

Fungus Garden Structure in the Leaf-Cutting Ant *Atta sexdens* (Formicidae, Attini)

M. BASS^{1*} and J.M. CHERRETT²

¹Trinity College, Carmarthen, Wales SA31 3EP, UK, Fax. +44-1267-230933;
and ²School of Biological Sciences, University of Wales, Bangor, Gwynedd
LL57 2UW, UK

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Abstract

Leaf-cutting ants have a mutualistic relationship with a fungus, which they cultivate on fresh plant material. For optimum efficiency, this 'fungus garden' must have a structure that combines a large area for the production of ant rewards ('staphylae'), with the smallest chamber volume in which it can be maintained, and with accessibility for workers. We investigated the structure of a fungus garden of *Atta sexdens* (L.) by sectioning. It contained many small cavities, most of which (74.7%) were only accessible to small 'minima' workers, excluding larger sizes. These cavities provided a large internal surface area, 74% of the total surface area of the garden examined. Internal surfaces had more staphylae per unit surface area than external surfaces, suggesting a heavy harvesting pressure from large workers on the latter. The problem of producing a garden structure capable of yielding large crops of staphylae may have been important in the evolution of the characteristically small minima workers, which have access to the smallest cavities. We also examined staphyla production in fungus gardens. Numbers of staphylae present increased with garden age, but few were lost with discarded substrate. This suggests that workers remove all staphylae before removing substrate, or that the oldest garden produces few staphylae anyway.

Keywords: *Atta sexdens*, fungus garden, minima sub-caste, staphylae

*The author to whom correspondence should be sent.

1. Introduction

The New World leaf-cutting ants (Tribe Attini) have an obligate mutualistic relationship with fungi, which they cultivate on fresh vegetable material. These fungi rarely produce reproductive structures and were placed in the *Mycelia Sterilia* by Kriesel (1972). However, Powell (1984) has since shown that they are Basidiomycetes. Leaves brought to the underground nest

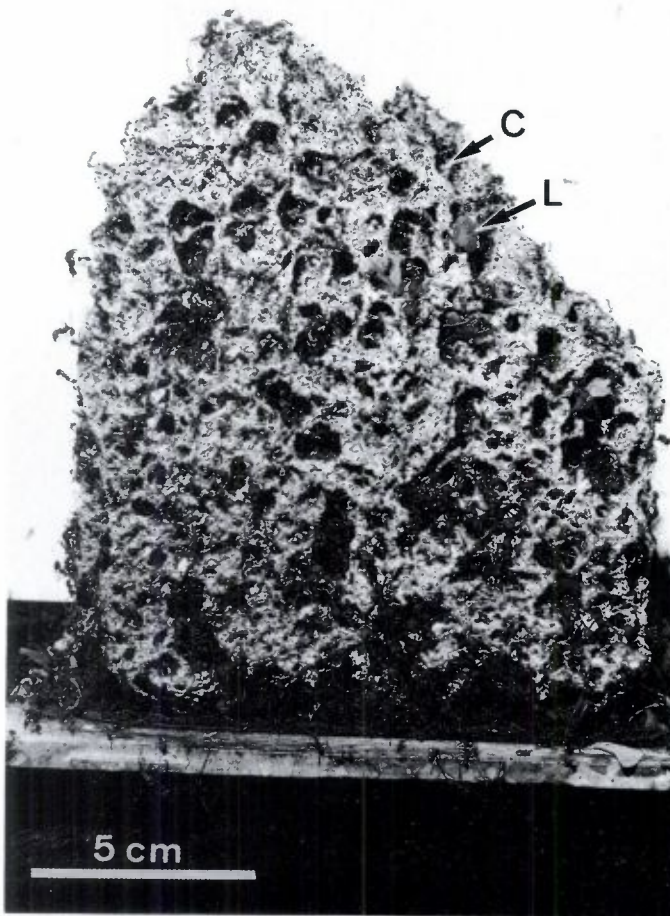


Figure 1. Fungus gardens of *Atta sexdens*. a) External appearance of a laboratory nest, with protective plastic cover removed. The upper regions of the garden, where new substrate is added, (young garden) appear to have large cavities (C), while the lower areas (old garden) appear much more compact. Pieces of leaf substrate (L) can also be seen being degraded by workers.

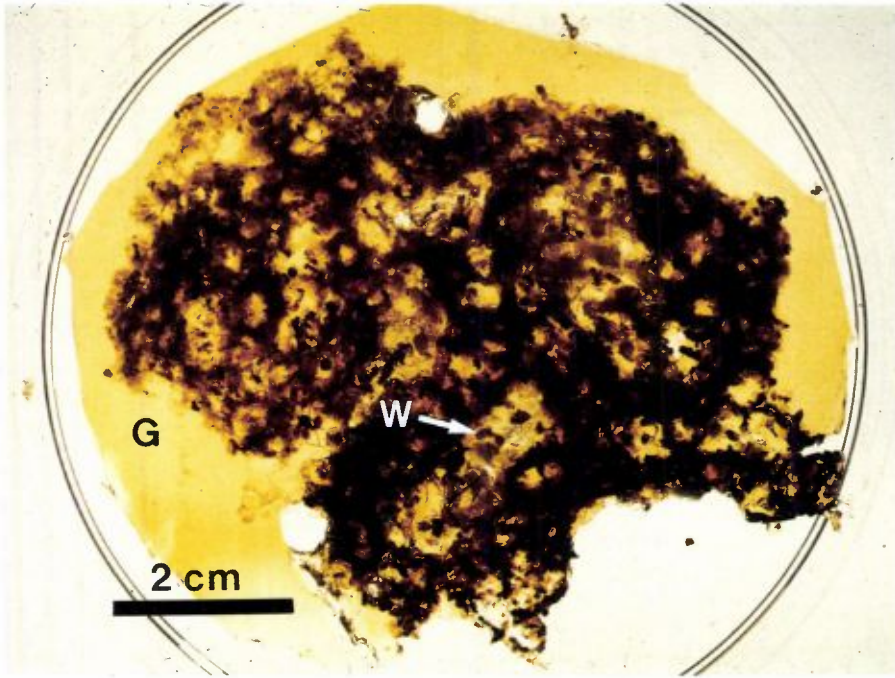


Figure 1. Fungus gardens of *Atta sexdens*. b) Internal appearance, shown by a section of fungus garden. Workers (W) can be seen within the cavities. G = gelatine carrier.

are reduced to a pulp by workers and inoculated with their fungus, to form a 'fungus garden' (Fig. 1a). The fungi produce bunches of swollen hyphae (staphylae), which the ants harvest as food for themselves and their larvae (Moeller, 1893; Weber, 1972).

Leaf-cutting ant workers are polymorphic, ranging from 2–15 mm in body length in *Atta*. In contrast, primitive Attines, e.g., *Cyphomyrmex*, which cultivate fungi on dead plant material and insect frass, are monomorphic (Hölldobler and Wilson, 1990). *Atta* workers have evolved from a monomorphic ancestor, with medium-sized workers (Oster and Wilson, 1978), partly because the efficient harvesting of fresh leaves requires large workers (Wilson, 1980a). However, it has never been fully explained why very small workers have evolved to look after the fungus gardens. Hölldobler and Wilson (1990) state that this activity requires very small workers, which can safely

manipulate the delicate fungal hyphae. However, much of this manipulation can be performed by medium-size workers (personal observation).

The fungus garden is a spongy, honeycombed structure, and all worker sizes have access to its outer surface. In contrast, the internal areas may be less accessible, in particular for larger workers. These internal surfaces are likely to make up a large proportion of the total surface area of the garden, and this has implications for staphyla production, as these are mainly borne on hyphae growing clear of the leaf substrate (personal observation). Theoretically, it would be most efficient for the ants to develop a compromise between accessibility and surface area available for staphyla growth, producing as many small cavities as possible without compromising worker access. The fungus gardens of many termite species also have complex cellular structures that increase the surface area per unit weight of fungus comb (Wood and Thomas, 1989).

Another important feature of fungus gardens is how long substrate material will support fungal growth, and how this relates to staphyla production. Weber (1972) found that one garden of *Atta cephalotes* had a life cycle of 7 weeks, while a second had one of 4 months. He also pointed out that the removal and addition of substrate is cyclic, triggered by seasonality and the length of time that the substrate can be used by the fungus. The most efficient strategy for the ants would be to keep substrate only for as long as it produced a worthwhile crop of staphylae.

In this paper we investigate two features of the fungus garden. First, we examine its internal structure and accessibility to workers. Secondly, we look at when and where staphylae are produced in the garden, and how this is linked with garden structure.

2. Materials and Methods

An *Atta sexdens* (L.) nest with 80 fungus gardens built in clear plastic containers, each of 2.5 l capacity, was maintained at 27°C and 80% relative humidity. Fresh leaves from a variety of British tree species were provided daily.

Assessing worker size

In *Atta* workers, there is a continuous size range from 2–15 mm in body length, with head size increasing disproportionately to body length (Oster and Wilson, 1978). In this study, minima were defined as those workers having body lengths of 2–3 mm and head widths of <1.2 mm.

Assessing garden age

Externally, the fungus garden appears heterogeneous. The upper regions are grey-green, with large cells, and this is young garden, where most fresh substrate is added. In contrast, the basal areas are yellow-brown and have small cells. This is the oldest part of the garden and Bass (unpublished data) has shown by means of time-lapse photography that the garden is constantly changing position and sinking down, so that older material is usually found at the bottom. Between young and old garden is a mature region, which appears intermediate. Fungus garden was therefore divided into three age types, according to its external appearance.

Determining the internal structure of a fungus garden

The internal structure of a fungus garden was examined by sectioning a garden embedded in a gelatine carrier. A similar technique was used by Campbell and Tomkeieff (1952) to study lung structure. A single small intact garden (250 cm³) was placed in a muslin bag and dropped into liquid nitrogen, immobilizing the workers present (the size of the nitrogen flask neck limited the size of the garden that could be studied). This frozen garden was placed in a 25% gelatine solution in distilled water heated to 75°C, then left to soak overnight at 50°C in an incubator, and was then cooled for several hours to allow the gelatine to solidify. Finally, the resulting block containing the garden was sectioned using a fine wire stretched on a fret saw frame. Sixteen slices, each about 4 mm deep, were produced. Ideally, sections should be as thin as possible, but thinner sections could not be produced easily. The garden was cut horizontally, so that sections 1-4 were from young garden, sections 5-11 were from mature garden, and sections 12-16 were from old garden. A typical section is shown in Fig. 1b.

Sections 1 and 16 were damaged and discarded, and the remaining sections were photographed with a back light. Transparencies were projected onto a screen and outlines of the visible cavities in each section, together with any workers or brood visible, were then traced onto paper (recording the scale of size increase).

Three-dimensional information about the internal composition of structures can be obtained by making two-dimensional sections of them and applying stereological principles (Williams, 1977). These have been widely used in geology and electron microscopy. Area to volume ratios were estimated for each section using the Delesse principle (1848). This states that the area fraction of a component lying in a random transverse section is equivalent to its volume fraction, expressed by:

$$\frac{Vc}{Vt} = E \frac{Ac}{At}$$

Vc is the total volume of cavities within the section, Vt is the total volume of the section, Ac is the total area of cavities within the section, At is the total area of the whole section and E is the theoretical mean.

The outlines of cavities were digitized, enabling both the areas and the circumferences of cavities to be calculated. The former were used in the above equation to calculate the percentage of air space within the fungus garden, one estimate being obtained for each section. Cavity circumferences were used to calculate surface areas per unit volume (S_v). S_v is the surface density of structures in sections and can be obtained by examining profile lengths (cavity circumferences). There are a variety of equations for calculating S_v , the one used in this study being:

$$S_v = 4 \frac{m}{\pi}$$

S_v is surface area per unit volume (mm^2 per mm^3) and m is the length of profile per unit test area (mm), calculated by dividing the sum of the cavity circumferences by the total area of the section (Williams, 1977). S_v was calculated by examining a large rectangular area inside each section, the sum of the enclosed profile lengths being fitted into the equation.

The thickness of the cavity walls in young, mature and old garden were also measured over the range of sections available. These were found by taking transects across the sections and measuring the distances between those cavities falling along the transects.

Estimating the rates of production of staphylae, and of garden turnover

Staphyla numbers on an external area of fungus garden was examined as the sample area aged. It was advantageous to make repeated observations on the same area, because different substrates support different levels of fungal growth, some even being toxic to the fungus (Mullenax, 1979; Pagnocca et al., 1990), and the laboratory nest used received a varied diet. However, once new substrate has been added to the garden, it is quickly overgrown by fungal hyphae and becomes indistinguishable from the rest of the garden. To follow a particular area of garden over time, it was therefore necessary to insert markers.

Marking fungus gardens

Artificial markers such as steel pins were quickly removed and discarded by the ants. However, Bottrell (1980) noted that the ants would cut holly leaves (*Ilex aquifolium*) and incorporate them into their gardens. These have lignified edges and spines that persist when incorporated into a fungus garden, remaining visible even in discarded nest refuse. Holly spines were therefore used to mark fungus gardens and were obtained by cutting off the outer 1 cm edges of fresh holly leaves.

Individual gardens were marked by placing them on small tables connected to the main nest by removable bridges. When the bridges were removed, holly spines were supplied to the isolated gardens and the ants constructed a distinct spiny layer. The bridges were then replaced. These bridges were vertical so that ants moving towards the main nest had to climb upwards. Workers discarding refuse seldom carry it upwards (personal observation), so the refuse from the marked garden was discarded from the side of the table and could be collected. The presence of spines in this refuse therefore indicated that the original marked layer was now being discarded.

Monitoring the production of staphylae and the rate of garden turnover

A fungus garden built in a clear perspex observation chamber (30 cm × 27 cm × 4 cm) was used to estimate the number of staphylae produced on a marked area. About 1000 holly spines, weighing 21 g, were supplied over 3 days, and the ants incorporated them into the garden in a 5 cm layer. From the first day of spine-incorporation until the spiny layer disappeared, up to fifty × 1 cm² areas on the marked layer were selected at random and the number of staphylae in each was counted using a binocular microscope. Simultaneously, four more gardens were supplied with 500 spines each and the number of spines discarded were recorded daily. This was done to confirm that turnover was similar for the different fungus gardens in the nest. The whole experiment was then repeated using five additional gardens.

Examining the production of staphylae on internal surfaces

In the above experiment, staphylae were only counted on the outside of the garden, but such external surfaces may be atypical of the garden as a whole, because it contains a large internal surface area. The differences between staphyla numbers on external and internal surfaces of the fungus garden were therefore studied. Pieces of mature fungus garden were examined using a binocular microscope with an eye-piece graticule. This graticule was marked with a square, which measured a 1 cm² area. External standing crops of staphylae per cm² were recorded for the fungus garden samples. The diameters

of 80 randomly selected cavities opening to the garden surface were also measured. The internal surfaces of these cavities were then exposed using dissecting needles, and the numbers of staphylae per 0.25 cm² of cavity wall area were recorded. This was the largest area that could be reliably examined in a small cavity.

Assessing staphyla wastage

The numbers of staphylae being discarded with refuse were assessed by collecting 50 refuse loads (of known weight) collected from workers, and mounting them in cotton-blue stain in lactophenol.

Statistical analyses

All data were checked for normality. Two-tailed F tests were used to test for differences between variances. Normally distributed data with equal variances were subjected to parametric tests (Analysis of Variance (ANOVA) and Tukey's multiple comparison). Non-normally distributed data were, where possible, transformed to achieve normality and equal variances. Where these conditions could not be met, non-parametric tests were used (Kruskal-Wallis and Dunn's tests, as described by Zar, 1984).

3. Results

The internal structure of a fungus garden

The sizes of the sections cut varied, because the garden was approximately spherical. Section areas ranged from 11–21 cm² in young garden, through 27–36 cm² in mature garden, to 3–20 cm² in old garden.

Cavity areas were pooled for sections 2–4 (young garden), 5–11 (mature garden), and 12–15 (old garden). As the data were not normally distributed, cavity areas for different garden ages were compared using a Kruskal-Wallis test, and this was repeated for cavity circumferences. No significant differences were found ($p > 0.3$, $n = 169, 216, 979$). Overall, cavities had a median area of 2.17 mm² (95% sign confidence limits 2.04, 2.33), and a median circumference of 5.71 mm² (95% sign confidence limits 5.51, 5.92). Of course, when sections are cut through cavities, some will be cut at angles or superficially, so that the resulting cavity areas in the sections appear too large or too small. This makes a simple comparison of cavity sizes between garden ages difficult. However, there were significantly more cavities per cm² of

section in old, compared with young garden, with mature garden being intermediate ($p = 0.05$, Kruskal-Wallis and Dunn's tests, carried out due to unequal variances, $n = 3, 7, 4$; Table 1). Old garden also had significantly thinner walls than young or mature garden ($p = 0.05$, ANOVA with Tukey's multiple comparison, carried out on data transformed to the \log_{10} values, $n = 38, 39, 41$; Table 1), although there were no differences between the latter.

Table 1. Data obtained from sectioning a fungus garden of *Atta sexdens*, including mean numbers of cavities per unit area of section, mean wall thicknesses between cavities, mean percentage air space and surface areas per unit volume (S_v).

Garden age	No. of sections used	No. of cavities present	Mean no. of cavities per cm^2 of section (\pm SE)	Mean cavity wall thickness* (mm) (\pm SE)	Mean % space (\pm SE)	Mean S_v (mm^2 per mm^3) (\pm SE)
Young	3	169	4.0 ± 0.82^a	3.14 ± 0.32^a	37.2 ± 1.7^a	0.33 ± 0.08^a
Mature	7	979	5.0 ± 0.21^{ab}	2.27 ± 0.15^a	32.2 ± 2.1^a	0.52 ± 0.03^{ab}
Old	4	216	7.0 ± 0.62^b	1.64 ± 0.16^b	28.4 ± 5.4^a	0.64 ± 0.05^b
Overall	14	1,364	5.4 ± 0.39	2.30 ± 0.12	32.2 ± 1.9	0.51 ± 0.04

*Back-transformed from the \log_{10} transformed values. Means bearing the same letter, when compared down the column, were not significantly different ($p > 0.05$).

Percentage air space did not vary significantly between garden ages ($p > 0.3$, ANOVA, $n = 3, 7, 4$), but S_v (surface area per unit volume) was significantly larger in old than in young garden sections, with mature garden being intermediate ($p = 0.05$, ANOVA with Tukey's multiple comparison, $n = 3, 7, 4$; Table 1).

The external surface area of the garden was calculated, assuming a section depth of 4 mm and multiplying this by the section circumference, to get an external area for each section. Sections 1 and 16 were assumed to have the same dimensions as sections 2 and 15. The sum of the external section areas and the areas of sections 2 and 15 (the top and base of the garden) gave an estimate of 455 cm^2 for the total external surface area. Using the S_v values calculated for the different garden ages, internal surface area was found to be $1,283 \text{ cm}^2$; 74% of the garden's total surface area.

Accessibility of the fungus garden to workers

In total, 139 workers and 36 juveniles (larvae and pupae could not be distinguished) were found in internal cavities. It was possible to measure head widths for each of the workers, and 89.9% were found to have head widths in the minima size range (< 1.2 mm).

Assuming that a worker of a given head width requires a cavity with a diameter of at least that size in order to enter it, a worker of head width 0.6 mm could only enter cavities with cross-sectional areas greater than 0.28 mm². Figures for the cavity areas obtained for each section could therefore be used to estimate the maximum numbers of cavities that workers of different sizes could enter. The smallest workers, of head width 0.6 mm, could enter a mean of 99.2% (SE 0.2) of available cavities; those of head width 1.2 mm, 74.7% (SE 1.5); those of head width 2.2 mm, 29.7% (SE 1.8); and those of head width 4.4 mm, only 8.4% (SE 1.0). Even though sectioning will make some cavities appear smaller or larger than in reality, these figures suggest that worker access to the interior of the garden is restricted to smaller workers.

Staphylae production over time in fungus garden

The first staphylae were produced 4–5 days after holly spines were incorporated into that part of the substrate. Peak staphyla numbers appeared on the fungus garden surface 20–30 days after spine incorporation, and then declined as spine output increased. Spines were collected from refuse for much longer than the spiny layer was visible in the marked fungus garden (Fig. 2). The first set of four marked gardens had a mean peak spine output 32.8 (SE 0.3) days post-marking (Fig. 2a), compared with 42.4 (SE 0.9) days for the second set, of five gardens (Fig. 2b). These times were significantly different ($p < 0.001$, T-test), illustrating how rate of turnover can vary seasonally.

Regressing \log_{10} staphyla numbers per 0.25 cm² of cavity wall area against the \log_{10} diameters of the cavities in which they were found, showed a significant negative relationship ($p < 0.001$, $R_{sq(adj)} 51.4\%$, $df 79$). Smaller cavities had more staphylae per 0.25 cm² wall than larger ones (Fig. 3). In cavities of less than 4 mm diameter, staphyla numbers ranged from 48–72 per cm² of cavity wall, while on external surfaces, there were 28.1 (SE 0.9) staphylae per cm². Cavities larger than 10 mm diameter contained only 24–32 staphylae per cm², a similar figure to that obtained for external surfaces. Presumably all worker sizes could enter and crop large cavities, while access to small cavities would be restricted to small workers.

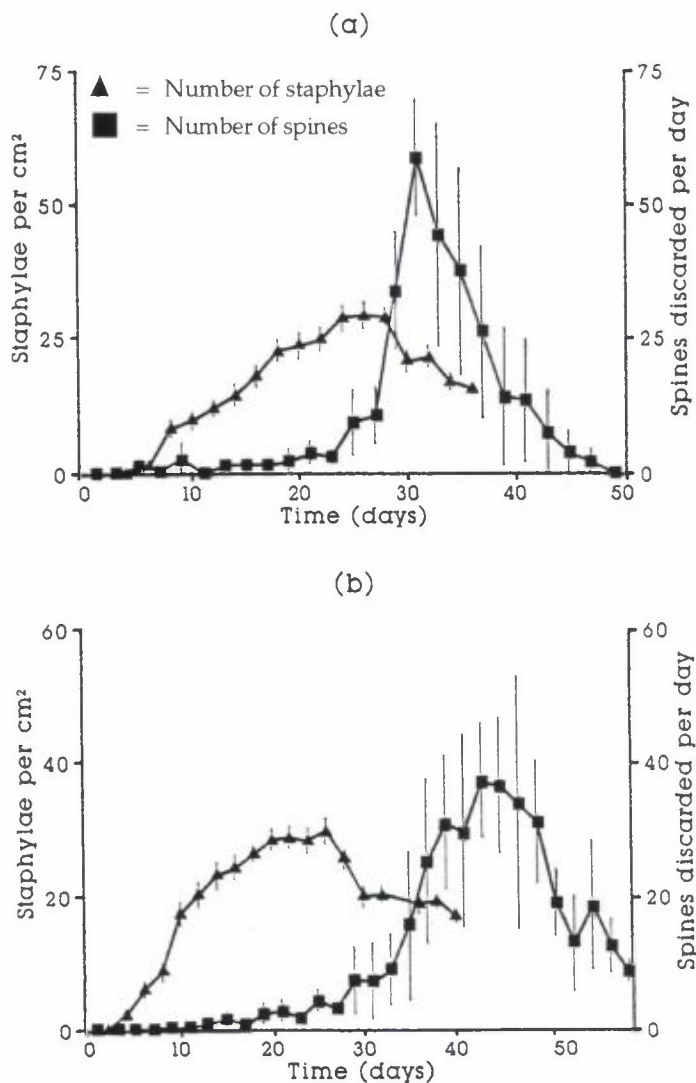


Figure 2. Mean numbers of staphylae present per cm² (\pm SE, $n = 50$) on a fungus garden marked with holly spines, compared with mean numbers (\pm SE) of spines discarded daily from simultaneously marked gardens. Two runs were carried out, with four simultaneously marked gardens in (a) and five in (b). Spines were collected from nest refuse for longer than the spiny layer was visible in the fungus garden.

A mean of 0.5 (SE 0.1) staphylae were discarded with each load of spent garden. These refuse loads had a mean weight of 3.6 (SE 0.1) mg. In contrast,

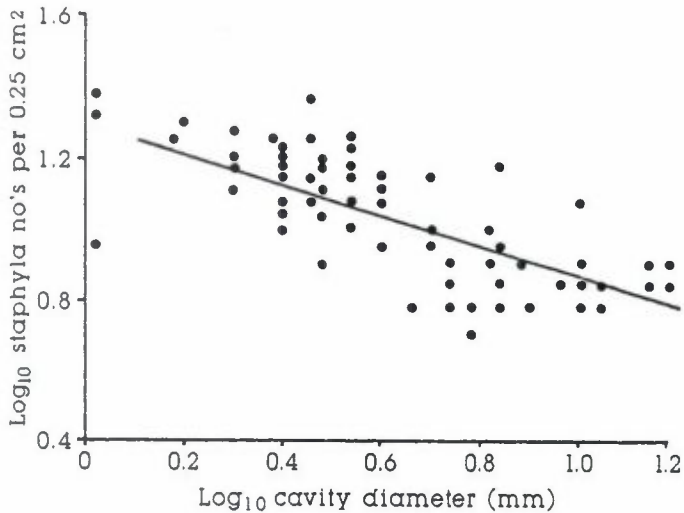


Figure 3. Log_{10} numbers of staphylda per 0.25 cm^2 area of fungus garden cavity wall, plotted against the log_{10} diameters of cavities in which they were found (regression equation, $y = 1.3 - 0.431x$, $p < 0.001$, $\text{Rsq}(\text{adj}) 51.4\%$, $\text{df } 79$).

five 0.1 g samples of mature fungus garden contained a mean of 81 (SE 4.0) staphylda each. Consequently, mature garden contained 5.8 times more staphylda than garden that was being discarded.

4. Discussion

One problem encountered when sectioning a spongy structure like a fungus garden, is that cavities are not all cut in true cross section. Some are cut at angles, or superficially, leading to areas and circumferences that are apparently larger or smaller than the original cavity. However, important information can be obtained from such data using stereological techniques. For example, percentage air space and S_v (surface area to volume ratio) can be calculated. Percentage space was remarkably consistent throughout the garden, although other characters varied. It might be expected that basal old garden would be dense, due to the weight of fungus garden above. However, old garden has tightly packed cavities with thin walls, while young garden has much thicker walls (Table 1). This probably reflects the way individual substrate particles are packed together. In young garden, they are loosely bound, while in old garden, they are tightly packed and bound together into a solid mass (Bass, 1993).

Numbers of cavities per cm^2 of section area increased with garden age as cavities became more closely packed together and had thinner walls. This led to an increase in surface area with garden age and was reflected in the calculated area to volume ratios (S_v ; Table 1), which also increased with garden age. This is advantageous for the ants, because staphyla production is related to garden surface area. Staphyla numbers on the garden surface also increased with garden age (until garden became very old). Mature and older garden therefore had an increased surface area of the most highly productive fungus garden.

Only one garden was used to make the observations on structure, and this was small, with a volume of only 250 cm^3 , and a large surface area to volume ratio. When its internal and external surface areas were calculated, these showed that at least 74% of fungus garden area was internal. In a larger garden, with a smaller surface area to volume ratio, the percentage of surface area that is internal would be higher.

Holly spines provided an ideal method for marking fungus garden. Workers readily accepted them as substrate and they were easily visible when incorporated into garden or when ejected in refuse. Different substrate types may affect the growth of the fungus in culture (Powell, 1984) and may therefore affect fungal growth in the garden itself, leading to differences in mycelial colonization of substrate or in the numbers of staphylae produced. Howard et al. (1988) suggested that secondary metabolites present in some plants may be harmful to the ants and their fungus. The holly used in this study appeared to be a good fungal substrate.

Marking garden with holly spines showed that the first staphylae were produced about 5 days after spine incorporation. Angeli-Papa and Eyme (1979) found that staphylae developed in agar culture after 20 days. However, in the fungus garden a large fungal inoculum is available to ensure rapid growth and development.

Few staphylae were discarded with refuse, showing that efficient removal of these from old garden must take place before it is discarded. Those that are discarded may be old and unpalatable. Staphyla numbers on the garden surface first increased with garden age, then, just before dumping of the spine-marked areas began, numbers declined. Either the garden was producing fewer staphylae or workers were harvesting them more intensely. Discarding garden after it has passed its peak productivity would be an optimal strategy for the ants. There is clearly a trade-off to be made between harvesting as many staphylae as possible when their quantity and perhaps their quality are falling and the need to clear space to make room for a new crop. One problem is that standing crops of staphylae were measured rather than production rates. The latter are difficult to assess because the ants continually remove

staphylae. Areas of garden with low standing crops of staphylae may simply be experiencing higher harvesting rates. The timing of staphyla production has presumably been selected by the ants. Crops can either produce over an extended period of time, producing some stability in production between subsequent sowings, or they can mature rapidly, producing a synchronized harvest over a short period which can be pulled out and resown to maximize productivity over time. The former type is often used by human subsistence farmers, while the latter is often selected for by industrial growers, who need a synchronized mass harvest, and have preservation techniques that obviate the necessity for stability of production between harvests. The inability to store staphylae may account for the extended cropping period shown by the mutualistic fungus.

Workers were found deep inside the fungus garden, indicating that most of the internal surface area was connected with the outside. Most of these workers (89.9%) were minima. However, Weber (1972) found that 60% of workers in a colony were minima. This suggests that most internal cavities were too small for larger workers to enter. Many staphyla-bearing areas inside the garden were therefore available only to minima, which are a specialist fungus-gardening sub-caste (Wilson, 1980b). Smaller cavities had more staphylae per unit area than did larger ones or outer surfaces, suggesting that harvesting pressure was lower in these areas. All worker sizes are found on external surfaces, and as a hungry worker will probably take the nearest available staphyla, a greater harvesting pressure may occur in large cavities and on external surfaces. The larger number of staphylae found in small cavities could indicate that more staphylae are produced here, but Bass and Cherrett (1996) suggest that the numbers of staphylae produced may be related to the amount of pruning by the ants, as this is a stimulus for staphyla production. Worker access to internal cavities is restricted, so a lower level of pruning is likely, compared with external surfaces. This strengthens the suggestion that the large numbers of staphylae in these small cavities reflect the lower harvesting pressure; staphylae simply build up.

There is probably a trade-off between maximizing the potential staphyla-producing surface area, maintaining accessibility for workers and keeping the gardens as compact as possible to maintain high humidity, and to use space efficiently, as the excavation of chambers has an energy cost. This has implications for the division of labor practiced by the *Attines*; small workers are gardener-nurses while larger ones forage, excavate or defend the nest (Wilson, 1980b). The evolution of the minima sub-caste may be a response to this need to access the honeycomb structure of the fungus garden.

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