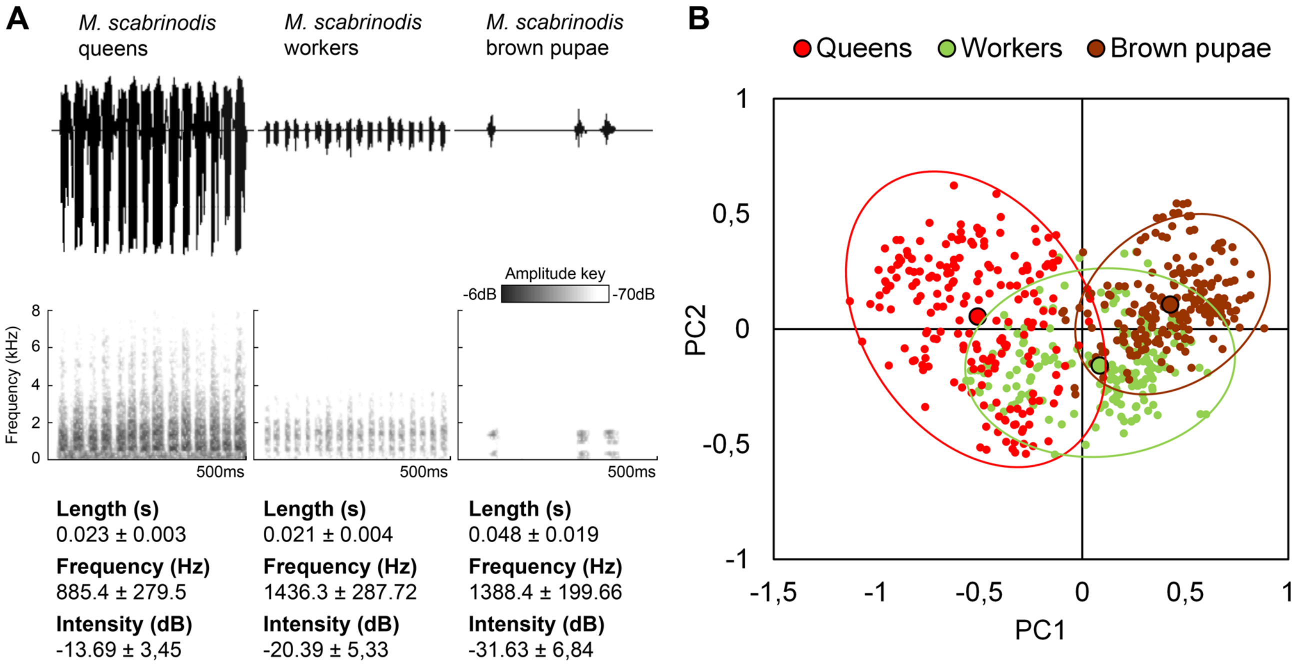
A
M. scabrinodis queens
M. scabrinodis workers
M. scabrinodis brown pupae
Amplitude key
-6dB
-70dB
Frequency (kHz)
8
6
4
2
0
500ms
500ms
500ms
Length (s)
0.023 ± 0.003
Frequency (Hz)
885.4 ± 279.5
Intensity (dB)
-13.69 ± 3,45
Length (s)
0.021 ± 0.004
Frequency (Hz)
1436.3 ± 287.72
Intensity (dB)
-20.39 ± 5,33
Length (s)
0.048 ± 0.019
Frequency (Hz)
1388.4 ± 199.66
Intensity (dB)
-31.63 ± 6,84
B
Queens
Workers
Brown pupae
1
0,5
0
-0,5
-1
PC2
-1,5
-1
-0,5
0
0,5
1
PC1

Figure 2

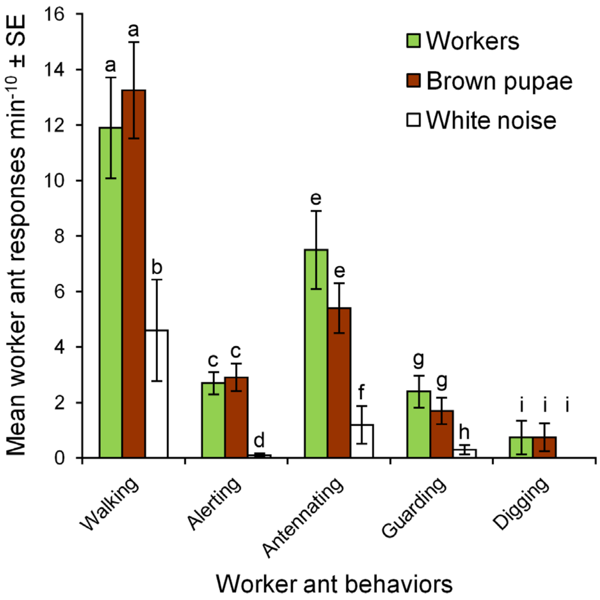
Mean worker ant responses min^{-10} ± SE
16
14
12
10
8
6
4
2
0
Workers
Brown pupae
White noise
a
a
b
c
c
d
e
e
f
g
g
h
i
i
i
Walking
Alerting
Antennating
Guarding
Digging
Worker ant behaviors

Figure 3

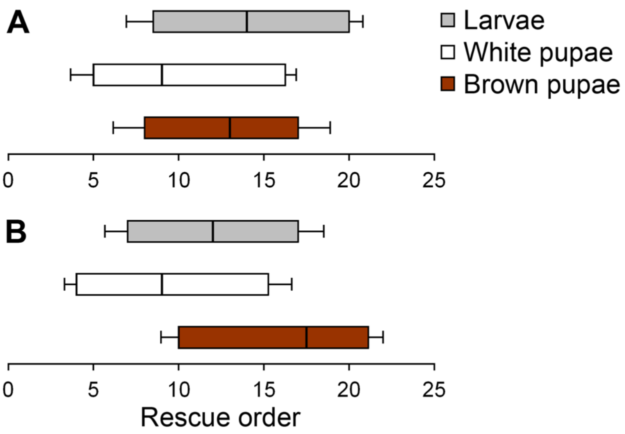
A
Larvae
White pupae
Brown pupae
0
5
10
15
20
25
B
0
5
10
15
20
25
Rescue order

Figure 4