

Evolution of acoustic communication in fungus-growing ant societies

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Abstract

Do ants ‘talk’? If so, how important is talking in ant societies? Chemical communication, through pheromones for example, was thought to be the main communication tool in ant societies; however, a major question is whether chemical substances alone can control highly differentiated social behaviours. In this study, we focused on the ‘talk’ of agricultural ants, key organisms in the Neotropical ecosystem, and conducted a detailed acoustic analysis. Our results indicate that in fungus-growing ants, acoustic communication is a much more frequent and critical factor than previously believed. The frequency of stridulatory sound-production recordings from the ants were found to be significantly correlated with social structure complexity. Structural analysis indicated that both the area and number of slits in the stridulatory acoustic organs were significantly correlated with body size. The ability of leaf-cutting ants to maintain fungus gardens was significantly lower in the sound-inhibited group than in the pheromone secretion-inhibited group. These results suggest that ants that have become ‘chattier’ may induce altruistic behaviours and create more complex societies. The findings of this study may be applicable not only to social evolution studies but also for effectively controlling ant behaviours.

Full Text

Social animals have evolved into highly developed societies through intimate cooperative and altruistic behaviours¹. For instance, the development of ‘agriculture’, managing other organisms for stable supply of food resources, requires highly sophisticated cooperative behaviours². Indeed, some non-human animals are engaged in agriculture, of which the best-known examples are fungus-growing ants (tribe Attini, subtribe Attina). While the origin of human agriculture was only ~10,000 years ago, fungus-growing ants can be traced back to 60 million years ago³.

Agricultural ants have diverse societies

Ant agriculture involves garden cultivation of fungi (order Agaricales) within their nests to nurture the queen and larvae with balanced symbiotic fungi containing high levels of nutrients⁴. Fungus-growing ants compose 17 genera containing ~250 species and maintain a wide array of social structures often studied with respect to various evolutionary theories⁴. In groups that have retained ancestral traits (10 genera, ~130 species), there are only 50–100 workers in a nest, with no physical differences among them⁵. Fungal gardens are restricted to small spaces, such as shallow soils, rotten wood, or underneath rocks⁶. The advanced group (5 genera, ~80 species) have colony sizes of several hundred to a thousand individuals, multiple fungal garden chambers, and two or three physically identical subcastes^{7,8}.

The leaf-cutting ants (genera *Atta* and *Acromyrmex*; ~40 species), have the largest colonies and most complex societies among ants, with millions of individuals and >10 subcastes, ranging from small (2 mm in length) to large workers (>15 mm in length)^{3,4,6}. As they require large amounts of vegetal substrates to

cultivate numerous mutual fungi, these ants play a critical role in the ecological succession of vegetation in Neotropical forests^{7,8}. When their vigorous agricultural activities are directed towards human society, they cause enormous economic and agricultural damage. For instance, *Atta texana* in Texas can destroy >5,000 ha·yr⁻¹ of pine seedlings, with annual control and management costs reaching ~US\$ 2.3 million⁹.

To maintain such a complex social structure and elaborate agricultural system, leaf-cutters must perform >30 time-sensitive tasks with the most appropriate members^{6,7,8}, and our preliminary research in which we recorded high-frequency acoustic signals in *Atta* led us to hypothesize that ‘talking’ (i.e. acoustic communication) is an important factor used to control such social behaviours.

Social evolution is based on communication

Eusocial insects, including fungus-growing ants, are conventionally referred to as ‘superorganisms’¹⁰, and the study of communication within these species is expected to provide a major breakthrough in the theory of social evolution¹¹.

Highly evolved social behaviours of insects are controlled by constant sharing of information through chemical^{12,13}, acoustic^{14,15}, visual^{16,17}, and tactile¹⁸ stimuli (Table 1). In ants, chemical communication is generally understood to be a foundational feature of their social evolution and cooperative behaviours^{12,13}. Although visual, acoustic, and tactile communications are postulated as the next evolutionary steps, those have yet to be experimentally revealed¹¹.

Barbero et al. (2009) published a revolutionary study on acoustic communication in ants¹⁹. It had been established that larvae of the lycaenid butterfly (genus *Maculinea*) used chemical mimicry to invade host ant (*Myrmica*) nests²⁰; additionally, the larvae of *Maculinea (Phengaris) rebeli* were found to mimic queen ant sounds of *Myrmica schencki* and received protection from the workers once inside the nest. The study revealed that queens sounds induce cooperative behaviours, bringing attention to the importance of acoustic communication among ants. Further examples of acoustic communication research are well known in leaf-cutting (genus *Atta*)²¹ and fire ants (*Solenopsis invicta*)²². Others are also known to produce drumming sounds by striking their abdomens against the ground^{23,24}; however, no quantitative studies have been conducted on these functions.

To establish the importance of acoustic communication in the evolution of sociality in fungus-growing ants, we proposed the following two hypotheses: (1) A significant correlation exists between social evolutionary levels and sound-production frequency and types; and (2) acoustic as well as chemical communication in agriculture-practicing ants require a high level of cooperative behaviour. To test these hypotheses, we obtained detailed stridulatory sound recordings with each independent behaviour of seven genera (eight species) of fungus-growing ants using a high-resolution recording device that we developed. Phonetic analyses of the recorded sounds and structural analysis of the sound-producing

organs were carried out via SEM, and the effects of chemical and acoustic stimuli on fungus-garden maintenance were examined through their experimental inhibition.

'Chattier' ants formed complex societies

Using a self-developed, high-resolution recording device, we recorded the acoustic signals of 3–10 workers and fragments of symbiotic fungi from their fungus garden for 15–30 min in attines and successfully obtained ant-derived stridulatory sounds for all species (Extended Data 1–3). The groups that retained ancestral traits produced acoustic signals <0.5 times per minute per individual (0.01 ± 0.002 in *Myrmicocrypta*, 0.07 ± 0.06 in *Apterostigma auriculatum*, 0.03 ± 0.03 in *Ap. mayri*, 0.26 ± 0.30 in *Cyphomyrmex*). In the moderately derived group, the average frequency was 6.37 ± 2.32 and 2.15 ± 2.24 times per minute per individual in *Trachymyrmex* and *Sericomyrmex*, respectively. The genera of the highly advanced group, *Acromyrmex* and *Atta*, were much 'chattier' (average frequency 19.15 ± 6.35 in *Acromyrmex* and 24.08 ± 24.21 in *Atta*) than those of the other groups. A Jonckheere–Terpstra test confirmed a significant positive correlation between the stridulatory sound frequency and estimated branch age of each lineage (Z-value, 2.97; $P < 0.01$).

We next recorded and phonetically analysed acoustic signals under independent stimuli and external conditions for the 'chattiest' leaf-cutting ant species, *Atta colombica*. The two independent external stimuli were (1) pinching with forceps and (2) burying with oats, whereas the recorded signals from independent conditions and locations were (3) cutting Leguminosae leaves, (4) cutting Clusiaceae leaves, (5) cutting Heliconiaceae flowers, (6) vigilant sound near a fungus garden, (7) bark-like sounds near a garbage dump, (8) bark-like sound near an entrance, (9) alarm sound on a trail, (10) larval care, and (11) a queen's alarm sounds were extracted and subjected to phoneme and canonical discriminant analyses. Each acoustic signal was discriminated at a rate of 60–100% (Fig. 1; Function 1: $\chi^2 = 750.225$; $P < 0.0001$, raw data: Extended Data 4, Supplementary Information). The variables that correlated best with axes 1 and 2 from the standardized discrimination coefficients were f0 duration (seconds) and pitch range, respectively. A 100% discrimination was found for four stimuli: (1), (3), (9), and (11). Although 14 types of stridulatory sounds were significantly discriminated in preliminary analysis, including those of workers of different body sizes (2.5–15.0 mm in body length), only the acoustic signals produced by medium-sized workers (~7.0 mm) and queens were recorded here; thus, the effects of the size of the stridulatory sound-producing organs due to differences in body size were eliminated, and only differences between situations, stimuli, and acoustic signal types were observed. Reanalysis of seven types of mixed acoustic signals (Fig. 1a, dashed circle) resulted in discrimination rates of 80–100% (Fig. 1b). A 100% discrimination was revealed for (2), (4), (5), and (7) (Function 1: $\chi^2 = 453.974$; $P < 0.0001$). From the standardized discrimination coefficients, the strongest correlations for axes 1 and 2 were f0 start (Hz) and pitch range, respectively. It was, thus, concluded that medium-sized leaf-cutting ant workers produced 10 significantly different acoustic signals depending on the situation and stimuli.

Accordingly, acoustic communication in fungus-growing ant societies was found to be more important than previously assumed. The evolutionary nature of ants was further revealed by two findings: the frequency of acoustic signal production increased with social evolutionary stage, and the complex society of leaf-cutting ants produced the highest number of eusocial insect sound types recorded thus far. Accordingly, acoustic forms of communication, much like chemical communication, are an important factor in the evolution and maintenance of social system in the ants, as 'chattier' communities appear capable of more complex societies than 'silent', small, and simple societies.

In leaf-cutting ants, there were 10 types of stridulatory sounds identified in the same caste, representing the highest number among eusocial insects such as bees (*Apis mellifera*²⁵, 7; *A. cerana*²⁶, 3; and other bees²⁶, 1–3 types) and termites²⁷ (higher termites: *Constrictotermes cyphergaster*²⁸, 1 and *Macrotermes natalensis*²⁹, 3; lower termites: *Mastotermes darwiniensis*³⁰, 2). Termites, including fungus-growing agriculture-based species, are known to use drumming sounds as alarm signals²³; however, there is no further evidence of complex acoustic signals for communication among insects.

The honeybee is a eusocial insect that is capable of complex acoustic communication despite the absence of a specific sound producing organ. They can produce vibroacoustic signals by shaking their thoraxes and wing vibrations³¹, and when workers combine figure-eight dances with acoustic signals, they can share information about both the distance and direction of nectar sources³¹. The tooting and quacking sounds of queen bees inhibit the hatching of new queens and inform nestmates of the presence and activity of enclosed queens³². In worker bees, a piping sound terminates the dance and helps recruit nectar receivers³³. Alternatively, the leaf-cutting ants here demonstrated 10 significantly different acoustic signals, indicating a potentially more complex communication system.

A subset of ant species are the only eusocial insects with stridulatory organs to produce sound. Only groups with two petiole segments have these organs, comprising just six of the 20 subfamilies, including Myrmecinae, Paraponerinae, and Ponerinae³⁴. Although there is some ant sound communication research in several ant species, no quantitative studies have been conducted on these functions. Accordingly, the high-resolution recording device we developed could be useful in facilitating discovery of more complex acoustic communication systems within eusocial insect societies in the future.

Evolutionary relations of sound organs

Head width and the stridulatory organ area were measured for 69 individuals of seven genera (eight species) of attines using SEM, and the following allometric equation was derived: $\log(\text{organ area}) = 14.41 \cdot \log(\text{head width}) - 19.54$ ($R^2 = 0.847$; Fig. 2a, Extended Data 5). When tested for Spearman's rank correlation coefficient, a significant positive relationship was observed between the stridulatory organ area and body size, with an S-value of 10,752 ($P < 0.0001$). The relationship between head width and slit number was also calculated: $\log(\text{slit number}) = 4.67 \cdot \log(\text{head width}) - 5.37$ ($R^2 = 0.61$; S-value = 23,899;

$P < 0.0001$; Fig. 2b). More specifically, the area of the stridulatory organ was not correlated with head width in the ancestral and middle-advanced groups but highly correlated in the two genera of leaf-cutting ants. The slopes (α) were 12.66 for *Atta colombica* (S-value = 80.71; $P < 0.001$) and 1.01 for *Acromyrmex octospinosus* (S-value = 514; $P < 0.001$), whereas the intercepts (β) were -15.73 and 1.94, respectively. Similarly, no significant correlation was found between the allometry of observed slit number and the ancestral or middle-advanced groups, although a significant positive correlation with *Atta* ($\alpha = 3.18$, $\beta = -2.19$; S-value = 138.12; $P < 0.01$) and *Acromyrmex* was observed ($\alpha = 1.09$, $\beta = 1.51$; S-value = 503.58; $P < 0.001$). The data thus suggest that even among two advanced genera, *Acromyrmex* followed a relative growth curve, whereas the more advanced genus *Atta* had slopes much greater than 1, indicating a highly variable structure.

These observations prompted an assumption regarding the physical evolution of acoustic communication in ants. The allometry indicated that the area and slit numbers of the stridulatory organ increased with body size. Body size, in turn, was regulated by habitat, queen egg-laying ability, and colony size⁶, suggesting that such ecological and social changes had driven the evolution of communication ability in ants.

In Hymenoptera, allometric analyses have shown that the slope of their reproductive organs is significantly >1 , whereas those for the brain and central nervous system are <1 ³⁵. Reproductive organ size is correlated with egg size, and egg size depends on the presence of parasitic organisms; thus, changes in the allometric slope can indicate an adaptive pathway for external factors. This suggests that the stridulatory organs of *Atta* were subjected to positive selection pressure, significantly increasing their function as a critical means of communication. In turn, it may affect the ecological factors and account for the 100-fold greater colony size and more complex social structure of *Atta* than of *Acromyrmex*.

Chemical vs. acoustic cues

To compare the efficiency of social behaviours and management among leaf-cutting ants, sound and pheromone inhibition experiments were conducted. Ten sub-colonies, each consisting of eight workers, were placed for 1 week near a small fungus garden, along with glued pheromone-producing organs, sound-producing organs, or upper part of the mesothorax. Daily garden weights and social behaviours were recorded. The mortality rates in each experimental treatment were negligible and did not significantly affect the experimental results. It was found that the fungus garden was significantly smaller in the sound-inhibition group ($48.4\% \pm 9.2\%$ of the initial garden size) than in the pheromone-inhibition group ($71.6\% \pm 10.9\%$ of the initial garden size; Extended Data 6); the control garden was $103\% \pm 9.19\%$ of the initial garden size. Significant differences among the groups were observed using a multiple comparison test (Steel–Dwass method: control vs. sound, $t = 3.81$; $P < 0.001$; sound vs. pheromone, $t = 3.66$; $P < 0.001$; control vs. pheromone, $t = 3.70$; $P < 0.001$).

Additionally, a behaviour comparison revealed that each inhibition group showed decreased fungus garden maintenance (FG), gathering of vegetal substrate (FOOD), dumping of garden waste pieces (DUMPING), nest digging (DIGGING), defensive behaviours (GUARD), and walking around (WALK) (Fig. 3). A generalized linear model analysis indicated that the behaviours with a significant effect on fungus garden maintenance were FG, FOOD, DUMP, and GUARD, (best model: FG, $P < 0.01$; FOOD, $P < 0.01$; DUMP, $P < 0.0001$; GUARD, $P < 0.01$, df, 6, AIC = -148.79).

These data indicate a reversal of conventional beliefs regarding ant communication, as chemical communication has historically been considered the most effective factor supporting intimate social behaviour, with all other factors being supplementary; the results here indicate that acoustic communication in leaf-cutting ants more effectively regulates social and cooperative behaviours.

Conclusion

Acoustic communication in attines not only conveys information but also serves as a form of advanced social grooming³⁶ to induce social behaviours within a relatively low kin-related society. Further study of the evolution of acoustic communication in ants will likely provide useful insights in many fields, including the evolution of altruistic behaviour, evolutionary linguistics, and social psychology. In addition, sound communication research in ants may be applicable for controlling the behaviour of agricultural pests (such as leaf-cutting ants and fire ants) in the future, potentially being both low cost and effective.

Declarations

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Author Contributions

T. M., S.H. and H.S. conceived the project, designed experiments and collected ants. T.M. and H.S. performed all experiments. T.M. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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Methods

Sample collection

The studied species of fungus-growing ants were collected with permission from Soberania National Park, Republic of Panama (9.128° N, 79.714° W). Species, number of colonies, sampling date, and recorded stridulatory data are summarized in Extended Data 1. A total of 87 fungus-growing ants, belonging to seven genera (eight species) were collected during September 2012, August 2013, and February 2015. Each sampling was conducted with the permission of the Panamanian Ministry of the Environment (ANAM). The permission numbers were SE/A-11-15, and SE/A97-13. The collected colonies were brought to a laboratory at the Smithsonian Tropical Research Institute and kept in artificial, plaster of Paris nests in plastic casing at 25 °C and 70% humidity. After the analyses, the ants were kept in Kyushu University.

Acoustic recording device and recording conditions

For the stridulatory acoustic recordings of fungus-growing ants, it was essential to have a small recording device with high resolution and could be safely carried to the research sites. However, the commercially available devices were too large and expensive. Therefore, we developed a small, high-resolution recording device that we designed. Hoshiden's KUC3523-04-0245 lead condenser microphone (Hoshiden, Osaka, Japan) was used to detect stridulatory cues, and it was connected via a 3.5 mm jack. The sound was amplified by a portable multimeter AT-PMX5P (Audio Technica, Tokyo, Japan) and imported into a computer. The recording device was covered with three layers of acrylic casing (the size of the outermost acrylic case is 15 cm x 15 cm x 15 cm), with a cork mat at the bottom, and a rubber sheet underneath to absorb vibrations and eliminate noise. In total, 945 min of acoustic data were recorded for seven genera (eight species) of attines (Extended Data 2). For the basal group, 10 individuals were recorded at a time, whereas for all the other groups, three individuals were placed in the recording device for 10-30 min each

in three conditions: with the fungus garden, larvae, and pupae; with the garden substrates (Leguminosae leaves, Clusiaceae leaves, and Heliconiaceae flowers); and with the garbage dump.

Acoustic analyses

Noise in the recorded stridulatory data, such as white noise, human conversation, and walking, was removed using the sound effect software Audacity (v.2.3.3), so that only ant-derived acoustic information remained. The acoustic analysis software Praat (v.6.1.40) was used to determine pitch range, the following factors were measured: F0 max (Hz), F0 start (Hz), F0 duration (s), F0 max - F0 start (s), no. pulse, F1 width (Hz), F2 width (Hz), F3 width (Hz), and F4 width (Hz).

Structural analysis of the acoustic organs

Images of the acoustic organs for 69 individuals from seven genera (seven species) of attines were obtained using a scanning electron microscope (JEOL JSM-6360LA). Image J (v.1.52) was used to measure the area and count the number of slits.

Sound and chemical inhibition experiments

Two plastic cases were prepared with 0.20 g of fungus garden fragments, and eight small-to-medium-sized leaf-cutting ants were introduced into one of the cases, whereas the other was filled with oats (0.50 g), a fungus garden substrate. Ten sub-colonies were prepared for each treatment, and three manipulation conditions were used: (1) fixation of the postpetiole with glue to prevent it from producing sound (soundlessness of all individuals was confirmed using a microphone), (2) fixation of the end of the abdomen with glue to prevent pheromone secretion (individuals were made to walk on filter paper for confirmation), and (3) the pronotum was glued as a control. Sub-colonies were kept at 25 °C and 70% humidity, and newly treated individuals were introduced daily to replace any mortalities (control, 0.1 ± 0.08 mortalities·day $^{-1}$; pheromone inhibition, 0.51 ± 0.3 ; sound-inhibition group, 0.30 ± 0.32). Worker behaviours were observed for 5-min steps at regular intervals daily, and the weights of the fungus garden and oats were measured after 1 week.

Statistical analyses

A Cochran–Armitage analysis was used to analyse the correlation between the stridulatory sound frequency and evolutionary stage. Canonical discriminant analyses were used for statistical processing of the stridulatory acoustic types in each species, and Pearson's product-rate correlation tests were used

for the allometric analyses of the stridulatory organ structure. Analyses of the manipulation experiments were performed using the multiple comparison of means method (Ryan method), and a generalized linear model analysis was used to assess the types of behaviour for each treatment. All analyses were performed in R (v.3.6.1). The raw data and the codes of statistical processes were saved in Kyushu University and is available if required to confirm reproducibility.

Table 1

Due to technical limitations, table 1 is only available as a download in the supplementary files section.

Figures

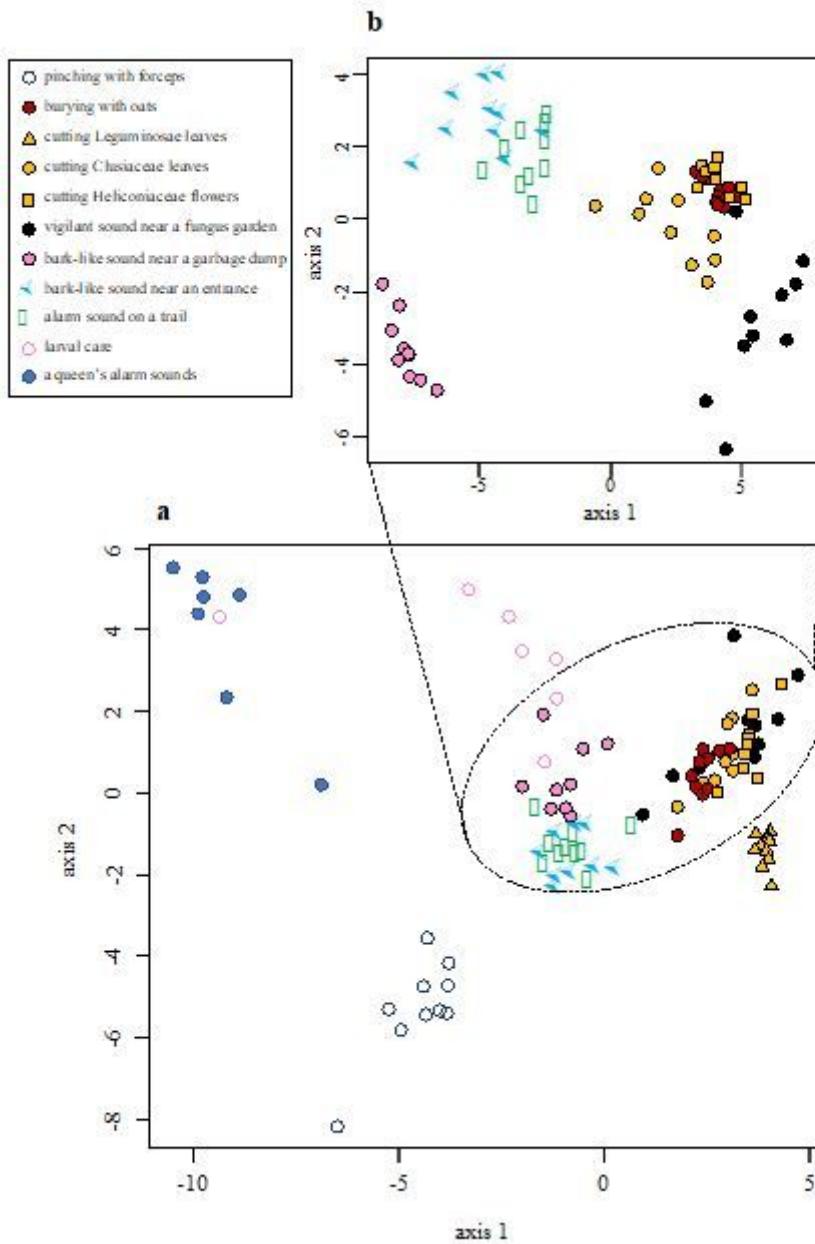


Figure 1

Discriminant analysis of 11 significantly different sound types from leaf-cutting ant (*Atta colombica*) mid-sized workers and queens. Discrimination rates ranged from 60% to 100%. Reanalysis of the seven types of acoustic signals that were unclear in (a) resulted in discrimination rates of 80–100% (b).

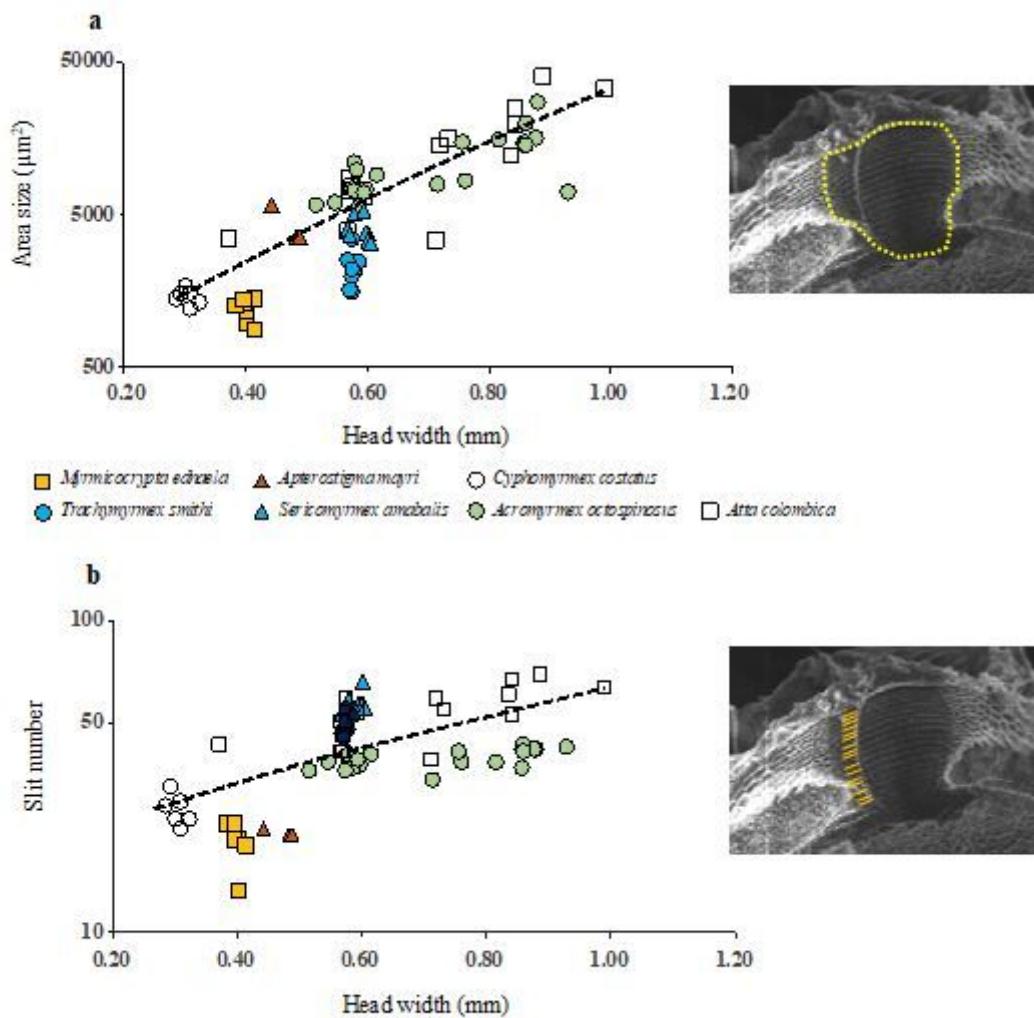


Figure 2

Allometric relationships between (a) stridulatory organ size and (b) slit number according to head width. Yellow square, *Myrmicocrypta ednaella*; orange triangle, *Apterostigma mayri*; blue triangle, *Trachymyrmex zeteki*; blue circle, *Sericomyrmex amabilis*; green circle, *Acromyrmex octospinosus*; green square, *Atta colombica*. The derived allometric equations were (A) $\log(\text{organ size}) = 14.41 \cdot \log(\text{head width}) - 19.54$ ($R^2 = 0.847$), and (B) $\log(\text{slit number}) = 4.67 \cdot \log(\text{head width}) - 5.37$ ($R^2 = 0.61$).

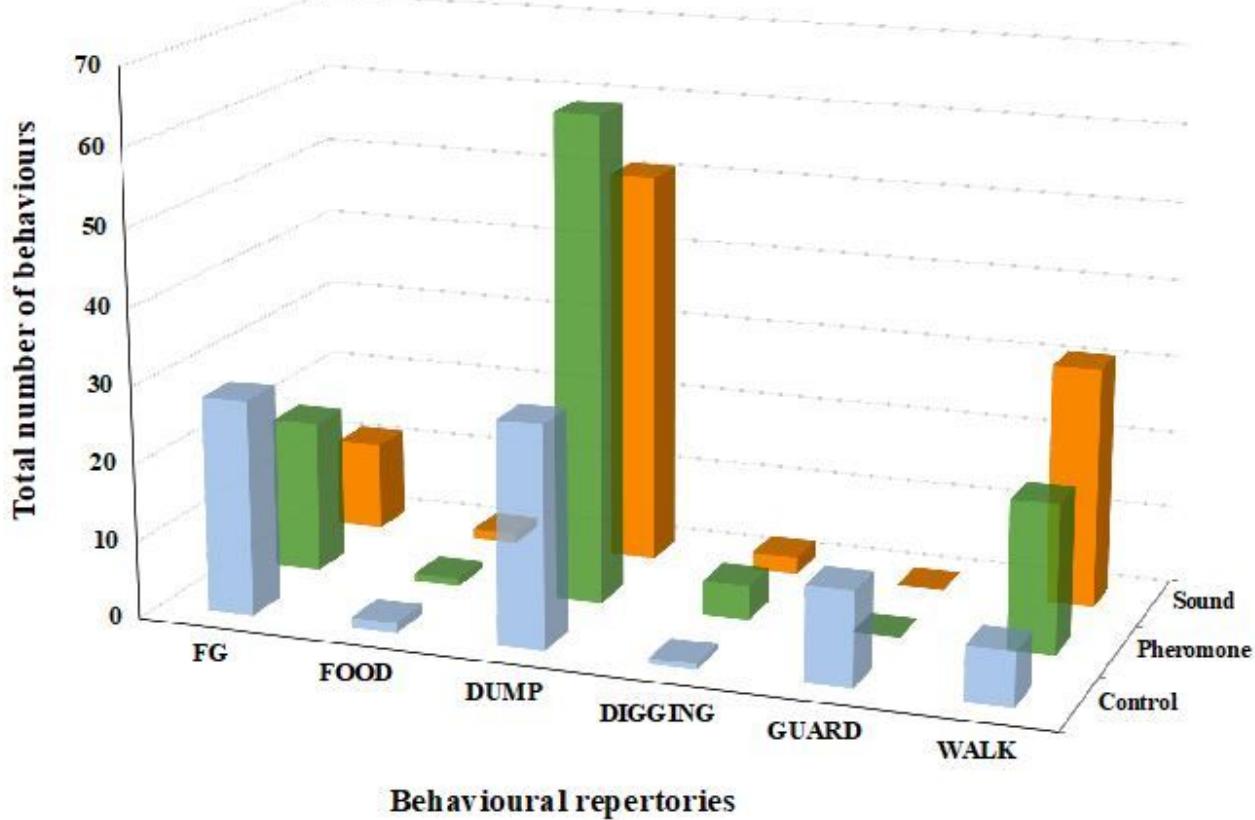


Figure 3

Behaviour frequency in the sound-inhibited (orange), pheromone-inhibited (green), and control (pale blue) groups. FG, fungus grooming, a specific behaviour to eliminate parasitic microbes for maintenance a fungus garden; FOOD, staying in a food arena; DUMP, dumping wasted garden pieces; DIGGING, digging a nest floor; GUARD, guarding nest entrance; WALK, walking around.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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- 7barklikesoundnearG.mp3

- 5CuttingHeliconianceaeflowers.mp3
- 3CuttingLeguminosaeleaves.mp3
- 11Queensalarm.mp3
- 4CurttingClusiacealeaves.mp3
- 10larvalcare.mp3
- table1.docx
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